Advances in Mucosal Immunology

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Preface

The immunology of mucosal surfaces is one of the most exciting and relevant areas of medical veterinary and dental research since it applies basic research to tissues involved in everyday defence against microbes and against environmental and food antigens. This book is based on the contributions presented at the International Congress of Mucosal Immunology, held in London in July 1989 and organised by the Mucosal Immunology Affinity Group of the British Society for Immunology. The meeting was attended by over 500 delegates from 27 countries, including virtually all of the leading investigators in the field. The contents give comprehensive and up-to-date information on such topics as antigen presentation and processing in the gut, mucosal vaccines in man and animals, HIV infection in the gut, the role of $\gamma\delta$ T cells in the gut epithelium, recent advances in inflammatory bowel disease and coeliac disease, the role of cytokines in the regulation of the IgA response, mucosal mast cells and cell migration. The contributions reflect the rapid pace of research in mucosal immunology, and the great strides which are taking place in the understanding of the immunology, molecular biology and biochemistry of host response at mucosal surfaces.

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Local immunity: The human mucosa in health and disease

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ABSTRACT. The secretory antibody system depends on local production and selective epithelial transport of polymeric immunoglobulin A (pIgA) and immunoglobulin M (pIgM). To this end there is a fascinating co-operation between the local immune system and the glandular epithelium by their production of two key factors, J chain and secretory component (SC), respectively. Mucosal immune regulation is normally geared for generation of early memory B-cell clones with prominent J-chain expression and preference for IgA production. Moreover, cytokines released from activated T cells and macrophages probably stimulate the secretory epithelium to perform enhanced SC-dependent transport of J chain-containing pIgA and pIgM when their local production is increased. There is also circumstantial evidence to suggest that interactions between the gut epithelium and T cells are involved in mucosal downregulation of systemic types of immunity to non-replicating luminal antigens. IgG (and IgE) responses and delayed-type hypersensitivity may thereby be minimized to avoid undue complement activation, increased mucosal penetrability, inflammation, and tissue damage. It is possible that abrogation of such hyporesponsiveness to usually harmless luminal antigens contributes to the pathogenesis of various mucosal disorders. Enhanced stimulation of local T lymphocytes, increased epithelial expression of HLA class II determinants, and disproportionate local overproduction of IgG, are features that may reflect such a deleterious development leading to disease. Expansion of a particular TCRγ/δ^+ intraepithelial T-cell subset may also be involved in coeliac disease.

1. Introduction

The secretory immunoglobulin (SIg) system is the best defined effector mechanism of mucosal immunity. It depends on cooperation between the local B-cell system and the secretory epithelium which contribute, respectively, J chain-containing polymeric Ig (pIgA and pIgM) and the epithelial pIg receptor called "secretory component" or SC [1]. Recent observations in our laboratory have also indicated that interactions between activated leucocytes (e.g., T cells and macrophages) and the secretory epithelium may upregulate the SC-dependent pIg transport as part of a local immune response [2]. Furthermore, lymphoepithelial interactions, perhaps involving the numerous intraepithelial lymphocytes (IEL) in

the gut, may participate in the modulation of mucosal immunity by normal downregulation and pathological upregulation of systemic types of immune responses to harmless luminal antigens [2,3]. This review will focus on recent progress in these aspects of local immunity with emphasis on current work in our laboratory.

2. Mucosal Immunobiology

2.1. CELLULAR BASIS OF SPECIFIC HUMORAL IMMUNITY

Exocrine tissues constitute quantitatively the most important mediator organ of humoral immunity. The generation of secretory antibodies depends on a striking local preponderance (70-90%) of IgA immunocytes (plasma cells and plasma blasts) in the absence of overt inflammation; the gut is the major contributor to this unique defence system. There are normally about 10^{10} Ig-producing immunocytes per metre of human small bowel [4]. Taking the total length of the intestines into consideration, a rough estimate indicates that about 80% of all Ig-producing cells are located in gut mucosa.

Immunocytes adjacent to exocrine glands produce mainly pIgA (dimers and larger polymers) containing a disulphide-linked polypeptide called the "joining" or J chain [1,5]. Such pIgA can be transported through the glandular epithelium along with J chain-containing pentameric IgM (pIgM) via the pIg receptor constituted by transmembrane SC [1]. J chain and SC may in fact be regarded as "lock and key" factors in secretory immunity; glandular pIg transport thus depends on an intimate interaction between the B-cell system and exocrine epithelia. More pIgA is in this way translocated to the gut lumen every day (40 mg/kg) than the total daily production of IgG [6].

Regulation of local humoral immunity has to be geared for generation of early memory B-cell clones with prominent J-chain expression and preference for IgA production [1]. The result is not only a "first line" defence mediated mainly by SIgA antibodies; in addition, the abundance of locally produced IgA antibodies is probably crucial for immunological homeostasis within the lamina propria. IgA may block the triggering of non-specific biological amplification mechanisms because it lacks potent effector functions such as classical complement (C) activation [4]. At the same time it would be conducive to preservation of health to dampen potentially phlogistic IgG and IgE responses and T-cell mediated hypersensitivity against harmless luminal antigens (Figure 1). Although there is little direct evidence that such "oral tolerance" exists in human, it most likely does so in view of the fact that the vulnerable gut mucosa normally shows virtually no IgG response and inflammation despite the influx of small amounts of intact dietary antigens after meals [4].

The concept of a "common mucosal immune system" has gained wide acceptance. Antigenic priming of organized gut-associated lymphoid tissue (GALT), mainly including the Peyer's patches (PP), may give rise to specific secretory immunity not only in the gut but also in the respiratory tract and in the lacrimal, salivary and lactating mammary glands [4,7]. Experimental evidence in animals has suggested that the dissemination of IgA-precursor cells to distant sites is much more limited from bronchus-associated lymphoid tissue (BALT) than from GALT [8]; and the regulatory mechanisms operating in GALT, BALT and tonsils may in fact differ in several ways [8,9]. Altogether, it seems

that GALT plays a fairly unique and dominating role in mucosal immunity.

2.2. MIGRATION OF MUCOSAL LYMPHOCYTES

An important basis for mucosal immunity is the migration of specifically primed T and B cells from GALT through lymph and peripheral blood and then primarily to the lamina propria surrounding the crypts of Lieberkühn, which are the glands responsible for pIg transport into the gut lumen. Extravasation of lymphoid cells seems to depend on receptors for endothelial determinants ("vascular addressins") which to some extent are tissue-specific [10]; but additional adhesion molecules and other local factors are also involved [11]. Small amounts of the PP-type of vascular addressins are present on ordinary venules in the gut lamina propria and probably contribute to the low-rate continuous mucosal extravasation of GALT-derived lymphoid cells [11]. High endothelial venules of mesenteric lymph nodes apparently share vascular addressins of peripheral lymph nodes and GALT [10]. This fact may explain that migratory spillover from the secretory to the systemic immune system seems to take place mainly in the mesenteric nodes as indicated from the relatively high expression of J chain and IgA2 subclass shown by IgA immunocytes terminating there [12]. However, some migration of T lymphocytes and B cells with activated J-chain gene, probably occurs from stimulated peripheral lymph nodes to secretory sites because parenteral immunization can lead to low-level priming of an intestinal immune response [4].

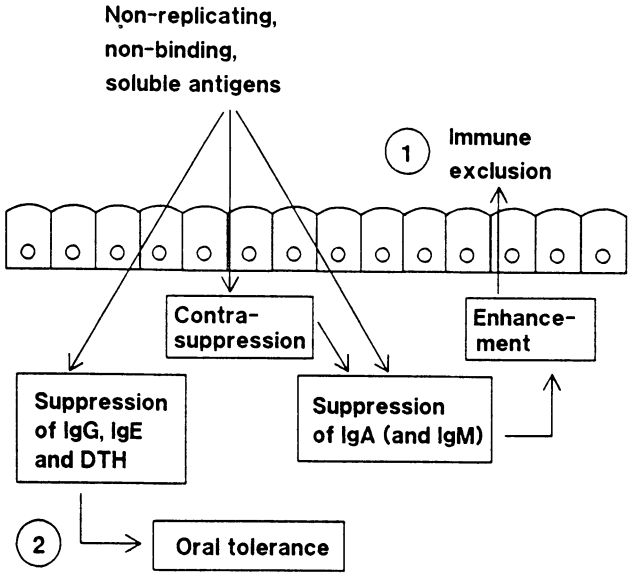

Figure 1. Schematic depiction of various putative mechanisms affording two levels of normal mucosal protection: (1) Secretory immunity performing immune exclusion (its enhancement against soluble dietary antigens may be afforded by contrasuppression releasing IgA- and IgM-producing cells from suppression); and (2) suppression of systemic types of immunity to dietary antigens (leading to "oral tolerance").

While B cells localize in the lamina propria and proliferate and differentiate there [4], many T cells find their way into the intestinal epithelium. The latter phenomenon may be partly antigen-independent because intraepithelial lymphocytes (IEL) are observed before birth; but luminal antigens clearly determine the magnitude of the intraepithelial migration [2]. The follicle-associated epithelium (FAE) covering the human PP contains particularly many T cells, especially near the antigen-transporting M ("membrane") cells [13].

2.3. PROPERTIES OF INTRAEPITHELIAL LYMPHOCYTES

Two as yet unproven functions have been ascribed to the IEL, namely MHC-restricted or unrestricted cytotoxicity and suppression of mucosal hypersensitivity reactions. Human IEL are mainly positive for the pan-T-cell marker CD3, and they show a striking predominance (80-90%) of T8 ($CD8^+$) cells ("cytotoxic/ suppressor" phenotype), whereas the lamina propria contains mainly the T4 ($CD4^+$) ("helper/inducer") subset [2,3]. Most human IEL are negative for the H366 antigen [14], which is a putative marker of MHC-restricted cytotoxic T8 cells, and they show no spontaneous cytotoxicity [15]. The FAE of human PP contains a much higher proportion of T4 cells than the ileal villous epithelium, i.e. 40% vs. 10% [13], perhaps reflecting a larger population of functional helper (Th) cells.

Human T4 cells that express large amounts of the CD45R/2H4 marker (CD45RA) apparently belong to a naive (unsensitized) population, whereas those high in the smallest (180 kDa) CD45/UCHL1 marker (CD45R0) probably represent memory (antigen-primed) cells [16]. The same distinction seems to hold true for T8 cells. Normal human intestinal mucosa has been reported to contain relatively fewer $CD45RA^+$ T cells than peripheral blood - that is, 38% vs. 68% [17], and our findings have indicated a still lower mucosal proportion of this putative naive subset [18]. Also studies of interleukin 2 (IL-2) receptor (Tac antigen or CD25) on intestinal T cells from normal monkeys have suggested a remarkable level of stimulation [17]. This observation harmonizes with the fact that many T cells in normal (and especially in diseased) human intestinal mucosa express CD45R0, both in the lamina propria and in the epithelium [18].

The IEL bear, in addition, a lymphocyte marker (HML-1) that was thought to be fairly unique for mucosal sites [19]; but HML-1 positivity may rather reflect a certain state of stimulation [20], which is in keeping with the CD45R0 expression [18] and morphological features of the IEL [21]. However, they generally seem to be negative for markers of recent activation such as MHC class II and IL2 receptor. Nevertheless, CD7 (RFT2) is normally expressed on most intraepithelial T8 cells, supporting the notion that this IEL subset is indeed stimulated [14].

There has been some recent controversy about the nature of the T-cell receptor (TCR) for antigen employed by the IEL. Two studies in mice indicated that intraepithelial T8 cells expressed exclusively the TCRγ/δ rather than the conventional TCR$\alpha/ß$ [22,23]; this finding was taken to support the notion that the IEL are mainly cytotoxic [24]. However, we have recently shown that most human IEL employ the TCR$\alpha/ß$ [18,25]; this feature along with considerable CD45R0 expression [18], suggests that they are largely traditional memory T cells. What remains unexplained is their remarkable enrichment of the T8 subset and its biological significance. Another intriguing feature is the small fraction (median 2%, range 0-39%) of TCRγ/δ^+ IEL which normally are mainly (~75%)

$CD4^-CD8^-$, employ the variable $\delta 1$ gene ($V_\delta 1/J_\delta 1$) much more frequently than their counterparts in peripheral blood [25], and increase intraepithelially in coeliac disease [25].

2.4. ANTIGEN UPTAKE, PROCESSING, AND PRESENTATION

The importance of particular antigen-presenting cells (APC) within GALT has been emphasized in several studies [4]. It is interesting that we have found a much greater proportion of APC of the interdigitating cell phenotype relative to L1 positive reactive macrophages beneath the FAE of human PP than in the intestinal lamina propria [26]. At the latter site macrophages may be involved in down-regulation of immunity, as recently shown to be the case in rat respiratory mucosa [27].

The way antigen normally enters the mucosa may have a profound effect on local immunity. The M cells of FAE lack SC and generally also HLA-DR expression [28]; they therefore are probably without an IgA-transporting and antigen-presenting function. The M cells rather seem to perform non-selective and to some extent receptor-mediated inwards transport of luminal antigens and immune complexes [29]. The remaining FAE, like the villous epithelium, shows a striking apical expression of HLA-DR and also a granular appearance of such protein in the cytoplasm [28]. In view of recent information about the peptide-binding properties of MHC molecules, it is tempting to speculate that antigen that has been adequately processed in the gut lumen is taken up by HLA-DR-expressing epithelial cells on a genetically restricted basis and perhaps subsequently presented directly to primed (memory) T lymphocytes [2].

2.5. EPITHELIAL TRANSPORT OF POLYMERIC IMMUNOGLOBULINS

Cloned cDNA of rabbit SC has demonstrated that it contains five extracellular domains with remarkable homology to each other and to variable or constant regions of Ig chains [30]. We have recently confirmed this for human SC by cloning of its cDNA [31]. Like other members of the Ig superfamily, SC expression can be regulated by lymphokines. Both recombinant interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) upregulate the intracellular pool and epithelial membrane expression of functional SC in the HT-29 cell line [2]. The effects of these two cytokines on SC expression are additive in a dose-dependent manner [32]. IFN-γ is secreted by T cells during immune responses whereas activated macrophages are the best recognized source of TNF-α. Our observations therefore suggest that both these cell types may promote the external transport of pIgA and pIgM and thereby enhance the efferent limb of the secretory immune system.

The availability of cDNA probes specific for human SC mRNA [31], has made it possible to confirm by Northern blot analysis that our previous immunohistochemical studies [1] have given a true idea about SC expression in various exocrine tissues. Impressive amounts of mRNA can be detected in various segments of the gut, in harmony with the prominent IgA-transporting requirement. Interestingly, no mRNA is detected by this method in the liver, confirming that there is no SC-mediated IgA transport through human hepatocytes [1].

2.6. SUPPRESSION OF MUCOSAL HYPERSENSITIVITY

When secretory antibodies are unable to perform adequate antigen exclusion, the internal body environment should preferably be protected against potentially harmful systemic types of immune reactions elicited by IgG, IgE or T cell-mediated delayed-type hypersensitivity (Figure 1). Experimental evidence in animals has suggested that such protection is afforded by suppressive mechanisms collectively called "oral tolerance" [33]. This phenomenon of hyporesponsiveness to harmless non-replicating luminal agents that do not bind strongly to the epithelium [34], probably involves multiple immunoregulatory events and may to some extent depend on the liver.

There is evidence in human that systemic IgG hyporesponsiveness to common dietary antigens develop over time [4], and it is tempting to propose that oral tolerance induced via the intestinal mucosa involves the numerous intraepithelial T8 cells. The experiments of Bland and Warren [35] with MHC class II positive columnar cells from rat villous epithelium, and those of Mayer and Shlien [36] with human colonic epithelial cells, have suggested that antigen presentation by gut epithelium may lead to suppression mediated by T8 cells. Interestingly, it has been possible by means of soluble antigen to induce cytotoxic class II-restricted T8 cells that apparently exerted clonal killing of B cells presenting the same antigen [37]. This could be one way IEL perform suppression after returning to the lamina propria. Alternatively, since a great proportion of the IEL apparently are primed [18] (and therefore express various adhesion molecules) the cells may have sufficient affinity to be retained in the epithelium and be stimulated by antigen in the context of MHC class II molecules, even without being CD4 positive. This might cause unresponsiveness (clonal anergy) rather than activation and therefore lack of some essential co-stimulatory lymphokine [38]. Class II positive keratinocytes have in fact been shown to render antigen-specific T cells unresponsive [39]. Moreover, idiotypic peptides derived from the α and ß receptor chains of retained anergic IEL, may subsequently be presented to anti-idiotypic T4 cells in the context of class II molecules; expansion of such autoreactive cells could in various ways result in antigen-specific suppression of both $CD4^+$ and $CD8^+$ clonal subsets [40].

It is puzzeling how local IgA responses can be elicited and maintained if suppression is a general phenomenon induced by soluble non-replicating antigens. One possibility is that GALT generates contrasuppressor T cells (Tcs) specifically interfering with the inhibition of IgA- and IgM-antibody production (Figure 1). Murine PP have been reported to contain a population of Vicia villosa lectin-adherent Tcs that preferentially support IgA responses [41]. Recent observations have also indicated the presence of Tcs of the CD8 phenotype in normal human gut mucosa; these cells augmented IgA and IgM production in an in vitro assay, apparently by acting on a $CD4^+$ subset when this was overrepresented [42]. The possibility therefore exists that the prominent intraepithelial T8 population may include Tcs cells.

3. Mucosal Immunopathology

3.1. PUTATIVE BREAK IN ORAL TOLERANCE

Abrogation of hyporesponsiveness to non-replicating luminal antigens may be

involved in the pathogenesis of coeliac disease, chronic gastritis, and inflammatory bowel disease (IBD) - as suggested by the disproportionate although highly varying local overproduction of IgG seen in all these disorders [4]. In experimental animals, antigen feeding combined with some sort of damage to the gut epithelium [43], seems to be incompatible with induction of oral tolerance. The same is true when APC are excessively activated by stimuli such as muramyl dipeptide, estrogen or a graft-versus-host reaction [33]. All these situations apparently favour general overstimulation of Th cells.

Also aberrant epithelial MHC class II expression may be involved in abrogation of oral tolerance. Preferential activation of T4 rather than T8 lymphocytes was observed after in vitro stimulation with antigen presented by colonic epithelial cells obtained from IBD [44], in which epithelial HLA-DR is markedly increased [4]. Enhanced and differential (DR>DP>DQ) epithelial HLA class II expression may likewise be involved in exaggerated immune responses to gluten and other dietary antigens in coeliac disease and to autoantigens in IBD [3]. Interactions between gut epithelium and T cells may in this way contribute to a class II-associated predisposition, which is particularly well documented in coeliac disease. The primary association seems to be related to a particular DQ polymorphism where peptide-binding residues of both the α and the ß chain may have functional importance for the pathogenesis [45].

3.2. IMMUNOPATHOLOGY OF COELIAC DISEASE

The first immunopathological feature observed in coeliac lesions after challenge with gluten, is a dose-dependent migration of lymphocytes into the jejunal epithelium [21]. The proportion of $CD3^+CD45R0^+$ IEL is significantly raised [18], and the epithelial density of such putative memory T cells is strikingly increased [46]. A recent study has reported raised numbers of T4 cells isolated from the lesion [47], and about three times more intraepithelial T4 cells show sign of stimulation by expressing the CD7 marker [48]. Noteworthy is furthermore an increased fraction of $CD3^+CD4^-CD8^-$intraepithelial cells [49]. These "double-negative" IEL obviously represent $TCR\gamma/\delta^+$ cells, which also are increased to at least 20% in coeliac disease [25]. For this subset, CD8 is limited to about 10% whereas a remarkably high expression (~67%) of the $V_\delta1/J_\delta1$ gene product is observed [25]. The possibility that the $TCR\gamma/\delta^+$ IEL are cytotoxic and contribute to villous atrophy is a fascinating challenge for further research, but a putative contrasuppressive function should also be explored.

Untreated coeliac lesions show increased epithelial expression of both SC and HLA-DR [50], most likely mediated by cytokines released from primed T cells and perhaps macrophages. Differential HLA class II expression in the jejunal epithelium is in fact related to the number of intraepithelial putative memory ($CD45R0^+$) T cells, and positivity for DP and DQ is seen only when this number is high [46].

Local overstimulation of T cells (virus-induced?) may therefore be an early event in the coeliac lesion. One consequence is probably cytokine-induced crypt hyperplasia [21,51] and another may be increased epithelial permeability [52] with ensuing overstimulation of the B-cell system. In untreated adult coeliac disease the average numbers of jejunal IgA, IgM and IgG immunocytes per mucosal "tissue unit" are raised 2.4, 4.6 and 6.5 times, respectively [4]. Overstimulation of T4 helper cells inducing undue local and systemic IgG responses to gluten and other dietary antigens, may cause altered immunological

homeostasis which precipitates important pathogenic mechanisms in the mucosa. The local immune response is, nevertheless, dominated by a typical secretory IgA and IgM response with preserved J-chain expression and a relatively high proportion of IgA2-producing immunocytes [53]. This fact probably constitutes the basis for the reversibility of the mucosal lesion usually seen after gluten elimination.

3.3. IMMUNOPATHOLOGY OF INFLAMMATORY BOWEL DISEASE

The aetiology of inflammatory bowel disease (IBD) is unknown. Regardless of initiating factor(s), however, much circumstantial evidence suggests that immunological mechanisms are involved in the pathogenesis of both ulcerative colitis (UC) and Crohn's disease. A significant increase of T4 lymphocytes in the lamina propria of severely inflamed UC lesions was recently reported, and the same authors described a striking increase of T4 and a decrease of T8 cells in the epithelium compared with normal colon [54]. Abrogation of oral tolerance to luminal antigens may thus be an early event. Activation of T cells and macrophages probably leads to aberrant epithelial HLA-DR expression [3,4]; and it has been suggested that antigen presentation by epithelial cells from IBD lesions results in stimulation of T4 helper rather than T8 suppressor cells [44]. This may be further conducive to intensified B-cell activation and increased Ig production. The established IBD lesion is in fact a typical B-cell lesion. We and others have found that the mucosal IgA- and IgM-cell populations are increased several times and that there is a disproportionate rise of IgG immunocytes depending on the severity of the lesion, both in Crohn's disease and in UC [4,54]. In addition, the IgA1-producing cells are increased to the extent that they become much more frequent than the IgA2 counterparts [55]. It is also noteworthy that J-chain expression is reduced, both in IgA1 and IgA2 cells [56]. This change apparently reflects local accumulation of mature B cells of systemic immunity and jeopardizes secretory immunity [1].

The consequences of the change to a systemic type of humoral immunity in IBD lesions are probably conflicting in terms of preservation of health. A "second line" of defence established within the gut wall may promote immune elimination and thereby limit dissemination of antigenic and possibly replicating agents, but it will at the same time disturbe the normal immunological homeostasis in the mucosa. However, the overall production of pIgA is quantitatively maintained in IBD because of the great total increase of mucosal IgA immunocytes [56]. Nevertheless, epithelial IBD lesions often show decreased SC expression [4], which probably results in patchy defects of SIgA (and SIgM) secretion and thereby topically reduced immune exclusion. The persistent immunopathological reactions may thus be maintained in IBD lesions by ubiquitous microbial components present in the gut lumen, but some antigens may be more important in Crohn's disease (? mycobacteria) and others in UC (? autoantigens). Recent observations showing relatively more colonic IgG1 production in the latter than in the former disorder, and the opposite finding for IgG2 [57,58], further attest to some disease specificity of the local IgG responses.

Localization of C3c in extensively washed tissue specimens indicates recent (within hours) or ongoing C activation, and the presence of terminal C complex (TCC) reflects formation of the cytolytic pore-forming C5b-9(m). We have recently found that walls of submucosal blood vessels contain significantly more C3c and TCC deposits in IBD than in histologically normal colonic control

tissue [59]. We have also observed C3c and TCC deposits, sometimes along with bound IgG1, on the luminal phase of epithelial cells in specimens from active UC [60]. These observations suggest that IgG-mediated C activation is involved both in epithelial destruction and in perpetuation of the inflammatory reaction.

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Part 1
Basic Mechanisms in Mucosal Immunology

SECTION A: ANTIGEN HANDLING AND PROCESSING AT MUCOSAL SURFACES

2
Intestinal processing of soluble protein antigen

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ABSTRACT. Food and environmental antigens entering the intestine are processed at several levels before interacting with class II molecules and, ultimately, with the antigen receptors of T cells which will regulate the appropriate immune response. Thus: intraluminal digestive enzymes; epithelial transcytosis mechanisms; lysosomal activity of lamina propria macrophages; and mechanisms acting downstream of the mucosa may all have a modifying effect.

In experiments in which pigs were weaned onto a diet containing ovalbumin as the protein source, the survival of the antigen in the intestine was found to be dependent on stomach emptying time and efficiency of intraluminal enzyme activity (both of which were age-dependent). In *in vitro* experiments, using isolated rat cells, ovalbumin was not degraded by enterocytes as assessed by SDS-PAGE and leupeptin did not suppress effective enterocyte processing mechanisms. However, chloroquine, ammonium chloride and monensin all inhibited "processing" by enterocytes.

The results indicate that the processing changes which take place in the ovalbumin molecule during absorption are dependent on functional endocytic pathways in the enterocyte and are more subtle than the degradative mechanisms characteristic of systemic systems.

1. Introduction

Although live, replicating antigens induce a vigorous immune response at mucosal surfaces, soluble dietary proteins on the whole are more likely to induce specific tolerance. The point in the induction of a response at which these effector mechanisms diverge is not known. However, recent studies have shown that the epithelium of the intestine has the potential to present soluble protein antigens directly to T cells, causing the induction of suppressor cells (Bland & Warren, 1986a,b; Mayer & Shlien, 1987). If these mechanisms operate *in vivo*, this may point to a direct role for the gut epithelium in the processing of foreign antigen and indicate that the divergence of effector mechanisms may take place very early in contact with antigen, i.e., at the epithelial interface.

It has been shown that many soluble proteins require "processing" in some way before they can act as effective ligands for the T cell receptor. The antigenic epitope for the T cell receptor for most soluble proteins consists of a short sequence of amino acids and in order for this sequence to bind successfully to the receptor, degradation or conformational changes must take place within the protein molecule. Such processing has been studied in several cell types, including macrophages, dendritic cells and B cells and has been shown to be dependent on energy-dependent mechanisms and on intact lysosomal enzyme systems.

Processing of antigens in the gut, however, is a compex, multi-step process involving modification by luminal enzymes in addition to processing steps which can take place in several cell types within the mucosal tissues. Here, the processing of ovalbumin by luminal enzymes in the pig and by epithelial cells isolated from the small intestine of the rat has been investigated.

2. Materials and Methods

2.1. ANIMALS

Male DA (RT1av1) rats, obtained either from Harlan-Olac Ltd (Bicester, Oxon), or from our own barrier-reared colony, were used when 8-10 weeks old. They were maintained under constant environmental conditions on CRM diet (Labsure Ltd., Poole, Dorset), which contains no egg proteins.
The litter from a Large White sow was weaned at three weeks of age onto an experimental diet containing 20% protein comprising 50% soya protein and 50% dried egg powder. Piglets were trough-fed and were used in investigations at the times stated after feeding.

2.2. ANTIGENS AND IMMUNISATIONS

Chicken ovalbumin grade V (Sigma Chemical Co., Poole, Dorset) was used without modification. Rats were immunised with 100ug ovalbumin emulsified with Freund's complete adjuvant by footpad injection. Ovalbumin was labelled with ^{125}I either by the chloramine T method (Hunter & Greenwood, 1962), or using Bolton-Hunter Reagent (Bolton & Hunter, 1972). Radiolabelled antigen was passed over Sephadex G-25 (Pharmacia, Uppsala, Sweden) immediately prior to inclusion in assays.

2.3. MEDIA

Hanks' balanced salt solution (calcium and magnesium-free) and RPMI-1640 were obtained from Gibco Laboratories Ltd. (Paisley, Scotland). RPMI was routinely supplemented with 2mM L-glutamine, 1mM sodium pyruvate, 50μM 2-mercaptoethanol, 100unit/ml penicillin, 100μg/ml streptomycin, 40μg/ml gentamicin, 20mM HEPES and, unless otherwise stated, 10% heat-inactivated foetal calf serum.

2.4. CELL ISOLATION

Enterocytes, ovalbumin-primed T cells and splenic adherent cells were isolated as previously described (Bland & Warren, 1986a).

2.5. ANTIGEN PULSING AND CELL TREATMENTS

Enterocytes or splenic adherent cells at 10^6 cells/ml were pulsed with ovalbumin at 1mg/ml in RPMI for 30 min at 37°C. They were then washed three times in RPMI or, if they were to be subsequently fixed, were washed once in serum-free RPMI and twice in PBS. Cells were fixed for one minute in paraformaldehyde in a water bath at 37°C, followed by neutralisation with an equal volume of ice-cold 0.06% glycyl-glycine. They were then washed once in PBS and twice in complete RPMI and included immediately in assays. In some experiments, cells were incubated for 30 minutes at 37°C with either 0.1mM chloroquine, 10mM ammonium chloride, or 1μM monensin (all Sigma Chemical Co., Poole, Dorset), or with 0.5mM leupeptin (Boeringer-Mannheim, W.Germany), and washed twice with complete RPMI before further treatment.

2.6. ANTIGEN PRESENTATION ASSAYS

In assays of antigen presentation by enterocytes, T cells were cultured (10^7 cells per dish) with enterocytes (10^6 or 2 x 10^6 cells per dish) in 35mm plastic tissue culture dishes for 18hr. T cells were then separated from enterocytes on Histopaque 1083 (Sigma), washed and replated as triplicates at 2 x 10^5 cells per well in 96 well flat-bottom tissue culture plates (Nunc, Gibco, Paisley) for 96 hr (Bland & Warren, 1986). Splenic adherent cells were cultured directly with T cells throughout the 96hr culture. Cultures were pulsed for the final 4hr with 1μCi/well ^{3}H-TdR (5.0 Ci/mmole,Amersham),and counted on a liquid scintillation counter. Control induction cultures included T cells alone and T cells with ovalbumin at 100μg/ml. Background (unstimulated) T cell proliferative responses were usually less than 1,000 cpm and have been included as a control in Figure 1 only.

2.7. ELECTROPHORESIS

Equal numbers of enterocytes or SAC (5-10 x 10^6 per tube) were incubated in 1ml ovalbumin in RPMI at 100μg/ml, trace-labelled with ^{125}I-ovalbumin to give a final specific activity of 10-15mCi/mmole, for the time periods stated. Cells were then pelleted and supernatents removed. Cell pellets were washed in RPMI and supernatents removed. Cell pellets were washed in RPMI and lysed in 0.5ml-1.0ml of lysis buffer (1mM EDTA, 1% NP-40, 0.1% SDS, 0.1% sodium azide and 1mM PMSF) for 30 min on ice. Nuclei were pelleted at 3,000g and lysates clarified at 12,000g for 5 minutes. Lysate and supernatent samples were then mixed with equal quantities of loading buffer containing 2-mercaptoethanol, heated to 100° for 3 min and loaded onto discontinuous 12.5% polyacacrylamide gels. Gels were run at 40v, dried and autoradiographed with Kodak X-Omat film in intensifying screens at -70°C.

3. **Results**

3.1. LUMINAL PROCESSING

In an effort to determine the extent to which the ovalbumin molecule is degraded in the intestinal lumen, piglets were weaned onto a diet, of which the 20% protein content comprised dried egg powder. The piglets were given access to food at time 0 and at half-hourly intervals beginning at two hours post-feed individuals were anaesthetised. Under geneeral anaesthesia, sections of intestine were tied off and the contents removed to a mixture of protease inhibitors on ice. The intestinal samples were subsequently run on SDS-PAGE, Western blotted with polyconal sheep anti-ovalbumin and the blots were developed with peroxidase-conjugated rabbit anti-goat and PAP.

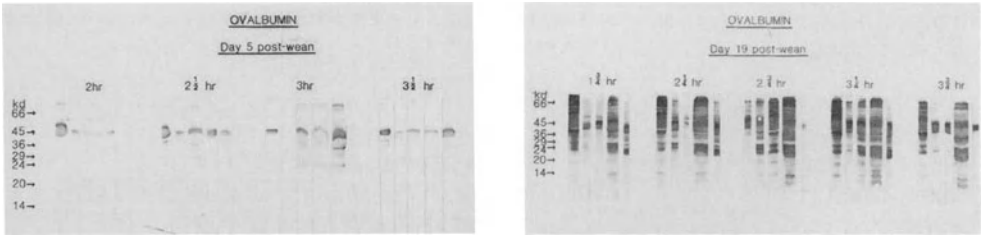

Figure 1. SDS-PAGE of intestinal contents of five individual piglets, 5 days or 19 days after weaning, at different times after feeding, Western blotted with polyclonal sheep anti-ovalbumin. From left to right in each set: stomach; proximal; mid; and distal intestine; and colon.

Figure 1 shows that in piglets 5 days after weaning, at 2hr post-feed the majority of ovalbumin of native molecular weight was located in the stomach, with only a small quantity in the small intestine. By 2.5hr post-feed increasing amounts of antigen were observed in blots of intestinal contents and some evidence of breakdown products due to luminal hydrolysis was evident. By 3hr post-feed, although more degradation products were seen in the distal small intestine, a similar profile was seen in the colon, implying that incomplete absorption was taking place in the small bowel of animals of this age. In piglets at day 19 post-wean a very different pattern was seen. Thus, stomach emptying time was more rapid, more extensive hydrolysis of the ovalbumin molecule took place and absorption of the resultant products was more complete with very little ovalbumin, native or fragments remaining in the colon.

3.2. PROCESSING BY ISOLATED ENTEROCYTES

3.2.1. Effect of agents interfering with endosome/lysosome activity. Providing fixation by paraformaldehde was carried out after the antigen pulse, subsequent presentation of antigen to primed T cells was unaffected. However, fixation before the antigen pulse significantly depressed subsequent presentation of the antigen (Figure 2). Treatment of enterocytes with the lysosomotropic bases, chloroquine and ammonium chloride prior to the pulse/fix regime effectively blocked presentation.

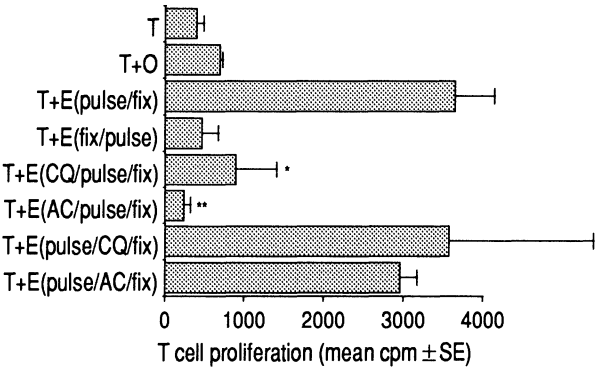

Figure 2. The effect of fixation and treatment with ammonium chloride (AC) or chloroquine (CQ) on presentation of ovalbumin by enterocytes to T cells.

Figure 3 shows the comparative effects of the cationic ionophore, monensin and the competitive protease inhibitor, leupeptin on processing by enterocytes and splenic adherent cells. It is clear from Figure 3a that both agents inhibit the processing of ovalbumin by splenic adherent cells. On the other hand, athough processing by isolated enterocytes was blocked by monensin treatment, leupeptin had no significant effect (Figure 3b). In the experiment shown in Figure 3b, leupeptin treatment was actually stimulatory, but this was not repeatable in other experiments.

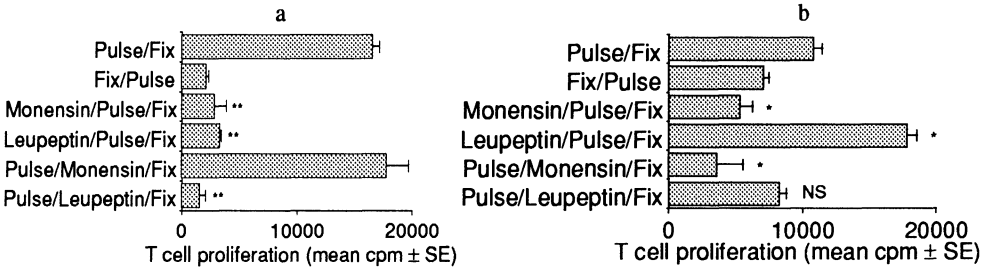

Figure 3. Effect of monensin and leupeptin on antigen presentation by splenic adherent cells (a) and enterocytes (b).

3.2.2. Analysis of degradative processing. Enterocytes and SAC were incubated with ^{125}I-Ova and then washed free of unbound label. Cell-free supernatents and detergent lysates of cells were then analysed by SDS-PAGE and autoradiography. After 4hr culture periods degradation products were seen associated with enterocyte supernatents, but these products were not different from those generated in cutures containing ^{125}I-Ova only (Figure 4a). However, culture of SAC with labelled Ova for the same culture period induced at least two additional degradation products at 13kd and 20kd. The 13kdproduct and a further product at 18kd were also seen associated with the SAC lysate proteins (Figure 4b). Eighteen hour cultures of SAC with ^{125}I-Ova induced an accumulation of very small cleavage products at the dye front of cell lysate gels, but this was never seen with enterocyte lysates (Figure 4c).

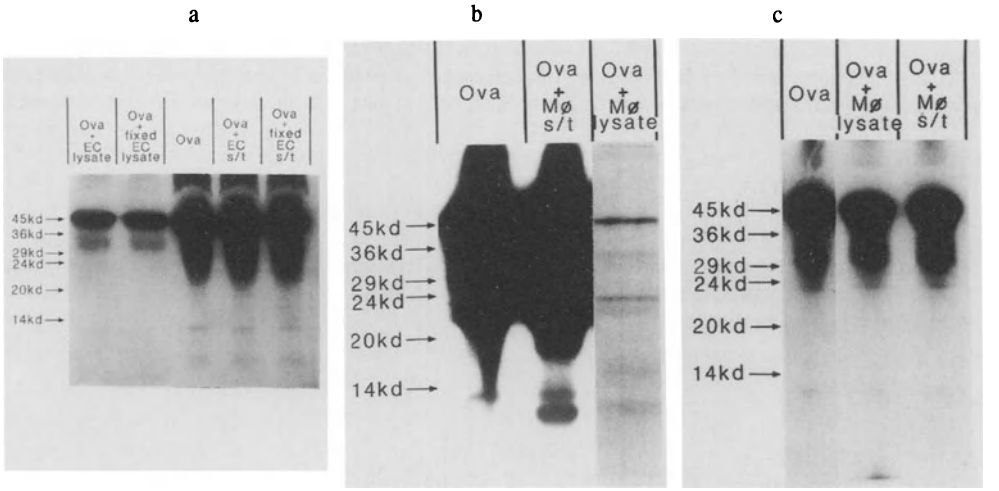

Figure 4. Autoradiographs of SDS-PAGE analysis of processing of ovalbumin by enterocytes (a) and splenic adherent cells (b,c).

4. Discussion

In these studies we have confirmed the complex, multistep nature of antigen processing in the gut. Thus, in the lumen of the intestine the gross degradation of dietary proteins by digestive enzymes and the subsequent absorption of resultant products involves a series of highly complex mechanisms. Processing at this level, then, is highly dependent on the maturity of enzyme systems, stomach emptying time, gut motility and absorptive capacity

Ingested antigen enters the second level of processing during absorption across the epithelium. Experiments using inhibitors of endosome/lysosome function showed that, to be effective, processing at this level required the maintenance of membrane mobility and low pH in this cellular compartment. These parameters have effects both on enzyme systems which are likely to be important in processing of endocytosed antigens, but are equally likely to affect post-translational glycosylation, effective assembly, and possibly antigen-binding capacity of the enterocyte class II molecule. These effects of ammonium chloride, chloroquine and monensin were also seen in SAC. However, although leupeptin blocked processing by SAC, it had no effect on processing by enterocytes, suggesting that acidic protease activity was less important in processing by enterocytes. This

concurs with the electrophoretic analysis of the products of processing which showed that antigen processed by the two cell types was fundamentally different. Thus, degradation products were detected in SAC cultures, but not in enterocyte cultures.

In summary, the results confirm data from other laboratories suggesting a requirement for degradative processing of ovalbumin for effective presentation by macrophages, B cells and dendritic cells to T cells, but show that processing of this same antigen by enterocytes, although still resulting in an epitope seen by the T cell receptor, is fundamentally different. Speculations on the reasons for this difference in processing might include: differences in the molecular structure and antigen-binding capacity of macrophage and enterocyte class II molecules; or an entirely different role for the class II molecule in the two cell types (we have already proposed that the major role of enterocyte class II may be as a recycling antigen receptor, enhancing selective and protected transcytosis of certain antigens during absorption)(Bland, 1988).

A third level of antigen processing in the gut - by the class II-bearing macrophage of the lamina propria - was not part of this study. However, we believe that processing and presentation of absorbed antigen by these cells plays a crucial part in the induction and regulation of immune responses to dietary and microbial antigens.

5.Acknowledgements

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3
Antigen handling in the intestine mediated by normal enterocytes

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INTRODUCTION

The co-existence of a myriad of foreign antigens in the intestinal lumen alongside immunocompetent cells has resulted in the evolution of a specialized mucosal immune system. A natural physical barrier to antigen has been the intestinal enterocyte joined to its neighbors by desmosomes forming tight junctions. Therefore, specialized antigen sampling cells, follicle associated epithelium or M cells, were thought to be the major sites for antigen entry into the mucosa associated lymphoid tissue (MALT). However, the majority of antigens which pass through these specialized cells are large and particulate. Recently, several groups have identified the existence of a class of immunoregulatory glycoproteins, class II antigens, on normal and inflamed epithelial cells (1-3). The presence of such glycoproteins has raised questions as to the functional nature and the role that these cells might play in normal mucosal immune responses. We and others have already documented that normal enterocytes from man (4) or rat (5-6) can function as antigen presenting cells, presenting processed antigens to immunocompetent T cells. However, unlike conventional antigen presenting cells (APCs), normal enterocytes selectively activate T suppressor cells (Ts). In this paper we will identify the various features that epithelial cells possess that permit them to function as antigen presenting cells, and, furthermore, describe the findings suggesting that the induction of Ts cells relates to the presence of a novel ligand for CD8 on normal enterocytes.

ABBREVIATED METHODS

Cells Peripheral blood mononuclear cells (MNC) were obtained after ficoll-hypaque density gradient centrifugation. T and non T cells were further isolated as previously described (4). Epithelial cells were obtained by a modification of the technique of enzymatic digestion of surgically resected mucosal strips. Dispase (Boeringher-Mannheim-3mg/ul) was incubated with 2mm mucosal strips for 30' at 37°C with intermittent vortexing. Liberated epithelial cells were 90-100% viability as assessed by propidium iodide staining and cells retained surface class II antigen expression and other epithelial cell markers. Although cell death occurred rapidly in culture medium (EMEM, 1% FCS and antibiotics or RPMI 1640, 1% FCS and antibiotics) over 24 hours, usually >50% viability was achieved for 48 hours. In contrast, epithelial cells isolated by EDTA (0.75mM) treatment had lower viabilities initially and typically failed to survive beyond 18-24 hours. Colonic tissues had few contaminating MNC (<1%), whereas small bowel epithelial cell preparations contained approximately 2-10% IELs. Lymphocytes were removed by Percoll density gradient centrifugation, with epithelial cells floating in the lowest density interface.

Cytokine Production by Epithelial Cells - IL-1 was measured by using the D10 T cell line as previously described (7). Epithelial cells or monocytes were stimulated with either LPS (250μg/ml) or γ-IFN (100 U/ml) for 48 hours. Cell free supernatants

were frozen and subsequently tested for IL-1 (or IL-6 activity). Control monocytes served as standards in each experiment.

In some cases, activated cells were spun onto poly-L-lysine coated slides and fixed for in situ hybridization. A standard protocol for in situ was performed as previously described (8) using the Oncor in situ kit. The IL-6 cDNA vector (pGEM 3Z-IL6#2) was obtained from the Cetus Corp. along with a map of the plasmid structure. Linearization with restriction enzyme Hind III and transcription with T7 polymerase in the presence of ^{35}S UTP generated specific probe. Linearization with Eco R1 and transcription with SP6 polymerase generated the control probe (nonsense) for non-specific hybridization. The correct identification of the probe will be confirmed by Northern blot analysis and detection of a 1.3kb mRNA in the poly-A+ RNA of the T24 bladder carcinoma line. A similar probe was constructed to detect IL1 mRNA. A human IL1 beta plasmid has been obtained from the ATCC . The 0.6kb cDNA insert was excised with Bam H1 and Sma I and electrophoresed into a block of low melt agarose. The agarose was simply melted, diluted and mixed with the pGEM3Z vector cut with the same two enzymes and ligated without further purification. New subcolonies were transfected into E coli and isolated by standard techniques. A Sma 1 digest and SP6 transcript were used to generate the IL1 probe and the T7 product off a Bam H1 cut vector was used as a negative control. The correct designation of these probes was confirmed by hybridization to a Northern blot of total RNA from LPS stimulated PB monocytes,annealing to an abundant 1.8kb RNA.

RESULTS AND DISCUSSION

Requirements for APC function in any cell type include (1) the expression of class II Ags (2) the presence of LFA molecules, (3) the ability to process Ag with subsequent re-expression of the processed petides on the cell surface, and (4) the presence of accessory cytokines (i.e. membranc or secreted IL-1, IL-6). Although our initial studies (4) as well as those of Bland et al(5,6) suggested that normal enterocyte were functional APCs by virtue of their ability to sustain Ag specific T cell proliferation responses, the unusual finding of Ts stimulation suggested that a subtle alteration in APC function might exist. We, therefore, examined each fundamental phase of APC function. Several groups have already described the presence of HLA-DR on normal enterocytes (2-3) although Ag density is roughly an order of magnitude less than conventional APCs. In addition, we and others have documented the absence of HLA-DQ despite the presence of mRNA for this glycoprotein. These findings by themselves cannot explain Ts stimulation as the more strongly DR^+ epithelial cell from patients with IBD also fail to express DQ, but are capable of stimulating Th rather than Ts cells. Furthermore, like monocytes, normal epithelial cells express cell adhesion molecules ICAM-1 and LFA-1 (data not shown) at a density comparable to peripheral monocytes. Thus, the first two criteria are satisfied.

Differences were detected, however, in antigen uptake and processing. As seen in Figure 1, uptake of fluoresceinated tetanus toxoid was slower in normal epithelial cells and internal acidification (within the endosome) was delayed when compared to normal monocytes. Inhibitors of endosomal acidification such as leupeptin (data not shown) could cause equivalent slowing of processing in normal monocytes, however, despite this similarity, no $CD8^+$ T cell stimulation was detected in leupeptin treated monocyte/T cell cultures.

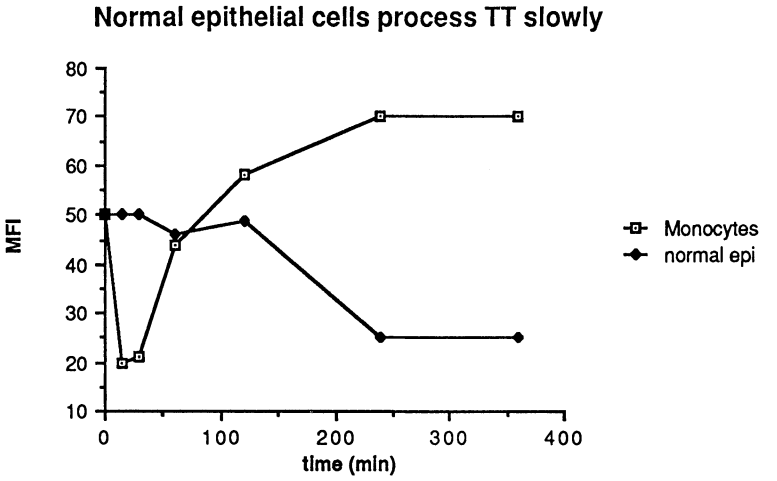

Fig.1

Lastly, normal epithelial cells were capable of producing and/or secreting accessory cytokines. Supernatants from LPS stimulated epithelial cell cultures displayed IL-1 like activity in the D10 assay. These findings were corroborated by in situ hybridization. Activated epithelial cells from a patient with CD contained specific mRNA for IL-1 (Figure 2) while no granules were detected using the nonsense probes. Normal epithelial cells stimulated with LPS contained mRNA for IL-6, although no functional assay has been performed to date. Thus, it appears that critical accessory cytokines can be produced and/or secreted by enterocytes under the appropriate culture conditions. Whether the amount and proportion of these cytokines play a role in Ts stimulations remains to be determined.

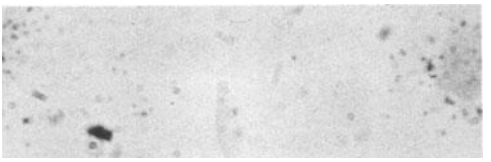

Fig. 2

The preceding experiments provide strong evidence that intestinal enterocytes can function as APCs and may do so in the physiologic state. However, the data fail to explain the selective induction of Ts cells.

We postulated, given the fact that differences in D region Ag expression and Ag processing did not correlate with Ts induction and that Ts cells proliferate in MLR cultures where Ag processing is not required, that there may be a surface molecule on normal epithelial cells which selectively binds to and stimulates $CD8^+$ cells. Preliminary evidence for such a possibility arose from the finding that antibodies to CD8, but not class I or CD4 inhibited $CD8^+$ T cell proliferation in epithelial cell/T cell MLRs (Figure 3).

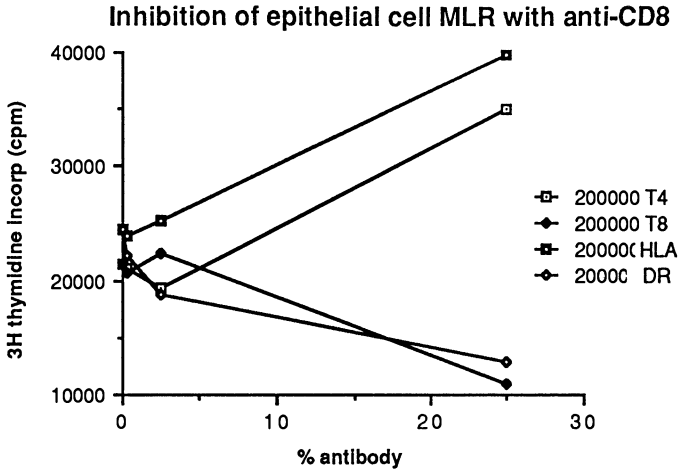

Fig. 3

In order to further investigate this possibility, we generated a series of epithelial cell specific mAb and screened them for their ability to inhibit CD8+ T cell proliferation. Four mAbs were identified (Table I) which varied in their staining patterns and in the molecular weight of the antigen recognized.

Table I

Characteristics of Anti-Epithelial cell Antibodies

Antibody	Mw (kd)	Staining profile
B9	68	surface predom luminal entire bowel thymus (Hassal corpuscles) thyroid epithelium nerve sheath (esophagus) skin negative
B39	60	intracytoplasmic entire bowel thymus medullary epithelium others negative
L12	45-60	large and small bowel surface
K3	?	large < small bowel surface

An example of one particular mAb, B9, is depicted in Table II. Addition of B9 to cultures inhibited $CD8^+$ T cell proliferation, reduced overall thymidine incorporation and allowed for class II restricted $CD4^+$ T cell proliferation to occur.

TABLE II
B9 INHIBITS ABILITY TO INDUCE SUPPRESSOR T CELLS

Cells	% staining	
	T4	T8
T only	66	32
T x Epi(normal)	12	68
T x Epi + B9	58	29
T x Epi + anti-DNP	16	71

* T cells incubated alone or in culture with irradiated normal epithelial cells +/- 30% or 3% B9 or isotype matched control mAb anti-DNP supernatant for 48 hours. T cells were isolated by Percoll density gradient centrifugation and stained .

Although not completely characterized, these data point towards a novel CD8 ligand which may activate $CD8^+$ T cells by the crosslinking CD8 on the cell and possibly result in activation through the src-like tyrosine kinase, p56 lck, known to be associated with the intracytoplasmic tail of CD8 as described by Veillette et al (9).

In summary, based on a compilation of data from various laboratories, we have proposed the following schema for antigen sampling in the intestine (Figure 4). Particulate antigens (as well as those with receptors on M cells) entering the lumen may preferentially enter the MALT through the M cell. Since this pathway involves antigen entry into the Peyer's patch, a Th response will result. In contrast, if Ag entry occurs through the enterocyte, $CD8^+$ T cells (despite the class II restriction) enter and proliferate. Should antigen enter through a paracellular route, tissue macrophages can process Ag and present it to local T cell populations. In this setting, one might envision the generation of both Th and Ts cells. The mode of antigen entry, therefore, may be the critical event in dictating the presence or absence of a systemic immune response to mucosal Ags.

Possible mechanisms for antigen entry in the intestine

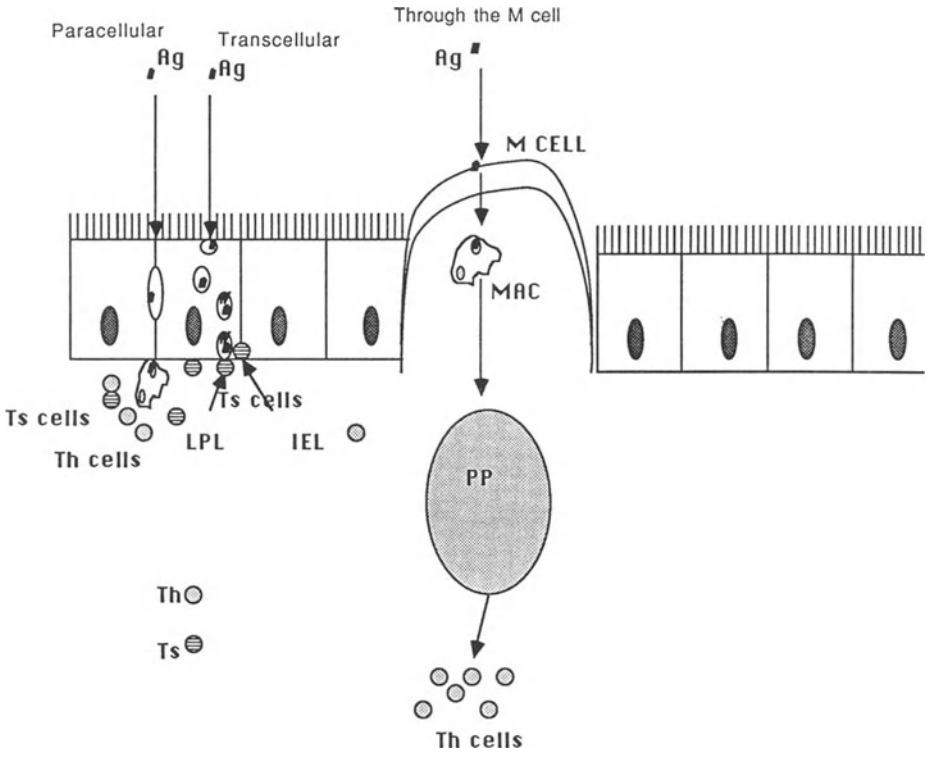

Fig. 4

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4

Ultrastructural distribution of Class II MHC molecules in mucosal antigen presenting cells and their relationship to antigen uptake and processing

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ABSTRACT. Expression of Class II MHC molecules by potential antigen presenting cells in the gut mucosa and afferent lymph has been examined at light microscope and ultrastructural levels by an indirect immunoperoxidase technique. Mature enterocytes in rat and human small intestine express cytoplasmic as well as cell surface Class II molecules. In rats, surface Class II molecules are restricted to the basolateral cell membranes of enterocytes in the midvillus region. Cytoplasmic Class II molecules are associated with multivesicular bodies and lysosomes in the apical cytoplasm. The pattern is essentially similar in human jejunal enterocytes, with the important exception that the brush borders also express Class II molecules. Large mononuclear cells in the lamina propria also contain Class II molecules in association with lysosomal bodies, but intracellular Class II molecules have not been detected in veiled cells in intestinal afferent lymph. We have failed to show uptake of horseradish peroxidase (HRP) from the lumen by enterocytes in normal gut but immature enterocytes absorb the marker from the extracellular fluid. The results are discussed in relation to antigen transport and presentation in the gut.

1. Introduction

The lamina propria of the gut contains many cells that express large amounts of Class II MHC molecules (1). Some may be dendritic cells but undoubtedly many are macrophages (2). These cells, together with gut-derived veiled cells in afferent lymph (3) and dendritic cells in Peyer's patches (4) are potential antigen presenting cells (APC) in the afferent phase of mucosal immune responses. However, mature enterocytes in the small intestine also express Class II MHC molecules (1,5). Enterocytes might themselves act as APC and recent studies indicate that of isolated rat (6) and human (7) cells can present antigen to primed T lymphocytes in a Class II-restricted manner.

Class II molecules may be present in the enterocyte cytoplasm as well as on the cell surface (1). Antigen is believed to be absorbed from the gut lumen by enterocytes and to enter lysosomes (8). It was therefore of interest that the intracellular distribution of Class II molecules resembled that of absorbed HRP (8) and of acid phosphatase (9), suggesting an association with the endocytic pathway. This appeared to be consistent with intersection of antigen with Class II molecules in the cytoplasm.

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We have examined Class II MHC molecules in rat and human enterocytes by ultrastructural techniques. For comparison, we have also examined Class II-positive cells in the lamina propria of rat gut and veiled cells in rat afferent intestinal lymph. The question of antigen absorption by enterocytes has been reviewed in normal and damaged rat intestine, using HRP as a marker.

2. Materials and Methods

The animals were specific pathogen free female DA rats and Balb/c mice. Human duodenum and jejunum samples were either surgical resection specimens or capsule biopsies of histologically normal tissue. Preparation of tissues for immunohistochemistry at light microscope and ultrastructural levels, the indirect immunoperoxidase technique, the primary antibodies used to detect Class II molecules (MRC OX4 plus MRC OX6 for rat tissue and FMC 4 for human tissue) and negative controls are described in detail elsewhere (10). Veiled cells from rat afferent intestinal lymph were semi-purified on Percoll step gradients (3), resuspended in re-calcified rat plasma and the clot was fixed in 1% paraformaldehyde plus 0.025% glutaraldehyde. Preparation of thick frozen sections from cryoprotected blocks, the pre-embedding immunoperoxidase technique and preparation for ultrastructural examination are described elsewhere (10).

HRP absorption by enterocytes was examined from the lumenal aspect and also from the direction of the baso-lateral cell membranes. Atraumatic infusion of the enzyme (5mg/ml, Type I, Sigma) into the jejunum was performed at 5ml/hour via a silastic-tipped cannula introduced 24 hours earlier. After infusion for 4 hours, jejunum was fixed in 4% paraformaldehyde - 3% glutaraldehyde. Ischaemic injury was produced in 10cm segments of jejunum containing 5mg/ml HRP by reversible occlusion of the superior mesenteric artery for periods of 5-15 minutes, followed by a further period of 2 hours with normal blood flow prior to sampling. Uptake of HRP from the circulation by jejunal enterocytes was examined in rats and mice at intervals after intravenous injections of 100mg and 10mg of enzyme respectively. All tissues were cryopreserved and frozen sections were examined for enzyme activity.

3. Results

3.1 CLASS II MHC MOLECULES IN ENTEROCYTES

The distribution of Class II molecules in rat jejunum was essentially as described elsewhere (1,10). Expression of Class II molecules is confined to the mature enterocytes, where anti-Class II antibodies appear to stain the basolateral cell membranes and cytoplasmic "granules" but not the brush border. As enterocytes approach senescence, they lose membrane staining and contain large stained bodies in the apical cytoplasm. Longitudinal studies along the normal rat intestine show that increasing proportions of crypts express Class II molecules. Stained cytoplasmic bodies are larger in ileal enterocytes. The distribution of Class II molecules in human duodenum and jejunum is similar to the rat, with the differences that the brush border appears lightly stained and cytoplasmic staining is concentrated in the region of the terminal web.

Ultrastructural studies confirm the expression of Class II molecules on the basolateral cell membranes in both species (Fig 1a). Class II molecules were also detected on the brush borders of human enterocytes (Fig 1b) but not in the rat (Fig 1a). Class II molecules were found associated with numerous multivesicular bodies (mvb) in the apical cytoplasm of both human and rat enterocytes (Fig 1a) and with lysosomes in the supranuclear cytoplasm of rat enterocytes. Class II molecules were lost from enterocytes near the tips of villi in rat intestine and these cells contained large stained lysosomal bodies (Fig 1c). We have not detected nascent Class II molecules in the endoplasmic reticulum or the Golgi.

3.2 DISTRIBUTION OF CLASS II MOLECULES IN LAMINA PROPRIA CELLS AND VEILED CELLS FROM AFFERENT LYMPH.

The lamina propria of the small intestine in rats contains many macrophages and/or dendritic cells that express large amounts of Class II molecules (1). At the ultrastructural level, these cells extend long processes between the connective tissue and lymphoid elements. The cell membranes stain strongly for Class II molecules and the cytoplasm contains Class II-positive bodies (Fig 1d). Afferent lymph veiled cells display surface Class II molecules but we have been unable to detect cytoplasmic Class II molecules (Fig 1e).

3.3. UPTAKE OF HORSERADISH PEROXIDASE BY ENTEROCYTES.

Despite clear evidence of absorption of HRP from the gut lumen through the Peyer's patch epithelium, we have found no evidence of binding of the enzyme to the brush border of normal villous mucosa nor absorption into enterocytes. Ischaemia for 5 minutes caused HRP to bind to the brush border and to penetrate scattered tight junctions. Increasing periods of ischaemia caused progressively greater binding and entry of HRP into the lateral spaces and lamina propria.

After intravenous injection, HRP was observed in the lamina propria and in the lateral spaces between enterocytes in all rats. There was no visible uptake of HRP into mature enterocytes within 1-2 hours, but a few small granules of reaction product were observed in crypt cells and in cells at the bases of villi. In mice, HRP was absorbed during the first hour after injection into numerous small vacuoles in the cytoplasm of crypt cells and within enterocytes on the basal third of the villi. Within 12-18 hours, the cohort of HRP-containing cells had moved distally by approximately the length of the crypts. In many cells the enzyme was condensed into large cytoplasmic vacuoles and by 24-36 hours most traces of enzyme activity were lost.

4. Discussion

The ultrastructural studies confirm that Class II molecules are present within the cytoplasm of rat and human enterocytes and that they are associated with the endocytic pathway. This suggests that antigenic macromolecules endocytosed by enterocytes will be directed to a compartment containing Class II molecules. These could salvage antigenic peptides and transport them to the cell surface, where they might be available to stimulate Class II-restricted T lymphocytes. Mid-villus enterocytes would be candidates for antigen presentation, although crypt cells could have a similar function in the lower intestine and in diseased mucosa. Class II molecules appear to be cleared from the surfaces of senescent enterocytes and may accumulate in large phagolysomes.

We have detected Class II molecules in the cytoplasm of lamina propria macrophages/dendritic cells. We interpret this as consistent with the site of intersection between processed exogenous antigen and Class II molecules. However, we have not detected intracellular Class II molecules in afferent lymph veiled cells, which are known to present antigen (3), suggesting that antigen presentation by these cells may not require endocytosis.

Our studies, and those of some other workers (11), suggest that enterocytes in normal gut do not absorb detectable quantities of antigen from the lumen. However, this is not to say that antigenic ligands such as cholera toxin are not absorbed by this route. Furthermore, the presence of Class-II molecules on the brush borders of human enterocytes raises the interesting possibility that antigenic peptides released by lumenal proteolysis might bind directly and in an MHC-restricted manner. The implications in the pathogenesis of coeliac disease are clear.

On the other hand, in confirmation of the work of Rhodes and Karnovsky (11), and Hugon and Borgers (12), our results indicate that antigens can reach the lateral spaces after minor gut damage and that they can be absorbed from this location. The cells most active in this process are the less mature enterocytes. This observation, together with the greater uptake of intravenously administered HRP by enterocytes in mice than in rats, suggests that the process may be linked to IgA transport. In other words, HRP (and any other fluid phase molecule) may be absorbed in the same endocytic vacuole as IgA. However, unlike the receptor-bound IgA, the non-bound HRP might be expected to be delivered to the lysosomal compartment.

We have observed transport of HRP to the mid-villus region by the movement of enterocytes, thus delivering the antigen to the region of constitutive Class-II expression.

The findings offer a morphological basis for antigen processing and presentation by enterocytes. Furthermore, they also provide a model for one route by which antigens can enter the enterocyte. This particular route may be important in situations where a disease process allows antigen to penetrate the normally impervious tight junctions. It might have important implications for understanding how epithelial damage could be perpetuated by immunological attack after a transient insult (e.g. virus infection) allows ingress of food antigen to the lamina propria.

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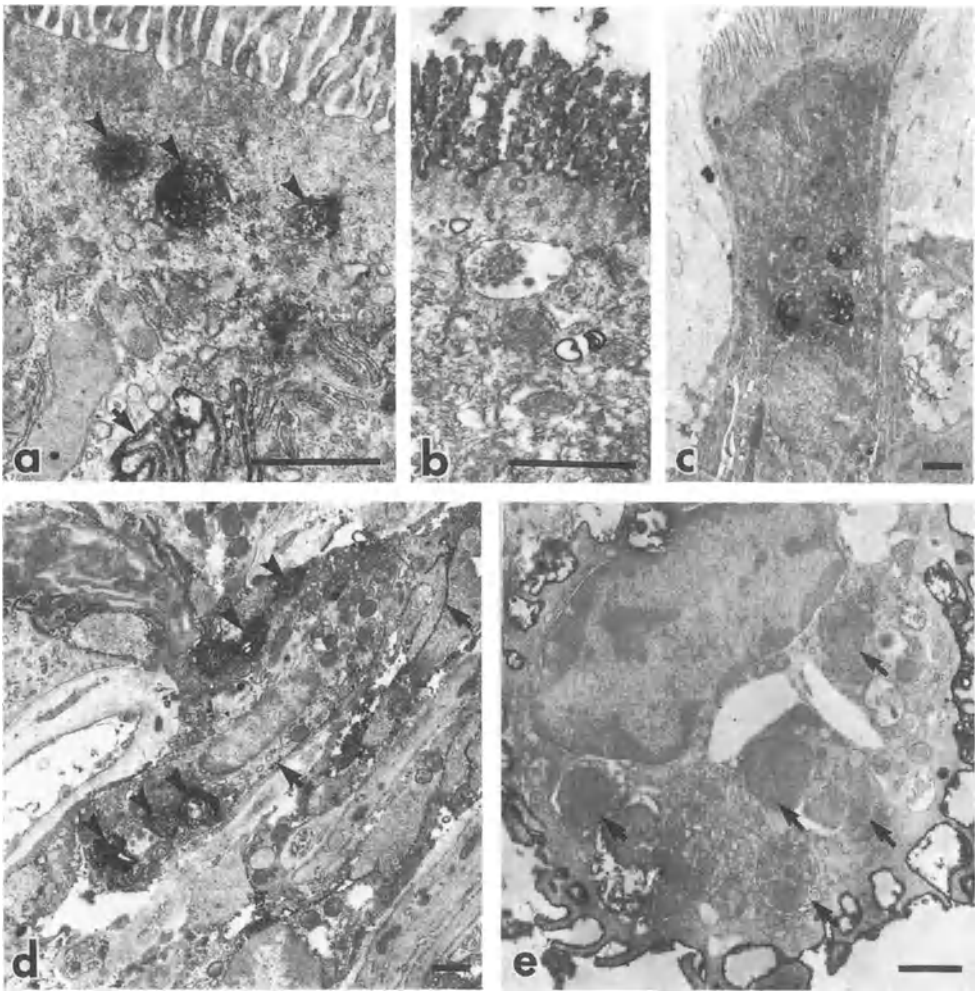

Figure 1. Ultrastructural localization of Class II molecules by the indirect immunoperoxidase technique. (a). Enterocyte from rat ileum. Class II - positive multivesicular bodies (arrow heads) and lateral cell membrane (arrow). Brush border negative. (b). Enterocyte from human duodenum with Class II - positive brush border. (c). Rat ileal enterocyte, flanked by goblet cells, near villus tip. Note negative lateral membranes and positive supranuclear phagolysosomes. (d). Class II - positive cell in lamina propria of rat ileum. Note Class II - positive cell membrane (arrows) and intracellular bodies (arrow heads). (e). Veiled cell from rat afferent intestinal lymph. Note Class II - positive surface membrane and negative phagolysosomes (arrows). Bar = 1μm.

5

Mature Ia$^+$ murine intestinal epithelial cells with APC activity share common antigens with gut interdigitating dendritic cells

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ABSTRACT. Immunohistochemical analysis of duodeno-jejunal epithelium *in situ* on tissue sections revealed that mature enterocytes of the villi expressed molecules commonly found on dendritic cells (DC) and skin Langerhans cells (LC), which seem acquired during maturation along the crypto-villus axis. These studies also showed the heterogeneity of murine gut epithelium and the presence of three phenotypically distinct subsets: crypt, Peyer's patch (PP), dome and villus epithelial cells (EC).

1. Introduction

Gut intestinal epithelium represents a physical barrier that separates immunocompetent cells of the lamina propria and PP from luminal antigen. One of the major role of gut EC is the secretory component mediated transepithelial transport of polymeric IgA (1), which ultimately prevents the attachement of luminal microorganisms to the mucosa. Recent data reported the capacity of villus EC to present soluble antigen in vitro to antigen specific CD8$^+$ T (2, 3) and CD4$^+$ (4) T cells, with an MHC class II-restricted mechanism. Considering that lympho-epithelial interactions in the gut mucosa might have immunoregulatory implications, we have examined the phenotype of intestinal epithelium covering distinct regions of the gut mucosal tissues. The present study describes the reactivity of various antibodies against dendritic cells with gut EC *in situ*.

2. Material and Methods

2.1 TISSUE SPECIMENS

Cryostat and paraffin-embedded tissue sections (4 μm thick) were prepared from duodeno-jejunum of BALB/C female mice (8 to 12 week old) bred in our facility.

2.2 ANTIBODIES

The following antibodies were used: 1/ the anti-Ia (monomorphic) rat IgG mAb (CD311) (5) (neat hybridoma culture SN) ; 2/ the NLDC-145 mAb (neat hybridoma culture SN) directed against a 145 kDa protein specific of murine DC (6); 3/ a rabbit affinity purified Ab specific for cow S100 alpha and beta proteins (Dako, Sebia, France) (dilution 1:50) and the anti-THAM mAb (7), which recognizes a neutral aminopeptidase was used at 5 µg/ml) of purified IgG.

2.3 IMMUNOHISTOCHEMICAL ANALYSIS

Indirect immunohistochemical analysis of Ia, NLDC-145 and THAM antigens was performed on cryostat sections. Binding of the specific Ab was revealed using a biotinylated goat anti-rat Ig specific Ab (Jackson Laboratories, Immunotech, France) and avidin-FITC (Vector Laboratories, France). S100 expression was detected by immunoenzymatic labelling of paraffin-embedded tissue sections using the PAP method (Ortho Diagnostics kits, Paris, France) and 3'-aminoethylcarbazole in the presence of H_2O_2.

3. Results

TABLE 1. Reactivity of the various antibodies with gut EC in situ

Antibodies to	GUT EPITHELIAL CELLS		
	Villus	Crypt	PP dome
Ia (CD 311)	+ + +	–	–
145 kDa (NLDC-145)	+ +	–	–
S100	+ +	–	–
THAM	+ + +	–	–

In situ analysis of murine duodeno-jejunal epithelium showed that mature EC covering intestinal villi strongly reacted with anti-Ia mAb, with an intracytoplasmic staining of the apical cytoplasm and a baso-lateral labelling of the plasma membrane as revealed by electron microscopy studies. These Ia + enterocytes constituted the upper two-thirds of the villi,

while the base of the villi, the crypts and the PP dome EC were unstained. Villi EC also expressed intracytoplasmic molecules recognized by the NLDC-145 mAb and by the anti-S100 Ab. Crypt EC precursors were not labelled by these two Abs; PP dome EC stained for the S100 protein only and remained unlabelled by NLDC-145. The neutral amino-peptidase recognized by the anti-THAM mAb was found exclusively on the brush border of villi EC, adjacent to or at distance from PP dome region, but not on PP dome EC, suggesting that EC from these two distinct anatomical sites had a different composition in proteolytic brush border enzymes.

4. Discussion

The present data indicated that mature gut EC covering the villi expressed molecules commonly found on interdigitating DC and skin LC, namely Ia antigen, the S100 protein and the 145 kDa molecule defined by the specific mAb NLDC-145. In view of the recent demonstration that purified Ia + villus EC are efficient APC in vitro (2, 3, 4), these data point to the possible functional role of these DC markers expressed by EC, and particularly with respect to antigen handling and intracellular processing mechanisms. Alternatively the fact that the DC specific 145 kDa antigen has also been found on Ia + thymic epithelial cells (6) raises the hypothesis that lympho-epithelial interactions which take place in the local microenvironment of gut villi may represent a site of local maturation of mucosal lymphoid cells interacting with Ia + NLDC-145 + THAM + EC.
In addition, comparative analysis of the reactivity of EC from different mucosal sites of the intestine with these Abs revealed a level of heterogeneity in the phenotype and allowed to identify three subsets whose phenotype was correlated to a specific location along the mucosa (table 1). These subsets of EC included: 1/ mature columnar absorptive cells of the villi with a Ia + NLCD-145 + S100 + THAM + phenotype and 3/ immature crypt EC with the Ia- NLDC-145- S100- THAM- phenotype. These data suggest that maturation of gut EC is associated with expression of Ia, NLDC-145 and S100 antigens, since EC differentiate from immature crypt EC precursors to mature villus EC. In conclusion expression of DC specific molecules is acquired by EC during their maturation along with the capacity to present antigen.

5. Acknowledgments

We are grateful to Dr. G. Kraal, Dr. M. Pierres and Dr. A. Glasebrook for their kind gift of the mAbs NLDC-145., anti-THAM and CD311 respectively. We also thank Mrs M. Blanc and M. Mutin for their skillful technical assistance.

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6
Murine gut epithelial cells present antigen to specific T cell hybridoma

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ABSTRACT. Highly purified MHC class II positive epithelial cells (EC) from murine small intestine were able to present a soluble antigen (KLH) to a specific class II-restricted CD4 + T cell hybridoma and to induce IL-2 secretion. Antigen presentation in this model was restricted to the H-2 haplotype and could be fully inhibited by anti-Ia antibodies.

1. Introduction

Peyer's patches (PP) are considered as the initiation site of the intestinal immune response after antigen penetration through specialized phagocytic EC covering PP dome i.e. M cells (1). Considering that the epithelium of gut villi are specialized in protein transport and degradation we have examined the immunological function of EC in antigen presentation to a specific class II-restricted T cell hybridoma *in vitro*.

2. Material and Methods

2.1 MICE.
BALB/C, CBA and C57BL/6 mice (IFFA/Credo, L'Arbresle, France), AKR and C3H/HeN mice (CSEAL, Orléans, La Source, France) were used between 8 to 12 weeks of age.

2 2 MONOCLONAL ANTIBODIES, ANTIGEN AND CELL LINES.
The following mAbs were used as neat hybridoma culture SN (2). A rat anti-Ia, mAb (CD311), specific for a monomorphic determinant of murine Ia, two murine mAbs: anti Ia15 (d, k, b haplotypes) (clone 17/227), and anti-Ia7 (d, k, p haplotypes) (clone 13/4) and the rat anti-T200 mAb. HLH was purchased from Sigma (La Verpillière, France). The cloned KLH-specific Ia^d-restricted T cell hybridoma CAK 1.22.3 was produced as previously described (3).

2.3 ISOLATION OF INTESTINAL EPITHELIAL CELLS AND ANTIGEN PRESENTATION SYSTEM
EC were isolated from murine duodeno-jejunum by a mechanical vibration procedure recently described (4). The resultant EC (98 %) (composed of 99 % Ia^+ $T200^-$ EC and excluding macrophages and dendritic cells) were cultured for 24h with CAK 1.22.3 T cell hybridoma (5×10^4) and and KLH (412 g/ml) as previously described (3). SN were assayed for the presence of IL-2 using the murine IL-2 dependent CTLL-2 cell line as previously reported (5). Results were expressed as cpm/culture.

3. Results

Mature Ia $^{+}$ EC are able to present KLH to the CAK 1.22.3 CD4 $^{+}$ T cell hybrid and stimulate IL-2 secretion. T cell stimulation by EC, obtained only in the presence of KLH, was optimal (12500 cpm/culture) for an EC/T cells ratio of 1/5. Comparable results were obtained with spleen accessory cells used instead of EC at the same dose. Only EC from H-2^{d} mouse strains (BALB/C, DBA/2) stimulated CAK 1.22.3 T cells for IL-2 production while EC of irrelevant haplotypes were inefficient. Addition of anti-Ia mAbs at the initiation of culture strongly inhibited IL-2 synthesis in this system (1000 cpm/culture).

4. Discussion

Recent studies in rat (6) and in man (7) showed that gut EC could present antigen to specific T cells ultimately resulting in the predominant expansion of CD8 $^{+}$ cells. The present data provide evidence that mature villi EC from murine small intestine express functional class II molecules and can activate CD4 $^{+}$ T cells for IL-2 production. Antigen presentation by EC is haplotype specific and requires Ia molecules. These findings suggest that handling of exogenous antigen *in vivo* could take place within gut villi by EC at distance from PP resulting in local activation of adjacent intra-epithelial or lamina propria CD4 $^{+}$ T cell for lymphokine synthesis. Although CD4 T cells represent only a minority of intra epithelial (IEL), interaction with a discret subset of CD4 IEL may bear relevance to induction and activation of antigen specific tolerogenic CD8 $^{+}$ T cells.

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7
The expression of Class II major histocompatability antigens in the murine small intestine is not influenced by the oral administration of cholera toxin

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1. INTRODUCTION

Feeding cholera toxin (CT) induces a strong mucosal IgG and IgA response. In addition CT can act as an adjuvant inducing a strong IgA response to an unrelated protein fed simultaneously. [1] Furthermore the normal oral tolerance induced by feeding proteins is abrogated by CT [2]. There is considerable variation between mouse strains in their ability to respond to different proteins fed with CT,and no correlation between antibody response to CT and that to the second protein [3].This has led to the proposal that CT acts as a pharmacological mediator of the mucosal immune system[3] a view supported by the observation that CT but not its B subunit has this effect[4].The mechanism whereby CT exerts its effect is not understood. However the necessity to feed CT simultaneously with KLH indicates that it acts locally within the intestine.
In graft versus host disease (GVH) there is also an increased antibody response to fed proteins and abrogation of oral tolerance [5]. GVH is also known to increase the expression of MHC antigens on intestinal epithelial cells [6]. Given the similarities between the effects of CT and GVH on the immune response to fed proteins,and the ability of epithelial cells to present antigen in vitro,it is of interest to examine the effect of CT on epithelial MHC class II antigens.

2. MATERIALS AND METHODS

2.1 Histology

Tissues were fixed in PLPG.[7] overnight then processed and wax embedded. 4 um. sections were cut and stained with monoclonal anti Ia (M5/114 Hybritech),followed by sheep anti rat biotin conj.(Jackson)and HRP streptavidin biotin complexes(Dako) colour was developed with DAB.

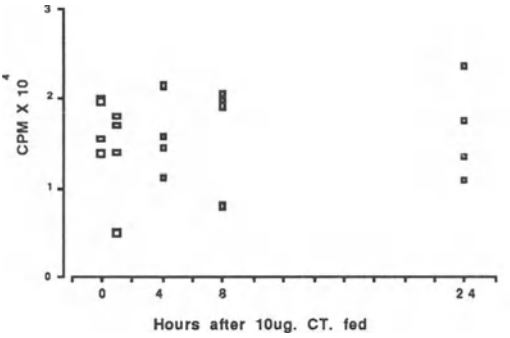
2.2 Radio binding assay.

Epithelial cells were isolated by stirring pieces of intestine in HBSS containing 0.37 mg/ml EDTA and 0.145 mg/ml dithioerithritol. The resulting cell suspension was fixed for 1hr. in PLG[7],.then washed in lysine phosphate buffer. Duplicate aliquots of 10^5 cells/well were incubated with M5/114 or normal rat serum,followed by sheep anti rat biotin conj and I ^{125} labeled streptavidin(Amersham). Samples were counted on a gamma counter.

3. RESULTS AND DISCUSSION

3.1 Effect of CT on epithelial MHC class II expression.

Mice were fed 10 ug of CT at time 0. At fixed intervals thereafter mice were killed and samples of intestine taken for histology .Class II MHC was also measured by radiobinding assay. Fig 1 shows the results from one such experiment, each point represents an individual mouse and is the mean of two replicate samples containing M5/114 minus the mean of two controls containing rat IgG. CT had no effect on the level of epithelial MHC class II expression.No effect of CT on the level or distribution of intestinal class II expression was seen in histological sections.These results indicate that the ability of CT to induce mucosal immune responses is not due to an effect on epithelial class II expression.



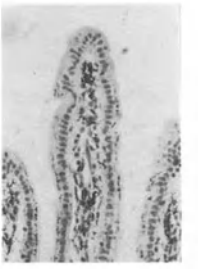


Figure 1 The effect of CT on epithelial Ia expression Figure 2a 2b

3.2 Environmental influences on epithelial class II expression

However we did find wide differences in the levels of epithelial class II expression between groups of mice of the same strain. This appeared to be related to the level of environmental antigenic challenge,isolator reared mice expressed little epithelial class II ,whilst those reared in conventional housing expressed more.Fig2 a. b.. The highest levels of expression were found in mice infected with intestinal parasites,this has been shown to be a cause of increased epithelial class II expression in rats.[6]. Taken together the results suggest that epithelial class II expression is a consequence of a mucosal immune reaction rather than an important factor in the primary induction of a response.

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8
Suppression of lymphoproliferation and interleukin-1 production by enterocyte-derived factors

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Recent studies demonstrated that enterocytes expressing surface Class II MHC antigens were capable of antigen presentation (1,2). In rodents, antigen-specific proliferation was observed only after T cells were separated from enterocytes following activation and re-cultured in the absence of further antigen stimulation (2,3). Furthermore, the inability to induce T cell proliferation was not reversible by the addition of recombinant IL-1 or IL-2 (3). This study shows that in primary culture the failure of enterocytes to stimulate antigen-specific proliferation of resting T cells was due to suppressive factors secreted by enterocytes.

Rat Wistar enterocytes and supernatants from culture of freshly isolated enterocytes and an established cell line (REC-2) suppressed the proliferative responses of ovalbumin (OA)-primed popliteal lymph node T cells stimulated with OA in the presence of peritoneal macrophage in a dose-related manner, but not dermal fibroblast supernatant. Indomethacin failed to block the release of inhibitory factor, indicating that it is not due to prostaglandin E2. There was no apparent loss in the efficiency of inhibition when higher concentrations of enterocyte supernatant were added at 0.24 and 48 hr following antigen stimulation. At lower concentration there was a time dependent loss of potency. Further, its inhibitory effects were reversible when the cells were washed and re-stimulated with OA at 8 hr after starting the reaction, but thereafter the cultures were fully inhibited. These results suggest that other cellular processes may also be necessary for enterocyte factor to exert its inhibitory effects.

The inhibitory effects of enterocyte supernatant were not totally removed by extensive dialysis (m.w. cut-off 4kD), indicating that the supernatant contained more than one active factor.

The m.w. estimates of the size of the factors in enterocyte supernatant were determined by gel filtration chromatography using AcA34 column (1.5x95cm). Fractions eluted with PBS, pH 7.2 were collected and combined to form four pools before testing for inhibitory effects on the proliferation of OA-printed T cells

stimulated by peritoneal macrophages, IL-1 production by LPS-stimulated macrophages, and the proliferation of IL-2 dependent NK-7 blast cells. Pools 44-157kD and 1.3-4kD suppressed the proliferation of OA-primed T cells, whereas all four pools suppresssed IL-1 production by macrophages as assayed by the proliferation of C3H/HeJ mouse thymocytes, with pool 44-157kD being strongly inhibitory. Pool 1.3-4kD markedly suppressed the proliferation of IL-2 dependent NK-7 cells compared with pool 44-157kD, indicating that pool 1.3-4kD affects cell division whereas pool 44-157kD exerts its effect by inhibiting IL-1 production. Together, these results indicate that the inhibitory influence of enterocyte factors was mediated through inhibition of IL-1 production and cell proliferation.

In conclusion, our findings add another role of enterocyte which is the regulation of T cell proliferation and reactivity. Such process may be important in controlling inflammatory response in the gastrointestinal tract where sustained Class II antigen expression by enterocytes and infiltraion of activated T cells may lead to chronic inflammation and tissue injury.

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9
Human colon epithelial cell lysates evoke monocyte chemotaxis

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INTRODUCTION: Factors governing phagocyte populations in colon mucosa are incompletely understood. In health, macrophages and eosinophils are present to the exclusion of neutrophils (N). In acute inflammation, all three cell types are present, with N predominating. We tested the hypothesis that colon epithelial cells (CEC) influence migration of N and monocytes (M) to the lamina propria.

METHODS: CEC were isolated from normal portions of 10 colon resections for cancer by sequential 1 h incubations in 1 mM EDTA. CEC were counted, assessed for purity, washed, resuspended at 1.5x 10^6 cells /mL, lysed and centrifuged. Protease inhibitor was added, supernatants were filtered and tested for chemoattractant activity for blood M and N in 48 well micro-chambers. Blood M and N were separated on Ficoll-hypaque, washed and suspended in Gey's balanced salt solution (GBSS). Migration of N was measured through filters with 3μ pores for 30 mins, and M chemotaxis was measured through filters with 5μ pores for 90 mins. Chemoattractant activity was expressed as percent of migration in response to x 10^{-5} M n-formyl-met-phe (FMP) using GBSS as a negative control. Chemoattractant filtrates were further analyzed by gel permeation chromatography (GPC) on Sephadex G-25 and G-50, by testing for solubility in chloroform, for heat and pH stability, and digestion by trypsin.

RESULTS: Chemoattractant activity was present in all specimens. It was greatest in lysates of cells dissociated during the fourth h, and greater for M than N (Fig 1).

Checkerboard analysis indicated chemotaxis rather than chemokinesis. GPC on Sephadex G-25 showed a peak of activity between insulin α-chain and bacitracin, indicating a factor with a MW of approximately 1800, and a heavier factor(s) coming off in some "break-through" fractions (Fig 2). GPC analysis on Sephadex G-50 confirmed the presence of two major activity peaks (Fig 2).

The 1800 dalton chemoattractant was digested in a dose dependent manner by trypsin, inhibited by boiling in 5 of 6 tests, and 80 percent extractable in chloroform.

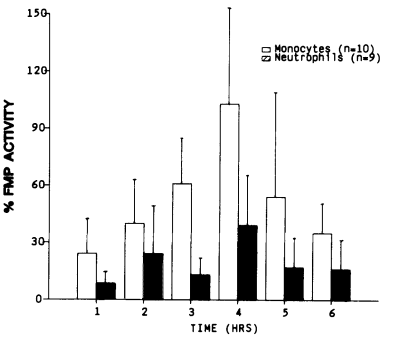

Figure 1. Chemotactic activity in lysates of CEC from 10 cancer resections.

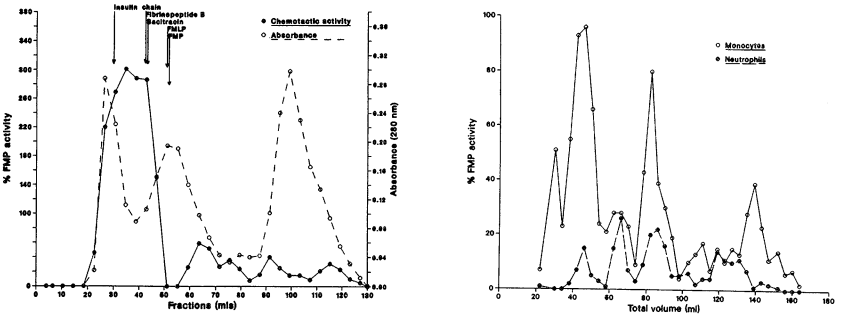

Figure 2. Chemotactic activity in 4 h CEC lysates eluted on Sephadex G-25 (left) and G-50 (right).

CONCLUSIONS: Chemoattractants for M are associated with human CEC. One appears to be an 1800 dalton hydrophobic peptide which may have a lipid component. The other(s) is a heavier factor of unknown nature. These factors could play a role in maintaining normal phagocyte populations in health, and in the induction of the inflammatory response in various forms of colitis.

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10
Antigen handling at respiratory epithelial surfaces in the rat: a highly developed network of dendritic APC revealed by a novel tissue sectioning procedure

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Recent studies from our lab suggest that Dendritic Cells (DC) represent a major (perhaps the major) antigen presenting cell (APC) population in the airway epithelium of rat, and a similar DC population appears to function in alveolar septal walls. Our evidence takes three forms. Firstly, collagenase digests of peripheral lung segments from lavaged/perfused rat lung, contains a cell type which is capable of restoring the capacity of APC-depleted immune lymph node cells to respond *in vitro* to specific antigen [1]. The lung APC's are non-adherent, non-phagocytic, IgE-FcR$^-$, Ia$^+$, and of ultra-low density on percoll, properties consistent with DCs, and their APC activity is grossly inhibited by endogenous lung macrophages [1]. We have employed the same approach to identify a comparable APC in tracheal epithelial digests, prepared as detailed in [2].

Secondly, immunoperoxidase staining of frozen sections of airway epithelium and peripheral lung shows the presence of substantial numbers of Ia$^+$ cells with a prominent dendritic morphology, the majority of which do not appear to co-stain with MoAbs against known macrophage markers [2,3].

Thirdly, the non-adherent fraction of dispersed tracheal epithelial cell preparations from animals exposed previously to aerosols of ovalbumin (OVA) antigens, transmit OVA-specific activation signals to immune T-cells *in vitro*. DC-like cells constituted virtually the sole source of detectable Ia in the fractionated tracheal preparations, and were hence the only available source of APC activity in the cultures; this strongly suggests that these cells had previously trapped and processed the inhaled OVA antigen within the airway epithelium [2].

While these results imply an important antigen trapping and presentation role for DCs in the respiratory tract, they do not **per se** rule out a potential contribution from other Ia$^+$ cell types which may be present within the epithelium e.g. monocytes. Indeed, close examination of longitudinal sections of airway epithelium does show the presence of apparently small Ia$^+$ cells [2,3]. However, the latter could equally represent isolated segments of Ia$^+$ dendrites (from DC).

It occurred to us that the potential solution to this dilemma lay in choice of the plane of section i.e. perhaps the situation in the airway epithelium was analogous to the epidermis, where the true nature of Langerhans cell (LC) network (shown in Fig. 1) is only evident in stained epidermal sheets which afford a plan view of local cell populations. We accordingly attempted to prepare tracheal epithelial sheets for immunoperoxidase staining, but were unable to obtain sufficient intact epithelium by the available methods. However, we reasoned that a possible alternative may be to section lengths of frozen trachea tangentially, thus providing strips of epithelium effectively sectioned in parallel to the underlying basement membrane.

As shown in Fig. 2, this procedure reveals for the first time, a highly developed network of DC within the epithelium, which compares closely with the LC network in the epidermis. It appears that virtually all of the Ia staining within the epithelium can be accounted for by these DC, and this observation strongly suggests that the DC network plays the primary role in surveillance for foreign antigens (including allergens and pathogens) within the respiratory epithelium.

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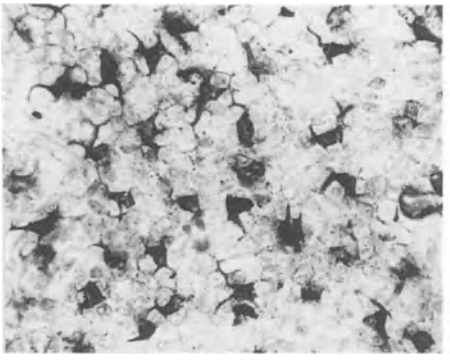

Fig. 1. Rat epidermal sheet

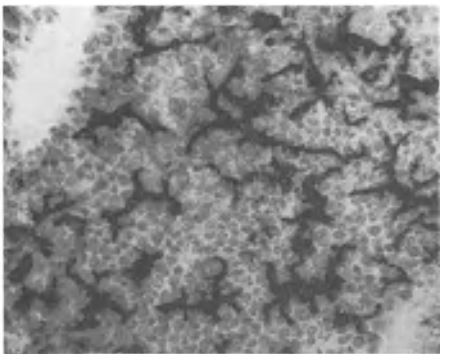

Fig. 2. Tangential section of rat tracheal epithelium

(Tissues from SPF Brown Norway rats; immunoperoxidase staining for Ia with the Ox6 MoAb as per reference 3)

11
Induction of Class II MHC antigens in the rat digestive system after systemic application of recombinant gamma interferon

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1. INTRODUCTION

Class II MHC antigens act as peptide-binding molecules in the presentation of foreign antigens to CD4 positive T lymphocytes. We investigated the normal distribution of class II molecules in the digestive system of rats and its alterations after systemic application of gamma interferon.

2. MATERIALS AND METHODS

Male LEW rats, aged 10 to 12 weeks, weighing approx. 300 g were obtained from the Zentrales Tierlabor of the Hannover Medical School, where they had been reared under SPF conditions. Six rats were continuously infused for 72 h with 0.5 mg/kg/d of recombinant rat gamma interferon via a catheter placed into the inferior vena cava. Six control rats received an infusion without interferon. Class II MHC antigens were demonstrated with an indirect immunoperoxidase technique on cryostat sections using mAbs Ox6, Ox4, Ox17 and F17-23-2.

3. RESULTS

3.1 Tongue, oesophagus and proventricle

Control rats: All keratinocytes were class II MHC negative. Only dendritic cells, located intraepithelially and in the lamina propria, expressed class II molecules.
Interferon treated rats: All keratinocytes turned class II positive.

3.2 Stomach

Control rats: Epithelial cells of the gastric mucosa did not express class II antigens. Only dendritic cells carried these molecules. These cells were especially numerous in the lamina propria surrounding the upper portion and the base of the gastric glands, leaving a sparsely populated region in between.
Interferon treated rats: Mucus-producing cells of the gastric surface epithelium stayed class II negative. Mucous cells at the glandular neck and parietal cells were, however, strongly reactive. In contrast, zymogenic chief cells at the bottom of the glands were not induced although they were directly adjacent to the parietal cells.

3.3 Small intestine
Control rats: Class II antigen staining of enterocytes increased in aboral direction. In the duodenum class II positive enterocytes only covered the upper parts of the villi. The villus bases and the majority of the crypts had class II negative epithelium. In the terminal ileum, however, all villus and crypt enterocytes including the epithelium of Peyer's patches strongly expressed class II antigens. Large numbers of dendritic class II positive cells were present in the lamina propria in all parts of the gut.
Interferon treated rats: In the duodenum all crypt enterocytes turned class II positive and class II expression in villi was slightly enhanced. Also in the jejunum class II expression in enterocytes increased. No alterations were observed in the terminal ileum, because all cells were already intensely stained.

3.4 Large intestine
Control rats: Enterocytes in the caecum and colon were entirely class II negative.
Interferon treated rats: All enterocytes and goblet cells were converted to intermediate class II reactivity.

3.5 Salivary glands
Control rats: In the submandibular and sublingual glands primarily interstitial dendritic cells were class II positive. However, in the intralobular and interlobular excretory ducts single class II positive epithelial cells and cell clusters were present. In the parotis some, but not all, striated ducts expressed class II antigens. The serous acini were not stained.
Interferon treated rats: All duct epithelia in the submandibular and sublingual glands expressed class II antigens. The previously unreactive or only faintly reactive granular convoluted tubules of the submandibular gland were heavily stained, while the serous acini stayed negative. The mucous cells in the sublingual gland showed variable and intermediate staining. All epithelial cells in the parotis including the serous acini were induced to express class II antigens.

4. DISCUSSION
Our study shows that class II MHC antigens are constitutively expressed only in certain enterocytes and salivary duct epithelia in normal LEW rats. Together with the observation of class II positive proximal kidney tubule epithelia in rats, this may indicate that class II molecules fulfill a non-immunological function in the transport or reuptake of proteins or peptides. Gamma interferon causes class II MHC expression in most epithelia. The inducibility of epithelial cells is, however, not uniform and appears to depend on their specialized differentiation, which might cause variable interferon receptor expression.

5. ACKNOWLEDGEMENT
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12
Rat intestinal lamina propria macrophages: characterisation and accesory role in ovalbumin-induced T cell proliferation

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INTRODUCTION

The MHC-restricted antigen specific interaction between T cells and antigen presenting cells(APC) is well documented. Macrophages expressing MHC class II antigens are extensively distributed in various tissues.However, little attention has been focused on intestinal macrophages as APC despite their strategic location at a site of primary antigen contact.In this study we have characterized isolated lamina propria macrophages by morphology , phenotype and by their ability to present ovalbumin to primed T cells..

METHOD

DA rats of both sexes aged 9 to 12 weeks and fed on CRM diet were used in this study.

Isolation of lamina propria macrophages

Intestines cut open and washed in HBSS.
Cut into 2 to 3 cm lengths and shaken in 1 mM EDTA at 37°C for 15 to 20 minutes x 3.
Mucosa scraped into RPMI +5%FCS +50 units of (each) collagenase type II and hyaluronidase (Sigma) and shaken for 45 minutes at 37°C
Cells fractionated on Nycodenz gradient 650 g at 20°C for 20 minutes.

Characterization

Morphology - cytospin smears stained with Diff-Quick(DADE).
Non- specific esterase activity.-using α naphthyl acetate esterase as substrate.
Adherence to various surfaces-FCS and Fibronectin coated dishes.
Phagocytosis -using yeasts.
Fc receptor expression -using SRBC.
Indirect immunoperoxidase/ antiperoxidase assay- staining of cytospin smears with various monoclonal antibodies(OX6,EDI,ED2,OX41,OX42,OX43 and W3 /25).

Antigen presentation assays

Nylon wool non- adherent cells were prepared from lymph nodes of ovalbumin(Sigma ,Grade V) immunized DA rats.Cells were co-cultured in different ratios with intestinal macrophages and splenic adherent cells in the presence of 100 ug/ml. of ovalbumin .OX6 was included in cultures for MHC restriction studies. Radioactivity incorporation into DNA synthesis was measured after 96 hrs. of culture.

RESULTS

Adherence,phagocytosis and Fc receptor expression Both unfractionated and fractionated cells showed none of these activities.

Antigen presentation assays

Figure1 shows that at a T- cell:macrophage ratio of 10:1, lamina propria macrophages usually inhibited T- cell proliferation whereas splenic adherent cells elicited optimal response. However, at high ratios(100 and 1000:1) a consistent T- cell proliferation was observed. Figure 2 indicates that the mechanism of T-cell proliferation is MHC class II restricted.

Figure 1 **Ovalbumin presentation by intestinal and splenic cells**

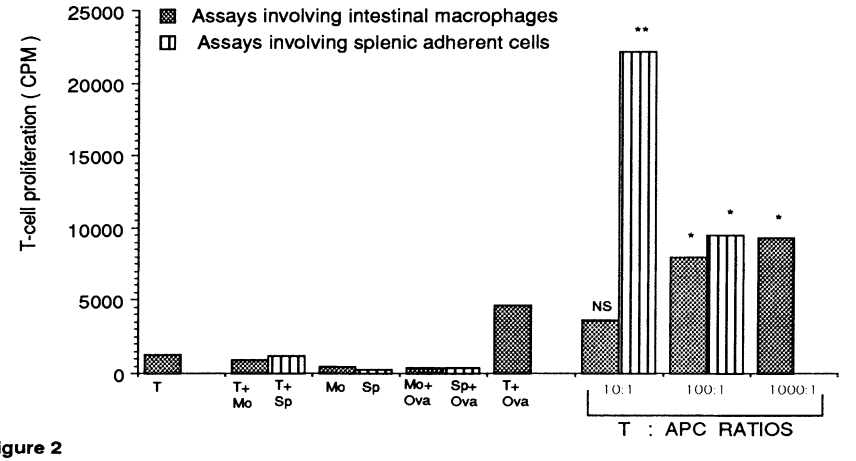
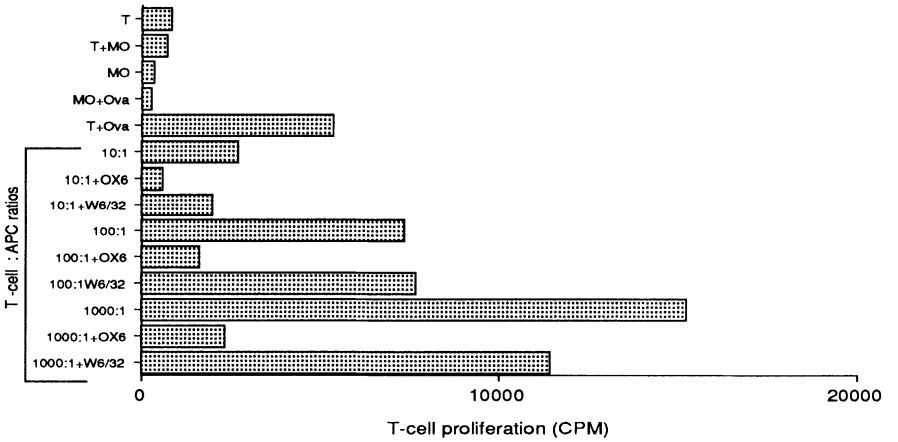


Figure 2
MHC restriction of ovalbumin presentation by intestinal macrophages



SUMMARY

1. A method for isolation of intestinal lamina propria macrophages has been developed yielding 0.8-1.5 x 10^6 cells per rat after Nycodenz gradient purification.
2.Purified cells were more than 85% macrophages as based on morphological and non specific esterase activity.
3. More than 70 % of the cells expressed MHC class II (OX6) and macrophage surface membrane antigens (OX43 and W3/25).
4.There was no evidence of adherence ,phagocytosis or Fc receptor expression. Whether the isolation procedure may have influenced some of these features needs further verification.
5.At high T-cell: macrophage ratios, intestinal lamina propria macrophages could function as APC and the abrogation of this MHC restricted cellular interaction observed at low ratios is partially mediated by secretion of prostaglandins(inclusion of indomethacin in cultures increased proliferation by more than 50%).

13
The effect of interferon gamma (IFN-γ) on MHC Class II (Ia) antigen by expression on the neonatal enterocyte

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INTRODUCTION: The class II major histocompatibility antigen (Ia) is a glycoprotein heterodimer expressed on the surface of antigen-presenting cells that is required for the presentation of exogenous antigens to T-cells (1). Ia is constitutively expressed in adult mice on the differentiated absorptive cells covering the upper 2/3 of the villus in the small intestine (2). Although no in vivo role has been confirmed for Ia on enterocytes, in vitro studies have demonstrated that enterocytes can act as antigen-presenting cells (3). Induction of Ia expression in crypts is associated with graft-versus-host disease in rodents and its expression in mature mice has been induced by administration of IFN-γ (4). In the rat, Ia is not expressed on intestinal epithelial cells until after weaning. In this study, we sought to determine the time-course of appearance of Ia during neonatal development and its susceptibility to induction by IFN-γ .

METHODS: Normal development of Ia: Untreated mice were sacrificed at 10, 15, 20, 25, 30, 35, 45 days after birth. The small intestine was removed, divided into 5 equal length segments, incised longitudinally, and samples obtained from the 2nd and 4th segments were examined. Effect of IFN-γ dose on Ia expression: At 16 days after birth, BDF1 mice were given a single ip injection of distilled water, 2×10^4 U IFN-γ , 4×10^4 U IFN-γ , 8×10^4 U IFN-γ , or 1×10^5 U IFN-γ ,and were sacrificed 24 hours later. Time course of IFN-γ response: On day 16, mice were given 1×10^5 U IFN-γ and were sacrificed 24 hr, 48 hr, and 72 hr later. Histochemical staining: Intestine was rapidly frozen in OCT. Five um sections were obtained, air dried, and fixed in acetone. Non-specific reactivity was blocked with normal rabbit serum and avidin. Sections were reacted with either M5/114 monoclonal rat IgG_{2b} anti-mouse Ia antibody or control, purified rat IgG_{2b} antibody. The second antibody, biotinylated rabbit anti-rat IgG, was added followed by avidin - biotin HRP complex, substrate, and counterstain. Tissue sections were evaluated for distribution and intensity of epithelial cell staining and were rated on a scale of - to 3+.

RESULTS: In untreated animals, Ia expression first appeared on small intestinal epithelial cells approximately 30 days after birth. Expression was more intense, more consistent, and included the crypt region in the distal intestine. In mice treated with IFN-γ on Day 16, Ia expression was inducible by 2 to 4 x 10^4 U IFN-γ ; this effect increased with dose. Twenty-four hr after treatment, expression of Ia was located predominantly in the crypt and adjacent lower portion of villi. By 48 hr, we noted less intense staining of epithelial cells in crypts, but spread of staining along the entire villus and tip. By 72 hr, staining for Ia was no longer apparent.

DISCUSSION: These studies show that Ia expression on intestinal epithelial cells is related to both age and location in the intestine and that it can be induced by treatment with IFN-γ prior to weaning. The absence of Ia before weaning suggests that substances within breast milk may inhibit expression of Ia. Substances, such as PGE_2, cortisol and TGF-beta, that are present in milk, were shown to decrease Ia expression (1,5,6,7,8). Although weaning occurred by Day 17, we did not see expression of Ia on epithelial cells until after Day 25; this observation suggests that, although milk may play a modulating role, expression of Ia may require additional signals. We observed that IFN-γ , a known stimulus for Ia expression (1), induced Ia in intestinal epithelial cells before weaning. The mechanism for induction of Ia by IFN-γ in enterocytes is unknown; in the macrophage, IFN-γ acts by altering the transcription rate of the Ia gene (8). The increased expression of Ia in the distal small intestine suggests that luminal factors may be important in controlling Ia expression in the intestine. In conclusion, we suggest that inflammatory stimuli such as IFN-γ or mucosal infection, may lead to Ia expression on neonatal enterocytes and may thereby alter the immune response to enteric antigens.

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SECTION B: INTRAEPITHELIAL LYMPHOCYTES AND LYMPHOEPITHELIAL INTERACTIONS

14
Subsets of gamma/delta T cells in human intestinal epithelium

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ABSTRACT. Immunohistochemistry has been used to investigate disulphide linked and non-disulphide linked forms of the T cell receptor γ/δ heterodimer (TCR γ/δ) in blood and epithelium of normal human small intestine, intestine of patients with untreated coeliac disease (in whom T cells expressing TCR γ/δ are disproportionately raised) and intestine of patients with tropical malabsorption. In blood from adult volunteers, 90% of T cells using TCR γ/δ use the disulphide linked form. In contrast, in the epithelium in normal small intestine, coeliac disease and tropical malabsorption, most of the T cells expressing TCR γ/δ use the non-disulphide linked form. This is especially prominent in untreated coeliac disease where the increase in TCR γ/δ T cells is mainly restricted to those using the non-disulphide linked form.

1. INTRODUCTION

Mature T cells in blood and secondary lymphoid organs predominantly express the α/β form of the T cell receptor (TCR α/β) (1). In addition a minority (approximately 10% in the blood) express the γ/δ form (TCR γ/δ) (2,3). It has been observed in mice that TCR γ/δ is preferentially expressed on cells associated with epithelia (4,5,6) namely the Thy1+ dendritic epidermal cells in the skin and the intraepitehlial lymphocytes (IEL) of the small intestine. In man however, cells expressing TCR γ/δ account for only approximately 11% of the CD3+ IEL poulation. IEL expressing TCR γ/δ increases to approximately 30% in patients with untreated coeliac disease, which is associated with an increase in cells which do not express the T cell subset markers CD4 and CD8 (7,8).

TCR γ/δ in man is found in at least two forms, a disulphide linked heterodimer and a non-disulphide linked heterodimer (9). Antibodies are available which distinguish between the disulphide linked and non-disulphide linked forms of TCR γ/δ (9). In this study we have used these antibodies to investigate the expression of TCR γ/δ in IEL compared to peripheral blood. We have studied jejunum from normal patients, jejunum from patients with coeliac disease, where IEL

density and the percentage of cells expressing TCR γ/δ is increased and patients with tropical malabsorption where IEL density is increased but the percentage of CD3+ IEL expressing TCR γ/δ is not.

2. MATERIALS AND METHODS.

2.1. Tissues studied.

Peripheral blood lymphocytes from 4 normal donors were isolated by centrifugation over Ficoll/hypaque and cytospin preparations were made.

All jejunal biopsies studied were taken using a Crosby capsule from patients suspected of having upper intestinal disease. Half of each biopsy was fixed in formalin and processed and half was frozen in liquid nitrogen.

Normal biopsies were from patients in whom coeliac disease was suspected but in whom no intestinal abnormality was found (n=16). The patients were 7 male, 9 female, aged 1.7-69 years , mean age 29.1 years.

Biopsies from patients with coeliac disease (n=7) had total villous atrophy (6 biopsies) or partial villous atrophy (1 biopsy). and an increase in the density of IEL. All patients showed improvement clinically and histologically when placed on a gluten free diet. Symptoms returned on gluten challenge. These were 3 male and 4 female patients, aged 1.1-6.8 years, mean age 3.5 years.

Biopsies from patients with tropical malabsorption (n=5) showed partial villous atrophy and an increase in IEL density. All patients had gastrointestinal symptoms after returning to the UK from the tropics. This group consisted of 4 male and 1 female patients, aged 25-47 years, mean age 39 years.

2.2. Immunocytochemistry.

Immunocytochemical studies were carried out on acetone fixed, cytospin preparations and 8μm cryostat sections of snap frozen jejunal biopsies. Four monoclonal antibodies were used: anti-CD3 against the CD3 trimer associated with all forms of the T cell receptor, was purchased from Dako UK Ltd. TCRδ1 against all forms of TCR γ/δ and δTCS1 against the non-disulphide linked form of TCR γ/δ were purcheased from T Cell Sciences Inc., Cambridge, MA, USA, and BB3 against the disulphide linked form of TCR γ/δ was kindly given by Dr. A Moretta. The indirect immunoperoxidase technique using peroxidase conjugated rabbit anti-mouse secondary antiserum (Dako UK Ltd) was used.

2.3. Quantitation of IEL

The density of IEL staining with any monoclonal antibody was determined by counting the peroxidase cells stained in the epithelium as a percentage of the total nuclei of stained and unstained cells in the epithelium. The percentages of CD3+ IEL expressing TCR γ/δ and TCR γ/δ in the disulphide linked and non-disulphide linked forms were deduced from counts in serial sections. Results are shown as means ± 1 SE in parenthesis.

3. RESULTS.

3.1. Peripheral Blood Lymphocytes.

In 4 normal adults, approximately 10% (9.9% ±0.8) of the CD3+ cells in the peripheral blood were TCRδ1+. The majority of these (9.4% ±0.7 of the CD3+ cells) were BB3+. Few were δTCS1+ (1.2% ±0.1 of CD3+ cells).

3.2 Expression of CD3 and TCR γ/δ in jejunal biopsies.

In normal jejunal biopsies, 14.5% (±0.3) of cells in the epithelium were CD3+. Of the total CD3+ cells in the epithelium, 11.8% (±0.6) were TCRδ1+.

In coeliac disease as previously reported (8), there is an increase in the density of IEL expressing CD3 (45.9% ±1.6). Of these, 31.6% ±1.3 express TCRδ1, which is a significantly greater percentage than in normal jejunum ($P<0.00001$, analysis of variance). In tropical malabsorption, there was also an increase in the density of CD3+ cells in the epithelium (45.5% ±2.2). However, only 5.5% of these were TCRδ1+, which is not significantly different to the percentage in normal jejunum.

3.3 Expression of disulphide linked (BB3+) and non-disulphide linked (δTCS1+) TCR γ/δ in jejunal biopsies.

In normal jejunal biopsies, δTCS1 was expressed by 3.8% (±0.3) of CD3+ IEL and BB3 on 1.3% (±0.1) of CD3+ IEL. In coeliac disease, δTCS1 was expressed by 21.6% (±1.4) of CD3+ IEL whereas only 1.2% (±0.1) of CD3+ IEL expressed BB3 (Figure 1). In tropical malabsorption, δTCS1 was expressed by 2.3% (±0.2) of CD3+ IEL and BB3 was expressed by 1.5% (±0.4) of CD3+ IEL.

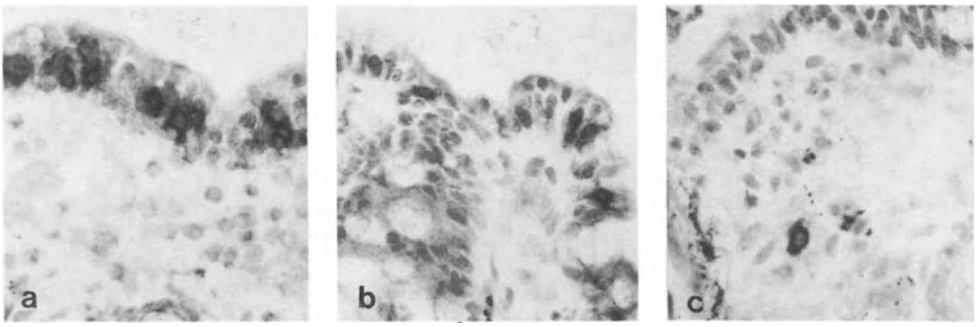

FIGURE 1: Cryostat sections of a jejunal biopsy from a patient with untreated coeliac disease stained using immunoperoxidase with antibodies TCRδ1 (a), δTCS1 (b) and BB3 (c). δTCS1+ IEL predominate over BB3+ IEL.

4. DISCUSSION.

In blood, most T cells expressing TCR γ/δ use the disulphide linked form (3,9), a result we have confirmed here. However, in the epithelium of

biopsies from patients with normal intestinal morphology, in biopsies from patients with untreated coeliac disease (in whom the TCR γ/δ cells are disproportionately increased) and in biopsies from patients with tropical malabsorption, T cells expressing TCR γ/δ in the non-disulphide linked form predominate over those expressing TCR γ/δ in the disulphide linked form. This is the reverse situation to the blood. In addition, we found that in the blood, BB3+ and δTCS1+ cells accounted for approximately all cells expressing TCRδ1. In the epithelium however, the sum of the cells expressing BB3 and δTCS1 did not account for the total TCRδ1+ population. This discrepancy was statistically significant in untreated coeliac disease ($P < 0.005$, paired Student's T test). It appears therefore that some TCR γ/δ + cells have either lost the epitope recognised by BB3 or δTCS1, or that a TCR γ/δ + population not recognised by either BB3 or δTCS1 is present in the gut epithelium.

Although the TCRδ1+ population is increased in coeliac disease, the BB3+ population appears constant and independent of changes in the TCRδ1 and δTCS1 populations. T cells in the thymi of children, like those in the epithelium express TCR γ/δ in the non-disulphide linked form. Also it has been shown that T cells in a patient with thymic aplasia expressed TCR γ/δ in the disulphide linked form (10). It has been suggested therefore that T cells expressing the disulphide linked form of TCR γ/δ mature extrathymically (9). The changes in the TCR γ/δ population in coeliac disease therefore appear to be associated with the thymus dependent subset.

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15
Increased numbers of T cell receptor gamma/delta bearing lymphocytes in the epithelium of coeliac patients

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ABSTRACT

We studied the numbers of T cell receptor-gamma/delta, CD3, CD4 and CD8 positive lymphocytes in jejunal specimens from 19 coeliac patients, in 13 control specimens. In the lamina propria of coeliac patients, the mean number of gamma/delta+ cells was significantly higher than in the controls during the gluten-free diet and after the gluten challenge ($p<0.01$). In the jejunal surface and crypt epithelium of coeliac patients, the number of gamma/delta+ cells was significantly elevated before and during gluten elimination and after the challenge test ($p<0.001$ in all comparisons). The absolute number of these cells remained constant during gluten elimination; therefore their proportion of the surface CD3+ cells rose from the mean of 8.0% before treatment to 20% during the gluten-free diet and, as CD3+ cells increased during the gluten challenge, the proportion of gamma/delta + cells fell to 7.2%.

INTRODUCTION

The intestinal intraepithelial lymphocytes (IEL) are a cell population whose significance in the immune defenses is presently unknown. The numbers of these cells increase in intestinal diseases characterized by increased epithelial cell turnover, resulting in crypt hyperplasia and villous atrophy (1). If the offending agent, such as gluten in coeliac disease, is eliminated, the number of IEL falls to normal (1-2). About two thirds of IEL are CD3+ T cells more than 90% of which express the surface CD8 antigen, with very few showing the CD4 antigen in normal human jejunal mucosa (3-4). In patients with coeliac disease, a considerable number of jejunal CD3+ IEL expresses neither the CD8 nor the CD4 antigen (3-5).

A recent study of the surface membrane proteins of murine intraepithelial lymphocytes suggests that the majority of these cells might bear T-cell receptor (TCR)-gamma/delta protein (6). In man, TRC-gamma/delta- bearing lymphocytes are rare, comprising 0-5% of peripheral and even less of thymic lymphocytes (7-9). Similar low numbers have been seen in the lamina propria of the intestine (10). As TCR- gamma/delta+ cells can cause non--MHC restricted cytolysis and also may act against Ig FC receptor-bearing target cells (8,11), they might have a role in the pathogenesis of diseases where mucosal

epithelial cell destruction is involved, as in coeliac disease.

PATIENTS AND METHODS

PATIENTS. 25 jejunal specimens from 19 pediatric patients with coeliac disease were used in this study; 9 were taken before any treatment at a mean age of 5.3 years, 8 were taken when the patient was on a gluten-free diet at a mean age of 12.1 years, and 8 were taken after a gluten challenge resulting in villous atrophy at a mean age of 12.2 years. From 6 patients the specimens both during gluten elimination and after the gluten challenge were available. As controls we studied 13 histologically normal jejunal specimens taken from children at a mean age of 8.2 years as a part of the evaluation of their asymptomatic growth failure.

TISSUE PROCESSING. Five µ cryostat sections were cut and dried at room temperature for one hour. They were fixed in acetone for 10 minutes, then in chloroform for 30 minutes, and washed three times in Tris buffer pH 7.4 (4).

IMMUNOHISTOCHEMICAL STAINING. After the buffer had been wiped of, sections were covered with the diluted monoclonal antibodies in Tris-buffer for 16 hours. To stain monoclonal antibodies, a Vectastain Elite ABC kit (PK-6102, Vectro Laboratories, Burlinghame, CA.) was used according to the instructions of the manufacturer. The fixed peroxidase was revealed with hydrogen peroxidase-activated AEC (3-amino-9-ethyl-carbazole, Sigma Inc, St. Louis, Mo.) incubated for 20 minutes. The slides were counterstained with hematoxylin for 30 seconds. As negative controls we used slides processed similarly, but without the first layer of monoclonal antibodies.

Peroxidase-stained cells in the slides were counted with a light microscope using 900x magnification. They were counted in a known length of epithelium or area of lamina propria through a calibrated graticule. The number of positive cells is expressed as cells /mm of epithelium or cells /mm^2 of lamina propria (4).

MONOCLONAL ANTIBODIES. Monoclonal antibody deltaTCS-1 (12)(T cell Sciences Inc. Cambridge, MA) was used in dilution 1:100. Monoclonal antibodies Leu4 (anti-CD3, Beckton-Dickinson, Mountain View, Ca), T4 (anti-CD4, Coulter Immunology, Hialeh, Fl) and OKT8 (anti-CD8, Ortho Diagnostic System, Raritan, NJ) were used in dilution 1:400.

RESULTS

TCR-GAMMA/DELTA-BEARING LYMPHOCYTES IN THE LAMINA PROPRIA OF JEJUNUM. In the morphologically normal intestine of the controls, the mean number of gamma/delta+ cells was 1.3% of the total number of CD3+ cells, varying between 0 and 12% (Table 1).

Table 1. Numbers of lymphocytes (cells/mm^2, the mean and SEM in paranthes are given) expressing various surface antigens in the lamina propria of jejunum of patients with coeliac disease and of controls.

Group	Gamma/ delta	CD3	CD4	CD8
Coeliacs: before	55	1471	1145	691
treatment (n=9)	(16)	(139)	(150)	(88)
during gluten free		69[1]	1517	1033649[2]
(n=8)	(13)	(179)	(122)	(62)
after gluten		65[1]	1520	1067709
challenge (n=8)	(12)	(107)	(153)	(47)
Controls		23	1650	1500946
(n=13)	(6)	(232)	(188)	(108)

[1] $p<0.01$ compared to controls, [2] $p<0.05$ compared to controls

The mean number of gamma/delta+ cells was significantly higher in the lamina propria of the jejunal specimens taken from coeliac patients during the gluten-free diet and after the

gluten challenge than in the controls, and the numbers did not seem to be affected by gluten elimination or challenge.
Gamma/delta+ cells represented on the average about 4% of the total number of CD3+ cells in the specimens from coeliac patients; in individual specimens they varied from 0 to 12%.

TCR-GAMMA/DELTA-BEARING LYMPHOCYTES IN THE JEJUNAL EPITHELIUM.
The number of gamma/delta+ cells was low in the surface epithelium of the control children with a normal jejunum; in nine specimens no cells were seen (density < 0.5cells/mm) and in the remaining four the numbers ranged from 0.7 to 3.0 cells/mm. Gamma/delta+ cells were seen in the crypt epithelium of only one of the controls.

In all jejunal specimens taken from the coeliac patients, intraepithelial gamma/delta+ cells were seen. The numbers of these cells were significantly increased both in the surface and in the crypt epithelium before treatment, during the gluten-free diet and after the gluten challenge as compared to controls ($p<0.001$ in all comparisions).

a. b.

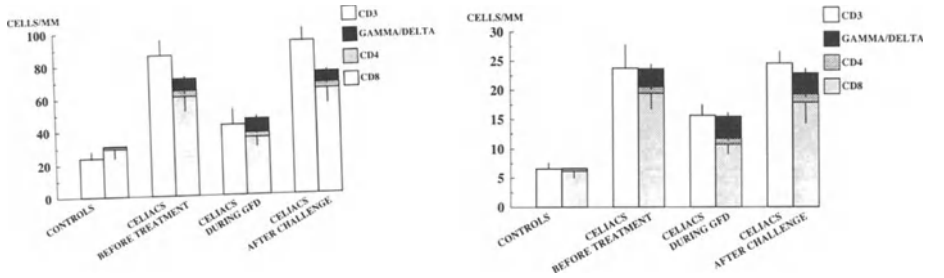

Figure 1a. Mean numbers of lymphocytes in the surface epithelium expressing surface antigens CD3, gamma/delta, CD4, CD8 of controls, and patients with coeliac disease before treatment, during gluten-free-diet (GFD) and after a gluten challenge. One SEM is indicated (I).
Figure 1b. Corresponding numbers of lymphocytes in the crypt epithelium of patients and controls.

In the surface epithelium, gamma/delta+ cells comprised 8.3% (range 2.5% - 15%) of CD3+ cells before the treatment, 20% (11% - 52%) during the gluten-free diet and 7.2% (3.5% -16%) after the gluten challenge. When compared with the mean numbers of CD3+CD4-CD8- cells, gamma/delta+ cells represented 34%, 165% and 26% of their counts at the respective phases of treatment of coeliac disease (Figure 1a).
In the crypt epithelium, gamma/delta+ cells represented 13% (range 3.6% -33%) of the mean number of CD3+ cells before the

treatment, 24% (12% - 37%) during the gluten free-diet and 14% (4.6% - 64%) after the gluten challenge. The corresponding percentages of the mean number of CD3+CD4-CD8- cells were 95%, 95% and 67% respectively (Figure 1b). In the jejunal specimens of all patients, the mean number of gamma/delta+ cells was unchanged at different phases of the treatment of coeliac disease both in the surface and the crypt epithelium (Figures 1a and 1b).

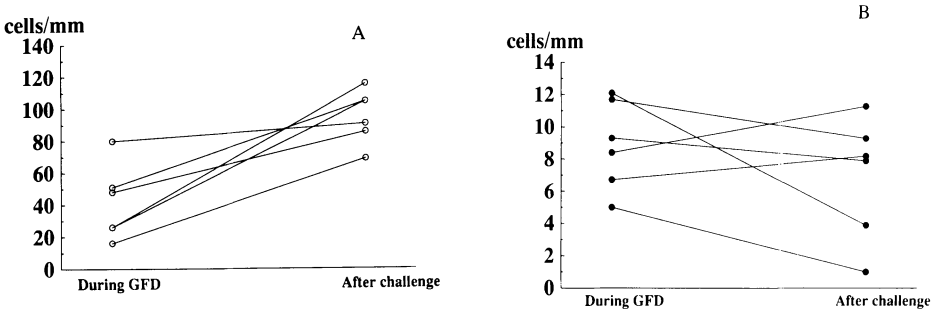

Figure 2a. Numbers of CD3+ cells in the surface epithelium of jejunal specimens from coeliac patients during a gluten-free diet and after a gluten challenge.
Figure 2b. Number of gamma/delta+ cells in the same specimens. In the six coeliac patients from whom both pre- and postchallenge biopsies were available, the numbers of CD3+ (Figure 2a) and CD8+ (data not shown) cells increased significantly ($p<0.01$) during the gluten challenge, while the number of gamma/delta+ cells remained constant (Figure 2b).

DISCUSSION

The recently recognized T cell type bearing TCR-gamma/delta as a part of the CD3 complex (13-14) has not been assigned a specific role in the lymphoid tissue of man. In mice, TVR-gamma/delta+ cells have been found in abundance among the dendritic epithelial cells (15-16) and analysis of the surface receptor proteins of the intestinal intraepithelial T cells suggested that the majority of them could bear gamma/delta receptors (6). In the normal human intestine, the number of TCR-gamma/delta-bearing cells seems to be low, both in the lamina propria and intraepithelially in the colon and jejunum, according to a recent study (10) and our results. Their number is of the same magnitude as has been described for the peripheral blood and thymic lymphocytes, only a small percent of the total number of CD3+ T cells (7,9-10,17).

In coeliac disease the fluctuation in the IEL numbers and the destruction of surface epithelial cells take place in concert: both depend on the presence of gluten (1-2). The role of intraepithelial lymphocytes in the pathogenesis of coeliac disease is still unknown. It was noted recently that

considerable a proportion of these intraepithelial lymphocytes expresses neither CD4 nor CD8 antigen, and that the number of these cells remains constant irrespective of the fluctuation of the total number of CD3+ T cells (3-4). This led us to speculate that there could be an increased population of gamma/delta+ lymphocytes in that location (4). In the present study, according to the quantitation of T cells bearing various surface receptors, the number of gamma/delta+ cells represents a sizeable proportion of the presumably double negative CD3+ cells (number of CD3+ minus the sum of CD4+ and CD8+ cells) in the epithelium of patients with coeliac disease. According to a recent study, only about 1/3 of TCR-gamma/delta-bearing cells in the peripheral blood are detected by the delta-TCS-1 antibody (7), antibody was used in the present study, and it is possible that we have missed a number of TCR-gamma/delta+ cells not reactive with this antibody (7,10,18). In the active stage of coeliac disease (before the treatment and after the gluten challenge), we found a considerable number of CD3+ cells, which were not stained with delta-TCS-1, CD4 or CD8 antibodies and may thus also bear a gamma/delta receptor. Some gamma/delta+ T cells express CD8 antigen (6,8,17), and this phenomenon may be accentuated in coeliac jejunal mucosa during gluten elimination. At that stage, we found quantitatively more cells bearing either gamma/delta receptors or CD4 or CD8 antigens than there were CD3+ cells.

Functionally, TCR-gamma/delta-bearing cells can exert cytolytic activity against tumor cells, which is not restricted by MHC class I and II molecules (9,11,19), and also toward IgG Fc receptor-bearing target cells (11) when first reacted with monoclonal antibody to CD3. Clearly, both types of cytolytic reaction could play a role in the destruction of surface epithelial cells characteristic of coeliac disease: mucosal B cells produce great amounts of antibodies to gluten when the patient consumes this antigen (20) and these antibodies may be fixed to surface epithelial cells. Coeliac disease is associated with specific MHC antigens (21), and even class II antigens are expressed on the epithelial cells (22) when the patient eats gluten-containing food. The MHC antigens may show specific affinity to gluten or its breakdown products and make the surface epithelial cells a target for cytolysis. In addition, other restricting surface molecules may function as a presenting antigen together with gluten for TCR- gamma/delta-bearing lymphocytes. The gamma/delta-receptor-bearing cells may also be precursors of autoreactive T cells (23). We infer that these cells have a specific role in the pathogenesis of coeliac disease, as they are constantly present in large numbers in the jejunal epithelium of patients with this disease.

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16
The gamma/delta T cell receptor (TCR) is expressed on less than 50% of intraepithelial lymphocytes (IEL) in human intestine

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Introduction: The γ/δ T cell receptor (TCR) is found on virtually all intestinal intraepithelial cells (IEL) and on dendritic cells of the epidermis in the murine system but only on a small proportion of peripheral blood lymphocytes (PBL) or in other lymphoid organs; thus a specific function of γ/δ T cells at epithelial surfaces has been discussed [1]. Very limited data exists so far on the distribution and phenotypic characteristics of γ/δ T cells in the human intestine. Therefore, we studied the expression of the γ/δ TCR and the coexpression with other surface antigens on human intestinal IEL.

Materials and Methods:

Serial cryostat sections of biopsies or resected specimens of 15 patients (6f, 9m; age 18-86, median 40 years; 8 controls, 4 HIV, 2 Crohn's Disease, 1 Celiac Disease; 9 small, 6 large intestine) were examined immunohistologically. Sections were stained by the APAAP technique using mAbs T3 (CD3), TCRδ1, βF1, and HML-1 (used as marker for IEL [2]). The number of stained intraepithelial cells per 100 epithelial cells was determined microscopically. Percentages of α/β or γ/δ HML-1+ T cells were calculated using these values.

Intraepithelial lymphocytes were isolated from uninvolved mucosa of 5 patients (2f, 3m; age 28-78, median 39 years; 3 small, 2 large intestine) undergoing surgical resection by incubation of the mucosa in $Ca^{++}Mg^{++}$ free medium and density gradient centrifugation of cell suspensions.

Isolated intraepithelial lymphocytes were stained using mAbs TCR1(WT31) FITC, TCRδ1 FITC, Leu4 PE (CD3), Leu3 PE (CD4), Leu2 PE (CD8), and HML-1 with goat anti-mouse Ig PE. Cytofluorometric analysis was performed using a FACScan cytometer.

Results were expressed as median (minimum-maximum).

Results:

By immunohistological examination of serial cryostat sections we found that 50%(20-79) of HML-1+ IEL expressed the α/β TCR, and 33%(10-50) the γ/δ TCR; there were no marked differences with regard to diagnosis, or between small or large intestine. Of isolated HML-1+ IEL 87%(75-90) were α/β T cells and 21%(10-30) γ/δ T cells as determined by cytofluorometric analysis. 7%(6-10) of IEL were double negative (CD4-CD8-) T cells, virtually all of these cells expressed the γ/δ TCR. Less than 5% of γ/δ T cells were CD4+, 29%(18-78) were CD8+, 60%(21-73) were double negative (CD4-CD8-), and 94%(85-100) were HML-1+. 46% of α/β T cells were CD4+, 46% (39-80) were CD8+, less than 5% were double negative, and 85%(57-98) were HML-1+. There were no marked differences between small or large intestine. Figure 1 shows a representative two-colour FACS profile of human IEL.

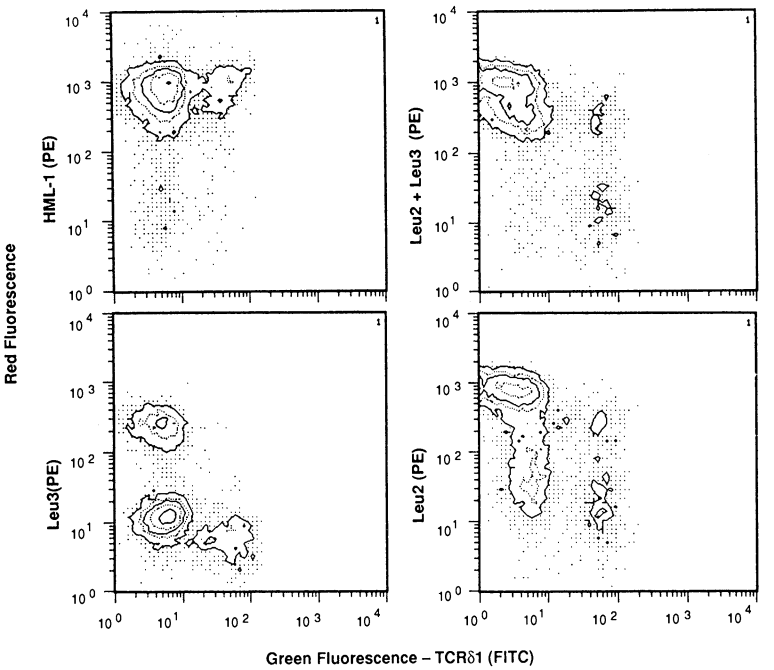

Figure 1. Two colour immunofluorescence profile of isolated IEL of human jejunum.

Discussion: In contrast to previous findings in the murine system our studies show that only one third of *human* IEL expresses the γ/δ TCR, two thirds are of the α/β type. γ/δ TCR+ IEL are either CD8+ or double negative. These findings were confirmed by both immunohistology and FACS analysis; the lower percentage of the γ/δ TCR seen in isolated IEL is probably due to contaminating lamina propria lymphocytes, which may also account for the CD4+ subpopulation found among α/β T cells. It has been speculated that γ/δ TCR-bearing T cells mediate immunological surveillance of epithelia by recognizing altered surface antigens on infected or transformed epithelial cells [1]. Our finding that at least in humans more than half of IEL are of the α/β TCR-type opens the question, whether the two subpopulations of IEL T cells definded by the type of the TCR carry out different functions in the local immune response at mucosal surfaces.

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17
Expression of T cell receptors TcR1 (gamma/delta) and TcR2 (alpha/beta) in the human intestinal mucosa

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Introduction

Recent attention has focused on T cells expressing the γ/δ-type T cell receptor (TcR1), although the functional role of TcR1+ cells remains speculative [1]. In human peripheral blood, TcR1+ γ/δ cells are a minority population of $CD4^-$ $CD8^-$ "double negatives". In murine intestinal mucosae, a high proportion of intraepithelial lymphocytes (IEL) are reported to be CD8+ T cells of the γ/δ type [2,3].

Materials and Methods.

Biopsy specimens of small bowel (4 from jejunum and 8 from duodenum) and colon (n=8) were obtained during routine endoscopy and cryostat sections were cut. All samples were histologically normal, with no evidence of an inflammatory reaction. Antibodies TCRδ1 and βF1 were used to investigate expression of TcR1 and TcR2, respectively. Other antibodies used comprised UCHT1(γ) (CD3), Dako T4(γ) and OKT4B(μ) (CD4), RFT8γ and RFT8μ (CD8), RFT1γ and RFT1μ (CD5) and MBG6(μ) (CD6). Tissue sections were double-labelled with combinations of one IgG-class antibody and one IgM class antibody, followed by incubation with class-specific anti-IgG/FITC and anti-IgM/TRITC. To detect low expression of TcR β-chain, anti-IgG-biotin and streptavidin/FITC were used. Cell counts of IEL and lamina propria lymphocytes (LPL) were performed by two independent observers.

Results

EXPRESSION OF TcR1 (γ/δ)

In the small bowel, very few cells were positive for TcR1, and virtually all TcR1+ cells were confined to the epithelial compartment. Between 2 and 10 TCRδ1+ cells per each biopsy section were observed out of a total of 200-250 CD3+ IEL. The median number of γ/δ cells was ≈2%. Duodenum did not differ from jejunum, and the results obtained in all 12 patients were very comparable.

In the colon, as in small bowel, virtually all γ/δ cells were in the epithelial compartment. The percentage of CD3+ cells expressing γ/δ chains was higher, with a mean value approximating 15%-20%. However, this reflected in part a much lower density of colonic IEL compared to small bowel, as absolute numbers of TCRδ1+ cells were in the range of 10 to 25 per entire biopsy section (median = 16). No major variability between different specimens was noted.

PHENOTYPES OF TcR1+ (γ/δ) CELLS

All TcR1+ cells were CD4$^-$. About half of the TcR1+ IEL were CD8+ and the other half were CD4$^-$ CD8$^-$ "double negatives". No TcR1+ cells were found to co-express CD5 or CD6. The same phenotypic distribution was observed in small and large bowel, and in both intraepithelial and lamina propria compartments (even though very few γ/δ LPL were observed).

EXPRESSION OF TcR2 (α/β)

All CD4+ IEL and LPL were βF1+. Of the CD8+ subset, 30-50% were positive with βF1, corresponding to the CD8+ CD5+ subtype. The non-γ/δ CD8+ CD5$^-$ cells (50-70% of IEL) were positive for βF1 only when biotin/streptavidin was used to increase sensitivity, and hence were considered as TcR2 "dull".

T CELL POPULATIONS OF THE HUMAN INTESTINAL MUCOSA

Five phenotypically distinct populations were identified. The TcR2+ subset comprised a) all the CD4+ cells, a population which invariantly co-expressed CD5 and CD6), b) the CD8+ CD5+ CD6+ cells and c) the majority of the CD8+ CD5$^-$ CD6$^-$ subset, although the latter group expressed the βF1 antigen at low intensity (ie. TcR2 "dull"). The minority TcR1+ (γ/δ) T cells were either a) CD8+ CD5$^-$ CD6$^-$, or b) CD4$^-$ CD8$^-$ "double negatives". All γ/δ cells, including the "double negatives", were CD5$^-$ CD6$^-$.

These T cell phenotypes occurred in small and large bowel, and in epithelial and lamina propria compartments (even though there were almost no γ/δ LPL), albeit in different proportions. There were no apparent deviations from these five basic phenotypes.

Discussion and Conclusions

At present, the role of T cells expressing the γ/δ TcR1 is unknown [1]. Their low incidence in the human intestinal mucosa suggests that they do not play a major role in the normal tissue. It has been suggested that TcR1+ cells may recognise "heat shock" (stress) proteins [5]. This would explain the higher incidence of TcR1+ cells in the colonic mucosa, where bacterial stress proteins occur in higher concentrations. Recent evidence suggests that γ/δ cells are increased in leprosy lesions [6] and in coeliac disease [in preparation]. In such conditions, increased production of the highly-conserved stress proteins would also be expected. Alternately, TcR1+ cells may be cytotoxic cells, or suppressor cells by virtue of cytotoxicity towards antigen-presenting cells and B cells [4]. Mucosal γ/δ T cells may be cytotoxic in either a class I-restricted (the CD8+ subset) or non-restricted fashion (the double-negatives) [1]. Thus, mucosal γ/δ cells could be suppressor/cytotoxic cells, whose role is to limit the inflammatory response.

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18
Analysis of intestinal intraepithelial lymphocytes in athymic (nude) and SCID mice

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1. INTRODUCTION

About 70 % of the intestinal intraepithelial lymphocytes (IEL) in euthymic mice expresses the CD3 associated $\gamma\delta$ receptor [1-5]. The remaining 30% of the $CD3^+$ IEL express the $\alpha\beta$ T cell receptor (TCR) [3,5]. The relative percentage of the $\gamma\delta$ and $\alpha\beta$ TCR positive cells in the IEL fraction, however, depends upon the gnotobiotic status of the animals [6, and our unpublished results].
In athymic mice the only receptor expressed on CD3 positive IEL is the $\gamma\delta$ receptor, as shown by northern blot hybridisation and negative staining results with monoclonal antibody (mAB) H57-597, which recognizes the $\alpha\beta$ TCR [3,5,7]. The phenotype of $CD3^+$ IEL in euthymic animals is $CD8^+Thy\text{-}1^-$ and $CD8^+Thy\text{-}1^+$ low for $\gamma\delta$ receptor expressing cells and $CD8^+Thy\text{-}1^+$ high for cells which carry the $\alpha\beta$ receptor [3,5]. In athymic animals the major phenotype of $CD3^+$ IEL is $CD8^+$ $Thy\text{-}1^-$. The presence of $CD3^+$ IEL in athymic mice indicates that these cells can differentiate in the absence of the thymus. Besides $CD3^+CD8^+$ cells we can also detect a population of $CD3^-CD8^+$ cells in the IEL fraction [5 and this report]. Although more dominant in the IEL of athymic mice this $CD3^-CD8^+$ population is also found in euthymic mice. Furthermore such $CD3^-$ $CD8^+$ cells are also present in SCID/SCID mice. Both this finding and the association with asialo-GM1 staining for at least part of these cells strongly suggest a relationship with non-lymphoid cells which express natural killer (NK) activity. In that case cells with a $CD3^-CD8^+$ phenotype would represent a novel phenotype for NK cells in the mouse.

2. MATERIALS AND METHODS

2.1. Animals

8-10 weeks old specific pathogen free (SPF) euthymic and athymic (nude) Balb/c mice were obtained from the Central Animal Facilities of the Radio Biological Institute TNO, Rijswijk, The Netherlands. CB 17 SCID/SCID mice of 12 weeks of age were obtained from the Jackson Laboratory, Bar Harbor ME, U.S.A.

2.2. Cell isolation and FACS analysis.

The isolation of IEL from the epithelial layers of the small intestine and the preparation of single cell suspensions from mesenteric lymph nodes was done as described in detail previously [4,5].
Monoclonal antibody (mAB) 145-2C11 (a gift of Dr.J.A.Bluestone), directed against mouse CD3 was used directly conjugated with FITC. mAB 53-6.7 (Becton & Dickinson, Sunnyvale, USA), directly conjugated with FITC or biotin was used for CD8 staining. Biotinylated mAB's were used in combination with Streptavidin-Phycoerythrin (Becton & Dickinson, Sunnyvale, USA). Asialo-GM1 was detected with a polyclonal rabbit anti-mouse asialo-GM1 antibody (Wako Chemicals, Dallas, USA) in combination with a FITC conjugated horse anti-rabbit polyclonal antibody (Miles, Elkhard, USA). For FACS analysis cells were washed once in PBS, 1% BSA and incubated with a mAB at the appropriate dilution for 30 minutes at 4°C. For the biotin- conjugates, the first step was followed, after washing twice in PBS, 1% BSA, by a second incubation step with Streptavidin-Phycoerythrin. After the final incubation, cells were washed twice in PBS, 1% BSA and resuspended in the same medium at a final concentration of 10^6 cells/ml. Labelled cells were analyzed using a FACScan equipped with the Consort 30 program and Paint-a-Gate software (Becton & Dickinson, Sunnyvale, USA).

2.2. Immunofluorescence histology.

The intestine was processed in 'Swiss rolls' and snap frozen in liquid nitrogen. 6µm cryostat sections were cut and incubated with mAB's against CD3 and CD8. After washing a second incubation with streptavidin-phycoerythrin was done for CD8 staining.
Fluorescent cells were visualized with a Leitz microscope, equipped for two-colour immunofluorescence.

3. RESULTS

IEL from euthymic as well from athymic mice, cells were isolated, stained with mAB's as indicated under materials and methods, and used for FACS analysis. Cells with a lymphoid appearance were selected, using forward and perpendicular light scatter gating.
The results of a representative double staining experiment of IEL with mAB's, specific for CD3 and CD8 is shown in Fig.1. As can be seen in Fig.1A the vast majority of IEL in euthymic animals is $CD3^+CD8^+$. In athymic animals, however, two distinct $CD8^+$ IEL populations can be detected: a $CD3^+CD8^+$ and $CD3^-CD8^+$ population (Fig.1B).
Similar results were obtained for nude mice using CD3 and CD8 immunofluorescence double staining on frozen sections of small intestine (Fig.2 A&B). Moreover, cells staining both for CD3 and CD8 can also be detected in SCID/SCID mice (Fig.2 C&D). The numbers of such double positive IEL, however, are reduced in athymic and SCID/SCID mice when compared to euthymic animals (results not shown). Also cells with the $CD3^-CD8^+$ phenotype are present in the epithelial cell layers both in the athymic and SCID/SCID mouse intestine (Fig.2 B&D). The numbers of these cells are similar in both mutant strains. These $CD3^-CD8^+$ cells can also be

found in the intestinal epithelial layers of euthymic mice. The numbers, however, are clearly lower than in athymic and SCID/SCID mice (results not shown).

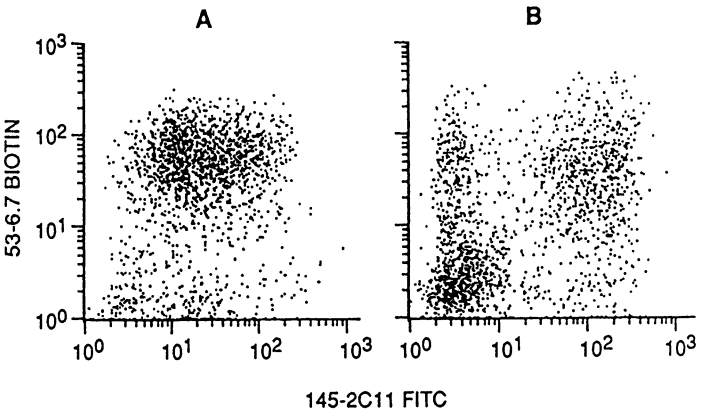

Fig.1. FACS double staining of IEL, isolated from euthymic (A) and from athymic Balb/c mice (B). The mAB's used are directed against CD3 (145-2C11-FITC) and CD8 (53-6.7-Biotin).

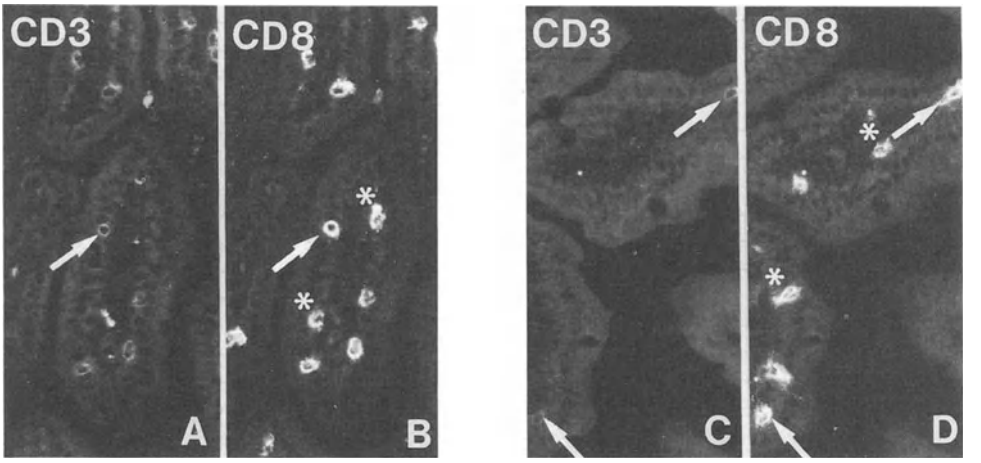

Fig.2. Immunofluorescence double staining of sections from athymic BALB/c (A&B) and CB17 SCID/SCID (C&D) mice with anti-CD3 (A&C) and anti-CD8 (B&D). Arrows: $CD3^{+}CD8^{+}$ cells; asterix: $CD3^{-}$-$CD8^{+}$ cells.

CD8 positive cells in the IEL fraction and mesenteric lymph nodes from euthymic and athymic mice were further analyzed using anti-CD8 and anti-asialo-GM1 antibodies. In Fig.3 the results are shown of a typical double staining experiment with these antibodies on the IEL fraction of athymic mice (Fig.3A) and mesenteric lymph node cells of euthymic mice (Fig.3B).

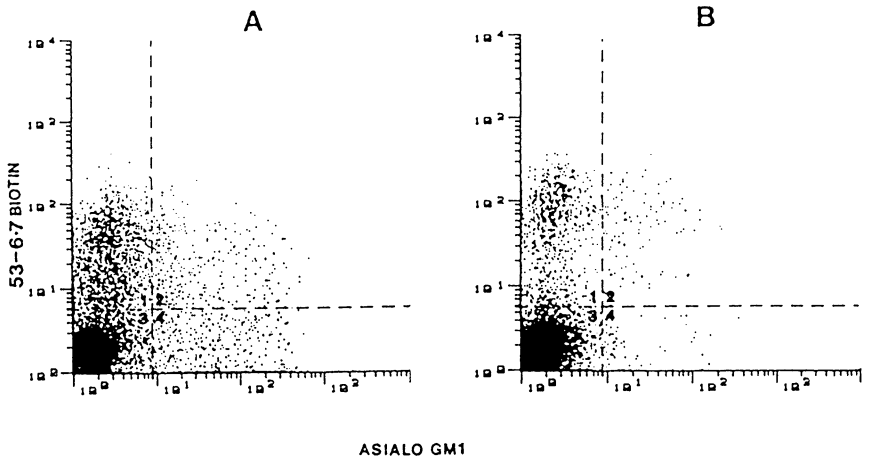

Fig.3. FACS analysis of IEL (A), isolated from the small intestine of an athymic mouse and cells derived from the mesenteric lymph nodes (B) of an euthymic animal. The vertical axis represents CD8 staining (53-6.7-Biotin), the horizontal axis represents asialo-GM1 staining (polyclonal rabbit anti-murine asialo-GM1). Staining with only second step reagents was negative.

In the IEL fraction of athymic mice two populations of IEL can be recognized which are positive for asialo-GM1: one, which is positive for CD8 and one, which is negative for this antigen (Fig.3 A). In the IEL fraction of euthymic mice we can also detect these asialo-GM1$^+$CD8$^+$ and asialo-GM1$^+$CD8$^-$ populations (results not shown). However, the cell numbers of these asialo-GM1$^+$ populations are significantly increased in athymic animals when compared to euthymic mice. In mesenteric lymph nodes of euthymic animals also small numbers of asialo-GM1$^+$CD8$^+$ cells can be found (Fig.3B). The asialo-GM1$^+$CD8$^-$ population at that site, however, is strongly reduced. In the mesenteric lymph nodes of athymic mice on the other hand, asialo-GM1$^+$CD8$^+$ and asialo-GM1$^+$CD8$^-$ can be detected in equal numbers (results not shown).

4 DISCUSSION

The vast majority of intestinal intraepithelial lymphocytes in euthymic mice is CD3$^+$CD8$^+$ (Fig.1A). We and others [1-5] have shown that these cells almost exclusively express the CD3 associated $\gamma\delta$ receptor. Such $\gamma\delta$ receptor bearing IEL are THY-1 negative or THY-1 low [4,5]. Also some $\alpha\beta$ TCR expressing cells can be found in the IEL fraction of euthymic mice [4].
The expression of the $\alpha\beta$ TCR in the intestinal IEL fraction, however, is dependent on the presence of viable microbiological antigens in the gut, since in germfree animals only Thy-1$^-$ CD8$^+$ CD3$^+$ IEL can be detected [6]. No THY-1$^+$ $\alpha\beta$ TCR expressing IEL are found in such animals. Moreover, exposure of germfree mice to a viable bacterial flora results in an increase of the percentage of $\alpha\beta$ TCR expressing cells in the IEL fraction [De Geus et al., manuscript in preparation]. Thus, studies in microbiologically

clean animals such as germfree or SPF mice will result in a relative abundance of $\gamma\delta$ receptor expressing cells in the IEL fraction, whereas $\alpha\beta$ TCR carrying IEL will only be found in contaminated (euthymic) mice, which are under an active microbiological pressure. Surprisingly, we and others [1,2,4] have not been able to detect α or β TCR chain expression by immunoprecipitation or RNA hybridization on IEL, not even in mice kept under conventional conditions. This is probably due to the relatively low expression of $\alpha\beta$ TCR on IEL [5, De Geus et al., manuscript in preparation].

Thus, in euthymic mice virtually all $CD8^+$ IEL do co-express CD3 and either the $\gamma\delta$ or the $\alpha\beta$ T cell receptor. In athymic (nude) mice, however, another $CD8^+$ IEL population can be observed: a $CD3^-CD8^+$ population (Fig.1B). Actually, cells with a similar phenotype, although not by FACS analysis, can be found in euthymic mice using immunehistological techniques (data not shown). Cells expressing this $CD3^-CD8^+$ phenotype represent a novel phenotype in the mouse. Cells with such a $CD3^-CD8^+$ phenotype have actually been reported in rat and man [8,9]. These $CD3^-CD8^+$ cells were shown to be cells with NK activity [8,9].

In order to determine whether these $CD3^-$ $CD8^+$ IEL are of lymphoid origin, we analysed IEL in CB17 SCID/SCID mice by immunofluorescence using mAB's specific for CD3 and CD8. The SCID/SCID mouse has a major recombinase defect, resulting in the absence of B and T cells [10]. NK cell reactivity and cell numbers in this mouse mutant are normal or even enhanced when compared to normal animals [M.Bosma, personal communication, and 10]. As can be seen in Fig.2 cells with a $CD3^-CD8^+$ phenotype can be found in the SCID/SCID mouse to a similar extent as in the athymic animals. This observation strongly suggests that the $CD3^-CD8^+$ cells in the IEL fraction are not of lymphoid origin.

To investigate whether these cells might be NK cells, we analysed IEL by double staining with anti-CD8 and anti-asialo-GM1 antibodies. Asialo-GM1 is known to be expressed on murine $CD3^-CD8^-$ cells displaying NK activity [11,12]. In the IEL fraction and mesenteric lymph nodes of euthymic and athymic mice we found two types of cells with a possible NK phenotype. One population, as described before [11,12], which is asialo-$GM1^+CD8^-$ and another population which is asialo-$GM1^+CD8^+$. In the athymic (nude) mouse the cell numbers of the asialo-$GM1^+$ populations are enhanced in the IEL fraction and mesenterial lymph nodes when compared to the euthymic animal. This observation is in line with other observations since in athymic (nude) mice and rats NK cell numbers and NK activity have been shown to be normal or even enhanced [13, 14]. One explanation for the fact that asialo-$GM1^+CD8^+$ cells have not been observed before in the mouse may be the fact that most studies have been performed on splenic NK cells. These splenic NK cells are indeed asialo-$GM1^+CD8^-$ [L. Nagelkerken, personal communication, and 11,12]. Further phenotyping and functional testing of the asialo-$GM1^+CD8^+$ IEL has to clarify whether these cells are $CD3^-$ and show NK activity.

Finally, it is also evident (Fig.3) that the SCID/SCID mouse contains low but significant numbers of $CD3^+CD8^+$ cells. It has been reported that 10-25% of the SCID/SCID mice are 'leaky' for their recombinase defect [10,15]. AS a result of that some B cells and some cells expressing the $\alpha\beta$ TCR can develop in these animals. This indicates that in such 'leaky' animals the recombinase de-

fect has been restored to some extent. We did not yet analyse receptor usage in the intestinal IEL fraction of SCID/SCID mice, but this may well be the first observation on the presence of γδ receptor expressing cells in SCID/SCID mice.

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Alpha/beta T cell receptor expression in the intestinal epithelium of rodents

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1. INTRODUCTION.

Although most T cells in the periphery express the alpha/beta heterodimeric form of the T cell receptor associated with the CD3 complex of proteins, recent reports have suggested that the majority of lymphocytes in mouse small intestine use the gamma/delta TcR [1,2]. These reports were based on the fact that after surface labelling IEL and immunoprecipitating with anti-CD3, anti-alpha chain or anti-gamma chain, the only polypeptide molecules detected were of a molecular weight similar to that of the gamma/delta chains of the T cell receptor. In the present study we have analysed intraepithelial lymphocytes for TcR expression, in normal and athymic BALB/c mice and in normal DA rats, by immunoperoxidase histochemistry on frozen sections. We provide evidence that a substantial fraction of IEL in normal mice and rats use the alpha/beta TcR. In IEL from athymic mice however there is no evidence of alpha/beta TcR expression. In addition we have studied TcR usage in the Thy1+ and Thy1- subpopulations of normal mice IEL by immunofluorescence on isolated cells. Double staining revealed a skewed distribution of TcR expression with the majority of Thy1+ IEL bearing the alpha/beta TcR. The frequency of alpha/beta TcR expression was greatly diminished on Thy1- IEL.

2. RESULTS.

2.1 TcR expression and phenotype of IEL in normal and athymic mice as determined by immunohistochemistry on frozen sections.

TcR expression and phenotype of IEL in normal and athymic mice was determined by immunohistochemistry on frozen sec-

tions. Differential counts of immunoperoxidase positive IEL and unstained IEL were made for a panel of T cell antibodies. In both groups most IEL were CD45+, CD3+, and CD8+. In normal mice 46.2% of IEL were Thy1+, 10.7% CD4+, 21.1% KJ16+ (Vβ8.1 and 8.2) (Figure 1), and 46.6% H57.597+ (pan alpha/beta). Cells staining with these antibodies were absent in athymic mice.

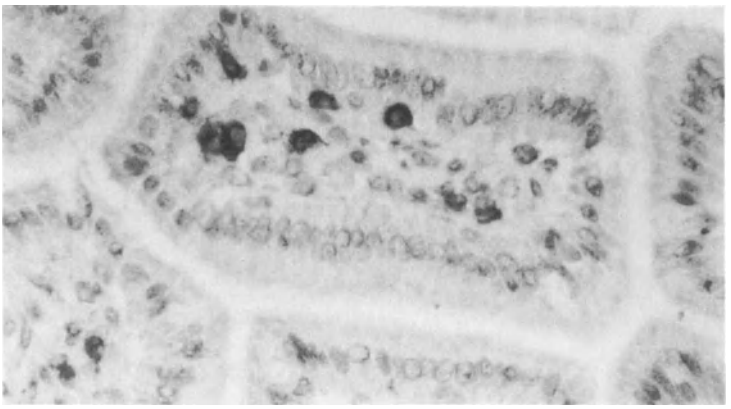

Figure 1. KJ16+ IEL in the intestinal mucosa from normal mice. Small intestine was snap frozen, sectioned and stained for Vβ8 expression using the immunoperoxidase technique. KJ16+ (Vβ8.1 & 8.2) cells can be seen in the epithelium. (Immunoperoxidase, original magnification x400).

2.2. TcR expression and phenotype of IEL isolated from normal mice.

IEL were isolated from normal mice, double stained for TcR expression and Thy1, and analysed by fluorescence microscopy. In both the Thy1+ and Thy1- IEL populations the majority of cells were CD45+, CD3+ and CD8+. CD4+ cells were restricted to the Thy1+ population. Similarly TcR expression was markedly skewed betweeen the two populations with the majority of alpha/beta positive cells also bearing Thy1 (Table 1).

Table 1. TcR distribution on Thy1+ and Thy1- IEL from normal mice.

	% Thy1+ IEL	% Thy1- IEL
KJ16	25.9 ± 9.7 (11.7-33.0)	4.1 ± 1.0 (2.9-5.2)
F23.1	32.7 ± 7.9 (23.8- 35.4)	12.1 ± 1.9 (10.0-13.6)
H57	94.0 ± 6.9 (86.0-100)	24.4 ± 12.4 (13.3-45.9)

Isolated IEL from groups of 3 normal mice were double stained for alpha/beta TcR and Thy1 and the number of Thy1+, $\alpha\beta$+ and Thy1-, $\alpha\beta$+ cells enumerated by fluorescence microscopy. The results are expressed as the proportion of alpha/beta positive cells as a percentage of either the Thy1+ or Thy1- cell population.

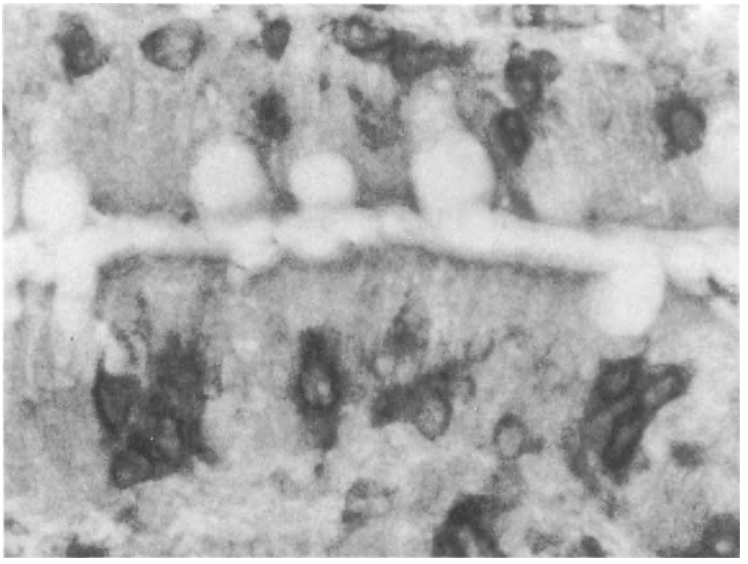

Figure 2. R73+ IEL in the intestinal mucosa from rat. Small intestine was snap frozen, sectioned and stained for R73 expression using the immunoperoxidase technique. Alpha/beta positive cells can be seen in the epithelium. (Immunoperoxidase, original magnification x1000)

2.3. TcR expression on IEL in rat intestinal epithelium.

Using immunoperoxidase histochemistry on frozen sections of DA ileum a mean value of 61.4 ± 13.2% (46.9-78.2) of IEL were stained by the monoclonal antibody R73 which is specific for an invariant determinant of the alpha/beta TcR (Figure 2). Analysis of isolated IEL by flow cytometry gave similar results and showed that 48.3 ± 11.9% (40-62) of cells expressed alpha/beta TcR.

3. DISCUSSION.

These results for alpha/beta TcR expression in mouse and rat intestinal epithelium do not support the view that this lymphoid compartment is dominated by T cells bearing the gamma/delta TcR. It has also been widely assumed that gamma/delta IEL are derived from foetal thymic emigrants that possess a tissue specific tropism, however this cannot be the case since IEL are present, albeit in reduced numbers, in thymus deprived and congenitally athymic mice. The observation that nude mice IEL express CD3 but not alpha/beta TcR is consistent with the view that gamma/delta IEL may be thymus independant. In view of the skewed distribution of TcR expression amongst the Thy1+ and Thy1- cell populations it does appear that IEL in rodents consist of two distinct lineages, thymus-dependant lymphocytes which utilise the alpha/beta TcR and thymus-independant, bone marrow derived, lymphocytes which express an alternative CD3-associated TcR [3,4].

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Do all murine intraepithelial leukocytes (IEL) have T cell receptor rearrangements?

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Introduction

Murine IEL express the γ/δ T cell receptor (TcR) [1,6] and have a host of cytolytic activities. The function and relationship to T cells of a major subgroup or IEL (50% $Thy1^-CD5^-CD8^+$) is unclear [4]. In C57BL/6 mice, IEL express $V\gamma7$ but dendritic epidermal cells (DEC) express $V\gamma5$ and adult peripheral $CD4^-$ $CD8^-$ T cells express $V\gamma4$ [1,7]. IEL are also distinct in that they have a granulated morphology, and express CD8 [5]. In order to further define the heterogenity in IEL with respect to the expression of the γ TcR V-genes, we examined several strains of mice for expression of $V\gamma7$ and $V\gamma4$ mRNA. To define the relationship of the $Thy1^-$ $CD8^+$ and $Thy1^+CD8^+$ IEL to other γ/δ T cells, we also examined $Thy1^-$ and $Thy1^+$ subsets of IEL for $V\gamma4$ and $V\gamma7$ gene rearrangement.

Material and Methods

Female BALB/c, CBA/J, C57BL/6 mice (Jackson Labs, Bar Harbor) were used between 8-14 weeks of age. Scid (scid/scid) mice were provided by Dr. L. Schultz (The Jackson Laboratory). IEL were isolated [8] and stained with FITC-anti-Thy1 (Becton Dickinson). The cells were sorted in $Thy1^-$ and Thy^+ subsets on an EPIC 541 FACS. Cellular DNA was extracted and digested with EcoRI and 7-10 μg electrophoresed through 0.6% agarose and transferred overnight onto Genescreen plus. Blots were hybridized with ^{32}P-dTTP and ^{32}P-dCTP labelled probes overnight at 42°C, washed with 2X SSC, 1.0% SDS at 60°C and 0.2X SSC, 0.1% SDS at 60°C for 30 minutes each and exposed on Kodak AKR X-Omat film overnight. Total cellular RNA was extracted using the guanidinium isothiocyanate-CsCl gradient method [3]. Ten μg was electrophoresed through 1.2% agarose containing 1.8% formaldehyde. Transfer to Genescreen plus was done with 2X SSC and hybridization with $V\gamma4$ and $V\gamma7$ cDNA probes [9] or $C\beta$ [2] performed as for Southerns. The blots were then washed with 2X SSC, 1% SDS at 60°C for 30 minutes and exposed.

Results

IEL from Balb/c, CBA/J and C57BL/6 mice expressed mRNA for both $V\gamma7$ and $V\gamma4$, but not $C\beta$ mRNA. IEL from SCID mice did not express mRNA for either of the $V\gamma$ region genes. Southern analysis of DNA showed that both $V\gamma4$ and $V\gamma7$ were rearranged in the $Thy1^-$ IEL; while in the $Thy1^+$ subset only $V\gamma4$ was rearranged.

Discussion

IEL from different strains of mice can express both Vγ4 and Vγ7 mRNA and this distribution of Vγ gene transcription is not strain related. The $Thy1^-$ subset alone has the ability to rearrange the Vγ7 gene. This difference in gene rearrangements in the two subsets of IEL suggests that the $Thy1^-$ IEL may be the source of IEL that express the Vγ7 T cell receptor. These two major subsets of IEL may represent separate pathways or different stages of differentiation of IEL. The absence of Vγ7 in $Thy1^+$ IEL suggests that this variable region gene is not responsible for the specific localization of IEL to the intestinal epithelium. This is consistent with the fact that SCID mice have granulated IEL with the $Thy1^-CD8^+$ phenotype and do not express γ-TcR mRNA. The relationship of the differential expression of these two variable region genes to the function of IEL is unknown.

Acknowledgements

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T cell receptor expression by CD8$^+$ intraepithelial lymphocytes from mouse small intestine

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Introduction

Intraepithelial lymphocytes form one of the largest populations of T lymphocytes in the body and probably play an important role in both immune defence and in immunopathology in the intestine. Nevertheless, their functions are unclear, partly because they have an unusual phenotype, with a large proportion of CD8$^+$ T cells, many of which do not express pan T cell markers such as Thy1 or CD5 (1).

Recently it has been suggested that most CD8$^+$ IEL in mice and chickens carry the $\gamma\delta$ form of the TcR, normally expressed on only a small proportion of mature T cells (2,3). In this study, we have tested this idea by examining directly the expression of different families of $\alpha\beta$ TcR on both CD4$^+$ and CD8$^+$ IEL from mouse small intestine.

Materials and Methods

Intraepithelial Lymphocytes: IEL were obtained from normal small intestines by mechanical disruption, followed by purification over Percoll gradients as described previously (4). These preparations were >90% lymphocytes.

Phenotypic Analysis of Lymphocytes: CD4$^+$ and CD8$^+$ T cells were detected using phycoerythrin (PE)-conjugated anti-L3T4 antibody (GK 1.5) and FITC-conjugated anti-Lyt2 antibody (3.16.8) respectively, while Thy1 expression was examined using rat anti-Thy1.2 antibody (13.4) plus PE-conjugated anti-rat Ig. Different V_β families of the $\alpha\beta$ TcR were detected using anti-$V_{\beta 6}$ (44.22.1), anti-$V_{\beta 8.1-3}$ (F23.1) or anti-$V_{\beta 8}$1.2 (KJ.16) antibodies followed by FITC- or PE-conjugated anti-mouse or rat Ig second step reagents. Cells were stained with these antibodies at 4°C and then subjected to flow cytometric analysis on a FACS. In most experiments, the staining patterns of IEL were compared with those of lymph node cells (LNC) from the same mice.

Results

All our preparations had the well-known phenotypic characteristics of murine IEL, with around 75% of the cells being CD8$^+$ and only 5-10%

$CD4^+$. There was usually only a small number of sIg^+ B lymphocytes (<6%), confirming that the IEL were not contaminated by lymphocytes from lamina propria or other sources. $Thy1^+$ cells were plentiful among IEL, but double staining revealed that 50% or more $CD8^+$ IEL did not express Thy1. In contrast, $CD4^+$ IEL and both $CD4^+$ and $CD8^+$ LNC were always $Thy1^+$.

The results of representative experiments on TcR expression by $CD8^+$ IEL are summarised in Table 1.

TABLE 1. Summary of phenotype of IEL T Lymphocytes

Cell Source	% Positive Cells			
	sIg	CD8	$V_{\beta 8}/CD8^+$	$V_{\beta 6}/CD8^+$
CBA IEL	5.8	73.0	14.6	n.d.
BALB/c IEL	6.0	70.0	18.2	6.2
CBA IEL	n.d.	60.9	14.0	5.7
CBA LN	n.d.	28.8	26.0	10.6

Numerous $CD8^+$ IEL always expressed $V_{\beta 8}$ or $V_{\beta 6}$ families of TcR and the $V_{\beta 8}$:$V_{\beta 6}$ ratio was identical to that for $CD8^+$ LNC, suggesting a normal repertoire of $\alpha\beta$ T cells among IEL. In some experiments, V_β antigens were expressed by as many $CD8^+$ IEL as LNC, but frequently IEL had somewhat fewer $\alpha\beta^+$ T cells. By extrapolation from the known proportion of $V_{\beta 8}$ or $V_{\beta 6}$ expressing cells among normal T cells, we estimate that at least 50-60% $CD8^+$ IEL are $\alpha\beta$ T cells. In parallel studies of $CD4^+$ IEL, the expression of $V_{\beta 8}$ or $V_{\beta 6}$ was identical to or even greater than that of $CD4^+$ LNC.

Summary and Conclusions

Using highly pure populations of isolated cells we have found that $\alpha\beta^+$ T cells are abundant among normal murine IEL. $CD4^+$ IEL were entirely normal in terms of their TcR expression and although $CD8^+$ IEL usually showed somewhat lower numbers of $\alpha\beta^+$ T cells than mature LNC, the majority of $CD8^+$ IEL seem to express $\alpha\beta$ TcR. These results confirm other recent findings on murine IEL (5) and we conclude that IEL are certainly not a homogenous population of $\gamma\delta^+$ T cells. Further work is required to clarify the apparent discrepancies over TcR expression by IEL, but in the meantime care must be exercised in attributing the functions of IEL to $\gamma\delta^+$ T cells in general.

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22
Tissue distribution and characteristics of an activated T cell sub-population from rabbit gut associated lymphoid tissues

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ABSTRACT. A monoclonal antibody (Mab) was developed against lymphoid cells dissociated from the dome area of rabbit appendix. By immunofluorescence microscopy, the Mab stained a large number of T-cells in the dome, all the T cells associated with M-cells, most T cells in the villous epithelium, some T cells in the gut lamina propria next to the epithelium, and a population of T cells in the periarterial lymphatic sheath of spleen (SPL) and in the interfollicular area of mesenteric lymph nodes (MLN). By analysis with a fluorescence activated cell sorter (FACS), the Mab(+) cells were identified as medium lymphocytes; their numbers in appendix, MLN, and SPL were in a descending order: 32, 28, and 19%, respectively. The Mab(+) cells were obviously mature T cell blasts because lymphoid cells from various tissues, when cultured with concanavalin A (Con A), showed a striking increase of Mab(+) cells both in the cell number and membrane fluorescence intensity. Since the Mab(+) cells were very rare in bronchus-associated lymphoid tissues, the characteristics and tissue distribution pattern suggest that this T-cell subpopulation may have a gut oriented migration pathway.

1. INTRODUCTION

In the rabbit, T-cells in the gut mucosa are present in the epithelium of the villi, the lamina propria and the gut associated lymphoid tissue (GALT) such as Peyer's patches and appendix. In the GALT, the vast majority of T-cells are present in the thymus-dependent area and the dome where they intermix with B-cells and macrophages. The function of T-cells in the GALT and their relationship with the T-cells in the villous epithelium and the lamina propria are still obscure. We attempted to study these aspects of the T-cells. We produced a Mab and describe here its reaction with a population of T-cell blasts which are distributed in such a pattern that they seem to have a gut-oriented migration pathway.

2. MATERIALS AND METHODS

A Mab, designated 93C6, and anti-M cell Mab were prepared as described previously [1]. A pan-T cell Mab produced by hybridoma L11-135 [2] was obtained from the American Type Culture Collection, Rockville, MD. Lymphocytes were cultured for 48 hours with Con A (10 ug/ml) in RPMI 1640 medium containing 10 % fetal bovine serum, 5 x 10^{-5} M 2- mercaptoethanol, and 20 ug/ml gentamicin. Thymocytes were cultured for an additional 24 hours in fresh RPMI 1640 medium supplemented with 30% IL-2 or Con A-pulsed spleen cell culture supernatant [3]. Eighteen hours before harvest, the cells were pulsed with 1 uCi/ml [^{3}H]-Thymidine. Frozen tissue sections were stained using indirect immunofluorescence techniques. Cells were stained indirectly with fluorescent antibodies and were analyzed in a FACScan (Becton Dickinson, Mountain view, CA).

3. RESULTS

Using 93C6 and the pan-T cell (L11-135) Mab, it was seen that 93C6 Mab stained only a subpopulation of T cells in the periarteriolar lymphatic sheath of SPL and in the interfollicular region of MLN. In gut-associated lymphoid tissues (sacculus rotundus, cecal patch, Peyer's patches and appendix), 93C6 also stained a subpopulation of T cells, which were present only in the dome and dome epithelium associated with M-cells (Table 1). In the absorptive villi of the small intestine, 93C6(+) cells were restricted to the epithelium and the lamina propria next to it. The 93C6(+) cells were scarcely seen in the thymus (THY) and were rarely seen in bronchus-associated lymphoid tissues (BALT).

TABLE 1. 93C6(+) cells in lymphoid tissues and among lymphocytes freshly isolated from tissues and from cultures stimulated with Con-A.

Tissue staining		FACS analysis (%)	
Tissue	Area	Fresh Cells	Con-A Stimulated
GALT	Dome & dome EP	32	73
MLN	IFR[a]	28	61
SPL	PALS	19	77
THY	fsc	5	15(72)[b]
GUT	EP & GLP	ND	ND

[a] EP, epithelium; IFR, Inter-follicular region; PALS, Periarteriolar lymphatic sheath; fsc, few scattered cells; GLP, gut lamina propria; ND, not done.

[b] ( ) are cells stimulated with Con-A plus IL-2.

By FACS and fluorescence microscopy, the 93C6(+) cells were mostly seen as medium size lymphoblasts. Their numbers in appendix, MLN, SPL, and

THY were in a descending order: 32, 28, 19, and 5%, respectively. All the 93C6(+) cells were also stained by the pan T Mab, suggesting that they are a subpopulation of T cells.

After 48 hour Con A stimulation in culture, the 93C6(+) cells from appendix, SPL, and MLN increased greatly in number and fluorescence intensity. The 93C6(+) thymocytes increased from 5 to 15% when stimulated with Con A alone; they increased to 72% after a further culture in medium containing IL-2. All these results suggest that 93C6(+) cells are mature T cell blasts.

4. DISCUSSION

The 93C6 Mab appears different from those made against intra-epithelial lymphocytes of the gut in that it reacts only with mature T cell blasts. This is evidenced by the cell size of the 93C6(+) cells and the increases in Con A stimulated cultures of lymphocytes from THY and secondary lymphoid tissues.

From their tissue localization, 93C6(+) cells could be the T cells activated in situ or the T cell blasts in a migration journey. The latter possibility is more likely because of the specific localization in tissues. The 93C6(+) cells could be derived from naive T cells activated by intestinal ligands while moving to M cells in the dome epithelium. After the activation, they move to the dome area and exit the GALT. They migrate to SPL via MLN, thoracic duct and blood circulation, and arrive at the villous epithelium via the postcapillary venules of the GLP. Because 93C6(+) cells were not seen in the BALT and lung mucosae, the 93C6(+) cells are obviously different from IgA blasts of MLN [3,4] in that they have a gut-oriented migration pathway while the IgA blasts migrate randomly to various mucosal tissues.

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HML-1, an antibody raised against intestinal lymphocytes, recognises an antigen appearing on activated peripheral blood lymphocytes

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Introduction:

The monoclonal antibody HML-1 [1] recognizes about 95% of intraepithelial lymphocytes and to a lower extent lamina propria lymphocytes (LPL) and mesenteric lymphoblasts. In order to characterize T cells expressing HML-1 in the lamina propria we investigated the number of HML-1 positive cells and their distribution on the T cell subsets CD4 and CD8. Since LPL are known to have an increased state of activation when compared to lymphocytes of other origin [2] we also investigated the *in vitro* inducibility of HML-1 on PBL.

Materials and Methods:

LPL were obtained from macroscopically uninvolved small or large bowel segments of 9 patients (5 female, 4 male; median age 56 (47 - 73) years) undergoing surgical bowel resection. PBL were from these patients and from healthy volunteers. PBL were isolated by Ficoll density gradient centrifugation, LPL by an enzymatic method described in [2].

For cytofluorographic analysis cells were stained with directly or indirectly FITC- or PE-conjugated mAbs and analysed by flow cytometry (FACSCAN) using a Consort-30 program.

PHA- and ConA-blasts were generated by incubating PBL for 18h with 1µg/ml PHA or 10 µg/ml ConA whereafter the mitogen was removed by washing the cells twice.

For stimulation studies, cells were held in culture with or without mitogens, antigens, phorbolester or rIL-2.

Results:

Whereas less than 2% (range 0 - 2) of PBL expressed HML-1, 38% (range 9 - 54) of LPL were HML-1 positive. While in the CD8 subpopulation of LPL the majority of cells expressed HML-1 (63% (range 40 - 85)), in the CD4 subpopulation the HML-1 negative phenotype predominated (22% (range 12 - 48) HML-1 positive cells).

In vitro studies of PHA-blasts held in culture for up to 12 days showed that HML-1 was expressed, but clearly delayed and more prolonged than the IL-2 Receptor (IL-2R). A decrease in HML-1 expression was not observed before day 8. In contrast to PHA-blasts, LPL held in culture for the same period without any stimulus showed only a slight decrease in the number of HML-1 positive cells.

Differences in the expression of HML-1 vs. IL-2R in response to the investigated stimuli are summarized in Tab. 1.

TABLE 1: Characteristics of HML-1 and IL-2 Receptor expression.

	IL-2R	HML-1
18h blasts: inducibility by PHA vs. ConA	ConA>PHA	PHA>ConA
Enhancing effect of rIL-2 in the culture medium on 18h blasts	++	-
Inducibility on PBL by		
antigens	+	+
rIL-2	+	+
PHA	+++	++
phorbolester	+++	++
Enhancing effect of coincubation with PHA and phorbolester	+	-
maximal expression under the conditions tested	up to 95%	<59%
Distribution on T cell subsets		
CD4 and CD8	CD4>CD8	CD8>CD4

Discussion:

The finding that HML-1 is expressed on less than 50% of LPL and its predominant expression on CD8 positive T cells indicate a functional role of this antigen in the intestinal mucosa rather than indicating a role as homing receptor [3].
Since HML-1 is inducible on PBL by *in vitro* activation it can be classified as an activation antigen. Differences in comparison to IL-2R expression suggest different signal transducing pathways.
LPL express HML-1 more stably than *in vitro* induced blasts. It therefore should be investigated if HML-1 is a new surface marker of memory T cell function.

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Lymphoepithelial interactions associated with proximal colonic lymphoid tissue in the mouse

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ABSTRACT - The proximal colon of the rodent contains an organized lymphoid nodule consistently located 25% of the distance from the cecum to the anus. We have undertaken a morphokinetic evaluation of crypts associated with this lymphoid tissue, as compared to crypts on adjacent plicae. Results demonstrated that the crypts associated with this lymphoid nodule are deeper, contain greater numbers of columnar and goblet cells and have a greater cell production than crypts of adjacent alymphoid plicae. The distribution of metaphases is shifted toward the upper part of lymphoid associated crypts and the binding of radiolabelled recombinant human urogastrone (rhUG/EGF) is greater even in mice treated with steroid to deplete the lymphoid elements. These results indicate a significant lymphoepithelial interaction associated with proximal colonic lymphoid tissue (PCLT), and suggest that this interaction may be mediated by a locally acting paracrine mechanism(s).

Introduction

Lymphoid tissue of the small and large intestine has both a diffuse intraepithelial component and organized lymphoid aggregates or nodules (1). The lymphoid nodules of the colon (2,3) are much less well described than the Peyer's patches of the small intestine, and their frequency has probably been underestimated (4). The proximal colon of rodents contains an organized nodule which is consistently located about 25% the distance from the cecum to the anus (2,3). This nodule is predominantly (>85%) comprised of immature IgM^+, $B220^+$ B cells with a small (mostly peripheral) T cell population. Germinal centers are rare and approximately 15% of the B cells in this nodule possess the $CD5^+$ phenotype (2). In addition, the epithelium covering this nodule has many of the features of follicle associated epithelium previously described overlying small intestinal lymphoid aggregates, including the presence of M cells (5).

During the course of these studies it became evident that the crypts associated with PCLT differed morphologically from crypts associated with adjacent plicae which did not contain any lymphoid aggregates. Consequently, this investigation was undertaken to characterize any differences in epithelial cell proliferation and differentiation associated with PCLT.

crypt with a significantly increased proportion of metaphases in the upper 1/3 of the crypt ($p \leq .001$) as compared to crypts in non-lymphoid containing plicae. These results clearly demonstrate that crypts associated with lymphoid tissue of the colon differ from crypts in alymphoid plicae not only in their size and proliferative activity, but also in the distribution of the proliferative compartment within the crypt itself.

The above results intimated a "microenvironmental" effect of lymphoid tissue influencing mucosal cell proliferation in the colon. Therefore, we examined the distribution of growth factor receptors in various regions of the proximal colon using radiolabeled urogastrone (^{125}I-rhUG/EGF). Results demonstrated that ^{125}I-rhUG/EGF uptake was significantly increased in the segment containing PCLT as compared to adjacent segments containing no large lymphoid aggregates ($p \leq .005$). The uptake remained high in the lymphoid containing segment even after prior treatment of the mice with 125 mg/kg hydrocortisone ($p \leq .001$), a treatment which has been previously shown to deplete PCLT by up to 70% (2,3). Results of these studies demonstrated increased rhUG/EGF binding to segments containing lymphoid aggregates, which correlates with the increased proliferative activity in the same segment. Taken together, these results strongly suggest that the increased cell proliferation seen in crypts associated with lymphoid tissue may be mediated through an increase in growth factor receptor expression in the epithelium of these areas.

TABLE 1

Comparison of Selected Parameters of Epithelial Cell Proliferation and Differentiation for Proximal Colonic Epithelium Associated with Lymphoid Aggregates versus Alymphoid Adjacent Plicae

Parameter	Ratio : Lymphoid Associated Mucosa / Adjacent Mucosa	p Value
Crypt Depth	1.46 ± 0.09	≤.001
Cells/Crypt Column	1.55 ± 0.02	≤.001
Goblet Cells/Crypt Column	1.42 ± 0.09	≤.001
Crypt Cell Production Rate (Metaphase Arrest Technique)	3.07 ± 0.43	≤.001
Metaphases in Upper 1/3 of Crypt / Metaphases in Lower 1/3 of Crypt	8.01 ± 2.25	≤.001
^{125}I-rhUG/EGF uptake		
Without Hydrocortisone	2.87 ± 0.47	≤.005
With Hydrocortisone	1.80 ± 0.12	≤.001

Discussion

The results of these studies establish that the crypts associated with PCLT are larger than those of adjacent alymphoid plicae, and have a greater crypt cell production rate. In addition the metaphases in these crypts are distributed along the entire length of the crypt rather than being confined to the lower two-thirds as is usually the case. In this regard the crypts associated with PCLT resemble the pre-neoplastic crypts that have been described in animals treated with carcinogens such as dimethylhydrazine (7), and in patients at increased risk for colon cancer (8). It is interesting to note that carcinogen induced tumors are frequently associated with sites of colonic lymphoid aggregates, particularly in the proximal colon (9). This locally increased proliferation may target the epithelium at this

Materials and Methods

Morphokinetic Studies

All studies were performed using male Balb/c mice (25-30 gm). For morphokinetic studies, animals were injected intraperitoneally with vincristine sulfate (1 μg/gm body weight) and killed by cervical dislocation 2 hours later between 1500 and 1600 hours to minimize diurnal variations. The colon was excised and divided along the mesenteric edge. PCLT was removed with adjacent plicae and fixed in Carnoy's solution. Serial sections (5 μm) from each sample were cut and stained with H&E. Sections which were complete and well organized, and which contained the lymphoid nodule and 3 adjacent plicae on each side were selected for analysis. Crypt depth (by micrometer scale), crypt cell production rate (CCPR), distribution of metaphases within individual crypts and the number of goblet cells per crypt section were determined for each of 60-100 sample crypts in each sample area.

Biodistribution Assays

For the studies involving biodistribution of ^{125}I-Urogastrone (^{125}I-rhUG/EGF; a gift from Chiron Corp. to Dr. Jon Thompson at our Institution), 2 mg of urogastrone was labeled with 5 mCi of ^{125}I using the chloramine-T method (6) with specific activity of 2-3 μCi/μg. The biochemical integrity of this product was confirmed by polyacrylamide gel electrophoresis and autoradiography, and its biological integrity by its ability to bind to the A431 cell line in a dose responsive manner. Mice were injected with 10 μCi of ^{125}I-rhUG/EGF and killed 2 hours later. Colons were excised and tissues isolated. PCLT was identified and removed by cutting along its margin. The segment immediately adjacent to PCLT was removed, along with 2 segments both proximal and distal. Tissues were fixed in Carnoy's solution and counted for radioactivity. The percent of injected dose per gram was compared in PCLT versus adjacent alymphoid plicae. All tissues were subjected to histological evaluation to confirm the presence or absence of lymphoid aggregates in the tissues counted. An additional group of mice was injected subcutaneously with 125 mg/kg hydrocortisone acetate to deplete the lymphoid element of PCLT (2), and biodistribution studies were performed 72 hours later as above.

Data Analysis

Results are presented as ratios of the absolute values obtained for the lymphoid associated plicae compared to those for adjacent alymphoid plicae. All errors were compounded and the results subjected to statistical evaluation using a two-tailed Student's t-test at the $p \leq 0.05$ level of significance.

Results

In these studies we have examined the proliferative and differentiative characteristics of the colonic mucosa associated with PCLT (Table1). It can be seen that crypts associated with lymphoid tissue were significantly deeper ($p \leq .001$), had a significantly greater number of cells/crypt column ($p \leq .001$) and contained significantly more goblet cells/crypt column ($p \leq .001$) as compared to crypts in alymphoid plicae. It should be noted however that the ratio of columnar cells to goblet cells was unaltered in these same crypts. Crypts associated with lymphoid tissue demonstrated a significantly increased crypt cell production rate ($p \leq .001$), and the metaphases were observed to have a different distribution within the

site for differential effects of chemotherapeutic agents and radiation (10). The epithelium associated with PCLT exhibits a greater binding of ^{125}I-rhUG/EGF, suggesting increased growth factor receptor expression on these cells. Clearly this may correlate with the increased cell proliferation and greater crypt size observed at this site. It appears that this binding is associated primarily with the epithelium, as mice treated with hydrocortisone to deplete the lymphoid elements of PCLT also have significantly increased (although numerically lower) binding of rhUG/EGF. Although there is not a statistically significant difference between the ratios in untreated and hydrocortisone treated mice, this result does not exclude the possibility that some of the rhUG/EGF is bound by lymphoid or other elements of this colonic segment. The pattern of differentiation into the major cell types of the mucosa (columnar and goblet cells), does not appear to be altered in the enlarged crypts associated with PCLT. However, this study did not examine possible differences in Paneth and enteroendocrine cell populations.

In summary, it is clear that there are lymphoepithelial interactions at this site. Although these interactions are clearly evident in the crypts intimately associated with PCLT, they do not appear to extend to adjacent plicae. This suggests that these interactions may have a paracrine basis, mediated by factors active over a short range. Further investigation will be needed to define the character of these effector factors and the mechanism by which they modify the epithelium. The role, in turn, of the epithelium (if any) in establishing or maintaining the lymphoid nodule also remains to be determined.

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Salmonella increases M cell numbers in mouse Peyer's patch follicle associated epithelium

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1. INTRODUCTION. It is now widely recognized that local immune reactions mediated by activated gut associated lymphoid tissue (GALT) increase crypt depth, crypt cell production rate and intraepithelial lymphocyte numbers (Mowat & Ferguson, 1982). Secondary selective changes affecting enterocyte development also take place (Lund et al., 1986; Phillips et al., 1988). Mechanisms controlling the primary cause of these events, involving transport of enteric antigens across M cells in the FAE for presentation to the GALT, have been less well defined. Present work uses a new cytochemical technique (Smith et al., 1987; 1988) to quantify Salmonella effects on M cells present in germ-free mouse FAE.

2. EXPERIMENTAL. Twelve-week old BALB/C germ-free mice, used as control or monocontaminated with live or dead non-pathogenic S. typhimurium aroA$^-$, were killed and Peyer's patch tissue removed for AP activity incubation (normal enterocytes red; AP-deficient M cells white) and staining of goblet cells with Alcian Blue. Subsequent measurement of M cell areas in dome FAE was by image analysis and microdensitometry. Other tissue samples were used to determine enterocyte migration rates (EMR) in ARGs after [^{3}H] thymidine injection, IELs in frozen tissue sections and crypt-villus structure in tissue containing no Peyer's patches. Bacterial counts were also carried out on samples of liver and spleen taken from control and Salmonella infected mice. Evidence showing Salmonella to induce a CMI reaction in tissue devoid of Peyer's patches is summarized in Table 1.

Crypt depth and EMR were increased after oral administration of live or dead S. typhimurium. CD8$^+$ and CD4$^+$ IELs also increased in number after S. typhimurium challenge. Villus height remained unaffected under these conditions. These results confirm the ability of S. typhimurium to induce a CMI reaction in the small intestine. M cell area in the FAE under these different conditions is shown in Table 2. M cell area in control mice was increased threefold after challenge with dead S. typhimurium and fivefold after challenge by live S. typhimurium in both naive and S. typhimurium primed animals. CD8$^+$ IELs decreased and CD4$^+$ IELs increased in the FAE after S. typhimurium challenge. Further measurements revealed a

fiftyfold variation in S. typhimurium counts in the livers and spleens of infected mice, with spleen counts being directly correlated with those found in liver ($P<0.05$). This variation was further investigated by plotting M cell frequency against the sum of S. typhimurium concentrations in liver and spleen, used here as a measure of antigen challenge to the GALT, The results of this comparison are given in Fig. 1.

Table 1 Salmonella induced CMI effects on germ-free mouse small intestine. Values give means ± SEM (No. of mice). (*); values significantly different from control ($P<0.03-<0.001$). Dead organisms were added to the drinking water (10^9/ml); live organisms were given as single oral injections (5×10^9 in 0.1ml). 7D and 28D; Bacteria given 7 and 28 days before experiment.

S. typhi -murium aroA⁻	Villus height (μm)	Crypt depth μm)	EMR (μm/hr)	CD8⁺ IEL (No./10mm)	CD4⁺ IEL (No./10mm)
None	282 ± 16 (8)	63 ± 2 (8)	2.8 ± 0.1 (8)	16 ± 5 (4)	18 ± 5 (4)
Live:7D	325 ± 21 (8)	87 ± 4* (8)	5.3 ± 0.6* (8)	26 ± 4 (5)	35 ± 7 (5)
Dead:7D	325 ± 15 (8)	73 ± 2* (8)	4.1 ± 0.3* (8)	-	-
Live:28/7D	-	-	-	17 ± 2 (6)	38 ± 6* (6)

Table 2 Salmonella induced effects on germ-free mouse FAE. Values give means ± SEM (No. of mice). (*); Values significantly different from controls ($P<0.05 - <0.001$). Dead organisms were added to drinking water; live organisms were given as single oral injections.

S. typhimurium aroA⁻	M cell (% area)	CD8⁺ (No./10mm)	CD4⁺ (No./10mm)
None	1.3 ± 0.3 (12)	122 ± 13 (4)	74 ± 9 (4)
Live: 7 days	5.1 ± 0.7 (13)*	53 ± 17 (5)*	79 ± 11 (5)
Dead: 7 days	3.4 ± 0.9 (8)*	-	-
Live: 28 & 7 days	5.0 ± 0.5 (5)*	58 ± 12 (6)*	107 ± 6 (6)*

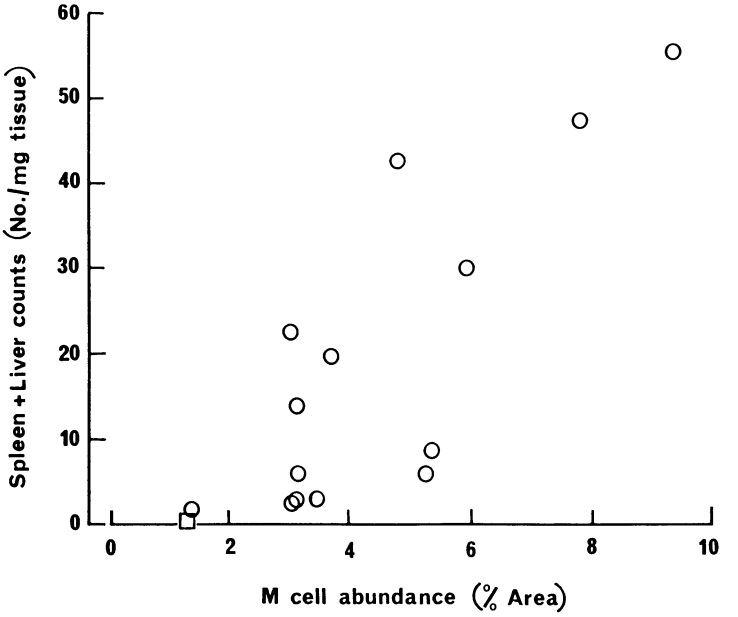

Fig. 1 Quantitative relation between Salmonella numbers and M cell frequency. Values for M cell frequency obtained from individual mice are plotted against spleen plus liver bacterial counts for control (□) and infected (○) animals. Linear regression of this data gives an intercept of -13.1 ± 0.6 and a slope of 7.0 ± 1.3 (corr. coeff. 0.82; $P<0.001$).

There was a highly significant linear relationship between M cell frequency and Salmonella counts ($P<0.001$). This relationship broke down for occasional mice showing much higher bacterial counts in spleen but not liver tissue (results not shown). It is concluded from this that M cell appearance is extremely sensitive to the presence of S. typhimurium but only up to a maximal value representing about 10% of the dome FAE area. Taking this analysis one stage further one can use this linear correlation to 'correct' M cell areas for individual variation in Salmonella counts. This type of manipulation halves the standard error associated with these measurements (4.4 ± 0.5% for raw data; 5.6 ± 0.3% for data corrected to represent a spleen + liver count of 25 bacteria/mg; pooled data from 7 and 28/7 day challenged mice).

3. DISCUSSION. Present work arose from the need to develop an animal model to test critically an already existing hypothesis that enteric antigens can increase M cell numbers in mouse FAE (Smith et al., 1987). Use of germ-free mice in this case eliminated all but dietary antigen challenge to the GALT under control conditions. Challenge of these animals with a non-pathogenic mutant (Dougan et al., 1987) of an organism which is known to penetrate mouse FAE selectively (Collins & Carter, 1978; Kohbata et al., 1986), then allowed one to study Salmonella effects on M cell formation under carefully controlled

conditions. S. typhimurium aroA$^-$ induces a CMI reaction in the small intestine and increases M cell formation in the FAE. How this latter effect is produced remains to be determined.

M cell formation is generally assumed to result from local interactions taking place between enteric antigens and the GALT affecting mature enterocytes (Smith & Peacock, 1980) or the slow differentiation of a new cell type (Bye et al., 1984). Future work involving selective deletion of defined parts of the GALT by antibody injection, followed by S. typhimurium challenge in germ-free mice, could help to decide which of these possibilities is more likely to be correct. Preliminary results showing cyclosporin A treatment of animal house BALB/C mice to reduce M cell area from 3.5 $\pm$ 0.7 to 1.1 $\pm$ 0.2% in the dome FAE strongly suggests that activated CD4$^+$ lymphocytes can stimulate the formation of M cells under so-called 'normal' physiological conditions.

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Immunohistological studies of lymphocyte subsets in normal cervix, HPV infection and cervical intraepithelial neoplasia

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Introduction

HPV subtypes 6 and 11 are associated with condylomata and CIN I and II. HPV subtypes 16 and 18 are consistently associated with CIN 3 and invasive carcinoma of the cervix.
HPV infections are common in immunosuppressed individuals who have a tenfold increased relative risk in developing cervical cancer.
From these observations it would appear immune responses play a part in the pathogenesis of cervical cancer.

Aim of the study

The distribution of lymphocyte subsets in the cervix and identification of any changes in distribution in HPV infection and CIN.

Materials and Methods

50 cervical biopsies were obtained from women attending a colposcopy clinic with abnormal smears and colposcopic appearances. They had no previous history of cervical disease or biopsy and their mean age was 28 years. 8 age matched control women having laparoscopic sterilisation were biopsied at the squamo-columnar junction. They had normal smears and colposcopic appearances, had no previous history of cervical disease or biopsy and their mean age was 32 years. The majority of women in the study and control groups had a smoking history.

Frozen and formal fixed tissue was obtained from both the study and control cases. Frozen sections of the frozen tissue were stained with a panel of monoclonal antibodies using an indirect immunoperoxidase technique and an avidin biotin complex. A wide range of T cell markers were used including HML1 and three large granular lymphocyte markers, Leu 19, Leu 11b and Leu 7.
On a proportion of cases the number of HML1 and Leu 19 positive cells in the ectocervix was counted.

Results - HML1

Number of HML1 Positive Lymphocytes per mm Epithelium

	Cells per mm	(95% confidence interval)	mean length/mm
Normal n = 7	45.5	(39.2, 52.8)	3.37
KCA n = 3	46.9	(34.0, 64.6)	1.65
CIN 1 n = 4	18.6	(13.4, 25.7)	3.00
CIN 2 n = 2	13.6	(8.2, 22.5)	3.42
CIN 3 n = 7	8.4	(5.5, 12.9)	2.22

HML1 positive cells were found to significantly decrease in number in CIN in comparison to normal and HPV infection and the reduction was more pronounced with increased severity of dysplasia.

Discussion - HML1 findings

HML1 is a surface marker preferentially associated with epithelial associated lymphocytes of the gut, bronchus and breast (1). Only rare lymphocytes are positive in lymph nodes, bone marow and blood.
The HML1 population has been identified in this study as CD2, CD3 and CD8 positive.
The HML1 results parallel the findings of a depletion of intraepithelial lymphocytes generally in HPV and CIN (2). This may be secondary to a reduction in Langerhans cells which has been found in HPV and CIN and has been confirmed in this study (3). This may be a direct HPV effect or acting in association with other factors such as smoking. The end result is impaired mucosal immunity with persistence of HPV and potential for oncogenic effect.

Results - Large granular lymphocytes

Number of Leu 19 Positive Cells per mm Epithelium

		Cells per mm	(95% confidence interval)	mean length/mm
Normal	n = 9	3.10	(1.74, 5.50)	2.55
KCA	n = 4	3.87	(2.16, 6.92)	4.46
CIN 1	n = 5	11.29	(7.83, 16.28)	3.10
CIN 2	n = 7	7.66	(5.14, 11.42)	2.74
CIN 3	n = 8	9.95	(7.24, 13.69)	2.90

Leu 19 positive cells were found to significantly increase in number in CIN in comparison to normal and HPV infection. No Leu 11b or Leu 7 positive cells were found in the ectocervix in normal, HPV infection or CIN. However all 3 markers, Leu 19, Leu 11b and Leu 7 were found in the stroma and in particular Leu 11b positive cells. Leu 11b positive cells were interestingly found to concentrate around endocervical glands with occasional cells extending into endocervical epithelium.

Discussion - LGL findings

Different phenotypes of LGL are seen in different sites in the cervix with only Leu 19 positive cells only being found in ectocervix and classical NK cells, Leu 11b cells, in the endocervix. This may represent a different immune response in the two sites. Circulating immature LGL's in the blood, under the influence of local environmental factors, may result in maturation and differentiation of different LGL phenotypes with localisation to different sites. Epithelial LGL's may have cytolytic function and may kill HPV infected cells and stromal LGL's may prevent stromal invasion by HPV and neoplastic cells (4).

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Phenotype of intraepithelial lymphocytes in oral lichen planus

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INTRODUCTION

Lichen planus (LP) is a chronic mucocutaneous disorder characterised by hyperkeratosis and basal cell destruction associated with a bandlike infiltrate of activated T lymphocytes and macrophages. Keratinocyte expression of the Class II Major Histocompatibility Complex Antigen HLADR in oral lesions is associated with a significant increase in density of subepithelial lymphocytes and seems likely to be the result of interferon gamma production by activated T lymphocytes (1). There is some evidence that lymphocyte migration into epidermis is influenced by keratinocyte expression of HLADR (2) and hence in LP it may be important in lesion development. The aim of this study was to determine whether the distribution and phenotype of lymphocytes in LP is related to keratinocyte expression of HLADR.

METHODS

Oral biopsies, which conformed to the WHO criteria for LP, from 7 patients and 7 age, sex and site matched controls were used.

Serial cryostat sections from three levels were reacted using an immuno-alkaline phosphatase technique to identify T (Pan T), B (Pan B) helper/inducer (T4), cytotoxic/suppressor (T8), memory (UCHL1) and virgin (Leu 18) lymphocytes, and HLADR. Positively stained cells were counted at x100 magnification in areas with and without keratinocyte HLADR expression. Only non-dendritic, nucleated cells completely surrounded by reaction product were counted. Cell number was expressed per mm epithelial surface length measured from photographs. Statistical analyses were performed using the Student's t-test.

RESULTS

Control Mucosa

No evidence of either B lymphocytes or keratinocyte HLADR expression was found. In all biopsies more CD8 than CD4 positive cells were present with a CD8:CD4 ratio of 2:1. The total number of CD4 and CD8 positive cells did not exceed the number of T lymphocytes indicating that few non-lymphocytic CD4 or CD8 positive cells were present (Fig.1). The majority of lymphocytes were UCHL1 positive.

Lichen Planus

HLADR expression was found in 6 biopsies and in total 9 areas were

assessed as being positive and 13 as negative. Significantly more T lymphocytes were present in areas of keratinocyte HLADR compared with areas of no expression (p 0.01) and with control mucosa (p 0.05) (Fig 1). Corresponding increases in the number of CD4 and CD8 positive cells were found but there was no change in the CD8:CD4 ratio compared with controls. The total number of CD4 and CD8 positive cells exceeded the number of T lymphocytes (p 0.05) suggesting there may be accumulation of non-lymphocytic CD4 and CD8 positive cells. The vast majority of lymphocytes were UCHL1 positive but a few Leu 18 positive cells were evident. No difference was found in the number of T lymphocytes, and CD4 and CD8 positive cells in areas without keratinocyte HLADR compared with controls.

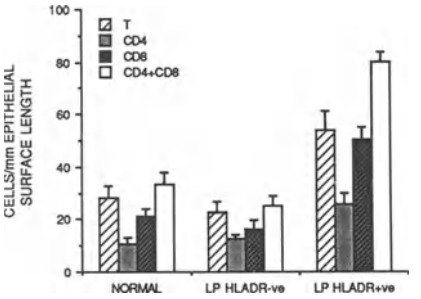

Fig.1. Intraepithelial cells in normal oral mucosa and lichen planus in areas with and without keratinocyte HLADR expression.

DISCUSSION

In normal oral mucosa only intraepithelial T lymphocytes are present and the majority are cytotoxic/suppressor and memory in phenotype. A similar predominance of memory cells has been observed in skin (3) but it is not known whether this represents specific cell accumulation or conversion from virgin to memory phenotype following exposure to antigen.

In LP only those areas showing keratinocyte expression of HLADR are associated with a significant increase in intraepithelial T lymphocytes, CD4 and CD8 positive cells compared with controls. This suggests that such areas are sites of immunological activity and is further supported by the finding that the majority of lymphocytes are memory in phenotype. The latter produce large quantities of interferon gamma upon activation (4) and may be responsible for the induction of keratinocyte HLADR expression.

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Thermolysin treatment; an improved dispersion technique for isolating functional lymphoid cells from intestinal tissues

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ABSTRACT. A novel two step enzymatic dispersion procedure has been evaluated for isolating cells from bioptic specimens of human duodenal tissue. The method is based on the sequential action of two different enzymes, a cold acting enzyme thermolysin, and collagenase/dispase. It is rapid, and yields highly viable and apparently functional mononuclear cells. (Supported by the Swedish Medical Research Council).

1. Introduction

Assessment of immune reactivity at local mucosal sites has proven to be rather difficult in humans. This is largely due to the relative inaccessibility of mucosal tissues and to the paucity of lymphoid cells extractable from small parenchymal tissues by conventional enzymatic dispersion procedures. In addition, such procedures are cumbersome in that they require repeated extraction of solid tissues and cell yields obtained are generally poor (1-3). In order to evaluate in functional terms local immune parameters at mucosal sites, we have devoted efforts to the establishment of an improved dispersion procedure for isolating cells from human mucosal tissues. We present preliminary results on the phenotypic and functional characterization of lymphoid cells isolated from biopsy specimens of human duodenal mucosa by means of a novel enzymatic dispersion procedure.

2. Material and methods

2.1. ISOLATION OF LYMPHOID CELLS FROM HUMAN INTESTINAL TISSUE

Based on preliminary experiments, the following standard procedure is routinely employed: A pool of 12 to 15 duodenal punch biopsies (3-4 mm Ø; approx. 200 mg) is extensively washed with isotonic Dulbecco's phosphate buffered saline. The tissue is sliced to 150 x 100 µm with a semi-automated tissue chopper (McIlwain) and washed thoroughly with chilled Hepes (25 mM) buffered Hank's balanced salt solution containing 1 mM $CaCl_2$, and 10 mM dithiothreitol, pH 7.4. (extraction buffer A). Tissue slices are then extracted for 30 min at 4°C under continuous shaking, in thermolysin solution, consisting of thermolysin (*Bacillus thermoproteolyticus*) diluted to 0.5 mg / ml in extraction buffer A. Single cell suspensions are collected by filtration through a nylon net (150 µm mesh), washed with Iscove's medium (IM), and kept at 37°C in IM+20% foetal calf serum (FCS). This initial fraction is referred to as fraction T. Remaining non dispersed fragments are re-extracted for 45 min at 37°C with prewarmed collagenase/dispase (3) diluted to 1 mg / ml in IM+20% FCS, under constant shaking. Single cell suspensions (fraction D) are then collected by filtration. Fractions T and D are pooled and incubated for 15-20 min with deoxyribonuclease (type IV, Sigma), diluted to 2 mg / ml in IM + FCS. Single cells are then collected by filtration through a thin (50 µm) nylon net. Cell suspensions obtained above are occasionally pelleted through a Percoll (35 %) cushion, by centrifugation (2000 x *g* , 15 min, 10 °C).

2.2. HISTOLOGICAL AND IMMUNOCYTOLOGICAL METHODS

Cytocentrifuge preparations of intestinal suspensions were stained with May-Grunewald and examined for relative distributions of mononuclear cells vs "other cell types" (epithelial, polynucleated, and undetermined cells). Isolated cells were also examined for cytoplasmic immunoglobulin content by indirect immunofluorescence staining using appropriate isotype specific

biotinylated anti-Ig reagents and FITC-conjugated streptavidin . Freshly isolated and cultured ($37^{o}C$, 16 hrs) intestinal cell suspensions were examined by dual flow cytometry for expression and/or co-expression of membrane antigens specified by the following monoclonal Ab reagents: CD3 (pan T cells), CD4 (T helper/inducer), CD8 (Tsuppressor /cytotoxic), CD 19 (B cells), Leu 7+ Leu 11 (NK cells), CD 14 (Leu M3; mostly monocytes,macrophages).

2.3. FUNCTIONAL ASSAYS

Isolated intestinal cells were assayed for numbers of IgA-, IgM-, and IgG-secreting cells by two-colour ELISPOT test (4); gamma-interferon-producing cells by two site reverse ELISPOT assay using unlabelled and biotinylated monoclonal anti-interferon gamma antibodies as solid phase capture system and probe, respectively (5); interleukin 1ß-secreting cells by amplified reverse ELISPOT assay using affinity purified Fab fragments of IgG anti-IL1ß antibodies and biotinylated anti-peroxidase antibodies as tracer (6).

3. Results

3.1. GENERAL CHARACTERISTICS OF ISOLATED CELLS

On average (20 experiments), the above procedure yields 2,1 x 10^6 nucleated cells (range 0.95 to 4.3 x 10^6) / pool of 15 punch biopsies (approximate weight 12 mg / biopsy) comprising 30-40 % mononuclear cells and 60-70 % epithelial and undetermined cells. Cell viability was > 90 % for mononuclear cells and 60-80 % for epithelial cells. Mononuclear cells could further be enriched up to 70 % by centrifugation through a single cushion of 35 % Percoll. Such enrichment can however reduce the final cell yield by almost 40 %.

3.2. PHENOTYPIC CHARACTERISTICS OF ISOLATED CELLS

T cells CD3 (Leu 4)+: 80 ± 13 % (N=8); the majority of T cells are CD45R+ (presumably "activated" and "memory" T cells); both CD4 and CD8 markers are destroyed by the enzymatic procedure. CD19+: ≤ 2 % (N=7). CD14 (Leu M3)+: 2 ± 3 %; (N=6). Leu7+,11+ : 2 ± 4 %; (N=7).

3.3. FUNCTIONAL CHARACTERISTICS OF ISOLATED CELLS

. Immunoglobulin-secreting cells: Ig A: 14 238 ± 5835 (mean ± S.E.M.)/ 10^6 MNC, N=7 (≈88%); Ig M: 1018 ± 398 /10^6 MNC, N = 7 (≈6%); Ig G: 1060 ± 413 /10^6 MNC, N = 7 (≈6%).

. Gamma-interferon-producing cells: 160 ± 60 /10^6 MNC; N = 7

. Interleukin-1ß-secreting cells: 590 / 10^6 MNC; N = 4 (range 175-1000).

4. Conclusion

A novel dispersion procedure has been developed for isolating highly viable lymphoid cells from small biopsy specimens of human mucosal tissues. Thermolysin treatment, being performed at low temperature, minimizes the action of cellular proteases, and therefore gives a good cell preservation. We have successfully employed this strategy for isolating functional lymphoid cells from a variety of human tissues, such as labial minor salivary glands and gingival tissue. Although the technique is not suitable for certain phenotypic analyses (as several markers may be removed by the enzymatic treatment), the method is rapid, and permit to isolate apparently functional lymphoid cells in sufficient numbers to perform multiple functional assays.

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A novel carbohydrate epitope on the surface of mouse intraepithelial lymphocytes

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Introduction Our objective has been to reveal new information about the surface of intraepithelial lymphocytes (IEL) to try to learn more about cellular interactions within the gut epithelium. Large panels of rat monoclonal antibodies were prepared against isolated mouse IEL and screened for selective reactivity with mucosal lymphocytes. Two antibodies have been studied in our laboratory, M290 (1) and M371 (2). The former indentifies a novel integrin which appears to be locally induced in mucosal T cells. The second antibody, M371, is the subject of this paper.

Results

Tissue distribution M371 was shown by immunoperoxidase staining to identify a subset of IEL in mouse intestine (Fig 1). Lymphocytes in the lamina propria were unstained and M371$^+$ lymphocytes were completely absent from peripheral lymph nodes, mesenteric lymph nodes, spleen, thymus, foetal thymus and bronchiolar epithelium. Lymph node cells that had been stimulated in culture once or repeatedly with mitogens or alloantigens also gave negative results. Some goblet cells, particularly those in the mid and distal gut, were stained by M371 but in the proximal gut M371$^+$ IEL were often seen in the absence of stained goblet cells. In athymic nude mice M371 stained at least 70% of IEL.

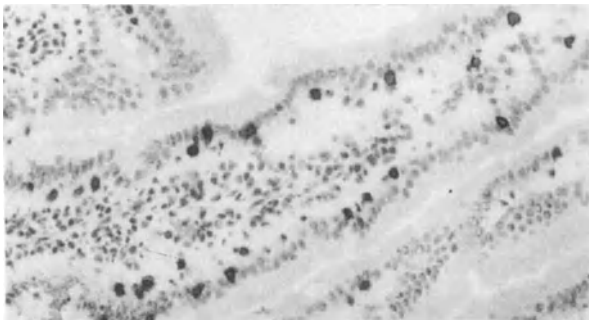

Figure 1
M371+ IEL in the duodenum of C3H mice

Subset distribution of M371$^+$ IEL Table 1 shows data pooled from fluorescent staining of gut sections and FACS analysis of isolated Balb/c IEL. M371 stained approximately 35% of IEL and essentially all

were Thy 1^-, Lyt 2^+. Expression of TcR utilising Vβ8 was confined almost entirely to Thy1+ cells, the same was true for Lyt 3. More than 80% of IEL, and therefore most M371+ cells, expressed CD3.

Table 1

Subset	IEL within subset (%)	Prevalence of markers within subset (% positive ± SD)	
M371	35 ± 5	Lyt 2	99.2 ± 1.2
		Thy 1	1.1 ± 0.8
Lyt 2	78 ± 6	Thy 1	31.5
TcRVβ8	21 ± 7	Thy 1	89.3 ± 3.3
Lyt 3	29 ± 4	Thy 1	91.7 ± 0.7
CD45	92 ± 3		

Immunoprecipitation M371 precipitated a surface molecule approximately 275 kDa in size. Pre-clearance of the IEL lysate with the antibody M1/9.3, specific for the leucocyte common antigen CD45, removed M371 reactive material. The reciprocal experiment in which pre-clearance was conducted with M371 removed a 275 kDa species from the CD45 precipitate.

Resistance to heat or periodate Heat treatment of fixed IEL at 100°C had no effect on immunofluorescent staining with M371 whereas 2mM periodate completely prevented it.

Relationship to CT antibodies Antibodies CT1 and CT2 detect a carbohydrate epitope (GalNAcβ1,4[SAα2,3]-gal) of CD45 that is expressed on mouse cytotoxic cells and IEL. Fortuitously, it is also present on Sd(a+) Tamm Horsfall glycoproteins (THG). M371 failed to react with Sd(a+) THG by ELISA or to block binding of CT1.

Discussion The absence of M371+ lymphocytes from the lamina propria and from other lymphoid tissues argues that this subset of IELs does not recirculate. Further, it implies that expression of the carbohydrate determinant identified by M371 is induced locally by factors in the epithelial environment. The epitope was found on thymus-independent IEL that express CD3 but not Vβ8. It is highly probably that these are γδ cells. Their origin is unknown but it is plausible that they differentiate directly from bone marrow precursors, possibly under the influence of the gut epithelium.

The antigen precipitated by M371 was found to be physically associated with CD45 but whether or not the association is covalent is unknown. M371 and the CT antibodies clearly differ in specificity.

So far no information is available about the relevance of this antigen to lymphocyte-epithelial interactions but in the immediate future M371 will be useful for isolating an unusual IEL subset for functional studies.

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SECTION C: CYTOKINES IN RELATION TO MUCOSAL IMMUNITY

30
Cytokine/lymphokine regulation of IgA B cell differentiation

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Abstract/Introduction

The factors regulating the differentiation of IgA B cells has been an area of great interest to mucosal immunologists as well as those interested in B cell differentiation generally. It is now clear that such differentiation involves two major steps, first, isotype switch differentiation of sIgM B cells (surface IgM-bearing B cells) into sIgA B cells (surface IgA-bearing B cells) and second, terminal differentiation of IgA B cells into IgA producing plasma cells. It has also become evident that both of these steps are regulated processes that are under the influence of various cytokines and lymphokines. In the present study we address this latter point by presenting data that further defines the role of cytokines and lymphokines in the regulation of IgA B cell differentiation. In particular, we show that IL-4 may play a role in the switch differentiation step of IgA B cells and that IL-5 and IL-6 are important in the terminal differentiation step of these B cells.

Switch Differentiation of IgA B Cells

Some years ago we showed that Peyer's patches contained cells that induce sIgM B cells to switch to sIgA B cells (1). These cells, called "switch" cells, could conceivably act by secreting various factors that induce IgA isotype switching. This possibility, plus recent evidence that the T cell-derived lymphokine, IL-4, can cause sIgM B cells to undergo isotype switch to sIgG1 B cells (or at higher concentrations to sIgE B cells) prompted us to consider IL-4 as a candidate switch factor in IgA B cell differentiation.

In an initial approach to this question, we cultured FACS-sorted sIgM-positive, sIgA-negative Peyer's patch B cells with lipopolysaccharide (LPS) and recombinant IL-4 (at various concentrations) to determine if IL-4 has IgA-specific switching effects. We found that IL-4 in such cultures (or for that matter combinations of IL-4 and IL-5) does not in fact lead to the occurrence of sIgA-positive B cells or cells that produce significant quantities of IgA. Whereas these data rule out the possibility that IL-4 alone induces IgA B cell switch differentiation, they do not speak to the question of whether IL-4 still plays a role in such differentiation, perhaps by acting as a factor necessary for completion of a switch process initiated by another, more primary factor.

To investigate this latter question we next conducted a series of studies of the effect of IL-4 (and other lymphokines) on the differentiation of CH12.LX B cells, a B lymphoma cell line that is composed of a majority population of sIgM B cells (98% of the cells) admixed with a minority population of sIgA B cells (2% of the cells) and that can thus be assumed to have undergone the earliest steps of IgA B cells differentiation (see further evidence relative to this point below) (2). In our first studies of CH12.LX B cells we cultured the cells with IL-4 and found that the latter does induce a dose dependent increase in surface IgA expression (from a baseline level of 2.4% sIgA positive cells to a maximal post-culture level of 15% sIgA cells). This effect was IL-4 specific, since it was blocked by the addition of monoclonal anti-IL-4 antibody (11B11). In addition, no other lymphokine had a similar effect including IL-1, IL-2, IL-3, IFN-γ or IL-6 and in none of the cases tested did these lymphokines augment the effects of IL-4. Of interest, while IL-4 caused the CH12.LX B cells to manifest increased expression of sIgA, it did not result in a significant increase in IgA secretion; this provided initial evidence that IL-4 was acting as a switch differentiation factor and not as a terminal differentiation factor in the CH12.LX system.

In further, more rigorous studies of CH12.LX B cells, we sorted the cells into sIgA-negative and sIgA-positive cell populations prior to culture with lymphokines in order to focus on IL-4 effects on cells bearing only surface IgM. In these studies we found that culture of the initial sIgA-negative cell population (i.e., the sIgM-positive cell population) with IL-4 led to an increase in sIgA expression (from a baseline level of 0.2% sIgA-positive cells to a post-culture level of 6.7% sIgA-positive cells); in contrast, culture of the sIgA-positive cells with IL-4 had no significant effect on the high percentage of cells bearing IgA in this population (pre-culture value of 80.1% sIgA-positive cells and post-culture value of 83.9% sIgA-positive cells). As in the earlier studies, IL-4 had no effect on the amount of IgA produced per cell in either cell population; on the other hand, culture of the CH12.LX B cells with IL-5 led to increased IgA production/cell in both the sIgA-negative and sIgA-positive cell populations, but had no effect on sIgA expression. This is consistent with other studies (described below) indicating that IL-5 acts as terminal differentiation factor during IgA B cell differentiation.

In yet additional studies, we evaluated the effect of IL-4 on CH12.LX B cell proliferation. First, we showed that culture of sorted sIgA-positive and sIgA-negative cells (sIgM-positive cells) with IL-4 for 24 and 48 hours led to equal rates of proliferation in the two cell populations; this indicates that IL-4 does not cause short-term selective proliferation of sIgA-positive CH12.LX B cells. Next we showed that a population of CH12.LX B cells enriched for sIgA-positive cells (obtained by panning and containing about 50% sIgA-positive cells) cultured with IL-4 undergoes a decrease in cells bearing IgA during cutlure (to 18%); this shows again that IL-4 does not cause selective long-term proliferation of sIgA-positive CH12.LX B cells. Third and finally, we showed that about 50% of the sIgA-positive CH12.LX B cells appearing after exposure to IL-4 were in fact dual bearing sIgM-positive/sIgA-positive cells; this suggests that the sIgA-positive CH12.LX cells that occur during IL-4 culture are derived from sIgM-positive CH12.LX cells. Taken together these data point quite clearly to the view that the

increase in sIgA expression in CH12.LX B cells upon culture with IL-4 is not due to effects of this lymphokine on the proliferation of sIgA-positive B cells in the starting population, but rather to true switch differentiation of sIgM-positive B cells to sIgA-positive B cells.

The above conclusion should not be taken to mean that IL-4 initiates switch differentiation of sIgM-positive CH12.LX B cells to sIgA-positive CH12.LX B cells. On the contrary, the fact that CH12.LX B cells can spontaneously differentiate into IgA-expressing cells indicates that these cells have received a switch differentiation signal earlier in their differentiation history, prior to exposure to IL-4 in vitro. In addition, a "downstream" role for IL-4 in CH12.LX B cell isotype differentiation is supported by our recent molecular studies of CH12.LX B cells before and after exposure to IL-4 (3). In these studies we have shown that unstimulated CH12.LX B cells contain "germline" Cα mRNA, i.e., mRNA transcribed from the Cα gene while the latter is still in an unrearranged germline state. Germline transcription of a particular heavy chain constant region (in this case the Cα constant region) appears to herald imminent isotype switch rearrangement in which the heavy chain constant region gene to which the B cells is switching is brought into juxtaposition with the recombined VDJ genes (4); thus, the presence of germline Cα transcripts in unstimulated CH12.LX B cells suggests that these cells have already initiated IgA switch differentiation prior to in vitro exposure to possible inductive influences such as IL-4.

In further molecular studies we have shown that IL-4 induces a new Cα germline transcript, i.e., a transcript which is different from the Cα transcript initially present by virtue of the fact that it has a unique start site. This indicates that IL-4 causes changes affecting the Cα gene at the transcriptional level, changes which may, in fact, be necessary for the firnal commitment of the CH12.LX cells to IgA differentiation. This latter point is favored by the fact that while most (if not all) CH12.LX B cells have activated Cα genes, only a small minority actually become IgA-bearing cells; hence, by inference, additional induction factors are necessary for maximal IgA isotype expression.

In summary of these various studies, one can say that whereas IL-4 does not act as a switch factor for "virgin" B cells that do not have any commitment to IgA switch differentiation, this lymphokine does have a switch factor role for cells (such as CH12.LX B cells) that have received initial IgA-specific switch signals and are partially committed to IgA switch differentiation. We would therefore posit that IgA B cell switch differentiation requires the interaction of switch factors from a variety of cell types including cells which provide the initial switching signals as well T cells which provide late switching signals such as IL-4.

Terminal Differentiation of IgA B Cells

Turning now to the question of (post-switch) terminal differentiation of IgA B cells, we come first to the now well-established finding that the T cell derived lymphokine, IL-5, has been identified as an important stimulator of IgA synthesis, both in mitogen- and antigen-stimulated systems (5,6). Two key characteristics of such stimulation should be mentioned. First, the IL-5 effect is not isotype-specific

(limited to IgA), although it may be more necessary for an IgA response than for other isotype responses. Second, we and others have shown that IL-5 induced enhancement of IgA synthesis occurs at the post-switch level, i.e., it involves the differentiation of B cells that have already undergone IgA isotype switch differentiation (7).

In a effort to further expand our knowledge of the terminal differentiation of IgA B cells, we have recently begun to examine the effects of other lymphokines on IgA secretion (8). To study the effects of IL-6, we measured the capacity of T cell-depleted B cell populations obtained from Peyer's patches to synthesize IgA (and other immunoglobulins) following culture in the absence of LPS. The reason for choosing this system was twofold: first, Peyer's patch B cell populations contain partially activated B cells that produce immunoglobulin in culture in the absence of a mitogenic stimulus; thus immunoglobulin production by such cells is indicative of antibody production that has been stimulated by "natural" antigen in vivo; second, Peyer's patch cell cultures not containing added mitogen do not contain endogenously produced IL-6 which would mask the effect of added IL-6, since such cultures contain few macrophages and the few present do not produce IL-6 in the absence of mitogen.

In the studies performed we found that whereas IL-6 alone (at optimum concentrations) had minimal to moderate enhancing effect on IgM and IgG (IgG3) secretion, this lymphokine in combination with recombinant IL-1 greatly enhanced IgM and IgG (IgG3) secretion. In contrast, IL-6 alone had quite modest effect on IgA secretion and this effect was not significantly increased by the addition of IL-1. Thus, the IL-1/IL-6 combination was an effective one for IgM and IgG (IgG3) but not IgA secretion. A different situation obtained for IL-5. In this case IL-5 alone brought about only moderate effects on IgM, IgG (IgG3) and IgA secretion and this effect was not augmented by IL-1. On the other hand, while the combination of IL-5 and IL-6 did not augment IgM and IgG secretion it did have an marked enhancing effect on IgA secretion. Thus, the IL-1/IL-6 combination was most effective in enhancing IgM/IgG secretion whereas IL-5/IL-6 combination was most effective in enhancing IgA secretion.

The above data allow certain hypotheses relating to the regulation of the terminal differentiation of IgA B cells. First, on the basis of the now well-established view that IL-6 is a relatively late-acting lymphokine, it is likely that whereas IL-1 induces IgM B cells and IgG B cells to be receptive to the effects of IL-6, it is IL-5 that induces IgA B cells to be receptive to IL-6. Second, it seems that whereas neither IL-5 nor IL-6 alone can lead to maximal IgA secretion, IL-5 has the more fundamental effect inasmuch as this lymphokine acts in the absence of IL-6 and IL-6 does not act in the absence of IL-5. Third, it appears that IgA class-specific effects of T cells on the terminal differentiation of B cells probably involves preferential IgA B cell signalling by more than one lymphokine. In the latter regard, it is now known that T cells can take various differentiation pathways, one leading to predominant IL-5 and IL-6 production (TH_2 cells) and one leading to predominant IL-2 and IFN-γ production (TH_1 cells); thus, the data suggest that TH_2 cells are particularly important in terminal differentiation of IgA B cells.

Finally, while we have been focusing on the role of IL-5 and IL-6 in IgA B cell

differentiation it remains possible that other lymphokines also play a role in this process. In this regard, there is some evidence in antigen-specific systems that IFN-γ and perhaps other lymphokines also augment terminal IgA B cell differentiation. Thus, one cannot rule out a role for TH_1 cells in the overall differentiation process.

Summary

The various studies discussed above suggest a model for lymphokine/cytokine regulation of IgA B cell differentiation. In this model, IgM-bearing B cells undergo an initial isotype switch differentiation as a result as yet unknown signals received from switch cells present in Peyer's patches. The partially committed IgA B cells thus formed would manifest Cα gene activation such as that seen in CH12.LX cells and could be acted upon by IL-4 to complete the isotype switch differentiation process to become fully IgA-committed B cells. The latter cells can then be activated by antigen and thus express receptors that allow IL-5 and IL-6 (and/or other factors) to induce terminal differentiation.

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Role for interleukins 5 and 6 in IgA synthesis

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ABSTRACT. Terminal differentiation of Peyer's patch (PP) IgA committed B cells to IgA secreting plasma cells is regulated by the cytokines IL-5 and IL-6. Both cytokines caused increased IgA secretion by PP B cells, as measured by radioimmunoassay, and IgA spot forming cells, as measured by ELISPOT assay. Neither cytokine caused significant B cell proliferation nor isotype switching of $sIgA^-$ to $sIgA^+$ B cells. B cells induced to secrete IgA by these cytokines were activated, $sIgA^+$ non-germinal center (PNA^{Lo}) B cells. This same PNA^{Lo} B cell population migrated to intestinal mucosa when adoptively transferred to SCID mice.

1. Introduction

Immune responses at mucosal sites are dominated by antibody of the IgA isotype. The majority of this IgA is secreted by local IgA plasma cells in mucosal tissue (1). We and others (2, 3, 4) have previously shown that supernatants from Peyer's patch T cells, T cell clones and T cell hybridomas preferentially support polyclonal IgA secretion by PP B cells and LPS stimulated splenic B cells. Because these supernatants contained multiple cytokines we have cultured PP B cells with 10 recombinant cytokines (IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IFNα, TNFγ and TGFβ) and looked for increased IgA secretion. PP-B cell subpopulations were also injected into SCID mice in order to determine if B cells from this IgA inductive site can home to mucosal effector sites.

2. Materials and Methods

2.1 PEYER'S PATCH B CELLS

PP B cells were prepared by enzymatic disaggregation, followed by panning on anti-Ig coated petri plates as described (5). B cells were further enriched or depleted of $sIgA^+$ cells by flow cytometry (5) or separated into PNA adherent/non-adherent B cells by panning (6).

2.2 RECOMBINANT CYTOKINES

PP B cells were cultured with rmIL-1α, rhIL-2, rmIL-4, rmIL-5, rmIL-6, rhIL-6, rmIL-7, rmIFNγ rmTNFα and TGFβ. All cytokines were first tested for activity in appropriate bioassays (3) and were then added to B cell cultures at concentrations of 5-200 biological units per culture.

2.3 *IN VITRO* CULTURES

B cell subpopulations were cultured with cytokines for 7 days as described (1). Antibody (IgM, IgG, IgA) in culture supernatants was determined by isotype specific radioimmunoassay (1). IgA spot forming cells in cultures were determined by enzyme linked immunospot (ELISPOT) assay as described. (3).

2.4 B CELL TRANSFER TO SCID MICE

Whole PP B cells or PNA^{Hi}/PNA^{Lo} B cell subpopulations (5-10 x 10^6) were transferred IV to SCID mice. After 10 days mice were sacrificed and gut, salivary gland, mesenteric lymph node and spleen removed. Tissues were fixed, sectioned and stained with FITC anti-mouse Ig and PE anti-mouse IgA. Control mice received no cells or splenic B cells.

3. Results and Discussion

Of all the cytokines tested, only rmIL-5 and both human and mouse rIL-6 enhanced IgA secretion by PP B cells (data not shown). Both cytokines also increased the number of IgA SFC in PP B cell cultures (Table 1).

Table I. Increase in IgA SFC in Interleukin Supplemented Cultures/Number of IgA SFC/10^6 PP B Cells (Day 3)

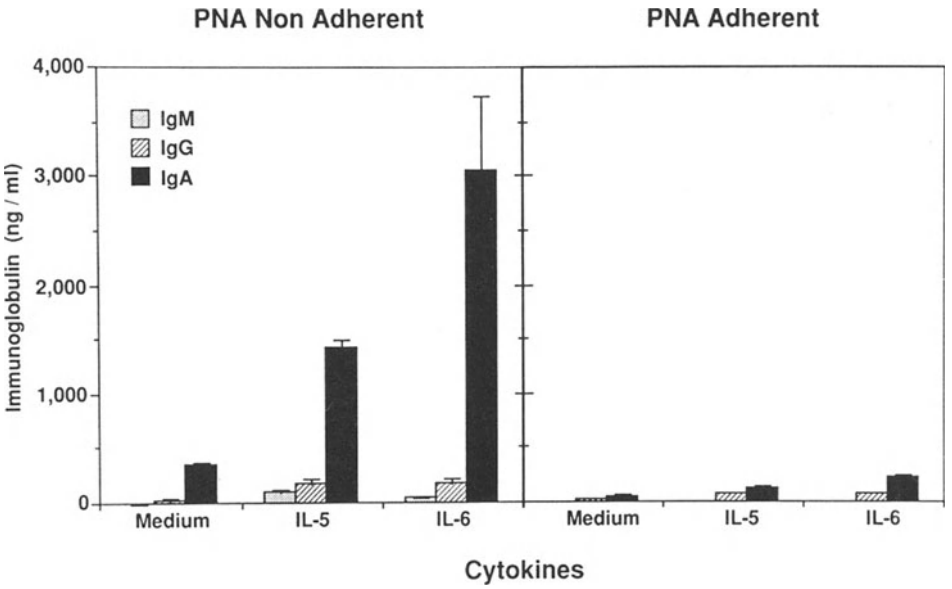
	Medium	IL-5	IL-6
Exp. 1	1200 ± 212	5475 ± 106	10475 ± 1025
Exp. 2	1920 ± 1149	4650 ± 1149	14397 ± 1621

Panning purified PP B cells were cultured with rmIL-5 (25 U/ml) or rmIL-6 (50 U/ml). After 3 days in culture, cells were washed, cell viability determined by Trypan blue dye exclusion and IgA SFC measured by ELISPOT assay.

IL-6 mediated increases in both IgA secretion and IgA SFC were always 2-3 fold higher than those in IL-5 supplemented cultures. IL-5 also enhanced secretion of IgM antibody by PP B cells, however IL-6 had little effect on the secretion of IgM or IgG. B cells responding to both IL-5 and IL-6 were $sIgA^+$ B cells. Removal of $sIgA^+$ B cells by FACS sorting abolished cytokine mediated increases in IgA secretion.

When PP B cells were separated into PNA adherent and non-adherent cells by panning and cultured with IL-5 and IL-6, cytokine mediated increase in IgA secretion was

only seen in the PNA non-adherent (non-germinal center) cells (Fig 1). Adoptive transfer of PP B cells to SCID mice demonstrated that B cells from this IgA inductive site can home to the gut mucosa (Table 2). Ten days after transfer to SCID mice, only the PNA nonadherent (PNA^{Low}) PP B cells homed to gut mucosa (Table 2).



PP B cells were separated into PNA adherent and non-adherent fractions by panning on PNA coated plates (6). Both populations (1 x 10^5/culture) were cultured with rmIL-5 (25 U/ml) and rmIL-6 (50 U/ml) for seven days. Immunoglobulin in culture supernatants was measured by RIA.

Table II. Homing Patterns of Peyer's Patch B Cells to Intestinal Mucosa of SCID Mice

Adoptive Transfer Of	Number of IgA B Cells/Field*	
	Surface IgA^+	Cytoplasmic IgA^+
PP B Cells (Total)	14	29
PNA^{Lo}	8	12
PNA^{Hi}	0	0

*Magnification x 150.

Whole PP B cells (1 x 10^7) or PNA adherent/non-adherent B cells (5 x 10^6) were adoptively transferred IV to SCID mice. After 10 days, the gut was removed and sections stained to reveal IgA plasma cells.

Transfer of splenic B cells to SCID mice did not give rise to IgA plasma cells in gut mucosa.

These results show that both IL-5 and IL-6 are important terminal differentiation factors for IgA secreting PP B cells. Both cytokines induce activated, sIgA$^+$, non-germinal center B cells to terminally differentiate to IgA plasma cells. Neither cytokine can induce isotype switching to IgA in PP B cell cultures depleted of sIgA$^+$ cells. The same B cell population induced to secrete IgA *in vitro* by IL-5 and IL-6 also homes to the gut mucosa when adoptively transferred to SCID mice. The majority of SIgA$^+$ B cells (75-80%) in PP are found in GC (8) and GC represent the site of B cell commitment to antigen and also to IgA secretion. Both processes are dependent upon interaction with T cells which regulate B cell activation and isotype switching via secretion of regulatory cytokines. Our studies, however, show that a minority population of non-GC, sIgA$^+$ B cells contain plasma cell precursors which can home to mucosal tissues *in vivo* and can be induced to secrete high levels of IgA *in vitro* when cultured with IL-5 or IL-6. These results support the role of the PP as a major IgA inductive site for IgA plasma cells found in mucosal tissues, and show that IL-5 and IL-6 regulate the terminal differentiation of these cells.

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Regulaton of intestinal immunity by soluble B and T cell products

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ABSTRACT. Previous work from this laboratory has shown that activated surface IgA-bearing ($sIgA^+$) B cells of mice develop into IgA-secreting plasma cells under the influence of IL-5 and IL-6. We have examined the regulation of the IgA response by analysis of messenger RNA (mRNA) in murine intestinal cells. T cell hybridomas isolated from Peyer's patch which have receptors for the Fc region of immunoglobulin α chain (FcαR) spontaneously secrete IL-5. The expression of mRNA for IL-5 in these cells is suppressed by incubation with IgA. Furthermore, we have found that intraepithelial lymphocytes of BALB/c mice also express mRNA for IL-5 in relatively high amounts compared to spleen cells. These results highlight unique features of intestinal cytokine production and may indicate the presence of a homeostatic mechanism whereby IL-5 synthesis by T cells in the intestine can be down-regulated by IgA via Fc receptors.

Experiments

Activation of T and B cells by antigen or mitogen is characterized among other biological changes by secretion of immunoactive components. The T cells which are activated release cytokines. In mice $CD4^+$ T helper cells (T_h) can be subdivided according to the cytokines they release (1). T_{h1} cells are characterized by secretion of interleukin 2 (IL) and IFNγ whereas T_{h2} cells are distinguished by secretion of IL-4, IL-5 and IL-6. IL-5 and IL-6 play key roles in mucosal immunity, since both IL 5 and IL 6 can drive B cells which are committed to immunoglobulin A (IgA) synthesis to become IgA secreting plasma cells (2,3; Table 1).

TABLE 1. Enhancement of IgA Synthesis by IL-5 and IL-6

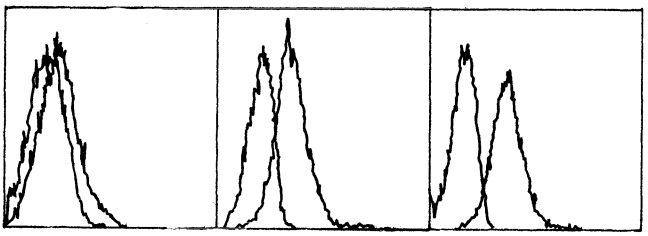
	Ig (ng/ml)		
	IgM	IgG	IgA
Medium	64 ± 11	121 ± 13	300 ± 19
IL-5 (25 U/ml)	463 ± 142	223 ± 49	1175 ± 75
IL-6 (50 U/ml)	144 ± 23	378 ± 103	2868 ± 55

Whole PP B cells (1 x 10^5/culture) were cultured with rmIL-5 (25 U/ml) and rmIL-6 (50 U/ml) for 7 days. Immunoglobulin in supernatants was measured by RIA.

T cells isolated from intestinal tissues (PP and intestinal epithelium) spontaneously secrete higher amounts of IL-5 and IL-6 than splenic T cells (data not shown). To further confirm our findings, we compared the amount of messenger RNA for IL-5 and IL-6 using cytoplasmic dot blots of freshly isolated intraepithelial lymphocytes (IEL). IEL exhibit strong IL-5 and IL-6 signals when compared to normal splenic T cells (data not shown). As a control hybridization we used a PstI fragment of the murine actin cDNA clone. These results support our current model, that Th2 subsets in gut tissues are important for IgA specific regulation at mucosal sites.

Besides interleukins, another important class of immunoactive molecules are membrane anchored receptors for Ig. Since Ig binds at the second constant region domain (Fc region of the molecule) this class of receptors are named FcR. In murine PP we have found a small proportion of $CD4^+$ Th that express an FcR specific for IgA (FcαR). To study the functional and molecular aspects of this FcαR we have established several T cell hybridomas, that express FcαR (Fig. 1) and secrete a biologically active form of the FcαR, called immunoglobulin binding factor (IBFα). Cell culture supernatants of $Fc\alpha R^+$ cell lines can provide help for activated spleen cells to become IgA secreting plasma cells (4). On the other hand, $Fc\alpha R^+$ T cells are reported to suppress IgA responses by reducing transcription of mRNA for IgA synthesis (5). This controversy may be due to the fact that $Fc\alpha R^+$ T cells are able to secrete both interleukins and IBFα. We find that $Fc\alpha R^+$ T cell hybridomas spontaneously secrete IL-5. This could explain the IgA promoting effect of cell culture supernatants of $Fc\alpha R^+$ T cells. On the other hand, affinity purification of cell culture supernatants on IgA columns yields a fraction that can suppress IgA responses. This fraction is thought to be enriched for FcαR or IBFα.

FIGURE 1. FACS staining (IgA binding) of FcαR positive T cell hybridomas PPT 4-16 and PPT 4-6 in comparison to the control T cell BW 5147



An additional indication for an immunoregulatory function of FcαR is provided by our finding that IgA incubation of $Fc\alpha R^+$ T cells with IgA reduces secretion of IL-5. However, the mechanisms of this regulatory pathway are not yet fully understood at the molecular level, since the amount of spontaneous IL-5 message in our $Fc\alpha R^+$ cells is low and a further reduction in message for IL-5 is therefore difficult to determine.

Immunoprecipitation of radiolabeled cell membrane proteins of $Fc\alpha R^+$ T cells results in a 38 kD protein band. Antibodies raised to 38 kD proteins of $Fc\alpha R^+$ cells bind to our $Fc\alpha R^+$ T cells as well as to $CD8^+$ T cells isolated from mice bearing IgA lymphoma. We are currently using this reagent to study the biological functions, physicochemical characteristics and to clone the FcαR molecule.

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Th1 and Th2 cells in mucosal associated tissues

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ABSTRACT. Cloned $CD4^+$ T helper (Th) cells may be divided into two subsets where Th1 cells produce IL-2, IFNγ and TNF β while Th2 cells secrete IL-4, IL-5 and IL-6. In this study, IFNγ and IL-5 specific ELISPOT assays were employed to examine the frequency of Th1 and Th2 type cells in gut-associated tissues. Analysis of lymphocytes spontaneously secreting IFNγ and IL-5 in IgA-effector sites, e.g., lamina propria (LPL), revealed that LPLs contained high numbers of IFNγ and IL-5 producing cells at the ratio of 1:3. On the other hand, lymphocytes freshly isolated from Peyer's patches (PP) contained smaller numbers of IFNγ and IL-5 producing cells in an equal distribution. When PP lymphocytes were stimulated with T cell mitogens, significant numbers of IFNγ and IL-5 producing cells were seen. These results suggest that T lymphocytes in mucosal tissues, especially LPLs, are activated *in vivo* in response to environmental stimulants, and higher numbers of IL-5 producing Th2-type cells reside in this site. On the other hand, the PP contain equal numbers of Th1- and Th2-type cells which may be important for regulation of the initial events for induction of the IgA response.

1. Role of Th1 and Th2 Cells in Isotype-Specific B Cell Responses

Murine $CD4^+$ Th cell clones have been divided into two subsets, Th1 and Th2 types according to the profile of cytokines produced [1]. Th1 cells produce IL-2, IFNγ and lymphotoxin (TNFβ) in response to antigen triggering via αβTCR-CD3, whereas cloned Th2 cells synthesize IL-4, IL-5 and IL-6. These different patterns of cytokine production by Th1 and Th2 cells may contribute to distinct functions in the regulation isotype-specific B cell responses. Th1 cell clones, upon allogenic or antigen-specific stimulation, produce IFNγ and support IgG2a synthesis in B cell cultures when compared with the same B cell population stimulated by Th2 cell clones [2]. In contrast, Th2 cells regulated IgE synthesis [1,2], a response largely due to the production of IL-4. Although IgG1 synthesis can be induced by an IL-4 independent pathway, it has been shown that Th2 cells and IL-4 preferentially augment IgG1 synthesis in both LPS-triggered B cell cultures and in antigen-specific systems [1,2].

In the regulation of IgA synthesis, IL-5 produced by the Th2 type cells has been shown to selectively enhance IgA synthesis in PP B cell or in LPS-triggered splenic (SP) B cell cultures [3]. Culture supernatants of activated $CD4^+$ T cell clones from murine PP or well-characterized Th2 clones which produce IL-4 and IL-5, resulted in enhanced IgA production when added to LPS-triggered SP B cell cultures [4,5]. Studies with purified B cells from PP which contain surface IgA^+ ($sIgA^+$) B cells, indicated that IL-5

predominantly affected sIgA$^+$ B cells [6,7,8] and thus do not support a switch function for this cytokine.

2. Quantitation of IFNγ (Th1) and IL-5 (Th2) Secreting Cells By ELISPOT Assay

To quantitate the frequency of IFNγ- or IL-5-specific spot forming cells (SFC), the enzyme-linked immunospot (ELISPOT) assay was employed [9]. For the IL-5 assay, the monoclonal antibody (mAb) TRFK-5 and biotinylated TRFK-4 were used in coating and detection, respectively, while the mAb R4-6A2 and biotinylated XMG 1.2 were similarly used for enumeration of IFNγ-specific spot forming cells (SFC) [10]. Specificity of each assay was tested by using concanavalin A (Con A) activated, cloned Th1 (H66-61) or Th2 (CDC-25) cells. Using mAb anti-IL-5 (TRFK-5) and biotinylated TRFK-4 as coating and detection antibody, respectively, Con A stimulated CDC-25 (Th2) cells formed high numbers of IL-5 specific spots while similarly treated Th1 cell clones (H66-61) did not produce IL-5 SFC [11]. Con A stimulated H66-61 cells gave significant numbers of IFNγ-specific SFC, however the Th2 clone CDC-25 did not form any IFNγ-specific SFC. Both IFNγ and IL-5 SFC were produced *de novo* since treatment of Con A-stimulated Th1 or Th2 cell clones with cycloheximide resulted in the inhibition of their respective cytokine response (85-90% reduction) [11]. Further, wells precoated with anti-IL-5 and incubated with Th2 cells did not form SFC when reacted with biotin-anti-IFNγ. Similar results were obtained with biotinylated anti-IL-5 as detection antibody in wells coated with anti-IFNγ and incubated with Th1 cells. Therefore, the IFNγ and IL-5 ELISPOT employed in this study is specific for the respective cytokine produced by either Th1 or Th2 cells [10,11].

3. Frequency of Th1 and Th2 Type Cells in IgA Inductive and Effector Sites.

It was important to determine the frequency of Th1 and Th2 cells in PP and LPL, since the PP contain a high frequency of sIgA$^+$ B cells but actual IgA synthesis does not take place in this tissue. On the other hand, IgA effector sites contain predominantly IgA producing plasma cells. In the initial study, the frequency of IFNγ and IL-5 SFC were compared between PP and SP. To do this, Con A stimulated and nonstimulated lymphoid cells or purified T lymphocytes from both tissues were subjected to IL-5 and IFNγ ELISPOT assay [Table 1]. Both PP and SP contained similar numbers of IL-5 secreting cells when stimulated with Con A. Although few IL-5 producing cells appeared in nonstimulated lymphocyte preparations, it should be noted that T cells isolated from PP contained some IL-5 specific SFC while splenic T cells possessed essentially no IL-5 producing cells [10,11].

A similar distribution pattern of IFNγ secreting cells was also seen with cells isolated from both PP and SP [Table 1]. Both Con A stimulated PP and SP lymphocytes contained significant numbers of IFNγ-specific SFC and, similar numbers of IFNγ SFC were seen with both SP and PP T cells [Table 1]. Interestingly, it was also noted that T cells freshly isolated from PP also had IFNγ producing cells, however these were essentially not detected in spleen. Taken together, these results show that both PP and SP contain similar numbers of T cells which are capable of producing IL-5 or IFNγ producing cells [10,11].

Based on the findings described above where freshly isolated PP lymphocytes contain low numbers of equally distributed IL-5 and IFNγ SFC, we next compared the frequencies of IFNγ and IL-5 producing lymphocytes isolated from the IgA-effector and -inductive tissues, e.g., LPL and PP, respectively. Lymphocyte preparations obtained from all mucosal associated tissues used in this study contained higher numbers of spontaneous IL-5 and IFNγ secreting cells when compared with spleen [Table 1]. Among gut-associated tissues, LPL, a major IgA effector site, contained the highest number of spontaneous IL-5 producing cells with significant but less numbers of IFNγ secreting cells [11]. In contrast, the PP possessed fewer numbers of spontaneous IL-5 and IFNγ producing cells. It should be emphasized that IL-5 producing (Th2 type) cells were always higher than IFNγ secreting (Th1 type) cells in the LPL subset [11].

Table 1. Frequency of IFNγ (Th1)- and IL-5 (Th2)-Secreting T Cells in Gut-Associated Tissues

Cytokine Production	Source of T Cells	Number of Spot Forming Cells/10^6 Cells	
		IFNγ (Th1)	IL-5 (Th2)
Spontaneous	LPL	9,600 ± 850	28,500 ± 3,450
	PP	3,150 ± 725	3,900 ± 650
	SP	475 ± 105	410 ± 95
Con A Induced	PP	20,150 ± 1,950	23,450 ± 3,500
	SP	24,400 ± 2,650	21,000 ± 2,350

4. Conclusions

The present study provides three major findings that may help us to better understand the potential importance of Th1 and Th2 cells *in vivo*. First, we have developed a single cell assay for enumeration of IFNγ and IL-5 secreting T cells. Second, the occurrence of Th1 and Th2 type cells among lymphocytes freshly isolated from lymphoid tissues of normal mice and activated *in vitro* for short periods have been assessed and, third, the frequency of these two subsets among $CD4^+$ T cells in gut-associated tissues was determined.

The studies described here used the IFNγ and IL-5-specific ELISPOT assay to enumerate Th1 and Th2 cells in various lymphoid tissues of normal mice. The specificity of these assays was verified by using the well characterized Th1 and Th2 cell clones, H66-61 and CDC-25. The Th1 clone, H66-61 produced IFNγ but not IL-5 SFC upon Con A stimulation, while Con A-treated CDC-25 cells produced IL-5 but not IFNγ SFC.

An important and potentially controversial finding was the detection of IL-5 SFC in T cells freshly isolated from lymphoid tissues including SP and PP of normal mice. Our study showed that primary stimulation of T cells with Con A for as little as 24 hr was sufficient to induce IL-5 production. Further, it was shown that although the actual number of IL-5 SFC was lower in non-stimulated than in Con A-activated T cells, spontaneous IL-5 producing cells were also found in naive T cells isolated from PP. These results suggest that T cells with Th2-like characteristics already reside in both PP and SP. It is also important to emphasize that >85% of the IL-5-producing T cells did not simultaneously produce IFNγ, strongly suggesting that the majority of IFNγ SFC were of the Th1 phenotype, while most IL-5 SFC were of the Th2 cell type.

Another unique finding was that both IgA inductive (PP) and effector (LPL) sites contain significant numbers of both IL-5 and IFNγ producing T cells. Our studies

showed that $CD4^+$ Th2 type cells producing IL-5 are present in both IgA inductive and effector sites, but freshly isolated LPL contained higher numbers of IL-5-producing cells when compared with PP. These findings may suggest that IgA induction by T cells is stringently regulated at the site of induction but not where IgA synthesis occurs.

5. Acknowledgements

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Recombinant murine IL-5 enhances IgA production by rat splenic B cells

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Recent reports using highly purified or recombinant interleukin 5 (IL-5) have determined that this interleukin induces enhanced IgA synthesis by lipopolysaccharide (LPS) triggered murine splenic B cells as well as unstimulated Peyer's patch B cells (1-3). The recombinant form of IL-5 is not yet available for the rat, however, a previous report by our group (4) has indicated that interleukin-containing culture supernatants from murine EL-4 and B151 K12 cell lines could support plaque forming cell responses by rat B cell cultures. Because of the importance of the rat models in mucosal immunity, we chose to study the effect of recombinant murine IL-5 (rmIL-5) on the polyclonal antibody response of rat spleen B cells.

Spleen B cells were derived by passing spleen cells over a Sephadex G-10 column prior to panning with rabbit anti-rat $F(ab')_2$ (4). Spleen B cells were cultured 7 days in the presence or absence of rmIL-5 (10 U/ml), LPS (10 ug/ml), or 10% rat spleen Con A stimulated supernatant (CAS). It was interesting that IL-5 enhanced significantly the secretion of IgA and IgG in unstimulated cultures yet only IgA secretion was enhanced in LPS stimulated cultures (Table 1). When splenic B cells were separated by density using a discontinuous percoll gradient (1), the low density, activated cells (above the 55% layer) also yielded enhanced levels of IgA as well as IgG in both unstimulated and LPS stimulated cultures supplemented with IL-5 (data not shown). To the contrary, IL-5 had a minimal effect on the secretion of any isotype from high density, resting cells (cells above the 70% layer yet below the 60% layer).

IL-5 alone stimulated some proliferation of rat spleen B cells (Table I), as measured by reduction of the dye MTT (5), yet rmIL-5 inhibited proliferation of LPS stimulated B cells. Using the uptake of tritiated thymidine to assess proliferation, we confirmed that IL-5 enhance proliferation in unstimulated cultures but also found that proliferation was enhanced in LPS stimulated cultures (data not shown). This discrepancy was probably due to a basic difference in the two methods for measuring proliferation.

These results suggest that IL-5 enhances the secretion of rat IgA and IgG. The mitogen, LPS, had very little effect on IgA secretion yet the presence of this mitogen masked the effect, if any, of IL-5 on IgG secretion. The fact that IL-5 exerted its effect mainly on the large, low density B cells suggests that IL-5 affected mainly activated B cells, similar to that as seen in the mouse (1). Of course, this enhanced secretion of Ig could be due to a proliferation of the cells since we have shown that IL-5 does indeed enhance proliferation of both unstimulated and LPS stimulated B cells. However, studies with murine B cells have shown that IL-5 stimulated only surface IgA^+ cells ($sIgA^+$) to

produce IgA (1, 6) yet it did not induce an increase in the number of Peyer's patch IgA producing cells (1) or enhance the proliferation of LPS stimulated sIgA$^+$ spleen cells (6). Experiments are currently under way to address this problem. Taken together, these results indicate that IgA secretion, and to a minor extent IgG secretion, by rat B cells is under the control of an interleukin similar to murine IL-5.

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TABLE I. Effect of rmIL-5 on rat spleen B cells.

	ng Ig/ml[a]			Proliferation (O.D. 590 nm)[b]	
Condition	IgA	IgG	IgM	Exp. 1	Exp. 2
Control	24 ± 8	7 ± 3	704 ± 354	0.031	0.084
IL-5	81 ± 22*	27 ± 9*	1,629 ± 494	0.050	0.102
CAS	279 ± 78*	27 ± 5*	565 ± 215	0.043	0.103
LPS	50 ± 9	33 ± 10	4,291 ± 1,314	0.084	0.226
LPS+IL-5	143 ± 22*	38 ± 9	3,288 ± 971	0.079	0.209
LPS+CAS	369 ± 75*	107 ± 23	3,972 ± 1,065	0.071	0.208

[a] Concentration of Ig in 7 day rat spleen B cell culture (2 x 10^5 cells/culture) culture supernatants. Values are the mean ± SE for 5 experiments.

[b] Proliferation of rat spleen B cells (1 x 10^5 cells/culture) as measured by the MTT dye assay (5). Values are the mean of triplicate cultures.

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Human interleukin-5 "switches" human IgA$^+$ B cells to express and secrete IgA

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INTRODUCTION In the murine and the human system there is evidence that T-cells regulate both isotype switching to IgA (1,2), and post-switch expansion of IgA$^+$ B-cells (2,3). The post-switch activity in the murine system appears to be mediated, at least in part, by Interleukin-5 (IL-5) (4). However, experiments examining the role of human IL-5 in IgA secretion failed to validate the results in the mouse (5).

We, therefore, investigated a number of human B-cell mitogens for their ability to induce IL-5 responsiveness in human B-cells. Branhamella catarrhalis (Bc) is a T-cell independent human B-cell mitogen (6). Using this B-cell mitogen we have been able to demonstrate for the first time that a purified recombinant human T-cell lymphokine (human IL-5) is able to switch IgM$^+$ B-cells to IgA$^+$ B-cells and also support the terminal differentiation of these cells to IgA secretion.

MATERIALS AND METHODS Interleukin-5 was kindly provided by Dr Colin Sanderson, of National Institute for Medical Research (Mill Hill, London).

B-cells were isolated from tonsillar lymphocytes by sheep red cell rosetting and macrophage depletion by plastic adherence at 37^{o}C. The resulting B-cell population contained greater than 97% B-cells and less than 2% T-cells and macrophages, as assayed by FACS analysis. In some experiments, the B-cells were fractionated into subpopulations of IgM$^+$ B-cells by using a panning technique. The resulting population contained greater than 80% IgM$^+$ B-cells and less than 3% IgA$^+$ B-cells as analysed by FACS.

B-cells (unfractionated and IgM$^+$) were preactivated with Staphylcoccal A Cowan 1 (SAC) or formalin-killed Branhamella catarrhalis for 24 hrs at 37^{o}C. The bacterial particles and non-viable cells were removed by Ficoll-Hypaque density centrifugation. 10^5 activated B-cells/well were cultured with various dilutions of IL-5. Supernatants were assayed for immunoglobulin after 8 days using an isotype specific ELISA.

IgM^+ B-cells that had been preactivated with SAC or Branhamella were cultured with or without IL-5 for 5 days and then stained for IgM and IgA surface expression.

RESULTS SAC-activated B-cells secreted increased amounts of IgM in the presence of IL-5, but IgG and IgA secretion were not altered (Fig.1a). However, IL-5 enhanced both the IgM and IgA secretion of Bc-activated B-cells (Fig.1b). These results demonstrate the importance of the B-cell activation signal in determining lymphokine responsiveness.

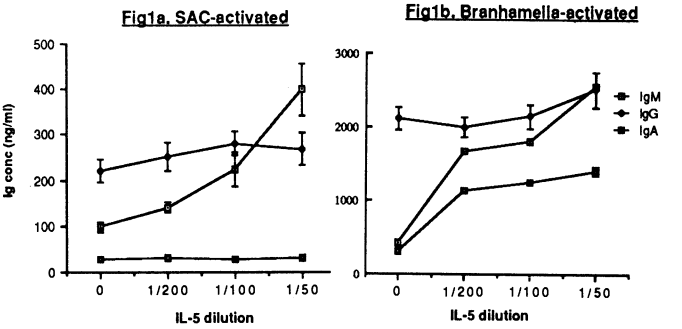

Fig 1. B-cells were activated with SAC (Fig.1a) or Bc (Fig.1b) and cultured with IL-5 for 8 days.

To determine whether IgA augmentation by IL-5 arises as a result of switching or post-switch help we isolated IgM^+ B-cells, stimulated with SAC or Bc and then cultured in the presence or absence of IL-5. Surface staining at day 5 revealed that SAC activated IgM^+ B-cells could not be induced to express surface IgA, but Bc activated B-cells did express surface IgA if cultured in the presence of IL-5. As IL-5 does not provide a proliferative signal to B-cells (data not shown) these results demonstrate that IL-5 is a switch factor for human B-cells activated with Bc.

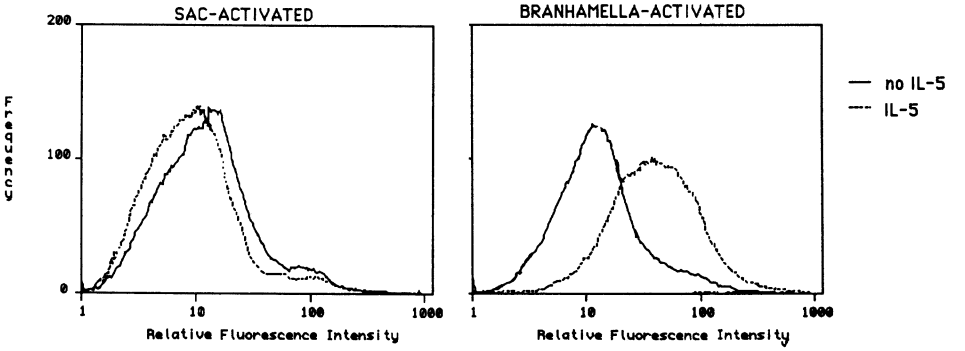

Fig.2. IgM^+ B-cells were pre-activated with SAC or Bc and cultured for 5 days. Surface IgA expression was then examined using a FITC-conjugated goat anti-human IgA antibody.

To determine whether these switched B-cells are also induced to secrete IgA, we measured the IgA secretion of IgM^+ B-cells activated with PWM, SAC or Bc. Bc activated IgM^+ B-cells secrete IgA in the presence of IL-5 (Fig.3).

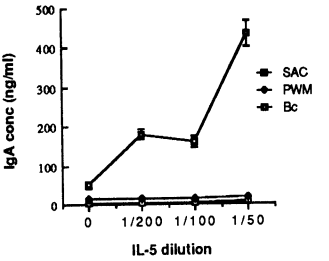

Fig.3. IgM^+ B-cells were activated with SAC, PWM or Bc and cultured with IL-5 for 8 days. IgA levels in the supernatant were then examined using an isotype specific ELISA.

CONCLUSIONS These results demonstrate that human IL-5 acts as a switch and terminal differentiation factor for B-cells, but also highlights the importance of the B-cell activation signal in determining the lymphokine responsiveness.

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Regulation of human IgA and IgA subclass synthesis by recombinant interleukin-6

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Immunoglobulin synthesis is mediated by several cytokines including IL-4, IL-5 and IL-6 which induce activation, proliferation and differentiation of B cells. Among these ILs, IL-6 has been cloned and described as a potent cytokine for the terminal differentiation of B cells to high Ig secreting plasma cells [1]. Further, a receptor for IL-6 has been cloned and was found only on activated and dividing but not resting B cells [2,3]. To this end, pokeweed mitogen (PWM) stimulated peripheral blood mononuclear cells (PBMC) were sensitive to recombinant human IL-6 (rhIL-6), where significant augmentation of Ig synthesis was noted in all isotypes [1, 4]. Furthermore, incubation of blast cells isolated from mitogen preactivated B cells with rhIL-6 resulted in enhancement of Ig synthesis.

Human mucosal surfaces cover an enormous body area (over 200 times larger than skin), and the major class of Ig present in external secretions protecting these surfaces is secretory IgA (S-IgA). In this regard, IgA production in humans is greater than 60% of total Ig produced [5]. Our recent study showed that both rhIL-6 and mouse rIL-6 induced high rates of IgA secretion in IgA committed B cells [surface IgA^+ ($sIgA^+$) B cells] isolated from murine Peyer's patches [6]. These findings supported the idea that IL-6 play an important role in promoting the terminal differentiation of $sIgA^+$ B cells to IgA secreting plasma cells.

We have studied the role of IL-6 for induction of human IgA synthesis, including IgA1 and IgA2 subclasses. In the first series of experiments, human spleens were obtained from patients with idiopathic diseases following surgery or at autopsy. Splenic (SP) B cells were enriched by removal of T cells and were further separated into high and low density fractions by Percoll gradients and cultured with rhIL-6. After 3 and 7 days incubation, the number of IgA, IgA1 and IgA2 producing cells were enumerated by ELISPOT assay. Although cultures containing whole B cells and rhIL-6 supported IgM, IgG and IgA spot forming cell (SFC) responses, enhancement of IgA SFC by IL-6 was largely confined to the low density, blast B cell population. Both IgA1 and IgA2 SFC were increased in the presence of rhIL-6 without affecting the IgA1 and IgA2 ratio (4:1). On the other hand, the small resting population revealed low numbers of Ig secreting cells even in the presence of rhIL-6. These results suggest that IL-6 affects IgA1 and IgA2 B cell blasts and induces their differentiation into Ig secreting cells [7].

Human PBMC contain more IgA1 SFC than IgA2 cells, however prolonged stimulation of PBMC with PWM, induced increased numbers of IgA2 positive cells [8]. Thus, PBMC freshly isolated from healthy donors were initially incubated with PWM for 48 hrs. These cells were extensively washed to remove residual PWM and then incubated with or without rhIL-6 for 3-7 days. Cells harvested from cultures containing preactivated PBMC and rhIL-6 resulted in increased numbers of all SFC isotypes. In terms of IgA subclass synthesis, although PWM-pretriggered cultures slightly augmented the number of both IgA1 and IgA2 SFC, the addition of rhIL-6 further enhanced the number of IgA1 and IgA2 producing cells. Interestingly, the number of IgA2 exceeded IgA1 SFC, indicating that IL-6 is particularly important for IgA2 responses in PWM-preactivated PBMC [7].

To further elucidate the effect of IL-6 on human IgA synthesis, purified B cells were obtained from the appendix. It is well known that GALT is an IgA inductive site and contains high frequences of $sIgA^+$ B cells when compared with systemic tissues (e.g., spleen) [5]. When rhIL-6 was added to cultures of purified B cells from appendix, PBMC or spleen, higher numbers of

IgA SFC were noted in appendix B cell cultures when compared with PBMC or spleen (Table 1). Further, rhIL-6 augmented both IgA1 and IgA2 producing cells in appendix B cell cultures. Taken together, our study shows that IL-6 acts on activated IgA1 and IgA2 B cells and enhances both IgA1 and IgA2 secretion [7].

Table 1. Comparison of IL-6 Induced IgA1 and IgA2 SFC in B Cells Isolated From GALT and Systemic Sites*

		SFC/10^6 Cells	
Source of B Cells	IL-6	IgA1	IgA2
PBMC	-	480 ± 30	141 ± 11
	+	486 ± 20	100 ± 30
Spleen	-	605 ± 153	262 ± 58
	+	890 ± 46	428 ± 122
Appendix	-	1,380 ± 46	2,050 ± 63
	+	3,100 ± 86	4,850 ± 86

* Purified B cells were cultured either in the presence or absence of rhIL-6. After 7 days of incubation, nonadherent cells were assessed for IgA1 and IgA2 SFC by ELISPOT assay.

** PBMC were isolated from normal subjects. Splenic B cells were obtained from patients with idiopathic diseases.

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Recombinant interleukin-6 enhances specific antibody secretion by *in vivo* activated human B cells

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Introduction

IL-6 promotes the terminal differentiation of lymphoblastoid B cells lines, mitogen-activated peripheral blood and tonsillar B cells *in vitro* (Muraguchi et al. 1988). In addition, it was recently shown that rhIL-6 augmented the antigen-specific response of murine B cells to sheep erythrocytes (Takatsuki et al. 1988). We investigated whether rhIL-6 stimulates *in vivo* activated peripheral blood B cells in an antigen-specific system.

Materials and Methods

rhIL-6. A cDNA for IL-6 was expressed in *E. coli* using a new expression-preparation system (Tonouchi et al. 1988). After purification, rhIL-6 with a specific activity of 10^5 U/19.3 µg/ml was obtained.

Anti-IL-6 antibody. Polyclonal antibodies were generated by immunizing a goat with rhIL-6. The antiserum neutralized 1 U/ml of rhIL-6 at a 10^{-5} dilution (Beagley et al. 1989).

Cell preparation. PBMC fractions enriched for B cells were obtained by removing monocytes/macrophages through adherence, and T cells by rosetting with AET-SRBC. Cells were further separated on a discontinous percoll gradient into high and low density fractions.

Immunizations. Healthy adult volunteers were systemically immunized with a 23 valent pneumococcal vaccine (PPS) (Pnu-Imune 23, Lederle, Pearl River, NY, U.S.A.), or diphtheria toxoid (DT) (Wyeth, Marietta, PA, U.S.A.), or a type 12F pneumococcal polysaccharide-diphtheria toxoid conjugate (Pn 12F-DT, kindly provided by Drs. Schneerson and Fattom, LDMI, NICHD, Bethesda, MD, U.S.A.).

Culture conditions. Unfractionated PBMC (10^6 cells/ml) or B cell-enriched fractions ($0.5x10^6$ cells/ml) were cultured in complete medium with or without rhIL-6 for 7 days.

Enzyme-linked immunospot assay (ELISPOT, Czerkinsky et al. 1988). A 96-well Millititer HA plate (Millipore, Bedford, MA,U.S.A.) was coated with specific antigen. PBMC was incubated on the plate for 4 hrs during which secreted antibodies bound to the solid phase. Then enzyme-labeled isotype-specific secondary antibodies were added. Spots that correspond to individual antibody-secreting cells (AbSC) were made visible with the substrate NBT/BCIP (Bio-Rad, Richmond, CA, U.S.A.).

Results and Discussion

PERIPHERAL BLOOD B CELLS RESPONSIVE TO IL-6

High density and low density B cells were stimulated for 7 days with rhIL-6. Stimulation with rhIL-6 increased the number of Ig-secreting cells predominantly in the low density fraction which represent *in vivo* activated B cells. The increase was not restricted to a certain isotype.

EFFECT OF rhIL-6 ON ANTIGEN-ACTIVATED LYMPHOBLASTOID B CELLS

Antigen-activated lymphoblastoid B cells were generated by systemic immunization with vaccines. No specific AbSC were detectable before immunization, and *in vitro* stimulation of preimmune B cells with rhIL-6 failed to induce specific antibody secretion. The same held true for PBMC isolated 3 days after immunization. Seven days after immunization with either PPS or DT or Pn 12F-DT, antigen-specifc AbSC representing lymphoblastoid B cells were present in the circulation (Stevens et al. 1979, Kehrl and Fauci 1983). When PBMC or B cell-enriched fractions were stimulated with rhIL-6 *in vitro* for 7 days, the numbers of AbSC specific for the respective antigen (PPS or DT) were increased in a dose-dependent fashion (Fig. 1).

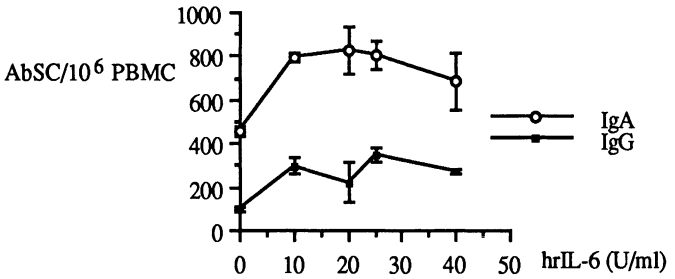

Figure 1. rhIL-6 increases the numbers of in-vivo activated antibody-secreting cells

After immunization with the complexed antigen Pn 12F-DT, Pn 12F- and DT-specifc AbSC were present among PBMC. rhIL-6 promoted the differentiation of lymphoblastoid B cells of both specificities. Stimulation with rhIL-6 did not change the isotype distribution of the AbSC.

INHIBITION WITH ANTI-IL-6 ANTIBODIES

To test whether the observed increase of AbSC was induced by rhIL-6, the cells obtained 7 days postimmune were cultured in the presence of rhIL-6 and anti-IL-6. The polyclonal antiserum completely neutralized the effect of rhIL-6 up to a concentration of 40 U/ml, while this inhibitory effect was reversed by higher doses of the cytokine.

SUMMARY

There is a subpopulation of peripheral blood human B cells which represents *in vivo* activated low density cells, and can be stimulated by rhIL-6 to differentiate into Ig-secreting cells without the use of exogenous mitogens. The differentiation of vaccination-induced lymphoblastoid B cells into plasma cells was promoted by rhIL-6. In our systems, rhIL-6 did not show preference for any antigen or isotype. IL-6 may play an important role in mucosal tissues, where antigen-activated B cells home and receive final signals to differentiate into high rate IgA-secreting plasma cells.

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The role of T-cell lymphokines in congenital immunoglobulin deficiency

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INTRODUCTION

It has become increasingly obvious that T-cell derived lymphokines play a vital role in B-cell proliferation and differentiation,. In view of this, we examined whether the defect in humoral immunodeficiencies could be modified by T-cell lympokines.

Infantile sex-linked agammglobulinameia (ISLA) is a congenital disease associated with early onset, markedly reduced serum immunoglobulin (Ig) levels, with a decreased number (1), or absence (2), of B-cells and plasma cells. ISLA with hypergammaglobulinaemia M (Hyper-IgM) (3) is a disease which presents similarly to ISLA but patients display normal to high serum levels of IgM. The precise defect underlying these diseases has yet to be elucidated (4,5).

The aim of this project was to determine whether T-cell derived lymphokines could alter Ig secretion by B-cells from patients with ISLA or Hyper-IgM.

MATERIALS AND METHODS

Peripheral blood lymphocytes were obtained from heparinized venous blood from both patients and normal healthy individuals. T and B-cell populations were separated by T-cell rosetting. The PWM stimulated B-cells from both patients and normals were then cultured with a variety of interleukins or supernatants from PHA stimulated gut-associated lymphoid tissue (GALT) T-cells. The interleukins tested were recombinant human interleukin 2 (IL-2), IL-4 and IL-5. Blocking experiments were performed on the GALT supernatants using anti-Tac, anti-IL-5 and anti-IL-6 antibodies. All cultures were incubated for 10 days and the supernatants tested for Ig by an isotype specific ELISA. Immunofluorescence was performed on peripheral blood B-cells from both patients and normal individuals. Cells were incubated with conjugated goat anti-human IgM, IgG and IgA , with normal goat serum and the cell line, CEM, as controls.

RESULTS

GALT T-cell supernatants have been shown previously to preferentially enhance IgA secretion of PWM-stimulated normal B-cells. This preferential enhancement of IgA is associated with switching of surface IgM positive B-cells to IgA secretion (6). We therefore examined whether the "switch" supernatant could enhance the Ig secretion of B-cells derived from patients with ISLA or Hyper-IgM. The addition of GALT T-cell supernatants to purified Hyper-IgM B-cells resulted in a significant increase ($p<0.05$) in IgG in all of the Hyper-IgM patients, and in 2 of the 4 patients there was also a significant increase in IgA ($p<0.05$) (Fig. 1). Similarly, the GALT T-cell supernatants enhanced the Ig secretion of B-cells from all of the 6 patients with ISLA (Fig 2.). In order to elucidate whether the augmentation of Ig secretion was due to one of the defined B-cell growth and differentiation factors,

we cultured patient B-cells with purified recombinant IL-2, IL-4 and IL-5. None of the lymphokines, either alone or in combination, could augment the Ig secretion of patients B-cells. Additional experiments with anti-TAC, anti-IL-5 and anti-IL-6 antibodies failed to inhibit the augmentation of Ig secretion in the presence of the GALT T-cell supernatants.

In order to more clearly define the B-cell substrate that the GALT T-cell supernatants were acting upon, we examined the patients B-cells for surface immunoglobulin (sIg). Although the Hyper-IgM patients had normal numbers of sIgM+ B-cells, they had no detectable B-cells with sIgG or sIgA. The patients with ISLA had reduced numbers of sIgM+ B-cells compared to normals and no evidence of B-cells expressing sIgG of IgA.

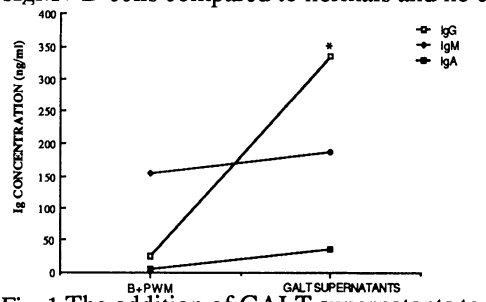

Fig. 1. The addition of GALT supernatants to Hyper-IgM B-cells resulted in a significant increase increase in IgG. Data representative of 4 patients. * $p < 0.05$.

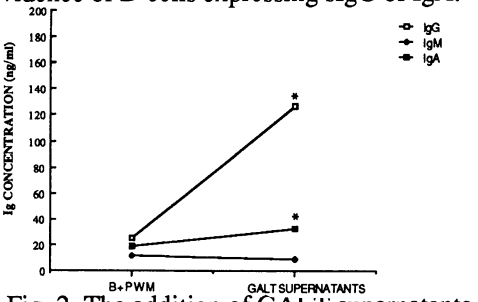

Fig. 2. The addition of GALT supernatants ISLA B-cells resulted in a significant in IgG. Data representative of 6 patients. * $p < 0.05$.

DISCUSSION

The results demonstrate that a product derived from GALT T-cells is able to augment Ig secretion of B-cells from patients with ISLA and Hyper-IgM. The augmentation of Ig secretion is the same order of magnitude as that seen on normal control B-cells. This activity does not appear to be due to IL-2, IL-4, IL-5 or IL-6, as the recombinant lymphokines do not mediate the effect nor do blocking antibodies reduce the activity in the GALT T-cell supernatants. Ig augmentation appears to be associated with a switch signal as neither patient group had detectable B-cells committed to IgG or IgA. The results suggest that the defect in patients with ISLA and Hyper-IgM may, at least in part, be corrected by the addition of T-cell lymphokines.

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A clonal microculture that supports IgA expression by murine B cells

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ABSTRACT. Immunoglobulin (Ig) isotype switching and commitment, generation of memory cells, and the functional potential of B cell subsets have been analyzed in two different clonal microcultures. One is antigen (Ag)-dependent, haplotype-restricted, and requires linked recognition of hapten and carrier. The other is Ag-independent and relies on the stimulation of single I-A^b cells by alloreactive D10 helper T cells. Immunising mice increased the frequency of clonal precursors among hapten-gelatin enriched cells as well as the proportion of clones secreting non-IgM isotypes. IgA was produced when either purified dendritic cells (DC) or IL-5/IL-6 were added to the T-B cultures. $sIgD^+$ cells could be induced to produce a wide range of isotypes including IgA whereas a majority of $sIgA^+$ cells produced IgA exclusively. Germinal centre (GC) cells could be stimulated in both systems to divide and produce antibody and this subset appears to contain a radiation-resistant population that secretes mainly IgA upon allo-recognition by D10 cells in the presence of DC.

Methods and Results

Priming affects frequency of antigen-specific splenic B cells and the isotype display. C3H/FeJ mice were primed with 100 µg of fluorescein-conjugated haemocyanin (FLU-Hy) in CFA i.p. and boosted 4 weeks later with 1µg of FLU-Hy on alum i.p. Six weeks later their spleens were harvested, panned on FLU-gelatin (1), and the cells plated out at 20 B per well with 3000 conalbumin specific, I-A^k restricted D10 G4.1 T cells (2) and FLU-conalbumin (50 ng/ml). The response was compared with that of cells from unprimed mice that had been panned and cultured in a similar way; the results are shown in Table 1. The frequency of hapten-gela-

Table 1.

			Clones secreting (%):				
B cells	Freq.(%)	Anal.(#)	Only IgM	No IgM	Some IgM	Some IgG	Some IgE
unprimed	0.83	24	45	5	95	50	0
primed	4.81	21	64	36	64	27	18

tin binding cells that could respond to specific Ag increased from 0.83 to 4.81% upon hyperimmunising the mice. The proportion of clones making no IgM increased from 5-36%; 18% made some IgE. No IgA was detected.

Dendritic cells support the expression of IgA in microculture. PP cells from mice primed intraduodenally (i.d.) 14 days earlier with N-acetyl-glucosaminyl (GlcNAc)-conjugated cholera toxin were panned on Petri plates coated with GlcNAc-gelatin and plated out at 30 B per well with 3000 T cells, 400 DC from PP or spleen (3,4) and GlcNAc-conalbumin (20 ng/ml). Cultures were harvested a week later and assayed for GlcNAc-specific Ab. As shown in Table 2, DC from both PP and spleen supported the expression of some IgA in culture and between 20-50% of clones produced only IgA.

Table 2.

		Clones secreting (%):			
Cells	# clones	Some IgM	Only IgM	Some IgA	Only IgA
B+T	1/120	100	100	0	0
B+T+PP-DC	10/120	80	60	40	20
B+T+Spl-DC	4/120	50	50	50	50

The germinal centre contains cells that can respond to specific antigen. $PNA^{high}\ sK^{low}$ (5) germinal centre cells from the PP of mice primed i.d. 1 wk. earlier with FLU-toxin were plated out at limiting dilution with 3000 T cells and 400 PP DC and the specific Ab response compared to that from $PNA^{low}sK^{high}$ non-germinal centre cells. The results are shown in Table 3. An Ag-specific response was seen in the germinal centre population with the expression of all isotypes, including IgA.

Table 3.

			Clones secreting some (%):			
B cell type	# clones	Freq.(%)	IgM	IgG	IgA	IgE
$PNA^{hi}sK^{lo}$ + Ag	9/120	0.01	100	56	11	33
- Ag	0/120	0	0	0	0	0
$PNA^{lo}sK^{hi}$ + Ag	17/120	0.02	100	65	6	24
- Ag	1/120	0.013	0	100	0	0

Allogeneic stimulation can be used to study the effect of accessory cells and lymphokines on the activation requirements and isotype potential of single B cells. It is known that D10 T cells can stimulate $I\text{-}A^b$ cells to divide and produce Ab (2). We used this property of the D10 cells to study the potential of single B cells in culture. PP cells from (C57BL/6 x C3H) F_1 mice were depleted of adherent cells and T cells and plated out at 1-2 per well with T cells alone, along with recombinant IL-5 or IL-6 or along with purified DC. The results are shown in Table 4. The addition of exogenous IL-5 led to the expression

of some IgA in the cultures. IL-6 added 2 days into the culture period was more supportive and even allowed the expansion of clones that produced only IgA. DC were extremely supportive of IgA expression and 13% of clones in this group secreted only IgA. All isotypes, including IgE, were expressed (data not shown).

Table 4.

		Clones secreting (%):			
Cells	#+ve/60	Some IgM	Only IgM	Some IgA	Only IgA
B+T	34	34	0	3	0
B+T+IL-5	31	42	3	7	0
B+T+IL-6	31	45	13	23	7
B+T+DC	30	65	10	20	13

An analysis of subsets of B cells from the PP obtained by fluorescence-activated cell sorting was then attempted using the B cell-T cell-DC system; the results are shown in Table 5. $sIgD^+$ cells could be stimulated to produce all isotypes, including IgA, suggesting that the culture supported intraclonal switching of these cells. $sIgA^+$ cells produced mainly IgA (44% produced only IgA). Germinal centre cells that are PNA^{high} could be stimulated in such single B cell cultures to produce a variety of isotypes although no clones produced IgA exclusively. However, when such cells were irradiated (ϟ), 73% of the responding cells produced only IgA.

Table 5.

		Clones secreting (%):			
Cells	#+ve/60	Some IgM	Only IgM	Some IgA	Only IgA
$sIgA^+$	16	38	6	56	44
$sIgD^+$	14	93	0	29	0
$PNA^{hi}{}_{SK}{}^{lo}$	33	76	21	20	0
ϟ $PNA^{hi}{}_{SK}{}^{lo}$	12	18	9	82	73

Discussion

The cells and lymphokines required for differentiation, maturation, and secretion of IgA by B cells has been the focus of intense investigation. However, most efforts have been hampered by the lack of a suitable culture system that allows the analysis of these events at a clonal level. The microcultures described here are extremely supportive of clonal expansion and IgA secretion by B cells at different stages of differentiation.

Addition of rIL-5 to minimal T-B cultures induced the expression of IgA from unfractionated B cells as well as $sIgD^+$ cells from the PP. IgA was always expressed in association with another Ig isotype, usually IgM, and this supports earlier observations (6) that IL-5 may be a switch factor. IL-6 promoted the exclusive expression of IgA in a small proportion of clones. It was more effective when added late in the

culture period and did not enhance IgA production from $sIgD^+$ B cells (data not shown). These observations suggest that IL-6 might act on cells that have been activated and have switched to the expression of non-IgM isotypes.

Utilising the alloreactivity of D10 cells and the supportive effects of purified DC allowed GC cells to be scored at the single B cell level. This had previously not been possible since panning for Ag-specific cells on haptenated gelatin consistently fails to yield PNA^{high} $s\kappa^{low}$ B cells. Some cells expanded in culture as shown by ^{3}H-thymidine incorporation and their clones produced a wide variety of isotypes, including IgA. However, cell division did not appear to be essential for the secretion of IgA by a proportion of GC cells. Indeed, when GC cells were irradiated (1600 rad) before culture, the only isotypes secreted were IgA and IgM and a great proportion of cells secreted only IgA. This subset of cells might represent the pre-plasmablasts that are ready to leave the germinal centre (and the PP) and may be the subset that has demonstrably high levels of mRNA for IgA in the cytoplasm (see Weinstein et al., this volume).

Distinct cell types can thus be identified in the GC. Some cells appear more immature and divide in culture to produce a variety of isotypes while others appear to more differentiated and secrete only IgA. It remains to be determined whether specific factors can trigger this subpopulation to secrete and whether IgA secretion is prevented by the prolific cell division that occurs in the germinal centre.

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Regulation of B cell differentiation by T cells obtained from mucosal and systemic lymphoid tissues

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Regulation of immunoglobulin (Ig) isotype has long been attributed to helper T cells and evidence is currently accumulating to suggest that different lymphokine/ cytokine combinations can directly influence the isotypes and amounts of Ig which B cells secrete. We [1,2] and others [3-5] have postulated that the differences in Ig isotypes which predominate in mucosal versus systemic immune responses are due, in part, to the combination of lymphokines produced by resident helper T cells. To address this question, we have isolated helper T cells from murine Peyer's patches, mesenteric lymph nodes, and spleens. We have measured lymphokine production by each T cell population and quantified the Ig isotypes produced by large, low density B cells cultured with the isolated T cells.

Lymphoid cell preparations were enriched for L3T4 bearing T cells by complement mediated lysis of Lyt-2 and MHC Class II bearing cells. The remaining cells were stimulated with Con A and cultured with T cell depleted,irradiated peritoneal exudated accessory cells (PEC) for either 40 hours or for 5 days with additional IL4 (10% supernatant derived from the X63Ag8-653 myeloma cell line which has been transfected with the murine IL4 cDNA [6]). T cells were cultured for 5 days and then washed extensively and restimulated for 40 hours with Con A alone. The supernatants were then analyzed for IL2 using the T cell line CTLL, blocked with anti-IL4, and for IL4 and IL5 using the B cell line BCL-1, blocked with either anti-IL4 or anti-IL5.

Supernatants of T cells isolated from all three organs and stimulated with Con A for 40 hours contain high concentrations of IL2 but do not contain either IL4 or IL5. Supernatants of T cells stimulated with Con A in the presence of IL4 and restimulated with Con A do not contain IL2 but do contain both IL4 and IL5. Peyer's patch T cells produce higher amounts of IL4 than do either mesenteric

lymph node or spleen T cells.

T cell stimulation of B cell differentiation was also determined. The T cells were mixed with low density (large) B cells from spleens or Peyer's patches. Ig isotypes, produced in the absence of added B cell mitogen, were measured. After stimulation with Con A plus IL4 for 5 days and restimulation with Con A for 24 hours, T cells isolated from both Peyer's patches and mesenteric lymph nodes are able to interact with large, low density Peyer's patch B cells in their differentiation to IgA producing cells while splenic T cells are only marginally effective. Peyer's patch T cells but neither mesenteric lymph node nor splenic T cells can drive large splenic B cells to produce IgA. When T cells are restimulated biweekly with Con A plus IL4, the profiles of T cell effects change. By two months after isolation, T cells from all three organs increased their ability to drive IgA production by large Peyer's patch B cells but the splenic T cells remained the least effective. Similar results are seen in analyses of IgM and of IgG subclasses. By four months after isolation, T cells from all three organs provide help for IgA production by both Peyer's patch and splenic low density B cells. This increase in ability to provide help for IgA producing B cells is accompanied by an increase in IL5 production. We conclude that prolonged in vitro stimulation favors the growth of T helper cells which provide the necessary signals for B cell terminal differentiation to become IgA producing cells.

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Spontaneous gamma-interferon production by human tonsillar lymphocytes: regulation of secretion by interleukin-1

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ABSTRACT. The frequency of gamma-interferon (IFN-γ) secreting cells was examined in tonsillar cell suspensions. Comparisons were made between tonsils from orally and/or parenterally immunized individuals and non-immunized controls, and between infected and clinically healthy tonsils . Our results show that the tonsils are the site of pronounced T-lymphocyte (and possibly natural killer cell) activation, irrespective of intentional immunization or clinical status. In addition, this study suggests that interleukin-1 is required not only as an inducer of IFN-γ synthesis, but also as a promoter of its secretion.(Supported by the Swedish Medical Research Council).

1. Introduction

The role of the tonsillar immune apparatus is poorly understood. In this regard, available information concerning T-cell function in the tonsils is scanty. Recently, we have observed pronounced T-lymphocyte (and possibly natural killer cell) activity in the intestinal mucosa associated with vigorous interferon-gamma (IFN-γ) production. This activity was substantially increased after a booster immunization with a prototype mucosal immunogen, i.e. oral cholera vaccine (see Quiding, M. et al, this volume).

Reasoning that the tonsillar system might constitute an extension of the mucosal immune network, we have examined the frequency of tonsillar mononuclear cells spontaneously secreting IFN-γ after parenteral and/or enteral immunization. In addition, the regulation of IFN-γ secretion by tonsillar accessory cells and/or monokines was studied.

2. Materials and Methods

2.1 IMMUNIZATION REGIMENS

Nineteen adult patients scheduled for tonsillectomy volunteered for this study. Six had clinically healthy tonsils (tonsillectomy indicated by "snoaring" problems) and 13 suffered from recurrent episodes of tonsillitis. Seven patients received a booster subcutaneous injection of tetanus/diphteria toxoids vaccine (TT/DT) and/or a booster dose of oral cholera B-subunit/whole cell vaccine (CTB/WC) (1), 7 days prior to tonsillectomy.

2.2 CELL ISOLATION

Tonsillar mononuclear cells (MNC) were isolated by teasing small pieces of tonsillar tissue through a nylon mesh, followed by centrifugation on a Ficoll-Isopaque gradient.

2.3 DEPLETION OF ADHERENT CELLS

Plastic adherent cells were depleted by 2 cycles of incubation (2 hours, 37^{o}C) in polystyrene Petri dishes.

2.4 ENUMERATION OF IFN-γ-SECRETING CELLS

Cell suspensions were assayed for numbers of IFN-γ-secreting cells by a two-site reverse ELISPOT technique using epitope specific monoclonal anti-IFN-γ antibodies (Ab) as capture and developing reagents (2). Briefly, intestinal suspensions containing various numbers of MNC were incubated for 20 hr at 37°C in wells coated with anti-IFN-γ Ab 1. Zones of solid phase bound IFN-γ secreted by individual MNC were revealed as spots by stepvise addition of biotinylated anti-IFN-γ Ab 2, horseradish peroxidase conjugated avidin, and suitable chromogen substrate.

3. Results

Tonsillar IFN-γ-secreting cells were detected in substantial numbers in all tonsillar cell suspensions examined (table 1). Booster doses of TT/DT and/or CTB/WC did not influence the frequency of IFN-γ-secreting cells. When comparing the frequences of IFN-γ-secreting cells in clinically healthy tonsils to inflamed ones, no significant differences were observed.

Unexpectedly, the relation between numbers of MNC assayed and numbers of detectable IFN-γ-secreting cells was not linear, but decreased dramatically below a treshold cell density of 10^6 MNC plated. This observation was confirmed by quantitative ELISA analyses of IFN-γ levels accumulated in supernatants from tonsillar MNC cultures performed at various cell densities. This lack of linearity was also observed with a human spleen cell suspension in contrast to intestinal cell suspensions and PHA- or anti-CD3-stimulated peripheral blood MNC cultures.

Depletion of plastic adherent cells from the tonsillar MNC culture resulted in decreased numbers (relative to the original cell density) of detectable IFN-γ-secreting cells by 52+/-7% (geom. mean+/-S.E.M., n=7), indicating that adherent cells (presumably macrophages or dendritic cells) provide an essential factor for supporting the secretion of IFN-γ. Addition of picogram amounts of purified IL-1α or IL-1ß partially restored the predicted linearity.

4. Conclusions

The tonsils constitute an organ with pronounced T-lymphocyte (and possibly natural killer cell) activation. This study establishes a dual regulatory role for IL-1 in the production of IFN-γ, in that it acts not only as a known IFN-γ inducer, but also as a promotor of IFN-γ secretion.

5. Refences

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Table 1

NUMBER OF IFN-γ-SECRETING CELLS IN VARIOUS HUMAN TISSUES

Tissue	Intestinal mucosa	Blood	Tonsils	Spleen	Bone marrow	Gingiva
SFC/10^6 MNC a)	97	13	116	278	8	21
Range	20-420	2-40	40-350		0-11	0-64
	n=7	n=13	n=13	n=1	n=9	n=7

a) Data are expressed as geometric mean of number of SFC (Spot Forming Cells)
Values for tonsilar and spleen SFC were determined at a cell density of 10^6 MNC/well

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Interleukin-6 is secreted by epithelial cells in response to Gram-negative bacterial challenge

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1. Introduction

Challenge of mucosal surfaces with Gram-negative bacteria results in a rapid inflammatory response. It is elicited by bacterial endotoxin and bacterial surface adhesins. In the urinary tract, adhesins attaching to Galα1-4Galβ-containing glycolipid receptors promote inflammation, whereas other adhesins specific for mannose-containing receptors do not (1). The inflammatory response, measured as the the activation of polymorphonuclear leukocytes (PMN's), requires an intact LPS gene of the host. C3H/HeJ mice (lps^d/lps^d) neither respond with inflammation to bacterial challenge, nor clear the infection, indicating that the inflammatory response is essential for bacterial elimination from the mucosal surface (2,3).

In this study we demonstrate that Interleukin-6 (IL-6) is secreted by epithelial cells in vitro , and that IL-6 is not directly involved in the in bacterial clearance from mucosal surfaces.

2. Results

2.1. IL-6 PRODUCTION IN RESPONSE TO MUCOSAL INFECTION.

Mucosal challenge with E.coli gives rise to an IL-6 response in both serum and secretions. This response was analyzed in a mouse model for urinary tract infection. An E.coli strain (Hu 734, O:75K:5H$^-$), expressing adhesins specific for Galα1-4Galβ and mannose containing receptors, with a smooth lipopolysaccharide (LPS) was instilled into the bladder via a soft polyethylene catheter (4). IL-6 levels were measured with the B9 hybridoma cell line (5) which requires IL-6 for growth. Urine samples were collected sequentially from the time of infection until sacrifice at 24 hours post infection (serum was collected at sacrifice). Infection was quantitated as the number of live bacteria in kidneys and bladder at 24 hours post infection.

IL-6 was measurable in urine within 30 minutes of infection, and reached a peak by two hours post infection, after which time the levels declined. The hypothesis that the urinary IL-6 was of mucosal origin was tested by comparing IL-6 levels in urine, serum, and peritoneal washes after either intravesical or intraperitoneal bacterial challenge. Intravesical infection elevated the urinary IL-6, but not the peritoneal levels. In contrast intraperitoneal challenge elevated IL-6 levels in serum and peritoneal washes, but not in urine (6).

2.2. IL-6 CAN BE SECRETED BY EPITHELIAL CELLS.

Epithelial cells dominate in the mucosa of of the urinary tract, there are few macrophages or lymphoid cells unless the mucosa has been stimulated by antigen. The rapid elevation of urinary IL-6 levels suggested that it was produced by epithelial cells. This hypothesis was tested by using a epithelial cell culture of renal origin (Moroff, kindley provided by Dr. Neil Bander, Cornell University, New York). 10^4-10^5 cells/well were stimulated with either whole attaching bacteria, or with isolated lipid A. Supernatants were tested for IL-6 at zero, two, six, and twenty four hours after stimulation (Table I.).

Challenge with attaching bacteria resulted in secretion of IL-6 from epithelial cells. Both the six and twenty four hour supernatants contained significant levels of IL-6 compared to samples from zero hours ($p<0.001$). IL-6 levels were also significantly increased between the six and twenty four hour samples ($p<0.01$). Lipid A stimulation resulted in only a low increase in IL-6 levels in the samples taken at six and twenty four hours.

2.3. LACK OF CORRELATION BETWEEN IL-6 LEVELS AND BACTERIAL CLEARANCE.

The high susceptibility to infection combined with the absence of both IL-6 and inflammation in the LPS non-responder mice suggested a causal relationship between IL-6 and bacterial clearance. This hypothesis was tested using anti-inflammatory agents. LPS responder mice were pre-treated with dexamethasone (a corticosteroid), diclofenac and indomethacin (non-steroidal anti-inflammatory agents), and cyclosporin A (Table II). Dexamethasone and diclofenac both significantly inhibited IL-6 secretion and bacterial clearance. Cyclosporin A inhibited IL-6 secretion, but did not influence bacterial clearance. Indomethacin inhibited bacterial clearance, but not IL-6 secretion.

3. Discussion.

Gram-negative infections are characterized by fever, a pronounced acute phase response, and eventually the induction of mucosal immunity. We have recently shown that IL-6 is a part of the mucosal immune response to Gram-negative infections (6,7). IL-6 was previously identified with only systemic effects such as pyrogenicity (8), as a stimulant of liver cells to produce acute phase reactants (9), and of B cells to produce immunoglobulins (10). More recently IL-6 was found to enhance IgA production (11). Epithelial cell secretion of IL-6 and the induction of enhanced IgA production by IL-6 suggests a possible role for this molecule as a infection site specific stimulator of mucosal immunity.

Inhibition of IL-6 did not correlate with a similar inhibition of bacterial clearance. IL-6 or bacterial clearance could be inhibited individually (by CsA, and indomethacin respectively), or they could be co-inhibited (by dexamethasone and diclofenac). This suggests that different mediators are involved in the induction of symptoms and clearance of infection, but that there are metabolic pathways common to their production.

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5. TABLES

Table I. IL-6 levels in culture supernatants following stimulation of a renal tubular cell line with either whole bacteria, or lipid A.

	IL-6 (UNITS/ml) [a]			
Time after cell stimulation	0	2hrs	6hrs	24hrs
E.coli Hu734 ($1.3x10^7$bacteria/well)	0	0	158(102)	365(129)
Lipid A (2ug/well)	0	0	15(15)	22

[a] mean (std deviation)

Table II. Dissociation between IL-6 levels and bacterial clearance after treatment of mice with anti-inflammatory agents, or Cyclosporin A.

TREATMENT[a]	IL-6(Units/ml)[b]	BACTERIA[c]
Dexamethazone (4 mg/ml)	88	1408
Diclofenac (10 mg/ml)	49	526
Indomethacin (5 mg/ml)	410	10747
Cyclosporin A (60 mg/kg)	88	51889
PBS	465	55

[a]All mice recieved intravesically 10^9 E.coli Hu 734 in 0.1 ml.
[b]From urine samples taken at 2 hours post infection.
[c]Mean no. of live bacteria/ml in kidney homogenate.

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IL-6 production by rat mast cell lines?

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Introduction

Inflammation induced by physical, chemical, bacterial, viral or parasitic means all have one common consequence, the dramatic change in serum concentrations of a group of proteins known as the acute phase reactants (APR's). The majority of APR's are of hepatic origin. An important mediator in the liver acute phase response is IL-6. Mast cells have recently been recognized as a potential source of several cytokines. Mouse mast cell lines have been demonstrated to produce IL-3, IL-4, IL-5, IL-6 and TNFα on activation with ionophore or cross linkage of FCεRI. Mouse bone marrow derived mast cells are known to produce both IL-3 and GM-CSF. We have no information concerning cytokine production by mast cells derived directly from an animal source.

We have examined the ability of mast cell supernatants and cell lysates to induce the production of acute phase reactants by rat hepatocytes. Specifically, we examined α2 macroglobulin and albumen production of over 72 hours in culture. Until very recently, this hepatocyte stimulating factor (HSF) assay was thought only to detect IL-6 like activity. However, the newly described leukaemia inhibitory factor (LIF) will also induce the activation of the IL-6 dependent acute phase response genes in this system (1). IL-6 can be assayed specifically in an alternate system, B9 murine hybridoma cells will proliferate in response to IL-6 but not LIF.

Materials and Methods

HSF assays were carried out according to the method of Koj et al (2). α2 macroglobulin and albumen content of supernatants were measured by Laurell rocket electrophoresis. The B9 hybridoma assay was performed in Iscoves modified Dubecco's medium on 24 well plates. 5000 B9 cells per well were labelled for 50-55 h with 0.2 μCi ^{3}[H] thymidine in the presence of samples or controls. RBL-2H3 and RCMC9 cells were a gift from Dr. A. Froese (Winnipeg). They were maintained in RPMI-1640 containing 5% FCS. Supernatants were harvested 3 days after passage of cells. Peritoneal mast cells (PMC) were obtained from male Lewis rats by peritoneal lavage. The PMC were purified on a discontinuous 30%-80% percoll gradient to ≥ 96% purity. They were acti-vated using a 1/40 dilution of ε specific sheep anti rat IgE at 37°C. Intestinal mucosal mast cells (IMMC) were purified from male Lewis rats which had been infected by Nippostrongylus brasiliensis (Nb), according to established methods, to a purity of 58-63%. They were activated using 30 worm equivalents /ml of Nb worm antigen at 37°C. Northern blot analysis was carried out by standard techniques. RNA was purified according to the method of Yang and Miller (3).

Results and discussion

Culture supernatants and cell lysates obtained by freeze thaw of RBL-2H3 and RCMC9 cells contained substantial HSF activity. While supernatants from activated or control incubated PMC and IMMC did not. The B9 hybridoma assay was used to examine IL-6 activity specifically in these preparations. The results demonstrated that IL-6 activity was not present in RBL-2H3 or RCMC9 supernatants.

Supernatant Cell Source	Activation	HSF units/ml	B9 assay Mean cpm
RBL-2H3	-	30	742
RCMC9	-	43	682
PMC	Anti-IgE	0	3 717
IMMC	Worm antigen	0	2 281
Media Control	-	-	2 488
Fibroblast	-	ND	29 393
Monocyte	LPS	233	ND

*Mast cell supernatants assayed at a 1/20 dilution positive control at a 1/12 800 dilution. Activations for 24 h at 37°C.

To further examine IL-6 production by RBL-2H3 and RCMC9 cells Northern blot analysis was carried out. Neither of the mast cell RNA preparations contained a detectable level of rat IL-6 mRNA using a cDNA probe for rat IL-6. Rat macrophages which produce a similar level of HSF activity gave a strong positive signal.

These results demonstrate RBL-2H3 and RCMC9 mast cell lines produce HSF activity. Our results suggest the observed activity may be due to LIF. Mast cells obtained directly from the rat, of either the CTMC or IMMC type, failed to produce any detectable IL-6 or HSF activity following activation or control culture for 10 minutes or 24 hours. This would suggest that the rat mast cell lines and freshly isolated mast cell differ substantially in their cytokine production.

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SECTION D: MUCOSAL T CELL RESPONSES and IMMUNO REGULATION

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T cell activation and inhibition by cholera toxin and its B subunit

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ABSTRACT. Cholera toxin (CT) and its B subunit (CT-B) are potent oral immunogens in vivo, although both strongly inhibit polyclonal lymphocyte activation in vitro. In order to help understand this paradox, we have studied the activation of CT-B specific T cells in vitro. CT-B subunit primed PLN T cells were used as responders, Con A stimulated PEC as antigen presenting cells (APC), and various forms of CT-B as antigen. There was a marked difference in the activation of CT-specific T cells when different forms of CT-B were used. Native CT-B did not stimulate T cell proliferation when co-cultured with primed T cells and APC's; in contrast, denatured CT-B or CT-B blocked by Gm1 ganglioside stimulated well. The degree of proliferation was proportional to the dose of antigen and APC's in the cultures, was antigen-specific, and was H-2 restricted. APC's from high responder strains to CT were significantly more effective than were APC's from low responder strains in presenting CT-B to F1 T cells. Addition of native CT-B to co-cultures of primed T cells plus denatured CT-B inhibited the CT-B specific proliferative response. Thus the inhibitory properties of CT-B and CT extends even to T cells specific for CT itself. The nature of this inhibition was further investigated using phorbol myristic acetate (PMA) and ionomycin as polyclonal activators, which trigger the two arms of the phosphatidylinositol system. CT-B inhibited T-cell proliferation induced by PMA and ionomycin and the kinetics of such inhibition indicate that CT-B and CT affect late events in T-cell activation. The inhibition occurred also after relatively brief pulses of CT. The CD8 T cell subset was more sensitive to inhibition than was the CD4. We conclude that, because of these inhibitory properties, processing of CT to non-binding molecular forms or fragments in vivo must be an important prerequisite for the induction of an immune response to CT.

From the work of many investigators, it is now well recognized that cholera toxin (CT) and its B subunit (CT-B) have remarkable properties as oral immunogens. Oral administration of CT induces strong secretory IgA and plasma IgG responses and does not lead to oral tolerance as is the case after the feeding of most other protein antigens (1,2). The murine response to the toxin is restricted by certain genes in the H-2 major histocompatibility complex (3), is T cell dependent and exhibits extended memory (4,5). Even more remarkable, CT can act as an adjuvant for unrelated protein or viral antigens given simultaneously with it into the intestine (2,6). These positive effects of CT and CT-B contrast markedly with their effects on lymphocytes in vitro which are strongly inhibitory (7). From previous work, we have postulated that the effects of cholera toxin in vivo are mediated by its effects on regulatory T cells,

particularly the inhibition of suppressor cells. In order to help understand the paradox of inhibition in vitro but stimulation in vivo, we have begun to study the activation of cholera toxin-specific T cells in vitro.

1. MATERIALS AND METHODS

Animals: Inbred mice of various strains were obtained from the Jackson Laboratories, Bar Harbor, ME.

Antigens: CT and CT-B were obtained from List Biochemicals. Denatured B subunit (CT-BD) was prepared by reduction and S-carboxymethylation using minor modifications of described methods (8). GM1 ganglioside was obtained from Sigma, St. Louis, Mo.

Antigen-specific lymphocyte proliferation: The method of Corradin et al (9) was used with some modifications. Mice were primed in the rear footpads and base of the tail with CT-B 50 μg in incomplete Freund's adjuvant as a mixed emulsion. Seven to eight days later the inguinal, periaortic and popliteal lymph nodes were removed and teased apart. Highly purified T cells were obtained by passage through a nylon column followed by incubation with magnetic beads coated with anti-mouse immunoglobulin followed by separation by a magnet. Antigen-presenting cells (APCs) were obtained from peritoneal exudate cells induced by the intraperitoneal injection of 30 μg of CON-A two days before sacrifice. In some experiments, CT-B subunit binding was blocked by pre-incubation with a 5-fold molar excess of GM1 ganglioside. Primed T cells were cultured and with antigen-presenting cells and varying amounts of antigen for 5 days in RPMI 1640, 10% fetal calf serum, and antibiotics. Lymphocyte proliferation was measured as the uptake of ^{3}H-thymidine into DNA during an 18-hour pulse at the end of the culture. In studies using polyclonal activators, unprimed spleen T cells were cultured for 48 hours with a pulse of ^{3}H-thymidine during the last 6 hours.

2. RESULTS AND DISCUSSION

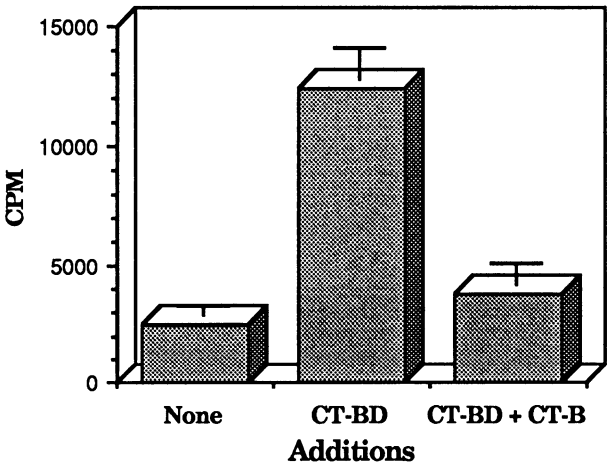
There was a marked difference in the activation of CT-B-specific T cells when different forms of CT-B were used. Native CT-B did not stimulate specific T cell proliferation when co-cultured with primed T cells and APCs. In contrast, denatured CT-B stimulated the same cells very well, as did CT-B blocked by GM1 ganglioside. A third effective method of antigen presentation was the pre-incubation of the native CT-B with APCs which were then washed and added to primed T cells. Interestingly, the dose response curve of such antigen-pulsed APCs was quite similar to the dose response in cultures in which CT-B blocked by GM1 ganglioside was simply added to the primed T cells and APCs for the duration of the culture. This infers that the ability of CT-B to bind to APCs does not increase its ability to be presented by APCs.

The degree of proliferation of CT-B specific T cells was proportional to the dose of antigen and the number of antigen-presenting cells in the cultures, similar to what is seen with T cells specific for other protein antigens. The proliferation was antigen-specific in that CT-B primed T cells did not respond to irrelevant protein antigens such as hen egg lysozyme or myoglobin. The T cell proliferation was H-2 restricted in that APCs from B-10 recombinant inbred strains could present antigen to B-10 T cells only if they were haploidentical. Consistent with findings previously demonstrated in vivo, APCs from mice from high responder strains to CT were significantly more effective than were APCs from mice from low responder strains in presenting CT-B to T cells from a cross between the high and low responder strains. In these experiments, C3B6F1 mice were primed with antigen and stimulated in vitro with APCs from C3B6F1 strain, C57Bl/6 high responder, or C3H/He low responder antigen-presenting cells. There was a significantly greater response to antigen

presented by the F1 and C57Bl/6 APCs than there was to antigen presented by the C3H/He APCs.

The forms of CT-B effective in co-cultures were not able to bind to GM1 ganglioside-coated plates in an ELISA, nor were these forms able to inhibit polyclonal T cell proliferation in vitro. Conversely, native CT-B, which binds to GM1 ganglioside and which we have previously shown to be capable of inhibiting polyclonal T cell proliferation, was found to actively inhibit the response of CT-B-primed T cells to the stimulatory forms of CT-B. In these experiments native CT-B was added back to co-cultures of primed T cells plus APCs plus denatured CT-B or CT-B blocked by GM1 ganglioside. In each instance the addition of native CT-B into such co-cultures inhibited the antigen-specific response to CT-B.

Figure 1. Native CT-B inhibits the proliferative response of primed T cells to denatured CT-B



Thus, the inhibitory properties of CT-B and CT involves not only mitogen and KLH antigen-specific responses as previously reported (7) but extends even to T cells specific for CT itself. The binding of native CT-B to the T cell surface is a crucial determinant of such inhibition of CT-specific T cells. This was demonstrated in experiments in which CT-B-primed T cells were stimulated with mixtures of native and GM1 ganglioside-blocked CT-B. There was marked inhibition in such cultures when native CT-B comprised more than 20% of the total antigen in the culture. This indicates that the antigen presentation of CT-B in vivo is likely to be quite complex with the degree of activation of T cells dependent on the ratios of stimulatory and inhibitory forms of CT-B or CT present in GALT.

There is increasing evidence that occupation of the T cell receptor complex and activation of T cells is transduced by the phosphatidyl inositol second messenger pathway. One possible mechanism by which CT and CT-B might be inhibiting T cells is by interfering with the phosphatidyl inositol system. This system can be bypassed by the use of ionomycin to increase intracellular calcium concentration and phorbol myristic acetate (PMA) to activate protein kinase C. Indeed, when one adds these two agents to purified splenic T cells at optimal doses, the T cells proliferate strongly as

they do with other polyclonal mitogens. Interestingly, both CT and CT-B inhibited T cell activation by PMA and ionomycin in a similar fashion and at similar doses as their inhibition of Con-A or PHA-induced polyclonal activation. Moreover, the kinetics of the inhibition indicate that CT and CT-B affect late events in T cell activation because they can be added 12-18 hours after the addition of PMA and ionomycin and still exert substantial inhibitory effect. This suggests that the inhibitory signal given by CT-B does not involve the phosphatidyl inositol system. Previous data proved that the CT-B signal did not involve the adenylate cyclase second messenger system (7). In regard to CT, the adenylate cyclase system probably is partially involved because CT is consistently a more potent inhibitor than is CT-B. Although the inhibitory signal can occur late after T cell activation, prolonged exposure to CT is not required: inhibition can occur after only short pulses of CT lasting only minutes.

The last set of experiments focussed on whether the different T cell subsets are equally susceptible to inhibition by CT and CT-B. This was examined in two ways. In the first, CD8 and CD4 subsets were negatively isolated from purified T cells by monoclonal antibody and magnetic beads. Each subset was then cultured in the presence of PMA and ionomycin with or without the addition of CT-B. The inhibition of the total T cells was 52%, of $CD4^+$ T cells 45% and of $CD8^+$ 75%. Thus, the CD8 T cells seemed to be much more sensitive to inhibition than was the CD4 subset. These results were confirmed by a second experiment in which purified T cells were cultured without separation of the subsets in the presence of PMA and ionomycin with or without the addition of CT-B. After the culture, the fractions of cells expressing CD4 and CD8 were measured by monoclonal antibody staining and flow cytometric analysis. Again, the CD8 subset was more markedly reduced than was the CD4 subset. These effects did not appear to be due to a greater amount of binding of CT-B to CD8 T cells as compared to CD4 T cells as measured by flow cytometry.

In summary, these studies have begun to define the activation of CT-specific T cells by this unusual antigen. The response was shown to be antigen-specific, H2-restricted and highly dependent on the form of antigen used to stimulate the primed T cells. The ability of native CT-B to bind to APCs did not seem to shift the dose response curve to more efficient presentation at low doses of antigen. Surprisingly, native CT-B was able to inhibit the specific T cell response to itself! The possible mechanism of such T cell inhibition was explored further in studies with non-primed splenic T cells polyclonally stimulated with PMA and ionomycin. Both CT and CT-B were able to inhibit this form of T cell activation by these agents, indicating that this inhibition involves late stages of T cell activation rather than membrane events and signal transduction, which are bypassed by these activators. In addition, kinetic studies show that inhibition can occur even when CT or CT-B is added many hours after polyclonal activation has begun. Inhibition can also occur after relatively short pulses with CT and CT-B in inhibitory doses. Lastly, the CD8 T cell subset is particularly sensitive to inhibition, much more so than the CD4 subset. This sensitivity of the CD8 subset <u>in vitro</u> is consistent with previous work done <u>in vivo</u> which suggested that some of the effects of CT as an oral immunogen and adjuvant was due to inhibition of suppressor cells in GALT. The finding that this inhibition can occur after relatively brief exposures of T cells to CT and CT-B <u>in vitro</u> indicates that these findings may be relevant to what likely occurs physiologically in GALT upon CT or CT-B exposure which is likely to be short. The <u>in vivo</u> effects of CT and CT-B are a complex net result of the final balance between these stimulatory and inhibitory effects.

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The role of cytophilic IgA and Fcα receptors in circulating T cells in autoimmune diseases

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Abstract

An investigation of T cells in systemic lupus erythematosus (SLE) and rheumatoid arthritis revealed a significant increase in circulating Vicia villosa (VV) binding CD8 cells in SLE and in synovial fluid CD8 cells of rheumatoid arthritis. $CD8^+VV$ binding cells can augment the immune response, despite suppressor activity and this might be involved in the pathogenesis of the central organs in SLE and of the joints in rheumatoid arthritis. However, circulating T cells in rheumatoid arthritis do not show an increase in VV binding cells but a significant increase in cytophilic IgA1 and a significant decrease in Fcα receptors. Cytophilic IgA bound to circulating CD8 cells may prevent the augmenting function of $CD8^+VV$ binding cells, consistent with the lack of exra-articular disease in most patients with rheumatoid arthritis. Further characterisation of the cytophilic IgA1 revealed that in some it is polymeric, with J chains and secretory component and in others the IgA1 appears to be monomeric. Hence, the source of the IgA1 might be either bone marrow or gut associated lymphoid tissue and the possibility of cytophilic IgA immune complex is under investigation.

Introduction

The function of circulating serum IgA, in contrast to mucosal secretory IgA is not clear. However, inhibitory functions have been recognised in chemotaxis of polymorphonuclear leukocytes (Van Epps & Williams, 1976; Abdulla & Lehner, 1979), IgG mediated bacteriolysis (Griffis, 1983), phagocytosis and in natural killer cell activity (Komiyama et al, 1986). In atuoimmune diseases IgA class of rheumatoid factor was described and a role for IgA-α1 antitrypsin complex was postulated in the pathogenesis of rheumatoid arthritis (Dawes et al, 1987).

Fcα receptors are found not only on polymorphonuclear leucocytes and macrophages but also on T cells (Lum et al , 1979; Fanger & Lydyard, 1981). Indeed, Fcα receptors can be up-regulated by polymeric IgA on cloned T cells (Briere et al, 1988). Recently, we have demonstrated that in vivo stimulation with tetanus toxoid in man will significantly up-regulate Fcα receptors and Vicia villosa (VV) binding of CD4 and CD8 cells (Fortune et al, 1989a). Furthermore, in rheumatoid arthritis the Fcα receptors of T cell subsets appear to be bound by cytophilic IgA1 (Fortune et al, 1989b).

The objectives of this paper were to attempt to find a relationship between Fcα receptors, cytophilic IgA and VV binding of CD4 and CD8 cells in circulating T cells of patients with

autoimmune diseases. The results suggest that $CD8^+VV$ binding cells which augment the immune response might be inhibited by cytophilic IgA bound to the Fcα receptors of T cells.

Materials and methods

Blood was collected from 51 patients with RA of whom 7 were males and 44 females. Within this group 19 patients had paired peripheral blood and synovial fluid samples taken. The age range was 36 to 66 years, with a mean of 56 years. The healthy control group consisted of 49 subjects of whom 39 were female and 10 male, with comparable ages to those of the patients. Blood was also collected from 20 patients with systemic lupus erythematosus.

SEPARATION OF CELLS AND DOUBLE IMMUNOFLUORESCENCE CYTOMETRY

The separation of peripheral blood and synovial fluid mononuclear cells by Hypaque-Ficoll and the double immunofluorescence method have been described before (Fortune & Lehner, 1988; Fortune et al, 1989b). In principal, the biotin avidin system, with streptavidin-phycoerythrin (Beckton-Dickinson, California) was used and the CD4 or CD8 cell subset was identified by monoclonal antibodies OKT4 or OKT8, followed by rabbit anti-mouse antiserum labelled with FITC (Miles Laboratories, Naperville, USA). Vicia villosa (VV; Sigma, Poole, England) was biotinylated and T cells binding VV were identified by means of streptavidin phycoerythrin. To detect cell bound IgA1, goat anti-human IgA1 antibody linked to FITC (Tago, CAlifornia) was used. Fcα receptors were identified by incubating secretory IgA (Sigma) with the cells, followed by FITC-labelled $F(ab)_2$ fragment of anti-human IgA.

Results

THE PROPORTION OF CD4 AND CD8 CELLS BINDING VICIA VILLOSA

In SLE there was a significant increase ($p<0.01$) in CD8 cells binding VV (8.9% $\pm$1.8), as compared with healthy control subjects (2.6%$\pm$0.5) (Table 1). The increased VV binding was almost entirely found in cells from 7 patients with active disease and all but 1 of the 13 patients with inactive disease showed a normal proportion of VV binding CD8 cells. In contrast, in rheumatoid arthritis the circulating cells showed only a small increase in VV binding CD8 cells (5.4%$\pm$0.8) or CD4 (6.9% $\pm$1.2) cells. However, the highest proportion of VV binding CD8 cells (17.6%$\pm$2.4; $p<0.0001$) or CD4 cells (13.1%$\pm$1.8; $p<0.01$) was found in synovial fluid (Table 1).

THE PROPORTION OF CELL-BOUND IgA1

In view of the lack of binding of VV to circulating T cells from patients with rheumatoid arthritis we investigated the possibility that cytophilic IgA1 might inhibit VV binding, as it is rich in N acetyl galactosamine (Fortune et al, 1989). Indeed, significant increases in IgA1 bound to CD8 (9.4%$\pm$4.0) and CD4 (5.2% $\pm$1.8) circulating cells were found in patients, compared with controls or synovial fluid cells (Table 2). The IgA1 was characterised as to the presence of J chains and secretory component (SC). In CD8 cells J chains and SC were found in 7 out of 14 patients with IgA1 (Table 2). However, in CD4 cells J chains were found in 6 out of 14 cases with IgA1 but SC only in 3 of the 14 patients.

Table 1

Double immunofluorescence flow cytometry of CD4 and CD8 cells binding Vicia villosa in control healthy subjects and patients with SLE; these were further divided into patients with active and inactive disease. A group of patients with rheumatoid arthritis was also examined and another was analysed with paired blood and synovial fluid T cells.

		CONTROLS		PATIENTS		ACTIVE		INACTIVE	
Disease	Cell Subset	(n)	Mean %†	(n)	Mean %†	(n)	Mean %†	(n)	Mean %†
SLE	CD4	(22)	2.7 (± 0.85)	(20)	4.5 (± 0.78)	(7)	6.4 (± 1.9)	(13)	3.3 (± 0.67)
	CD8	(22)	2.6 (± 0.55)	(20)	8.9 (± 1.8)*	(7)	18.2 (± 2.6)**	(13)	5.8 (± 0.67)
				Blood	Synovial fluid				
RA	CD4	(49)	3.7 (± 0.6)	(51)	6.9 (± 1.2)	(19)	6.7 (± 1.1)	(19)	13.1 (±1.8)*
	CD8	(49)	3.6 (± 0.6)	(51)	5.4 (± 0.8)	(19)	6.9 (± 1.6)	(19)	17.6 (±2.4)***

* $p < 0.01$ † - sem in parentheses.
** $p < 0.001$
*** $p < 0.0001$

Table 2

Double immunofluorescence flow cytometry of (A) CD4 and CD8 cells binding IgA1 in rheumatoid arthritis and (B) J chains and secretory component (SC),the results are given as the mean % ±s.e.m.

	CONTROLS		RHEUMATOID ARTHRITIS			
T Cells	(n)	Blood	(n)	Blood	(n)	Synovial Fluid
(A)						
$CD4^+IgA1^+$	(25)	0.8±0.2	(14)	5.2±1.8*	(14)	2.8±0.8
$CD8^+IgA1^+$	(25)	1.1±0.3	(14)	9.4±4.0*	(14)	5.5±1.2
(B)						
$CD4^+J^+$	(12)	1.0±0.3	(14)	4.5±1.2	(14)	6** 43%***
$CD4^+SC^+$	(12)	1.1±0.4	(14)	6.8±1.8	(14)	3 21%
$CD8^+J^+$	(12)	1.2±0.5	(14)	4.2±1.0	(14)	7 50%
$CD8^+SC^+$	(12)	2.1±0.5	(14)	7.1±1.5	(14)	7 50%

* $p < 0.001$
** number *** % greater than mean plus 2 SD of the corresponding control cells.

Fcα RECEPTORS ON THE T CELL SUBSETS

The proportion of Fcα receptors, as assayed by the binding of colostral IgA, was 3.3% (±0.4) in CD4 cells and 4.8% (±0.7) in CD8 cells from control subjects. However, in rheumatoid arthritis there was a significant decrease in Fcα receptors of circulating T cells; CD4 cells 1.3% (±0.6) and CD8 cells 0.9% (±0.1).

Discussion

The rationale for investigating T cells in human autoimmune diseases for the presence of VV binding cells, was that these cells augment antibodies and delayed hypersensitivity in the face of T suppressor cell activity (Green et al, 1981; Ptak et al, 1986; Lehner and Jones, 1984; Brines and Lehner, 1987; Kelly and Nielson, 1987). Indeed, we have found a significant increase in circulating $CD8^+$ VV binding cells, especially during disease activity of SLE (Fortune & Lehner, 1988). We were therefore surprised that in rheumatoid arthritis (Table 1) we were unable to detect a significant increase in circulating VV binding CD8 cells (5.4% ±0.8), as compared with healthy controls (3.6% ±0.6). However, examination of mononuclear cells from synovial fluid of patients with rheumatoid arthritis revealed a very significant increase in $CD8^+$ VV^+ binding cells (17.6% ±2.4; $p<0.0001$), compared with the corresponding circulating $CD8^+$ VV^+ cells (6.9% ±1.6; Fig. 2). A similar increase was found with $CD4^+$ VV^+ binding cells (13.1% ±1.8; $p<0.01$).

The discrepancy between synovial fluid and circulating VV binding T cells might be ascribed to the augmenting, VV binding T cells expanding in the synovium and not entering the circulation in a significant number. An alternative view, that an inhibiting agent in the circulation might affect the VV receptors on T cells was examined. As the lectin VV is affinity purified to Gal Nac and VV binding to T cell is specifically inhibited by Gal Nac, we searched for some serum factor rich in this sugar. We considered serum IgA1 which contains 7-11% Gal Nac located in the CH_2 domain and inhibits VV binding to erythrocytes. Indeed, IgA1 proved very effective in inhibiting VV binding to T cells, unlike IgA2, IgG, IgM or IgD (Fortune et al, 1989b). We have then found a significant increase in IgA1 bound to circulating CD8 and CD4 cells ($p<0.01$) from patients with rheumatoid arthritis, as compared with those in healthy controls. Furthermore, the bound IgA1 could be readily eluted from the T cells by incubating them in 10% FCS for 12 hours at 37°C. This suggests a low affinity binding between IgA1 and the corresponding Fcα receptors. However, the desorbed T cells which lost the bound IgA1 were now able to bind VV (Fortune et al, 1989b). Conversely, adsorption of serum IgA1 on to synovial fluid T cells resulted in a decrease in VV binding cells. These studies support the concept that IgA1 inhibits T cells from binding VV. We tested then the most likely possibility that IgA binds T cells by engaging the Fcα receptors. Indeed, we found a significant decrease in Fcα receptors on circulating $CD8^+$ cells ($p<0.0001$) and $CD4^+$ cells ($p<0.01$), from patients with RA, as compared with control cells, supporting the concept that the Fcα receptors of these T cells are engaged by IgA.

The nature of cytophilic IgA1 was then characterised, with special reference to J chains and secretory component. In 7 of the 14 patients investigated the CD8 cell-bound IgA1 contained J chains and the secretory component but CD4 cell-bound IgA1 showed both components only in 3/14 patients. This suggests that in half the patients the CD8 cell-bound IgA is of the polymeric secretory type and in the other half the IgA is monomeric. As secretory IgA is preferentially induced by the mucosal associated lymphoid tissue (Mestecky & McGhee, 1987), it is of considerable interest to find out if the IgA antibody represents an immune complex, originating from the mucosal associated lymphoid tissue. The other possibility is that the IgA might be rheumatoid factor of the IgA class. We suggest that $CD8^+$ VV binding cells can

augment the immune response, in the face of suppression and this regulatory mechanism might be involved in the immunopathogenesis of the central organs in SLE and joints in RA. However, the central organs in RA may be protected from damage by the blocking effect of IgA1 which prevents the circulating VV binding CD8 cells from augmenting the immune response.

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Analysis of IgA binding to FcαR$^+$ T cells and characterization of anti-FcαR antiserum

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Abstract

Subpopulations of CD4 positive (CD4+) T cells from murine Peyer's Patches (PP) express an Fc receptor for IgA (FcαR). A high frequency of FcαR+, CD8+ T cells were also found in spleens of mice bearing an IgA myeloma. We have established FcαR+ T cell lines to study the isotype specific regulatory role of this receptor in IgA responses. We have tested the expression of interleukins (IL) by FcαR+ T cells both at the level of mRNA and secreted proteins. These T cells secrete IL-5, a major cytokine for regulation of IgA responses. To better understand the precise regulatory role of FcαR+ T cells in IgA synthesis, we have analyzed the FcαR in more detail. Immunoprecipitation of membrane proteins from FcαR+ T cells with IgA resulted in a 38 kD protein as a major receptor component and antisera were raised against this 38 kD protein. The anti-FcαR antibodies blocked IgA binding to FcαR+ T cell hybridomas and to splenic CD8+ FcαR+ T cells of IgA myeloma-bearing mice, but did not interfere with the staining of CD3 and other surface markers. Furthermore, the anti-FcαR antibodies specifically stained FcαR+ T cells. Thus, our results indicate that FcαR on T cell lines derived from PP is a 38 kD protein. Further, an antiserum has been produced which is either specific for this FcαR or else recognize determinants which are in close proximity to this receptor and should be useful for cloning the FcαR.

Introduction

Previous studies have shown that a subpopulation of Peyer's patch (PP) CD4+ T helper cells (Th) express an IgA specific receptor [1,2]. The binding of the IgA to the receptor occurs through the Fc part of the immunoglobulin (Ig) as has been analyzed by rosette assays. Further, IgA myeloma bearing mice show an increased number of splenic CD8+ FcαR+ T cells [3,4]. Since FcαR expression on T cells is found on CD4+ T as well as on CD8+ T cells, we analyzed the molecular characteristics of the FcαR and the regulatory function of this receptor. To this end, we established several FcαR+ T cell lines, e.g., ThHA$_1$ #9 and PPT 4.16 from PP T cells by fusion with T cell lymphoma R1.1 and BW 5147 respectively [5,6].

Binding pattern of IgA to FcαR

To analyze the effect of IgA conformation on binding to FcαR$^+$ on T cells, FcαR positive and negative cells were incubated with different molecular forms of IgA followed by fluorescence conjugated anti-mouse IgA and then subjected to flow cytometry. Monomeric and dimeric IgA (mIgA, dIgA) specifically bind to the FcαR$^+$ hybridomas (e.g., PPT 4.16, ThHA$_1$ #9) and not to control cells (e.g., thymocytes, R1.1, BW 5147) (Table 1) [6]. dIgA shows a slightly higher binding when compared with mIgA. Polymeric IgA (pIgA) however binds nonspecifically to all T cells tested. The nonspecific binding of pIgA to T cells may be explained by a cooperative binding effect of the pIgA and by a carbohydrate-protein binding of the J-chain to a cell surface enzyme, β 1-4 galactosyltransferase (β 1-4 GalTase). This enzyme is expressed on a wide variety of FcαR$^-$ and FcαR$^+$ T cells (M. Tomana, personal com.) and can use glycoproteins with terminal glucose residues to add a galactose unit. Our data so far do not support a model where β 1-4 GalTase is identical to the FcαR on T cells since FcαR$^+$ and FcαR$^-$ T cells both express this enzyme at comparable levels [7]. Furthermore, the apparent molecular weight of murine β 1-4 GalTase is ~47 kD whereas immunoprecipitation of cell membranes from FcαR$^+$ T cells with radiolabeled IgA resulted in a 38 kD protein band which we could not detect in the control cell preparation [6].

TABLE 1. Analysis of the influence of the IgA configuration on the binding to FcαR$^+$ T cell hybridomas ThHA$_1$ #9 and PPT 4-16 compared to FcαR$^-$ Lymphoma R1.1

IgA		T Cells Tested		
Preparations	FITC-anti-α	ThHA$_1$ #9	PPT 4-16	R1.1
Monomeric	+	86.9%*	59.7%	0.7%
Dimeric	+	99.2%	91.1%	11.2%
Polymeric	+	95.6%	94.0%	96.0%
None	+**	1.6%	1.5%	1.9%

*The values are given as the percentage of the positive population by FACS.
**The background represented by staining with the second antibody only.

Polyvalent antibody to murine T cell FcαR

To obtain antibody which recognize FcαR, we prepared membrane proteins of FcαR$^+$ T cell hybridomas and electroeluted the 38 kD protein since immunoprecipitation studies with IgA revealed that IgA binds to a 38 kD protein. Isolated 38 kD proteins were used as antigen for the immunization of rabbits. Rabbit IgG F(ab')$_2$ fragments where prepared and extensively adsorbed with thymocytes and R1.1. This antiserum shows specific staining of FcαR$^+$ T cells and no staining of controls [6]. Furthermore, preincubation of T cells with the rabbit anti-38 kD F(ab')$_2$ fragment did not interfere with the staining of other T cell markers like CD3. Since IgA can induce an FcαR expression in splenic T cells *in vivo* and *in vitro*, we also compared the binding of the anti-38 kD antisera to these CD8$^+$ FcαR$^+$ splenic T cells. In this experiment, splenic lymphocytes or CD8$^+$ T cells were isolated from mice bearing IgA myeloma. These mice possessed 15-20% of the CD8$^+$ T cells expressing FcαR [3,4,6]. When splenic lymphocytes or CD8$^+$ T cells were incubated with biotinylated anti-38 kD antibody followed by fluorescein-avidin,

approximately 20-22% of cells stained positive (6). This antibody also blocks the IgA binding to FcαR (Table 2) and IgA preincubation can reduce the anti-38 kD antibody staining. For example, preincubation of $ThHA_1$ #9 or PPT 4-16 with anti-38 kD antibody eluminated IgA binding (Table 1). Furthermore, treatment of FcαR$^+$ T cells with IgA prior to incubation with anti-38 kD antibody also reduced FcαR specific antibody binding [6]. Similar results were also obtained by using CD8$^+$, FcαR$^+$ T cells isolated from spleen of mice bearing IgA myeloma. These results show that the 38 kD protein is a key molecule for FcαR on T cells or a part of the FcαR complex, and will provide a useful reagent for molecular characterization of this receptor.

TABLE 2. Blocking of IgA binding to FcαR$^+$ T cell hybridomas by rabbit $F(ab')_2$ anti-FcαR

	T Cells Tested	
Incubation of Cells	$ThHA_1$ #9	PPT 4-16
IgA + FITC-anti-IgA	75.8%*	44.0%
Anti-FcαR (38 kD) + IgA + FITC-anti-IgA	9.2%	7.5%
FITC-anti-IgA	0.6%	0.8%

*The values are given as the percentage of the positive population by FACS.

Biological function of FcαR expressing T cells

The precise biological function of FcαR on T cells is still unclear. First, FcαR is expressed on both CD4$^+$ Th cells as well as on CD8$^+$ T suppressor cells. Second, cell culture supernatants from FcαR$^+$ T cell hybridomas can provide either helper function to enhance IgA responses in cultures containing IgA-committed B cells [surface IgA$^+$ (sIgA$^+$)] [2,5] or suppressor effects on sIgA$^+$ B cells (8,9). Further, it was recently shown that IgA binding factors from FcαR$^+$ T cells can reduce the level of mRNA for IgA when added to cultures containing MOPC 315 lymphomas (10). It should also be pointed out that recent findings provided by our group as well as others indicated that interleukin 5 augments IgA synthesis in Peyer's patch B cells or LPS triggered splenic B cell cultures [11]. Further, IL-6 has been shown to induce terminal differentiation of sIgA$^+$ B cells to high Ig secreting cells [12]. Therefore, it was important to examine whether FcαR$^+$ T cells possess the ability to produce these cytokines.

To address these questions, we analyzed our FcαR$^+$ T cell hybridomas for their expression of interleukins. All FcαR$^+$ T cell hybridomas which were derived from PP CD4$^+$ Th cells were capable of secretion of biologically active IL-5 since culture supernatants of FcαR$^+$ T cells enhanced proliferation of dextran sulfate stimulated B cells. Further, significant levels of IL-5 specific mRNA was also detected in FcαR$^+$ T cell lines by using an appropriate cDNA probe. On the other hand, we could neither detect IL-4 expression in FcαR$^+$ T cells by anti-μ B cell co-stimulation assay nor by probing with the murine cDNA for IL-4. In this regard, FcαR$^+$ T cells may influence B cells in at least in two ways: The released form of FcαR (or IBFα) may act on B cells to reduce or enhance

the message for IgA. On the other hand, IL-5 produced by FcαR$^+$ T cells specifically act on surface IgA$^+$ (sIgA$^+$) B cells to induce them to become IgA secreting plasma cells.

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Functional potential of gut T and B cell subsets from mucosally primed mice

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ABSTRACT. We have developed a number of in vivo and in vitro systems for testing the functional potential of gut T- and B- cells primed in vivo via acute or chronic stimulation of the intestinal mucosa with reovirus or Morganella morganii (M.m.) respectively. Reovirus type 3 given orally to neonatal, normal mice or adult severe, combined immunodeficient (scid) mice results in a disseminated fatal infection due to meningoencephalitis at 10-12 d in the pups and to focal hepatitis and liver failure at 4-6 wk. in the scid mice. IELs (10^6) from adult, immune mice given to pups challenged one d later can prevent development of brain lesions. The protective effect of IELs is decreased after treatment with anti-Thy1 + C' and eliminated after treatment with anti-CD8 + C'. Transferred Peyer's patch (PP) cells from immune, congenic normal donors can likewise prevent dissemination of viral infection in scid mice challenged 2 d later. The most effective subset of PP cells is $Thy1^+$, $CD8^+$, and GCT^+.

Acute or chronic gut stimulation of germ-free (GF) mice following oral infection with reovirus or colonization with M.m. respectively was used to initiate de novo germinal center (GC) reactions in PP. The predominant non-IgM/IgD sIg on B cells at 6 d after reovirus was $sIgA^+$, while sIgG1 was not detected. In order to assay the functional potential of subsets of B cells in developing PP we have developed both Ag dependent and independent versions of T dependent, haplotype restricted clonal B cell microcultures that express any and all Ig isotypes. These cultures can be used to assess the growth requirements and functional potential of even very few B cells from subsets with a particular surface phenotype.

Introduction

Peyer's patches of the gut are sites of specific antigenic priming of both B- and T- lymphocytes. A long term goal has been to determine whether and why gut mucosal exposure to either replicating or non-living antigens is particularly efficacious for generating a protective humoral and cellular mucosal immune response compared with parenteral

introduction of antigens. Bases for advantages conferred by mucosal application of antigens could include special features of the microenvironment of PP that could favor the development of subsets of B or T cells that function especially well in mucosal and exocrine tissues and the exposure of lymphocytes to stimulation that have the propensity to selectively lodge in or recirculate through mucosal tissues. To assess the possible advantages of local mucosal priming we have developed a number of *in vivo* and *in vitro* assays for functional potential of subsets of B or T cells, defined by surface phenotype, that could test relatively few cells. Particularly, we have utilized reoviruses, serotypes 1 and 3, which cause transient, asymptomatic infections of the murine intestine when given intraduodenally (i.d.) or orally. Reovirus 1, especially, acutely primes both B and T cells in PP leading to IgA-committed B cell clonal precursors and virus specific, MHC haplotype restricted, serotype non-specific precursors for cytotoxic T cells (pCTL) (1). Another replicating antigen we have used is M.m., which can colonize the intestine of GF mice and results in the hypertrophy of PP, the development of GC in PP and the generation of IgA-committed memory B cells (2). Cells primed by such procedures have been separated into subsets by fluorescence activated cell sorting (FACS) and tested *in vivo* by protection assays or *in vitro* using clonal analyses (1, see George et al., this volume) and by *in situ* hybridization methods to discern mRNA for Ig polypeptides or lymphokines (see Weinstein et al., this volume).

Methods

Mice used to test for protective effects of primed lymphocytes include *scid* mice, maintained in germ-free isolators, and neonatal SPF mice, born in our colony, both of which are susceptible to fatal infections by reovirus given orally. The limiting dilution assay for pCTL has been described (1) as has the clonal microculturing of Ag-specific B cells enriched on haptenated gelatin (3) or B cells responding to Ia^b alloreactive T_H2 cells (see George et al., this volume).

Results and Discussion

We will present some examples of the application of our *in vivo* or *in vitro* functional assays to assessing the events occurring in PP during gut mucosal priming and the efficacy of primed subsets of lymphocytes in conferring some level of specific resistance during the course of infection.

Primed, congenic $Thy1^+$, $CD4^+$, GCT^+ PP cells can protect *scid* mice from fatal hepatitis induced by reovirus given orally. CB.17 *scid* mice were infected by the oral route with either 10^5 pfu of reovirus type 1 or type 3. The mice appear well 2 wk. after infection but infectious virus can be recovered from the brain, lung, liver, spleen, intestine, intestinal wash, and bile. Chronic, discrete inflammatory lesions appear in the liver of infected mice, are restricted largely to this organ and

are associated with hepatocytes containing demonstrable levels of viral antigen. The number of lesions increases with time but they remain discrete. Mice begin to die by 4 wk. after infection and all are dead by 6 wk. The adoptive transfer of 1, 5, or 15 x 10^6 PP cells taken from immunocompetent mice 7-14 d after oral infection progressively lowers and eliminates recoverable virus from intestinal wash and all tissues at 7 d if given 48 h before oral challenge. Non-immune PP cells offer little if any protection vs. infection and dissemination of virus. Depletion of lymphocyte subsets prior to transfer of immune PP cells indicated that the protective effect was most susceptible to anti-Thy1 and the MAb GCT and least to Ab vs. most B cells (J11d). Further depletions prior to transfer indicated that viral dissemination was most widespread if $CD8^+$ or GCT^+ cells were removed and virus could be most contained by PP cells lacking in $CD4^+$ T cells and B cells. Previously we have shown that reovirus specific pCTL and effector CTL were $Thy1^+$, $CD8^+$, and GCT^+ (1). These results are consistent with the thesis that this subset of lymphocytes is important in resistance and immunity to reovirus infections.

Intraepithelial lymphocytes (IELs) from immune mice can protect neonatal mice from a disseminated infection leading to a fatal meningoencephalitis after reovirus 3 given orally. We have previously shown that reovirus specific pCTL appear in the IEL population 1 wk. after i.d. infection and that these are MHC haplotype restricted (4). The effector CTLs are $Thy1^+$, $CD8^+$. About 75% of IELs are $CD8^+$, 50% $Thy1^+$, and 15% GCT^+ both before and after reovirus infection. Upon <u>in vitro</u> restimulation of pCTL from IELs the T cell population that grows out and relatively increases is $Thy1^+$, $CD8^+$ and bears the α/β TCR. The ability to grow out effectors from pCTLs in bulk culture made frequency analyses by limiting dilution possible. Surprisingly, we have found that 7 d after i.d. infection the CTL frequency among IELs is about 15-fold greater than in PP and, after normalization to $Thy1^+$, $CD8^+$ content is still about 3-fold higher. Thus, given the representation of specific pCTL in IELs (316-374/10^6), we sought to determine whether they could be protective vs. an oral reovirus infection. Previously we had found that most neonatal mice given 3 x 10^6 pfu reovirus 3 orally 48 h after birth died 8-10 d subsequently of meningoencephalitis (5). Immunization of mothers orally or subcutaneously with either reovirus 1 or 3 saved offspring from an otherwise fatal challenge although, depending on route and serotype used in mothers, virus could still disseminate to liver and brain and cause brain lesions (5). In several experiments 10^6 IELs from orally immunized or non-immune adults were transferred intraperitoneally into neonates 1 d before oral challenge, Most neonates given immune IELs, depleted or not for $Thy1^+$ or $CD8^+$ cells, survived the challenge (13/15). However, mice receiving depleted populations had pathogenic lesions in their brains. Recipients of non-immune IELs fared worse (4/8 survivors) and all survivors also showed brain pathology. Finally, many fewer recipients of immune IELs had detectable liver virus titers than did those given non-immune IELs. Since this assay system seems especially sensitive for testing protective or ameliorating effects of IELs at many levels - on initial gut infection, virus dissemination,

development of brain lesions, and death - it may be informative for testing subsets of IELs such as Thy1$^-$ CD8$^+$, CD8$^+$ γ/δ TCR$^+$, GCT$^+$, etc. Such an assay may circumvent the need for *in vitro* culture conditions allowing growth and/or differentiation.

Transient GC development in PP of GF mice orally infected with reovirus or colonized with *Morganella morganii*. Following acute gut stimulation of GF mice with reovirus 1 given orally, B cells with GC markers appear transiently in PP at 6-12 d. Initially these GC cells, which bind high levels of peanut agglutinin (PNA^{high}), are sκ^{high} but by d 8 the subset common in conventionally reared mice that is PNA^{high}, sκ^{low} appears and then declines. Over this same time period sIgA$^+$ but not sIgG1$^+$ B cells appear in PP and persist there for at least 2.5 wk. When the gut of GF mice is colonized with M.m. the ensuing chronic stimulation causes a similar, transient perturbation of PP B cells except that it becomes evident only at 12 wk. and subsides by 20 wk. During this period IgA anti-phosphocholine Ab is secreted in fragment cultures of PP taken from these colonized mice. Since the PP of GF mice do not possess chronically activated GC they may be likened to the cortical region of quiescent lymph nodes. Our findings of early expression of sIgA$^+$ but not sIgG1$^+$ by B cells in PP developing *de novo* GC contrasts with findings by others that GC arising in lymph nodes generate B cells expressing sIgG (6). These observations suggest that the lymphoid tissue microenvironment of PP rather than their usually activated state may account for the propensity of their B cells to switch sIg expression to sIgA$^+$.

An Ag dependent and independent clonal microculture for B cells that is T dependent and haplotype restricted. We have developed a microculture assay to assess the functional potential of small numbers of B cells at the clonal level. This microculture permits both determination of frequency of B cells responding to Ag, cytokines, T cell encounters, etc. by producing Ab with or without clonal expansion and the Ig isotype display of the responding B cell. Small numbers (10-20) of B cells (H-2k) enriched on haptenated (H) gelatin (3) are cultured with non-limiting numbers (3000) cloned T_H2 cells (D10, Ia^k restricted and conalbumin specific) in 10 µL with H-conalbumin (50 ng/mL). If the D10 cells are appropriately rested in culture and the B cells are enriched on low coupled H_1/H_3-gelatin the responses are limited to those induced by cognate T/B interactions. The frequency and complexity of isotype display by the responding cells increases with *in vivo* priming of the B cell donors. An Ag independent version of this assay exploited the alloreactivity of D10 for Ia^b, allowing 1 of every 2-3 B cells to respond in microcultures containing 0.5-2.0 F_1 (Ia^k x Ia^b) B cells/well. These cultures scored both primary and memory B cells and supported the expression of IgM, IgG1, and IgE Abs. In order to detect expression of IgA in these clonal microcultures, exogenous lymphokines and/or dendritic cells had to be added as described (see George et al., this volume) and then even GC cells responded with Ab secretion.

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Role of intestinal T cells in pulmonary immunity to non-typable *Haemophilus influenza*

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Abstract

Intestinal T cells have been shown to be an important component of respiratory tract immunity in the rat. The role of these cells may be in part to influence the behaviour of lung polymorphs. Lymphocytes were collected from the thoracic duct of rats primed by intra-Peyer's patch (IPP) inoculation with nontypable *Haemophilus influenzae* (NTHI). Initially, whole washed thoracic duct lymphocyte (TDL) populations were transferred to recipient rats. These rats cleared bacteria from bronchial spaces faster than non-transfused rats, and rats transfused with non-immune lymphocytes. Rats given a reduced number of lymphocytes did not clear NTHI faster than controls. When lymphocytes depleted of B cells were transferred, NTHI clearance rate was accelerated. Spleen cells were also capable of conferring protection to transfused rats. Polymorphs from immunised rats were shown to be recruited faster and have a higher activity level than non-immunised rats. Immunised nude rats are less able to accelerate clearance of NTHI. These data suggest that immune clearance of NTHI from the respiratory tract following gut immunisation is dependent upon antigen primed mucosal T cells which may modulate the function of lung polymorphs.

Introduction

The success of an orally presented killed nontypable *Haemophilus influenzae* (NTHI) vaccine in reducing the number of acute infection episodes in chronic bronchitis sufferers supports the concept that this microbe is a major factor in the aetiology of acute bronchitis and demonstrates the importance of activated gut associated lymphoid tissue (GALT) in the induction of distant mucosal immunity in man [1]. A rodent model has shown that GALT-bronchus associated lymphoid tissue (BALT) stimulation is essential for protection [2]. In both rodent and human systems, no correlation between immunity and specific antibody in serum and bronchial washings was demonstrated. The object of the present study was to assess the cellular basis of enhanced respiratory immunity in intestinally immunised rats.

Materials and Methods

6 to 8 week old male DA and Rowett rats were kept in SPF conditions until the commencement of the immunisation protocol. Rats were immunised with formalin killed NTHI, prepared to a concentration of $2x10^{10}$ colony forming units (CFU)/ml. NTHI was emulsified in an equal volume of complete Freund's adjuvant (CFA), and distributed via a 27g needle in 2-5µl volumes subserosally adjacent to each Peyer's patch. 5 days after immunisation, thoracic duct cannulations were performed, and lymphocytes were collected over 4 days. Recipient rats received 4 daily doses of lymphocytes that had been previously washed in Dulbecco's phosphate buffered saline pH 7.2 supplemented with 5% (v/v) foetal calf serum (DPBS/FCS). At the commencement and end of the lymphocyte transfer killed NTHI ($5x10^8$ CFU in 50µl) was administered intratracheally. Control rats which either received non-immune lymphocytes, or were not transfused, also received intratracheal NTHI. An enriched T cell preparation was obtained by passage over nylon wool columns before panning on anti-immunoglobulin coated dishes. Spleens were aseptically removed from donor rats 5 days after IPP immunisation, finely chopped and passed gently through a fine wire mesh into sterile PBS/FCS. Debris and clumps were removed and the cells washed. T cell deficiency of Rowett rats was confirmed by their failure to reject DA skin grafts after 9 weeks. These rats were immunised by a GALT/BALT regime which previously had been successful in conferring protection to DA rats [2]. To assay phagocytic behaviour, groups of rats were immunised according to the same protocol without CFA. At the conclusion of the protocol, bronchial washings were performed, and centrifuged at 400xg for 10 min. The cell pellet was resuspended in PBS/FCS and total and differential cell counts performed. The cells were then diluted to $2x10^6$ cells/ml and luminol enhanced chemiluminescence activity was gauged in a Wallac luminometer. Groups of rats within each experiment were compared by a Mann-Whitney test for non-parametric data. P values <0.05 were considered significant.

Results

One group of recipient rats were infused on four consecutive days with whole washed TDL populations. Rats received, on average, $2\text{-}3x10^8$ TDL per day collected from the pooled yield of an equivalent number of immunised donors. Rats receiving immune cells were compared with rats receiving non-immune cells and also with non-transfused rats with respect to their ability to clear NTHI from the respiratory tract. Rats receiving immune cells had significantly fewer (5%) bacteria remaining in their respiratory tracts than rats receiving non-immune cells and non-transfused rats ($p<0.05$) (Table 1). Because non-transfused rats and rats receiving non-immune lymphocytes cleared NTHI at the same rate, the former treatment was used as a control in all subsequent experiments. In some experiments recipient rats were infused over 4 consecutive days with $4\text{-}5x10^7$ immune TDL per day, and compared to a group of non-transfused rats. Each group received the two intratracheal immunisations in the transfusion period. Rats receiving the reduced number of immune cells did not show significantly improved clearance of NTHI ($p>0.05$) (Table 1). In a further experiment TDL were collected from immunised rats over four consecutive days and depleted of B cells (<5% surface Ig positive). Animals receiving these cells ($8\text{-}9x10^7$ per rat) were able to demonstrate significantly improved clearance ($P<0.05$), of the same magnitude as those receiving whole populations of unfractionated lymphocytes (Table 1). Finally, recipient rats were infused over four consecutive days with washed spleen cell populations, receiving $2\text{-}3x10^8$ cells per day. When compared with non-transfused rats, these rats demonstrated significantly accelerated

Table 1. Adoptive transfer experiments

Experiment	Clearance Index (mean ± SEM)	p value
Rats receiving whole populations of immune TDL (n = 6) ($2\text{-}3 \times 10^8$ per day)	5.0 ± 0.9	$p<0.05$
Rats receiving reduced numbers of immune TDL (n = 4) ($4\text{-}5 \times 10^7$ per day)	29.0 ± 6.3	$p>0.05$
Rats receiving immune purified T cells (n = 4) ($8\text{-}9 \times 10^7$ per day)	7.0 ± 7.1	$p<0.05$
Rats receiving splenic cells (n = 4) ($2\text{-}3 \times 10^8$ per day)	9.0 ± 4.4	$p<0.05$

n = number of animals SEM = standard error of mean

Clearance index = [mean number of bacteria harvested from transfused rats] x 100
[mean number harvested from control rats]

Table 2. Immunity in T cell intact and T cell deficient rats

Rat strain	Clearance index (mean ± SEM)	p value
DA rats (T cell intact) (n = 5)	4.6 ± 1.1	$p<0.05$
Nude rats (T cell deficient) (n = 5)	16.6 ± 5.1	$p>0.05$

n = number of animals SEM = standard error of mean

Clearance index = [mean number of bacteria harvested from immunised rats] x 100
[mean number of bacteria harvested from control rats]

Table 3. Recruitment of activity of lung polymorphs 2 hours after challenge

	Total Cell Count/ml ($\times 10^6$)	% Polymorphs	Chemiluminescence (mv at 5 mins)
Immunised (n = 6)	4.8 ± 0.6	65 ± 2	122.5 ± 28.3
Control (n = 6)	2.8 ± 0.4	28 ± 2	64.1 ± 14.5
	($p<0.05$)		($p<0.05$)

clearance of NTHI ($p<0.05$), similar in magnitude to rats receiving whole unfractionated TDL (Table 1). Athymic T cell deficient rats did not enhance clearance of NTHI from the respiratory tract compared to non-immunised athymic controls (Table 2). However, numbers tested were small, and a trend toward accelerated pulmonary clearance was evident, with immunised nude rats having on average a little less than a log fewer bacteria remaining in the lungs four hours after challenge. Immunised rats were shown to recruit neutrophils to the lungs faster than non-immunised rats, and these neutrophils were shown to have a higher level of activity (Table 3).

Discussion

This series of experiments has demonstrated that T cells activated following immunisation of Peyer's patches are able to relocate and provide functional immunity in the respiratory tract. Here, their effector function may cause accelerated recruitment and subsequent activation of polymorphs to the bronchial spaces. These findings have particular relevance in the understanding of immunity against nontypable *Haemophilus influenzae* respiratory infections, following accumulating evidence which questions the role of specific antibody in protection against this organism. Clearly, alternate mechanisms for immune clearance of this organism need to be investigated. Thoracic duct cannulation provides the opportunity to compartmentalise the rat immune system into mucosal and systemic components. In the experiments described above, the thoracic duct was cannulated at a point just above the cysterna chyli, where the majority of lymphocytes are of gastrointestinal origin. These data therefore support the concept of a mucosally restricted pattern of distribution of intestinally derived T cells consistent with the mucosal restriction of intestinal helper T lymphocytes previously demonstrated. That cells derived from the spleen conferred the ability to enhance clearance could reflect either dissemination of antigen from the injection site with subsequent systemic priming, or the seeding of mucosal effector cells in the spleen. It is relevant that rats immunised systemically (subcutaneously) have been shown incapable of clearance acceleration [2]. The observation that athymic rats had a greatly diminished response to vaccination stresses the importance of T cell immunity in protection against this organism. The trend toward enhanced clearance in these rats, however, does suggest that additional mechanisms may contribute to respiratory defence. These studies show that intestinally derived antigen activated T lymphocytes can function *in vivo* at a distant mucosal site. The demonstration that T lymphocytes mediate immune clearance of bacteria from the bronchial lumen suggests a major role for activated T lymphocytes in the interaction with luminal polymorphs and subsequent phagocytosis of bacteria, an event previously linked more closely with opsonisation due to antibody.

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Mucosal immunoregulation by $CD4^+$ ($L3T4^+$) T cells

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ABSTRACT. The role of L3T4+ T helper (Th) cells in mucosal immunoregulation was examined in mice given weekly injections of 200 μg GK 1.5 (anti-L3T4) either throughout their development or in adult life only. Depletion of L3T4+ T cells was confirmed by FACS analysis of peripheral blood lymphocytes. FACS analysis and ELISPOT assays were then employed to study T cell subpopulations and numbers of spontaneous antibody (Ab)-forming cells respectively in spleen, Peyer's patches and the intestinal lamina propria and epithelium. Following treatment with anti-L3T4, CD4+ cells were depleted in every tissue studied. A concomitant decrease occurred in the numbers of CD3+ cells and a relative increase in CD8+ cells was observed. CD3+ "double negative" cells were increased in the lamina propria and intestinal epithelium. Numbers of spontaneous Ab spot-forming cells (SFC) in the spleen were unchanged or in some instances increased with respect to IgM and IgG. In lamina propria, the IgA response was selectively suppressed. Preliminary studies in Peyer's patches revealed no change in the numbers of IgA forming cells following anti-L3T4 treatment. Thus different effects were observed in different tissues.

1. Introduction

T cells of the CD4+ subpopulation fulfill a number of crucial functions in the induction and regulation of immune responses. In order to assess the role of the CD4+ T cell in regulating antibody responses in the murine small intestine *in vivo*, mice were studied which were rendered deficient in CD4+ (L3T4+) T cells by injection of monoclonal anti-L3T4 antibody (1).

2. Materials and Methods

2.1 MOUSE TREATMENTS

Adult BALB/c mice were injected i.v. with 200 μg IgG purified from ascites of GK 1.5 hybridoma cells by ammonium sulphate and caprylic acid precipitation on 3 consecutive days and weekly thereafter. In some experiments, mice were exposed to GK 1.5 during development by injection of female mice prior to mating and during pregnancy and nursing. Offspring from these mice were injected weekly from birth with 200 μg GK 1.5 IgG i.p. The day of injection was called day 0.

2.2 EXPERIMENTAL PROTOCOL

On day 2 after injection some mice were bled and peripheral blood lymphocytes stained with fluorescent anti-L3T4. Once depletion of $L3T4^+$ T cells was established, mice were routinely sacrificed on day 3 after injection and various tissues obtained for analysis by FACS and for enumeration of spontaneous antibody forming cells.

2.3 CELL ISOLATIONS

Spleen cells were prepared by mechanical dissociation on a metal strainer. Peyer's patch cells were prepared by enzymatic digestion as described previously (2). Intraepithelial and lamina propria lymphocytes were prepared using a modification of the method of Davies and Parrott (3) in that 1.5 mg/ml Dispase ® was used for enzymatic digestion of the small intestine.

2.4 CELL SURFACE STAINING

T cell subpopulations were identified by FACS on the basis of surface staining with fluorescent monoclonal Abs. $CD3^+$ cells were identified by staining with anti-T3 Ab (clone 145 2C11). $CD4^+$ cells were identified by staining with anti-L3T4 (GK 1.5) and $CD8^+$ cells were determined by staining with anti-Lyt 2 (clone 53.6-72).

2.5 ANTIBODY-FORMING CELLS

Numbers of spontaneous Ab producing cells were determined by an enzyme linked immunospot (ELISPOT) assay (4).

3. Result

Mice were found to have a profound deficiency of $L3T4^+$ T cells in peripheral blood following anti-L3T4 injection for 3 weeks in adults or by 5 weeks in litters born to injected mothers (data not shown). Since similar results were obtained throughout the experiments regardless of whether mice were treated in adulthood or throughout development, the results are summarized without distinguishing between treatment groups.

FACS analysis of cell suspensions obtained from spleen, lamina propria, intestinal epithelium and Peyer's patch showed a deficiency of $L3T4^+$ T cells which was reflected in a decrease in the numbers of $T3^+$ cells in these tissues. A relative increase in $Lyt\ 2^+$ T cells was observed in the spleen, lamina propria and Peyer's patches. Cells of the $CD3^+$, $CD4^-$, $CD8^-$ (double negative) phenotype which are found in small numbers in the lamina propria and the intraepithelial site in normal mice were found in relatively higher numbers in mice treated with anti-L3T4 Ab (Table 1).

TABLE 1. T cell subpopulations (by FACS) in BALB/c mice: effect of anti-L3T4 treatment.

	% $CD3^+$	% $CD4^+$	% $CD8^+$	% $CD3^+$, $CD4^-$, $CD8^-$
SPLEEN				
untreated	**40.45*** (34.7-45)	**24.96** (19.7-27.2)	**12.65** (9.8-17.4)	
anti-L3T4	**18.86** (13.4-28.2)	**2.86** (0.1-7.4)	**14.9** (12.1-17.3)	-
LAMINA PROPRIA				
untreated	**45.09** (40.5-47.8)	**17.24** (12.6-20.6)	**12.79** (8.3-18.7)	**4.7** (pooled cells)
anti-L3T4	**35.59** (22.7-42.5)	**2.68** (2.3-2.9)	**15.15** (9.0-26.9)	**14.35** (10.6-28.6)
INTESTINAL EPITHELIUM				
untreated	**82.48** (74.3-91.6)	**11.97** (9.4-14.9)	**69.72** (58.5-77.7)	**8.9** (3.9-14.3)
anti-L3T4	**64.77** (56.9-72.7)	**1.92** (0.1-5.6)	**56.55** (44.5-68.0)	**14.42** (10.2-21.6)
PEYER'S PATCH (one experiment)				
untreated	**30.52**	**21.65**	**4.09**	
anti-L3T4	**21.46**	**1.32**	**9.69**	-

*Mean percentage with range in parentheses. Results summarized from multiple determinations.

3.1 SPONTANEOUS ANTIBODY SPOT FORMING CELL RESPONSES

Treatment with anti-L3T4 led to an increase in numbers of SFC for all isotypes in spleen. In lamina propria, IgM and IgG SFC were in most cases increased in number while IgA was suppressed. In Peyer's patches, the numbers of IgM and IgG SFC were decreased while IgA SFC were not significantly different in number. Thus different effects were seen in different tissues (Table 2).

4. Discussion

These results indicate that L3T4 T cells are crucial for the appropriate regulation of antibody formation *in vivo*. The regulatory influence of this cell sub-population is clearly distinct in different tissues as seen by the nature of the dysregulation of antibody responses of different isotypes in the different sites. Reduction in the numbers of IgA forming cells in lamina propria highlights the T cell dependency of this response however, the number of these cells in Peyer's patches is not altered and this may suggest some underlying role for $CD4^+$ T cells in directing traffic of IgA^+ lymphocytes out of PP

and into lamina propria. Further, CD4+ T cells may not be required for B cell commitment to IgA in Peyer's patches. That the spleen cell Ab response should be augmented by this treatment is surprising. Experiments are in progress to test the following hypotheses: I) that the increased numbers of SFC are specific for T independent antigens; II) that these cells are being activated by lymphokines produced by cells other than L3T4+ T cells which are perhaps also producing lymphokine in an uncontrolled manner in the absence of regulatory signals from CD4+ T cells.

TABLE 2. Spontaneous antibody spot-forming cells in BALB/c mice: effects of anti-L3T4 treatment

	SFC/10^6 cells ± SD		
	Spleen	Lamina Propria	Peyer's patches+
IgM			
No treatment	1367 ± 46	367 ± 153	360 ± 57
Anti-L3T4i	5400 ± 721**	600 ± 12*	33 ± 42*
Anti-L3T4ii	4933 ± 462**	347 ± 61	
IgG			
No treatment	433 ± 58	2933 ± 503	293 ± 70
Anti-L3T4i	1367 ± 379**	5300 ± 1752	37 ± 32**
Anti-L3T4ii	1367 ± 208**	4467 ± 551*	
IgA			
No treatment	167 ± 115	73,600 ± 2263	1453 ± 410
Anti-L3T4i	247 ± 103	22,600 ± 283**	1260 ± 191
Anti-L3T4ii	133 ± 70	21,300 ± 707**	

+Pooled PP cells from 4 mice. Results from one representative experiment.

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T-Cell subset distributions in spleen, thymus and mesenteric lymph nodes from immunodeficient wasted mice

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ABSTRACT. Mice bearing the autosomal mutation "wasted" (wst) develop neurologic abnormalities and immunodeficiency at secretory sites by 21 days of age. Initial experiments showed that spleen and mesenteric lymph nodes from wasted mice have normal distributions of cells bearing the surface markers Lyt2, L3T4, and Lyt2 L3T4 relative to littermate and B6C3 controls. Studies of thymocytes, however, revealed that Lyt2+ cells were predominantly L3T4- in wasted mice, while in controls most Lyt2+ cells also expressed surface L3T4. Cell volume experiments revealed different volume distribution patterns for T-cells subpopulation in wasted mice relative to controls for all tissues studied.

1. INTRODUCTION

"Wasted" mice (bearing the autosomal recessive mutation wst) spontaneously develop a disease that resembles ataxia telangiectasia in humans, with features that include faulty DNA repair following exposure to ionizing radiation, neurologic abnormalities, and immunodeficiency [1-3]. Numerous immunologic abnormalities including deficient secretory immunity, and low IgA levels have been reported in these mice [2-3]. We designed experiments aimed at examining potential T-cell abnormalities that might contribute to the altered B-cell responses in wasted mice.

2. MATERIALS AND METHODS

Wasted (wst/wst) mice were bred in the sterile, hooded animal facility at Argonne National Laboratory from wst/+ breeding pairs obtained from the Jackson Laboratory (Bar Harbor, ME). Lymphocytes stained for immunofluorescence were analyzed with forward angle light scatter to estimate cell volume.

3. RESULTS

T-cell subpopulations in lymphoid tissues of wst/wst mice were examined relative to controls by using both one-color and two-color

immunofluorescence (Table 1). In both splenic and thymic populations, we observed an increased percentage of total $L3T4^+$ cells in wst/wst mice relative to controls, which caused an increase in the Th:Ts ratio. Results of cell volume studies demonstrate that all populations of T-cells examined from wst/wst mice have a different volume distribution pattern than that obtained from T-cells derived from control animals.

TABLE 1. Lymphocyte Subpopulations in Wasted and Control Mice

Lymphocyte Source	Percentage				Th:Ts ($L3T4^+$:$Lyt2^+$)
	$Lyt2^-L3T4^+$	$Lyt2^+L3T4^-$	$Lyt2^+L3T4^+$	$Thy1^+$	
wst/wst spleen	25.8	8.0	1.5	44.7	2.9
wst/● spleen	13.6	6.2	1.4	43.4	1.9
BCF_1 spleen	16.3	6.8	1.2	43.2	2.2
wst/wst MLN	14.9	39.6	1.5	43.3	.4
wst/● MLN	14.2	46.0	1.8	51.3	.3
BCF_1 MLN	14.9	39.5	2.3	61.3	.4
wst/wst thymus	57.5	18.8	15.9	70.7	2.1
wst/● thymus	18.3	9.6	64.8	68.8	1.1
BCF_1 thymus	23.0	7.3	43.2	77.1	1.3

4. DISCUSSION

Our experiments defined several T-cell abnormalities evident in wasted mice, including a high Th:Ts ratio in both spleen and thymus, decreased percentages of $L3T4^+$ $Lyt\text{-}2^+$ cells in thymus, and differences in volume distribution patterns of T-cells derived from all tissues. These results suggest the presence of a T-cell abnormality in wasted mice that may underlie many of the reported B-cell-derived immune deficiencies.

5. ACKNOWLEDGMENTS

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Postnatal appearance of lymphocytes in the lamina propria of the gut mucosa of pigs: number, proliferation and T lymphocyte subsets

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The gut immune system controls the specific reaction to antigens coming from the gut lumen. In the lamina propria (LP) of the gut mucosa there are many lymphocytes. These are the effector cells for the gut immune system. Little is known about the development of these cells. In the early postnatal period the gut is exposed to a high amount of nutritional and microbial antigen for the first time. Therefore we studied the LP lymphocytes in young pigs in that period to answer the following questions:-

1. What is the number of LP lymphocytes in normal and gnotobiotic pigs at different ages?
2. Do lymphocytes proliferate in the LP?
3. Which phenotype do the LP lymphocytes express in the postnatal period?

MATERIALS AND METHODS

German landrace pigs, reared conventionally (n=67) or germfree (n=6), aged 1 to 91 days were examined. The cell number was determined using routine histology (fixation in Schaffer's solution, glycolmethacrylate embedding, 4 µm Giemsa stained sections). With an ocular grid the cell number was determined at a magnification of 625X in the crypt and villi region of the jejunum and ileum. Only cells with all morphological characteristics of lymphocytes were counted.

The proliferation of LP lymphocytes was studied with the metaphase arrest technique using vincristine and the mitotic rate/h was calculated (described in Pabst et al. (1988)).

The T subsets of lymphocytes were studied in immunohistology (three step APAAP technique) with monoclonal antibodies against pig T cells (reviewed in Lunney and Pescovitz (1988)). The number of positive stained cells per mm^2 was determined.

RESULTS

Cell number: During the first weeks of life the number of LP lymphocytes increased from 1300 $\pm$ 120/mm^2 on day 1 to 2100 $\pm$ 140/mm^2 on day 21

and changed only slightly until day 91. Surprisingly the number of LP lymphocytes was comparable in germfree animals (2313 ± 227/mm^2). There was no difference between the crypt and villi region or between the jejunum and ileum.

Proliferation: The mitotic rate of LP lymphocytes ranged from 0.32 to 0.62%/h in the different age groups. Only in 1 day old animals were significant differences observed: the mitotic rate in the villi (0.13 ± 0.04%/h) was lower than in the crypt region (0.50 ± 0.07%/h).

T cell subsets: A dramatic increase in the number of CD2$^+$ cells was seen between day 1 (76 ± 20.7/mm^2) and day 42 (1432.5 ± 146.6/mm^2). No differences were observed between the crypt and villi region or jejunum and ileum. On day 1 only a small number of cells was positive for CD4 (4 ± 5/mm^2) or CD8 (21 ± 2/mm^2). Their sum was much lower than the number of CD2 positive cells. From day 12 onwards the sum of CD4$^+$ (642 ± 165/mm^2) and CD8$^+$ (300 ± 68/mm^2) cells equalled the number of CD2$^+$ cells (915 ± 93/mm^2). The subset pattern of 49 day old germfree pigs (cells/mm^2: CD2: 815 ± 80; CD4: 61 ± 14; CD8: 98 ± 32) was comparable to that of 5 day old animals.

DISCUSSION

The increase in the number of LP lymphocytes in the postnatal period is probably dependent on nutritional and not microbial antigens in the gut lumen, because the cell numbers are comparable in age matched conventional and germfree animals. The amount of LP lymphocytes produced in the LP is comparable to that in the dome or interfollicular region of the Peyer's patches (Pabst et al. (1988)). The higher number of lymphocytes in older animals is partially due to local proliferation in the lamina propria. Only 30% of all T cells on day 1 and day 5 and in the 49 day old gnotobiotic animals are membrane positive for the CD4 or CD8 markers. So far this early postnatal subset pattern has not been reported in other species.

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T cells associated with transfer of mucosal immunity to *Trichinella spiralis*

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ABSTRACT.T-cell lines specific for *T.spiralis*-derived antigens, i.e. crude worm antigens (CWA) and excretory/secretory (ES) antigens, were generated *in vitro* using mesenteric lymph node cells (MLNC) from infected mice at various antigen concentrations in the presence of varying lymphocyte/ antigen presenting cell (APC) ratios. These T-cell lines were used in transfer experiments for assessing their roles in the induction of mucosal immunity to this parasite at gut level. Cultured T-cell lines were predominantly L3T4-positive. A pilot study performed with transferred 8-weeks-old T-cell lines indicated that only the T-cell lines generated in the presence of 50 μg CWA/ml and a lymphocyte/APC ratio 1:20 were able to transfer immunity to *T.spiralis*. In addition the transferred T cells were apparently capable to induce increased numbers of intestinal mastcells and IgA-plasma cells. Based on this pilot experiment we concluded that L3T4-positive T cells are indeed central to the transfer of immunity to *T.spiralis* and that T cells are apparently implicated in mediating cellular responses such as intestinal mastocytosis and IgA-plasmacytosis.

1 Introduction

Studies with *T.spiralis* have contributed to our understanding of immune mechanisms involved in gastrointestinal helminth infections. Adoptive transfer of immunity, using *in vitro* cultured *T.spiralis*-specific T cells have been employed for analysing mechanisms of resistance in the *T.spiralis*-mouse model (1,2). Additionally, it is now known that L3T4-positive T cells are implicated in the transfer of immunity in this system (2,3).

Since resistance to reinfection with *T.spiralis* ensues from primary oral infection as well as from priming parenteraly with CWA and ES (4) it may be argued that antigens present in ES suffice to generate the T lymphocytes necessary for mediating development of immunity to *T.spiralis*. In an attempt to analyse the roles of T cells mediating the induction of protective immunity and pathology during primary infection, T-cell lines specific for *T.spiralis* CWA and ES were prepared.

2 Materials and Methods

7 Days after oral infection of Balb/c mice with 200 *T.spiralis* infective larvae, MLNC were isolated and incubated at $1x10^6$ cells/ml in the presence of 10, 25 and 50 μg CWA/ml. Subsequent restimulation was carried out every 7 days using $1x10^6$ responder cells, $10\text{-}20x10^6$ irradiated syngeneic spleen cells (2500 rads) and 10, 25 and 50 μg CWA per ml culture. ES-specific T-cell lines were generated in the presence of 1 and 5 μg

ES/ml using the same protocol.

CWA-specific T-cell lines,cultured for 8 weeks were adoptively into naive recipients (day 0) by injection of $1x10^6$ cells in 0.3 ml PBS via a lateral tail vain. On day 1 these mice were each challenged with 200 *T.spiralis* infective larvae. On day 7 the animals were sacrificed and assessed for: worm burdens in the small intestine and mean numbers of mast cells and IgA-plasma cells per villus (mean number in 20 villi).

3.0 Results

CWA-specific T-cell lines generated at the various antigen concentrations and lymphocyte/APC ratio 1:20 were stable in culture up to 9 weeks, retaining their antigen specificity. These T-cell lines were all predominantly L3T4-positive (95%). Only the T-cell lines generated in the presence of 50 μg CWA/ml were able to transfer immunity and also mastocytosis and IgA-plasmacytosis (Fig.1). We were able to culture ES-specific T-cell lines for 3 weeks, currently we are optimizing the culture scheme.

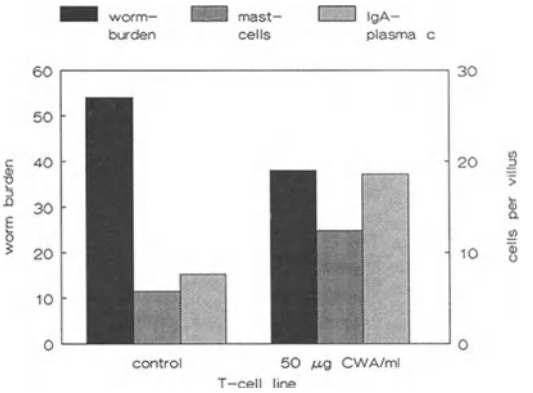

Figure 1. Adoptive transfer of T-cell lines generated in the presence of 50 μg CWA/ml and a lymphocyte/APC ratio 1:20.

4 Discussion/Conclusion

This pilot experiment seems to confirm that L3T4-positive T cells are involved in mediating both protective immunity to *T.spiralis* and cellular responses such as mastocytosis and IgA-plasmacytosis.

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Rat T cell subpopulations: Effect of environmental and microbial antigens

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Extensive studies by Mosmann et al. [1,2] using cloned murine T cells have defined two different subsets of $CD4^+$ T cells based on their lymphokine production and functional properties. However, the existance of similar subsets of $CD4^+$ cells in other systems is still unclear. In the rat, it has been reported [3-5] that the monoclonal antibody OX22 which exhibits specificity for a high molecular weight form of the leukocyte common antigen (L-CA) divides $CD4^+$ T cells into two subpopulations, with the OX22 monoclonal antibody binding to two-thirds of the T helper (Th) cells in the thoracic duct lymph. Recently, Powrie and Mason [6,7] also suggested that upon exposure of $OX22^+$, $CD4^+$ cells to antigen, the OX22 marker is lost and that these $OX22^-$, $CD4^+$ T cells represent memory cells.

Therefore, if the OX22 marker of $CD4^+$ cells defines a maturational stage that is dependent upon the presence of antigen, it was of interest to determine if the phenotype of T cells in the gut-associated lymphoid tissues (GALT) and in the spleen varied under different environmental conditions. In this study, we report the phenotypic characterization of lymphoid cell populations, including the distribution of $OX22^+$ T cells, in Peyer's patches (PP) and spleens of germfree, gnotobiotic and conventional Fischer rats.

Inbred germfree, gnotobiotic and conventional Fischer female rats used in this study were 12-15 weeks-of-age. After the rats were sacrificed by CO_2 asphyxiation, the PP and spleens were aseptically removed. Single cell suspensions were obtained by gentle dispersion of the tissues through sterile wire mesh into medium. Cell suspensions were assessed for viability by trypan blue exclusion. In all cases, >95% viability was found. Aliquotes of the single cell suspensions (~1 x 10^6 cells) were dispersed into tubes and incubated with the appropriate fluorescein-isothiocyanate (FITC) and/or biotin-conjugated anti-rat monoclonal antibody reagents followed by incubation with avidin-phycoerytherin, when appropiate. The cell preparations were analyzed using a FACStar® fluorescence-activated cell sorter.

Analysis of the lymphocyte populations in the PP and spleens showed a higher percent of Ig^+ cells and a lower percent of T cells in tissues from germfree rats than seen in tissues from gnotobiotic animals (Table 1). In both animal models, PP contained a higher percent of Ig^+ cells and a lower percent of T cells than was seen in the spleen. In addition, differences were seen in the percent of the T cell populations which were $OX22^+$ (Table 2). Tissues from gnotobiotic rats showed a lower percent of T cells that were $OX22^+$,

especially Th cells, than seen in the PP and spleens of germfree and conventional rats. The PP compared to the spleen of germfree rats also had a lower percent of $OX22^+$ T cells.

Table 1. Distribution of Lymphoid Cells in Peyer's Patch (PP) and Spleen (Sp)

	Percent Lymphocytes			
	Germfree		Gnotobiotic	
Cell Type	PP	Sp	PP	Sp
B Cell (Ig^+)	76	61	62	40
OX19 (T)	33	48	48	53
W3/25 (Th)	12	20	25	29
OX8 (Ts)	9	13	12	23
OX22 (L-CA)	89	87	79	74

Table 2. Distribution of $OX22^+$ Cells in Peyer's Patch (PP) and Spleen (Sp)

	Percent OX22+ Cells					
	GF		GN		CONV	
Cell Type	PP	Sp	PP	Sp	PP	Sp
Ttotal (OX19)	84	80	67	65	76	79
Th (W3/25)	50	59	32	39	57	61
Ts (OX8)	86	88	71	75	100	90

The lymphoid cell populations of germfree, gnotobiotic and conventional Fischer rats were assessed. The results showed that in gnotobiotic rats there were less $OX22^+$ Th cells than in germfree and conventional rats. This may indicate a loss of the OX22 marker upon recent monoinfection of these animals. When the results from germfree animals were compared to those from conventional rats, essentially no difference was observed in the percent of $OX22^+$ Th cells in PP and in spleens. These findings may be due to a need of the lymphoid cells in the tissues of both of these animal models to maintain a stable ratio of Th cells with the OX22 marker due to the continuous exposure to particular antigens in their environments.

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Mucosal injury in athymic nude rats following infection with *Nippostronglylus brasiliensis*

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1. Introduction

Several theories have been proposed to explain mucosal injury during gastrointestinal infections. Damage has been attributed to direct actions of the infectious agent or to defence mechanisms of the host. Inflammatory cells such as T lymphocytes produce cytokines which can alter epithelial kinetics [1,2]. In addition, activated mucosal mast cells (MMC) secrete potent mediators which can cause sloughing of enterocytes [3,4].

We previously described in detail the course of mucosal injury during infection in the rat with the nematode parasite, Nippostrongylus brasiliensis (Nb) [5]. The changes included early (day 4) stimulation of proliferation in the crypts with mucosal thickening, and later edema (day 7) and obvious cell loss at villus tips with consequent impairment of digestive function (day 10). At the latter times, mast cell activation was indicated by release of rat mast cell protease II (RMCP II) and leukotriene production, suggesting that enterocyte detachment was due to the action of MMC mediators. Increased epithelial permeability was evident at days 4-10 [6]. Worm expulsion was evident between days 10 and 16. To clarify the role of T cell factors in these various aspects of injury, we carried out a similar investigation in athymic nude rats. These rats lack functional T cells but have MMC. [7].

2. Methods

Nude rnu/rnu rats (200-300g) were infected with 3000 Nb larvae and studied at days: 0, 4, 7, 10 and 21. Permeability was measured by injecting a 15 cm ligated segment of proximal jejunum with ^{51}Cr-EDTA (125 μCi) and ovalbumin (100 mg) [6]. Blood and urine were sampled over 5 h. ^{51}Cr-EDTA was gamma counted; ovalbumin was determined in serum by radioimmunoassay. Distal to the loops, sections of intestine were obtained for histology and for assay of enzyme activitities. Damage was scored based on a 0-4 rating (0 = normal, 4 = villus atrophy). Lymphocytes were obtained from spleen and mesenteric lymph nodes for assays of functional T cells.

3. Results

Early in the infection, day 4, no change in crypt length or epithelial proliferation (increase in thymidine kinase activity) was apparent; uptake of the probes was normal. However, beginning at day 7, epithelial changes were apparent at the villus tips (damage score 0.7±0.2 vs 0.2±0.1) and 5 h recoveries of both ^{51}Cr-EDTA (blood and urine) and ovalbumin (serum) were significantly increased (200% at day 7, 350% at day 10). Mast cell activation was indicated by increased serum levels of RMCP II at days 7 and 10 (1.5±0.3 at day 10 vs 0.4±0.0 μg/ml). Worm redistibution was evident at day 10, but even at day 21 numbers were still ~30% of those originally present. Significant villus atrophy (and decreased activities of digestive enzymes) and crypt hyperplasia were present only at this latter stage.

No thymus gland was present in any rat. Absence of functional T lymphocytes was confirmed by the lack of proliferation of splenic or mesenteric lymph node cells or IL2 production in response to stimulation with Conconavalin A.

4. Discussion

Increased epithelial permeability began at day 7 and coincided with morphological alterations at the villus tips. As in conventional rats, these abnormalities were associated with and most likely due to mast cell activation. It is unlikely that the injury was due to a direct action of the parasite, since 1) no epithelial changes were apparent at day 4 although large numbers of worms were present, and 2) the greatest damage occurred at day 21 when the worm burden was significantly reduced.

When compared with Nb infection in conventional rats, the absence of T lymphocytes in nude rats did result in differences. Proliferation of enteroblasts and crypt length did not increase early in the infection; these changes occurred only after and perhaps in response to sloughing of villus cells. Although mast cell activation began at the same time and may have resulted in worm redistribution, in athymic nude rats parasite expulsion was slow and incomplete.

We conclude that mucosal damage during Nb infection involves at least two components, one mediated by activated T cells and another mediated by activated mast cells. Both components appear to be involved in host defence mechanisms which result in parasite elimination.

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Cytotoxic T cells in gut associated lymphoid tissue of the rat

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Introduction We have shown that after an appropriate immunization schedule antigen-specific B lymphocytes committed to IgA production and antigen-specific T helper cells appear in Peyer's Patches (PP) and migrate via mesenteric lymph nodes (MLN) and thoracic duct lymph (TDL) to various mucosal sites but not to peripheral lymph nodes[1],[2]. The aim of this project was to investigate whether cytotoxic T(Tc) cells could be generated using similar mucosal immunization schedules and whether they migrate to mucosally restricted sites in a similar fashion.

Methods Wistar-Firth (WF) rats were used as responders and the unrelated Dark Agouti (DA) strain was used as stimulator strain. DA thymocytes were emulsified in an equal volume of Complete Freund's Adjuvant (CFA) for i/P and i/PP injection. Thymocytes suspended in PBS were used for i/D immunization. Lymphocytes from various tissues were assayed using a standard ^{51}Cr release assay[3] using blast cells prepared from the stimulator strain by culturing MLN lymphocytes with Con A for 48 hours. Effector: target ratio from 200:1 to 6.25:1 were used for all assays.

Specific lysis was derived from the formula

$$\text{Specific lysis (\%)} = \frac{(\text{Test CPM} - \text{background CPM})}{(\text{Total CPM} - \text{background CPM})} \times \frac{100}{1}$$

The unpaired "t" test was used to assess significance of differences between test and control groups.

Results The results of i/P, i/PP and i/D immunization are shown in Table 1. Specificity of the response was demonstrated by assaying effector cells using MLN blasts from an unrelated strain of rat (PVG), (data not shown).

TABLE 1

Cytotoxic Activity in Various Tissues after i/P, i/PP and i/D Immunization

Tissue		(% Specific Lysis ± S.E.) i/P (Day 7) E:T = 200:1	i/PP (Day 5) E:T = 50:1	i/D (Day 14) E:T = 100:1
Spleen	Test	33.2 ± 7.1**	9.1 ± 1.3*	8.0 ± 1.4** (n=4)
	Control	4.4 ± 1.0	4.6 ± 1.1	3.2 ± 0.3 (n=7)
MLN	Test	4.7 ± 0.7	2.6 ± 1.5	
	Control	<1	<1	
PP	Test		1.0 ± 0.7	
	Control		<1	
PLN	Test		<1	
	Control		<1	

n=6 unless otherwise stated. Control rats were immunized with CFA (i/P) or unimmunized (i/PP, i/D). Rats immunized i/P had 2 injections 10 days apart; i/PP and i/D had 1 injection.
* $p<0.05$, ** $p<0.005$ (t test)

Discussion We have developed a model system for the stimulation of Tc cells in the rat using a variety of immunization routes which have been shown previously to stimulate a gut response (i/P, i/PP and i/D). Responses after immunization occurred in spleen and MLN but not PLN or PP. The response was shown to be specific for the stimulator strain. This demonstrates that Tc cells are generated by presentation of antigen to mucosal surfaces and implies that the T_C migrate in a manner analogous to gut-derived B cells and T_H cells. Further studies will investigate T_C generation and migration in MALT by assaying T lymphocytes from IEL and lamina propria.

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Small intestinal glycoprotein synthesis and secretion: effects of T lymphocyte activation

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Introduction

Mucus glycoprotein secretion in the small intestine is an important component of intestinal defences [1]. Studies in animal models have shown that immune mediated events [2] will stimulate glycoprotein secretion in pulmonary or intestinal tissues. T lymphocytes have also been implicated in inducing goblet cell hyperplasia associated with intestinal helminth infections in rats [3]. Recent studies have shown that in patients with untreated coeliac disease, a condition in which there is strong evidence of alterations in mucosal T cell activation [4], intestinal glycoprotein synthesis and secretion is increased [5]. The aim of this study was to examine the effects of T cell activation on human small intestinal glycoprotein biosynthesis during short term culture in vitro.

Methods

Duodenal biopsies were obtained from the second part of the duodenum of patients undergoing diagnostic endoscopy. Biopsies were cultured in duplicate in RPMI 1640 containing 10% FCS for 24 hours, as previously described [5]. Endogenous mucosal lymphocytes were activated by the addition of the mitogenic anti-CD3 monoclonal antibody (OKT3) or pokeweed mitogen (PWM). A non-mitogenic isotype-matched murine monoclonal antibody was used as a control antibody. Glycoprotein synthesis and secretion were measured using a modification of the methods of MacDermott et al [6]. Biopsies were cultured in medium containing 5μCi of D-[1-^{3}H]-glucosamine hydrochloride (specific activity 5.8Ci/mmol, Amersham). Total incorporation of radiolabel into acid-precipitable glycoproteins was determined by combining the tissue and secreted counts and was expressed as dpm/mg biopsy protein.

Results

Culture of duodenal biosies with OKT3 significantly increased ($p<0.01$) the total incorporation of glucosamine into tissue and secreted glycoproteins relative to paired control cultures (Table 1). PWM did not significantly increase the total incorporation of ^{3}H glucosamine into glycoproteins, but the secretion of in vitro radiolabelled glycoproteins was significantly increased by culture with PWM ($p<0.05$) as well as anti-CD3 ($p<0.001$).

TABLE 1. ^{3}H-glucosamine incorporation into glycoproteins in the presence of anti-CD3 antibody and pokeweed mitogen

DPM/mg protein x 10^{-3} (Mean ± SEM)

	Tissue	Secreted	Total
Control	133 ± 8.9	52.5 ± 10.4	185.5 ± 15
Anti-CD3 (n = 10)	** 83.9 ± 6.6	*** 177.2 ± 47.6	** 261.1 ± 17
Control	125.5 ± 16.6	55.1 ± 14.5	180.7 ± 25.3
PWM (n = 7)	103.4 ± 11.7	* 113 ± 24.5	221.4 ± 23.4

Wilcoxon's paired ranking test, *$p<0.05$, **$p<0.01$, ***$p<0.001$ from controls.

Discussion

Activation of endogenous small intestinal T lymphocytes with the mitogenic monoclonal antibody OKT3, resulted in a significant increase in intestinal glycoprotein biosynthesis. Whilst these results might reflect increased cell turnover on T cell stimulation, the marked increase in secretion of in vitro radiolabelled glycoproteins on T lymphocyte activation suggests that T lymphocyte products could be capable of moderating non-specific intestinal defences such as mucus glycoprotein secretion. T lymphocyte products have also recently been shown to modify epithelial differentiation, enhancing HLA-DR on fetal intestinal epithelium in vitro [7] and have been strongly implicated in intestinal enteropathy [8]. The observed changes in intestinal glycoprotein secretion in coeliac disease [5] may well be a consequence of T lymphocyte activation.

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Phenotypes and functions of lamina propria lymphocytes from swine gut

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1. INTRODUCTION

In viral enteritis, such as transmissible gastro-enteritis of swine (TGE), where viruses replicate in gut epithelial cells, there is the possibility that a true local humoral immune response may be initiated and/or amplified by the lamina propria lymphocytes (LP), since lymphocytes of the epithelium appear to be of cytotoxic-/suppressor phenotype. This view was also supported by our immunohistological studies showing the presence of T, B and plasma cells ; Thus the aim of this study was to look at the in vitro response of isolated LP cells.

2. MATERIALS AND METHODS

2.1 Isolation, purification and characterization of LP lymphocytes:

After removal of the whole gut epithelium by EDTA, the gut fragments were digested by Collagenase (50 UI/ml, one hour at 37°C, two runs). The resulting cell suspensions are cleared of dead cells by nylon wool filtration and further purified over Ficoll-Triosil gradient.
Membrane and cytoplasmic markers were assayed by indirect immunofluorescence using various monoclonal antibodies. Membrane receptors were determined by rosette formation.

2.2 Proliferative response and IL2 production:

LP cells were cultured in 96-wells , flat bottomed plates for 3 days with mitogens (PHA, conA, soluble Staphylococcal Protein A, PWM and LPS) and proliferation was evaluated by thymidine incorporation. IL2 was titrated on mouse T cell line.

2.3 In vitro immunoglobulin synthesis and secretion:

LP cells were cultured in P-24 wells plates in presence of PWM,

Protein A and LPS, for a total culture period of 13 days. At intervals of 3 days, one half of the supernatant was removed to be evaluated for IgA and IgG, using ELISA tests, and replaced with fresh medium.

2.3. Production of specific antibody:

LP cells originating from a sow previously oraly immunized by a attenuated strain of TGE virus were co-cultivated with UV-inactivated virus. Antibody was checked by viral neutralization.

3. RESULTS

3.1 Characteristics of LP lymphocytes:

LP lymphocytes comprised 59% T cells (22% T4 and 28% T8), 31% plasma cells (the majority IGA+), 12% SIg+ cells (predominantly SIgA+) and only 2% C3b+ cells.

3.2 Proliferative response and IL2 production:

LP cells cultured alone exhibited spontaneously a proliferative response without IL2 production. PHA, con A, Protein A and PWM induced a comparble level of cell proliferation, although IL2 was produced mainly in presence of PWM. In contrast LPS elicited a very low response if any.

3.3. IgA, IgG and antibody productions:

LP cells alone released spontaneously IgA (5 micogr/ml) in the culture supernatants (in contrast to IgG (0.09 microgr./ml) and Protein A increased this level to 10 microgr/ml. During the next 10 days of culture, there was a further increase in IgA and IgG productions in presence of Prtotein A and LPS, respectively.
Neutralizing antibodies occurred only in viral stimulated cultures.

4. DISCUSSION

The proportions of the various lymphocytes subsets isolated from the swine gut LP are close to those seen in tissue sections, thus validating the isolation procedure. These cells are fully functionnal as judged by their responses to mitogens, (proliferation, IL2 production and Immunoglobulin synthesis) and elaboration of specific antibody. The bulk of increase of IgA production occurred in the first 3 days of culture. It did not seem to be caused only by the IL2, but rather by a increase in the plasma cell numbers. On other hand, the effect may be caused by contaminant, such as Stapphylococcal enterotoxin. Whatever, increase in IgA and IgG production joined to the ability to respond to antigen support the view on the existence of positive regulation of immunoglobulin synthesis in the LP itself.

SECTION E: CELL MIGRATION IN THE MUCOSAL IMMUNE SYSTEM

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Effector cell migration in mucosal defence

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ABSTRACT. Experiments are described investigating the role of T cells in the induction of IgA responses, the way in which antigen deposition and subsequent cell migration patterns influence inductive events, and the role of antigen in effector migration to submucosal sites. The production of IgA antibody-containing cells (ACC) from precursor cells in PP is dependent on cognate T cell help and the ability of T_h cells to traffic between PP ensures distribution of helper activity to regionally applied antigens. The subsequent migration of ACC to submucosal effector sites is an antigen-dependent phenomenon and relocation of gut-derived effectors to the urinary tract occurs after concomitant gut/bladder immunization. Although ACC distribution is antigen-dependent, antigen is not the only signal required.

1. INTRODUCTION

The response by gut associated lymphoid tissue (GALT) to antigens occurring in the lumen of the intestine is characterized by the production from precursors in Peyer's patches (PP) of IgA-committed ACC which subsequently migrate via intestinal and thoracic duct lymph (TDL) to submucosal effector sites in the gut. This relocation of B cell effectors has been shown to be antigen dependent and indeed antigen-specific IgA-containing cells can be encouraged to relocate to the respiratory tract by concomitant gut/lung immunization (1,2). The role of antigen in IgA B cell migration is extended here to the urinary tract by demonstrating that concomitant intestinal and urinary tract immunization results in an enhanced traffic of IgA ACC from the intestine to the urinary tract.

The T dependence of IgA responses (3,4) raises the question of factors controlling the location and subsequent migratory potential of T cells providing help to IgA-committed precursors. The experiments reported in this paper extend previous studies by demonstrating the requirement for cognate help within PP for IgA B cell responses and the potential for T_h cells to migrate between PP providing help in tissues distant to their sites of interaction with antigen.

2. MATERIALS AND METHODS

2.1. Animals

Male rats of the inbred PVG strain were used at 12-16 weeks of age for all experiments.

2.2. Immunization

Intraperitoneal (IP) and intra-Payer's patch (IPP) immunizations were performed by injection of antigen in Freund's complete adjuvant (FCA) as previously described (8). Intraduodenal (ID) and intrabladder (IB) immunizations were achieved by intralumenal injection via laparotomy of 0.5ml antigen in saline. Intraurethral (IU) immunization was achieved by inserting a fine catheter into the penile urethra through which was injected 0.1ml of antigen in saline.

2.3. Assays

TD lymphocytes were collected and cell smears and sections of selected tissues were prepared for fluorescent histology as previously described (1). The preparation and labelling of T_h cells and the assay of functional helper activity has also been described previously (5,6).

3. RESULTS

3.1. Induction of IgA responses in GALT

The data in Table 1 summarize experiments comparing routes of primary and secondary immunizations for the generation of an IgA ACC response in the gut. Whereas a single ID immunization failed to generate a substantial response when assayed 5 days after injection (Expt.1), injection of a single PP with antigen in FCA 14 days prior to ID injection resulted in a substantial ACC response, the majority of which were IgA-specific (Expt. 3). Single PP injection alone failed to stimulate a substantial response (Expt.2). Similar enhancement of the response to ID injection is also achieved by prior IP delivery of antigen (Expt. 5), presumably providing antigen access to PP in a manner analogous to that achieved by subserosal injection in the IPP model. Both IPP+ID and IP+ID immunization regimes generated a substantial population of ACC in TDL 4 days after ID injection (Expts. 6 and 7) and chronic TDL drainage following ID injection prevented the appearance of ACC in the duodenum when assayed 5 days later. These results establish that IPP or IP immunizations prime the gut for an effective IgA-specific ACC response to subsequent ID immunization, and that these cells migrate via TDL to the gut LP as previously reported (1,7,8).

3.2. Cell migration and inductive events in GALT

The above immunization model has allowed the investigation of the

Table 1. Role of route of primary (1^0) and secondary (2^0) immunization with ovalbumin in the generation of an IgA-specific antiovalbumin-containing cell (AOCC) response in the intestine.

1^0 Route	2^0 Route	Jejunum		TDL	No. Rats
		AOCC/cm	%IgA	AOCC/hr x 10^{-3}	
1. -	ID	1.30±0.40	na	na	14
2. IPP	-	5.12±1.12	na	na	7
3. IPP	ID	158.08±22.86	78.69±8.45	na	9
4. IP	-	1.99±0.99	na	na	7
5. IP	ID	102.30±5.40	79.40±2.10	na	7
6. IPP	ID+TDL	9.37±3.25	na	158.69±21.24	4
7. IP	ID+TDL	2.31±1.55	na	243.12±119.11	7

IPP=single PP injected only; IP=intraperitoneal; ID=intraduodenal; 1^0 injections in FCA; 2^0 injections in saline. TDL=thoracic duct lymphatic cannulation (established at the time of 2^0 injection); AOCC/hr data is for Day 4 after cannulation.
Protocol: Day 0 1^0, Day 14 2^0, Day 19 Assay.

requirement for cognate help in the production of IgA ACC from PP and the functional significance of the ability of T_h cells to migrate between PP (5). This has been achieved by exploitation of a hapten-carrier antigen model in which the B cell response to a hapten linked to a protein carrier is dependent on carrier-specific T cell help. These data are shown in Table 2. Expt. 8 demonstrates that (as in Expt. 1), a single ID immunization with hapten-carrier conjugate is insufficient to generate an anti-hapten response in the jejunum. In Expt. 10 however, prior injection of PP in the proximal region of the gut with hapten-carrier conjugate promotes an anti-hapten response in the jejunum after ID instillation of the same conjugate. If the ID 2^0 immunization is preceded by IPP priming with hapten linked to an irrelevant carrier (Expt. 9) an anti-hapten response in the jejunum does not occur. However, Expt. 11 demonstrates that if KLH-specific T cells are generated in proximal PP and hapten-specific B cells are generated in distal PP (by injection with hapten linked to an irrelevant carrier) an anti-hapten response occurs after ID injection of hapten conjugated to KLH.

These experiments indicate not only that cognate T cell help is required for a B cell response in PP to lumenal antigen, but that this help can be provided by T cells generated in distant PP, presumably by virtue of their ability to migrate between PP (5).

3.3. Cell migration to submucosal effector sites and the role of antigen

Previous experiments have demonstrated that the relocation of IgA cells from their induction site (PP) to their effector sites in the gut lamina propria (LP) is a highly antigen-dependent event (1,8) and this has been exploited to enhance a respiratory tract IgA response by concomitant immunization of respiratory and intestinal mucosae (2). It

was of interest to determine if this principle could be extended to the urinary tract.

Table 2. Requirement for cognate help in responses to 1^0 and 2^0 immunization

	1^0		2^0		
	Proximal IPP	Distal IPP	ID	Anti-DNP ACC/cm Jejunum	No. Rats
8.	-	-	TNP-KLH	1.2 ± 0.6	12
9.	DNP-BGG	-	TNP-KLH	0.3 ± 0.3	10
10.	TNP-KLH	-	TNP-KLH	37.9 ± 1.0	10
11.	KLH	DNP-BGG	TNP-KLH	12.1 ± 4.2	9

IPP = multiple PP injected in either proximal or distal regions of the small intestine; 1^0 injections in FCA; 2^0 injectionsin saline; TNP = trinitrophenyl; DNP = dinitrophenyl; KLH = keyhole limpet hemocyanin; BGG = bovinegamma-globulin.Protocol as for Table 1

Table 3. Antiovalbumin-containing cell (AOCC) response in intestine and urinary tract after immunization by the routes shown.

		Jejunum		Bladder		Urethra	
Treatment	No. Rats	AOCC/cm	%IgA	AOCC/cm	%IgA	AOCC/cm	%IgA
12. IP+ID	7	102.30 ± 5.40	79.40 ± 2.10	0.90 ± 0.90	na	0.00	na
13. IP+ID+IB	13	112.12 ± 8.90	78.68 ± 3.71	38.06 ± 3.07	65.04 ± 3.28	16.62 ± 3.42	76.05 ± 4.75
14. IP+ID+IU	3	112.30 ± 9.67	73.58 ± 4.19	1.72 ± 1.72	na	47.78 ± 3.31	77.19 ± 9.19
15. IP+ID+IB +TDL	7	2.31 ± 1.55	na	1.04 ± 0.94	na	0.00 ± 0.00	na

Rats were given OVA by 1^0 immunization intraperitoneally (IP) followed by 2^0 immunization by intraduodenal (ID), intrabladder (IB), or intra-urethral (IU) routes 14 days after IP and tissues collected 5 days later. In Expt 16 the thoracic duct was cannulated at the time of 2^0 injection.

The results in Table 3 indicate that IP priming following by ID immunization 14 days later with ovalbumin (OVA) (Expt. 12) failed to stimulate the appearance of ACC in the bladder or urethral mucosa after ID challenge, although a substantial response in the jejunum occurred. When IB instillation of antigen was performed at the time of ID challenge substantial numbers of ACC appeared in both the bladder and urethral mucosa 5 days later (Expt. 13). If however antigen was administered only at the level of the urethral mucosa a response in the bladder did not occur but a substantial response in the urethra was observed (Expt. 14). This is consistent with the premise that antigen is essential for effective relocation of gut-derived cells into the urinary tract mucosa since antigen administered at the urethral level would not provide retention and proliferation signals for cells extravasating into "upstream" submucosal sites proximal to the urethra.

The gut origin of ACC in the urinary tract was confirmed by demonstrating in similarly immunized rats, abrogation of the urinary tract response by chronic drainage of TD lymph during the post challenge period (Expt. 15) and by demonstrating the appearance of radiolabelled ACC in the urinary tract after injection of labelled autologous TD cells collected during the post challenge period (Table 4, Expt. 16).

Table 4. The appearance of ^{3}H-labelled antiovalbumin-containing cells (AOCC) in jejunum ,bladder and urethra

	No. Rats	% ^{3}H-AOCC		
		Jejunum	Bladder	Urethra
16.	5	14.63 ± 1.51	4.86 ± 0.85	0.27 ± 0.27

Cells collected from the thoracic duct of rats given IP 1^{o} and ID+IB 2^{o} immunizations with OVA were labelled with ^{3}H-thymidine and reinfused iv. The appearance of labelled ACC in gut and urinary tract tissues was assessed by autoradiography.

In view of the ability of antigen to promote retention and proliferation of ACC at mucosal effector sites, and the recognised T dependency of IgA responses, the question arises of whether T_h cells are involved in B cell proliferation at extra-PP effector sites. The data presented in Table 5 indicate that adoptively transfered AOCC are

Table 5. The appearance of AOCC from syngeneic donor source in unimmunized recipients or recipients immunized by ID alone or IPP+ID routes.

	Recipient Treatment:				
	Day 0 1^{o}	Day 14 2^{o}	Day 18 Donor cells	AOCC in Jejunum (cells/cm)	No. Rats
17.	-	-	+	1.52 ± 0.30	5
18.	-	ID	+	0.97 ± 0.61	6
19.	IPP	ID	+	18.18 ± 6.06	3

All recipient rats were cannulated on Day 14 (to divert endogenous supply of AOCC arising after 2^{o} immunization) and were killed on Day 19. Donor TD cells were collected from syngeneic rats given 1^{o} IP, 2^{o} ID 14 days later and cannulated 4 days after the ID injection.

no better able to localize in the gut LP of recipient rats given only ID antigen (Expt. 18) than in unimmunized recipients (Expt. 17), but if an IP priming dose is given prior to ID challenge then localization is enhanced (Expt. 19). Since ID immunization alone provides an antigen signal without stimulation of antigen-specific T_h cells (9), signals other than those provided by antigen are required, and antigen-specific T_h cells may also be involved in ACC proliferation at the effector site level.

4. CONCLUSION

Data presented in this paper establish the importance of cell migration in maintaining IgA-mediated mucosal defence both at the induction and at the effector level. Cognate T cell help is required at induction sites and T_h migration between PP ensures adequate distribution of helper activity. The role of antigen in determining the distribution of IgA-specific B cells at mucosal effector sites has been confirmed. T_h cells which migrate from PP to LP effector sites may be important in this regard although other studies have shown that they do not localize to LP in response to antigen-specific signals (6). These experiments reinforce the concept of the intestine as the cornerstone to mucosal defences at a variety of extraintestinal sites by providing a pool of antigen-specific T and B effector cells capable of migrating within various levels of the intestine and between intestinal and extraintestinal sites.

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Search for B cells ready to emigrate from sheep ileal Peyer's patch

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ABSTRACT. Flow cytometry was used to compare B cells in the sheep ileal PP with those in the emigrant population. The phenotype (PNA^{lo}-sIg^{hi}) of the emigrants corresponded to that of a small subpopulation of follicular B cells. We speculate that these cells may be about to emigrate having survived unknown selection events. Comparison of these cells with the major follicular population might give insight into the cause of the extensive destruction of B cells happening in the PP follicles.

1. Introduction

The life expectancy of B cells is generally much shorter than that of T cells and, associated with this, newly-formed cells enter the B cell compartment at a much faster rate (1). Proliferating B cells are found throughout the immune system, but the size of the peripheral B cell pool is most likely maintained by the foci of vigorous B cell proliferation found in bone marrow, in the gut-associated lymphoid follicles, and in germinal centres in the spleen and lymph nodes. Osmond and his colleagues estimated in rodents (2) that approximately 20% of splenic lymphocytes were produced in the bone marrow over the previous 24 h. However, the dominance of the bone marrow in maintaining the peripheral B cell pool in rodents is unlikely to be a general phenomenon in all species. In birds, a gut-associated tissue, the bursa of Fabricius, is the focus of B cell genesis during development (3). In sheep, the ileal Peyer's patch (PP) makes a major contribution to the developing B cell system and may be functionally equivalent to the avian bursa (4). Both the bursa and the ileal PP involute by the time of sexual maturity (4), thus other sites of B cell lymphopoiesis must make an increasingly significant contribution as the animal ages. Clearly, both the species and its age are important factors when considering sites that influence the development and maintenance of the B cell compartment.

Our interest in the sheep ileal PP has been heightened by its similarity with the bursa and by the opportunities afforded in large animals to carry out detailed physiological studies of the ontogeny, origin and fate of lymphocytes. The ileal PP becomes morphologically mature during fetal life when vigorous B cell genesis occurs in the absence of any of the various hallmarks of an ongoing immune response (5,6). The life span of the newly-formed PP B cells is very short, measured in hours rather than days, in fact the birth and death of

most PP B cells seems to occur within the follicle (7). Jerne, referring to the T cell destruction in the thymus remarked (8) that "it is puzzling, why an organ should exist that entertains an enormous proliferation of cells for the apparent purpose of killing them". We are also puzzled and intrigued by the occurrence of a similar phenomenon with B cells in the ileal PP. The experiments summarized here will amplify some of the above points and outline our approach to investigating the biological relevance of the B cell genocide in PP.

2. Contribution of ileal PP to the B cell compartment in sheep

The ileal PP spans the last 1-2 m of small intestine in lambs and comprises approximately 100,000 follicles (5), each contains about 5 x 10^5 B cells, a total of about 5 x 10^{10} B cells. Every hour 5% of these cells divide (6) producing 2.5 x 10^9, or about 1 g of new cells. The technique used to study the fate of these cells involved a brief extracorporeal perfusion of the ileal PP region of intestine (9). This enabled the selective *in situ* labelling of all cells with FITC or the S-phase cells with bromodeoxyuridine (BrdU); the fate of the cells could then be followed in conscious unrestrained animals. In one series of experiments (Fig. 1) the contribution of ileal PP emigrants to the B cell compartment of various tissues was determined 3 days after extracorporeal perfusion. The values were 25% for ileal mesenteric lymph node (MLN), 9% for blood, 7% for spleen, 4% for lymph nodes (10) and 2-3% for the gut epithelium and lamina propria (Gyorffy and Reynolds, this volume). Thus, large numbers of B cells emigrate from the ileal PP to all lymphoid tissues with no apparent preference for GALT.

Because most lymphocytes leave the ileal PP via the lymphatics (9), we have been able to examine the rate of B cell emigration by cannulating the ileal lymphatic after labelling the ileal PP with BrdU. As mentioned, the rate of B cell production in the ileal PP is extravagant, 2.5 x 10^9 B cells/h. The release of so many cells into the lymphatics would rapidly overload

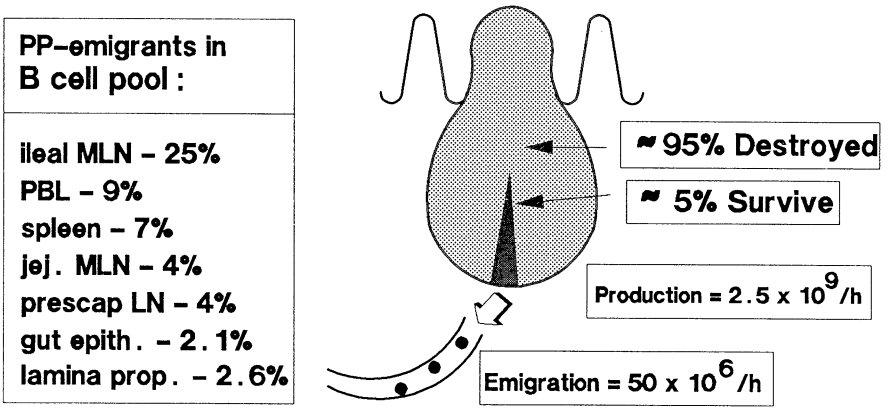

Figure 1

the immune system, especially the ileal MLN through which the cells must pass. However, we found that only about 5 x 10^7 newly-formed cells/h leave the ileal PP. This is enough to have a major impact on the animal's B cell pool, but it represents less than 5% of ileal PP B cell production. Pyknotic cells and tingible-body macrophages are common in the follicles and are probably associated with the B cells eradication. The reason why so many cells perish is not known although it is likely to pertain to some selection process, perhaps involving an interaction between the Ig expressing B cell and some other cell in the follicle, or, the B cells may undergo self destruction. Our ultimate goal is to understand the wisdom of this process and our initial strategy is to characterize the cells that are released from the follicle as they have endured the selection process. The cellular and molecular attributes of these cells will be compared with the follicular cells from which they originate, as any differences may prove informative.

3. Phenotype of B cells emigrating from ileal PP.

PP emigrants were identified in ileal lymph following extracorporeal perfusion to label all PP cells with FITC. The phenotype of the emigrants was determined by dual-colour flow cytometry using antibodies and lectins recognising various cell surface molecules. In addition to expressing high levels of surface Ig the emigrant B cells had little or no receptor for peanut agglutinin (PNA). The emigrant B cells were thus characterized as sIg^{hi}-PNA^{lo}.

4. A follicular B cell population has a similar phenotype to emigrant B cells.

The B cells in the ileal PP were analyzed with the same assays used for the PP-emigrants. Figure 2 shows the results of a simultaneous analysis of the expression of PNA receptor and sIg. Most ileal PP cells are PNA^{hi}-sIg^{lo} in contrast to the PP-emigrants. In addition, however, there was a distinct subpopulation of B cells with the same phenotype as the PP-emigrants, i.e., PNA^{lo}-sIg^{hi}. Some differentiation markers have also been discovered that may help distinguish between the cells destined to emigrate from those destined to be eliminated. For example, most follicular B cells, but few emigrant B cells, express a molecule recognized by the monoclonal antibody BAS9A. Another marker recognized by monoclonal antibody BAQ44A is present on many emigrants but few follicular cells (both antibodies were gifts from W Davis (11), Washington State University).

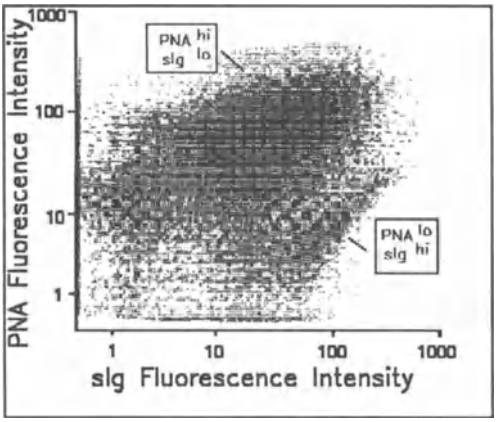

Figure 2

5. Conclusions

A high proportion of lymphocytes produced in the thymus are also destroyed and with the identification, isolation, and molecular and functional characterization of the various thymocyte subpopulations, a differentiation pathway for T cells is being outlined (12) and

evidence is being sought to indicate the stage at which T cells are destroyed. The ability to identify B cell subpopulations in the PP follicle is an important step in constructing a differentiation pathway for these cells. We have found a small subpopulation within the follicle with a similar phenotype to that of the emigrants and these may represent cells that have passed the still to be determined screening process. The reason for cell death in the thymus remains puzzling even though it was first recognized 20 years ago. For a number of years there have also been indications that extensive cell destruction is associated with B cell lymphopoiesis (2); the basis is also unknown. The sheep ileal PP affords some unique opportunities for investigating various questions associated with a phenomenon that seems likely to have a major impact on B cell biology, perhaps even representing a crucial step in the expansion and selection of an appropriate and diverse antibody repertoire.

5. Acknowledgements

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Role of antigen in migration patterns of T cell subsets arising from gut associated lymphoid tissue

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ABSTRACT. The migration of regulatory T cell subsets isolated from thoracic duct lymph (TDL) of rats following gut immunization was studied to determine their migratory behavior and the role of antigen in their localization. In initial experiments lymphocytes collected after immunization of Peyer's patches (PP) with Keyhole Limpet Hemocyanin (KLH) were enriched for T helper cells (Th) or T supressor/cytotoxic cells (Ts/c) and labelled with the fluorochrome H33342. In other experiments a higher frequency of antigen-specific Th was achieved by short term culture of the enriched Th in the presence of KLH and the blast cells were labelled with ^{3}H-thymidine. The distribution of these populations was determined after injection into immunized and unimmunized syngeneic recipients. Whereas the uncultured population (predominantly small T cells) localized almost exclusively in follicular lymphoid tissues the cultured Th (predominantly blasts) appeared initially in the lamina propria (LP), then in PP and mesenteric lymph nodes (MLN). However the distribution of these populations was not influenced significantly by antigen, in contrast to previous findings with regard to IgA-committed B cells.

1. INTRODUCTION

Antigen specific B cells migrate from PP of intestinally immunized rats via the TDL and blood circulation to the LP of the gut where the cells extravasate independently of antigen but are retained and proliferate if antigen is present (1). The presence of functionally active Th in the PP and LP has been demonstrated (2) and the necessity of Th in the induction of an IgA response in vivo has been established (3). It is not known however whether Th have a role only in induction of antigen specific B cells in the PP or also in B cell retention and differentiation in the LP, and whether Th retention is affected by antigen. We have therefore developed a strategy to look at the migratory behavior of antigen-specific Th generated in PP after intra-PP (IPP) immunization. TDL was collected from immunized rats, the Th (or Ts/c) purified, labelled and infused into immunized and

unimmunized recipients to determine the distribution of these cells and the effect, if any, of antigen.

2. MATERIALS AND METHODS

Male PVG donor rats were immunized by IPP injection of KLH in Freund's complete adjuvant given 14 days prior to thoracic duct cannulation. The immunized recipient rats were given an intraduodenal (ID) immunization with 1 mg KLH in 0.5 ml PBS on day 14 after IPP immunization and the donor cells were infused the next day. TDL collected overnight from donor rats was separated on nylon wool to enrich for T cells (4). Th were then purified by removal of Ox8 +ve (Ts/c) cells by panning (5) to obtain a population considerably enriched for Th (90% W3/25+, 1% Ox8+, 4% B cells) compared with the starting population (51% W3/25+, 3% Ox8+, 42% B cells). The frequency of antigen specific Th determined by limiting dilution analysis in an assay for functional T help was 1/9000. The cells were labelled with the fluorochrome H33342 (Hoechst) (6). Ts/c were prepared by removing W3/25 +ve (Th) cells by two panning steps. The population obtained was 62% Ox8+, 20% W3/25+, 29% B cells. To prepare a population with a higher frequency of antigen specific cells, purified Th were cultured for three days in Costar tissue culture plates ($5x10^6$/well) with mitomycin c treated spleen cells ($2.5x10^6$/well) and KLH (10 Ug/ml). Dividing cells were labelled by addition of 1 uCi methyl-^{3}H-thymidine (5 Ci/mmol, 21 mCi/mg) per well for the last 16 h of culture. These cells had a frequency of 1/90 antigen specific Th cells and were 86% W3/25+.

3. RESULTS

$2.5x10^7$ H33342 labelled Th were infused intravenously (IV) into immunized or unimmunized recipients. At various times the rats were killed and tissue samples frozen in liquid nitrogen. Frozen sections were cut and the number of fluorescent cells per field determined. The distribution of H333342-labelled Th is shown in Fig.1. Only occasional cells appeared in the LP and these had large lymphocyte morphology. Most of the labelled cells localised in follicular lymphoid tissue (PP, MLN, spleen and PLN) and these were predominantly small lymphocytes. No significant difference was seen in the distribution of cells in immunized and unimmunized recipients. There was no difference in distribution of H33342-labelled Ts/c at 18 hours after infusion compared to the distribution of Th cells at the same time (data not shown) and no significant effect of antigen on the distribution was observed.

The lack of effect of antigen on the distribution of Th cells labelled with H33342 could have been due to the low frequency of these cells in that population. To obtain a population with a higher frequency the cells were cultured with KLH and labelled with ^{3}H-thymidine to ensure labelling of stimulated cells only. These cells were infused into the mesenteric artery to provide the maximum opportunity for the cells to reach the gut vasculature and therefore maximize the detection of any antigen-specific effects in the gut. The distribution of these cells at

various times is shown in Fig.2. At 1 hr after infusion the majority of labelled cells were in the LP but they disappeared rapidly and were absent from this site by 12 hr. Labelled cells accumulated more slowly in PP and MLN with a peak incidence at 12 hr after which the numbers declined until by 24 hr post-infusion no cells were detected. There were no significant differences in the numbers of labelled cells in the immunized and unimmunized recipients despite the higher frequency of KLH specific Th cells in this inoculum.

4. CONCLUSION

Consistent with previous reports (7,8) a marked difference in the migratory behavior of small Th cells and large, activated Th cells was observed. The small cells migrated to organized lymphoid tissue (PP, MLN, PLN and spleen) whereas the large activated cells migrated predominantly to LP. This may reflect a change in expression of cell surface receptors during activation. However, no effects of antigen on the retention of any of these T cell populations was observed in any of the tissues examined although we have previously observed an effect of lumenal antigen on the migration of B cells to the gut LP (1) and on the distribution of Th functional activity in PP (2) in vivo.

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Figures.

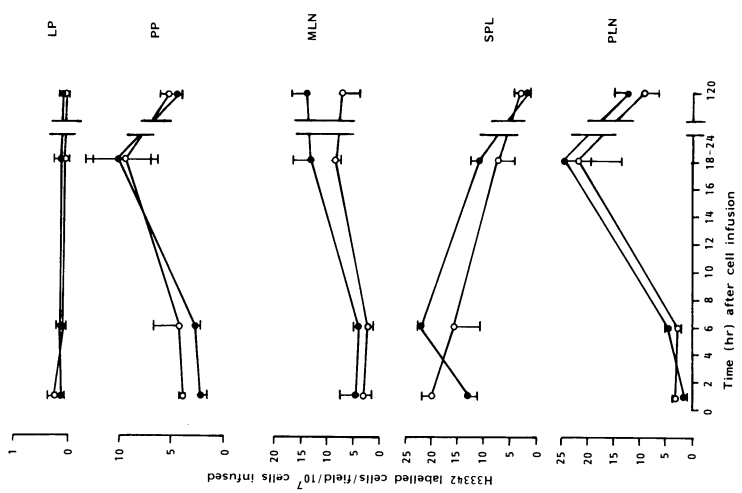

Figure 1. Distribution of H33342 labelled Th after infusion into intestinally immunized (●) or unimmunized (○) recipients.

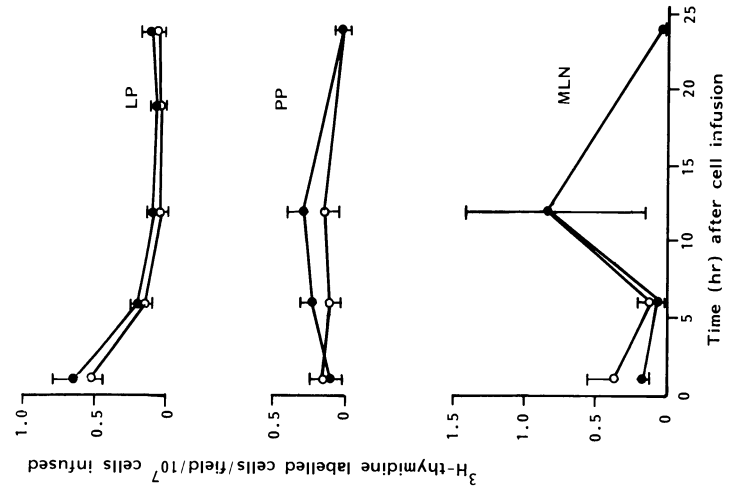

Figure 2. The distribution of ^{3}H-thymidine labelled, in vitro restimulated Th after infusion into intestinally immunized (●) or unimmunized (○) recipients.

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Emigration of lymphocytes from the ileal Peyer's patch to the intestinal mucosa in sheep

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ABSTRACT. Lymphocytes in the intestinal epithelium (IEL) and lamina propria (LPL) of sheep were characterized and their relationship with ileal PP was investigated. Three days after FITC labeling of ileal PP by extracorporeal perfusion, IEL and LPL were isolated from distant jejunum. T-cells in IEL were predominately $CD8^+$ (CD4:CD8 = 0.2) with < 5% B-cells, whereas the LPL included more B-cells (12%) and T-cells were predominantly $CD4^+$ (CD4:CD8 = 2.0). Ileal PP emigrants were found amongst both IEL (0.6%) and LPL (1.2%); their phenotype differed significantly, reflecting the overall composition of the LPL and IEL. Ileal PP emigrants contribute to both T- and B-cell populations in the epithelium and lamina propria, accounting for a similar proportion of $CD4^+$ and $CD8^+$ cells but slightly more to sIg^+ cells. The distinct distributions of ileal PP derived $CD4^+$, $CD8^+$ and sIg^+ cells suggests that their traffic in the gut wall is not random but varies with the cell's phenotype.

1. Introduction

The functional integrity of the mucosal immune system is dependant on the continued traffic of antigen reactive cells to mucosal sites. Peyer's patches (PP) make a major contribution to this process both as a source of B-lymphoblasts and a site where mucosal T-cells recirculate. Many PP emigrants preferentially traffic to gut [1] but the overall contribution of the PP to lymphocyte populations in the gut epithelium, lamina propria and other lymphoid organs remains unclear. The intraepithelial lymphocytes (IEL), which are phenotypically and functionally distinct from the lamina propria lymphocytes (LPL), are predominately $CD8^+$ although their exact function, lineage and derivation, including the role of PP, is not known [2].

The ileal PP in sheep is a continuous PP, 1-2 meters in length, that has many characteristics of a primary lymphoid organ. We developed a method using extracorporeal perfusion to selectively label the sheep ileal PP with fluorescein isothiocyanate (FITC) then follow the migration of the fluorescent cells to distant tissues [3]. We have previously shown that ileal PP emigrants contribute to the lymphocytes in a variety of lymphoid organs, including the intestinal mucosa [4]. The present study was designed to; i) compare the localization of ileal PP emigrants in the epithelium and lamina propria, iii) characterize the phenotype of the ileal PP derived cells and iii) determine whether ileal PP emigrants preferentially traffic to the gut.

2. Materials and Methods

2.1. Animals and extracorporeal perfusion of ileal PP with FITC.

Healthy 8-12 week old lambs of either sex were maintained in standard metabolic cages. The extracorporeal perfusion and FITC labelling of approximately 1.5 meters of ileal PP was performed as described previously [3].

2.2. Isolation of IEL and LPL from mid-jejunum.

The lamb was euthanized 3 d after ileal PP perfusion and fresh mid-jejunum, free of PP, was collected. The IEL and LPL were isolated by a modification of published methods [5]. Briefly, the intestine was cut into 2-3 cm^2 pieces and stirred in $Ca^{++}Mg^{++}$ free Hanks balanced salt solution (HBSS) with 2.0 mM EDTA. The supernatant was centrifuged and the IEL enriched on Percoll (Pharmacia, Uppsala, Sweden) gradients. LPL were isolated by stirring the remaining epithelium-free tissue in 250 U/ml collagenase (Gibco, Type XI). The resulting cell suspension was centrifuged and LPL were isolated on Percoll gradients.

2.3. Monoclonal antibodies, immunofluorescence staining and flow cytometry.

Monoclonal antibodies to sheep CD5, CD4 and CD8 [6] were the kind gifts of Dr Charles Mackay (Basel, Switzerland). Cells with surface immunoglobulin (sIg) were enumerated using rabbit anti-sheep Ig (Cappel, West Chester, PA). For immunofluorescence staining, 2 X 10^6 cells were washed in HBSS with 0.1% azide and reacted with 50 μl of mAb at optimal concentration at 4 °C for 30 minutes. A indirect labelling procedure was performed using biotinylated developing antibodies followed by phycoerythrin labelled avidin.

Quantitative dual colour flow cytometry was performed using an Ortho 50 H Cytofluorograph (Westwood, MA). Dead cells and granulocytes were excluded on the basis of forward and 90° light scatter. 5 X 10^4 cells were analyzed per sample. The contribution of ileal PP emigrants ($FITC^+$) to the total IEL and LPL populations and major lymphocyte subsets were quantified after correction for background autofluorescence of unlabelled cells.

3. Results

3.1. Phenotypic analysis of IEL and LPL.

Mucosal lymphocytes from sheep intestine showed distinct compartmentalization similar to that reported in other species (Fig. 1). A high percentage of IEL were $CD5^+$, many were $CD8^+$, few $CD4^+$ (CD4:CD8 = 0.2) and 5% sIg^+ . A high proportion of LPL were also $CD5^+$, but $CD4^+$ cells exceeded $CD8^+$ cells (CD4:CD8 = 2.0), and 12% were sIg^+.

3.2. Traffic of ileal PP emigrants to epithelium and lamina propria.

Ileal PP emigrants were found in both IEL and LPL populations (Fig. 1). Three days after ileal PP perfusion, 0.6% of IEL and 1.2% of LPL were ileal PP emigrants (Table 1). The phenotype of the PP-derived cells in the IEL and LPL differed significantly and reflected the

composition of the overall population. Thus, ileal PP emigrants in the epithelium were predominately $CD5^+$ and $CD8^+$ with few (12%) sIg^+ cells, whereas those in the lamina propria included more $CD4^+$ than $CD8^+$ cells and 28% were sIg^+.

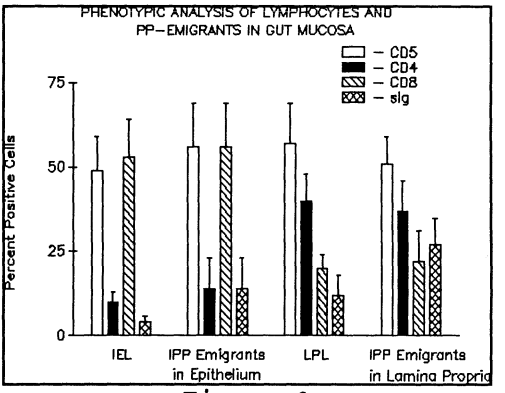

Figure 1

3.3. Contribution of ileal PP emigrants to lymphocyte subsets in the gut mucosa.

Ileal PP emigrants accounted for a similar proportion of the overall population of $CD5^+$, $CD4^+$ and $CD8^+$ cells (Table 1). The ileal PP emigrants contributed consistently more to the sIg^+ population than to the T-cell subsets although the difference did not reach statistical significance.

TABLE 1. Contribution of ileal PP emigrants to lymphocyte subpopulations in gut epithelium and lamina propria.

	% Ileal PP Emigrants				
	Total	CD5	CD4	CD8	sIg
IEL	0.6 ± 0.3	0.6 ± 0.3	0.6 ± 0.3	0.6 ± 0.4	2.1 ± 1.4
LPL	1.2 ± 0.5	1.2 ± 0.5	1.1 ± 0.6	1.2 ± 0.6	2.6 ± 1.0

Mean ± standard deviation of 4 animals.

4. Discussion

In this study, we analyzed the localization of ileal PP emigrant subpopulations in the epithelium and lamina propria of the intestinal mucosa. The exact destination of ileal PP derived cells is important in terms of their ultimate effector function. Most studies on lymphocyte traffic to the gut have dealt primarily with lymphoblasts and did not distinguish between the epithelium and lamina propria. However, it is clear that IEL and LPL differ significantly in their function, phenotype and T-cell receptor usage and it is likely that patterns of lymphocyte traffic differ as well. McDermott et al. [7] provided evidence of this by showing that mesenteric lymph node derived T-lymphoblasts localized predominately in the epithelium whereas B-lymphoblasts localized exclusively in the lamina propria.

Our results showed that there is selective, non-random traffic of ileal PP emigrants to the epithelium and lamina propria. The percentages of ileal PP emigrants found in IEL and LPL were relatively small, 0.6% and 1.2% respectively. When comparing percentages of ileal PP emigrants in various lymphoid organs, we found no evidence of preferential traffic to gut mucosa (accompanying paper by Reynolds *et al.*,). This provides further evidence that the

influence of the ileal PP is widespread and not focused on the gut. For both the IEL and LPL, the traffic of ileal PP emigrants paralleled the phenotype of the overall population, and contributed to all major T-cell subsets. The relatively greater contribution of ileal PP emigrants to sIg^+ cells, although not reaching statistical significance, is in keeping with the function of the sheep ileal PP as a major site of B-cell lymphopoiesis.

The extracorporeal perfusion of ileal PP labels all ileal PP cells including proliferating cells and those cells in the recirculating pool. Therefore, it is not possible to determine from this study if the emigrants found in the distant gut mucosa were produced within the ileal PP or trafficking through the ileal PP at the time of perfusion. One other consideration is that some rapidly dividing cells may go undetected as the FITC is diluted with successive cell divisions. Nevertheless, ileal PP emigrants traffic to the intestinal mucosa and contribute to both T- and B-cell populations. Within the intestinal mucosa a selective, non-random pattern of localization of ileal PP emigrants between epithelium and lamina propria was seen. The functional implications of this are unknown, but it is likely that T-cells migrating from ileal PP have important effector or regulatory functions throughout both mucosal and systemic immune systems. The cellular and molecular mechanisms controlling this pattern of migration await elucidation. It is unlikely to involve the interaction with high endothelial venules which have not been observed in gut mucosa [8]. It may involve other accessory molecules directing the tissue specific sorting of lymphocyte subsets after entry into that particular microenvironment.

5. Acknowledgements

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Computer assisted analysis of lymphocyte migration

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ABSTRACT. Computer assisted nonlinear regression analysis has been used to examine the time course of the in vivo accumulation of labelled lymphocytes in lymphoid tissues after their intravenous transfer to syngeneic recipients. We examined the time course of rat thoracic duct lymphocytes (TDL) reported by Smith and Ford (1), and the localization of murine CD4 enriched lymphocytes in our own experiments. Quantitative estimates for the clearance rate of these lymphocyte populations from the blood and the residency time of the lymphocytes in spleen and Peyer's patches were obtained using a simple model of lymphocyte migration kinetics and commercially available software.

1. Introduction

Lymphocyte migration is a highly regulated and dynamic process in the intact animal. A major objective of migration studies is to understand what controls the assembly of migrating lymphocytes within particular tissues, and central issues in this assembly are differences in the probability that given lymphocytes will enter different tissues from the blood stream, and differences in the probability that the migrating cells will continue to reside there after admission. Although a wealth of information regarding the molecular determinants on lymphocytes and endothelial cells that control the initial stages of lymphocyte migration has emerged (2,3), we need to rely upon studies of migration in vivo to assess the contribution of these factors to the physiology of migration.

The interpretation of in vivo studies of lymphocyte migration is frequently qualitative and provides only indirect assessment of the physiology of migration. We have previously tested ways by which the time course of tissue localization of labelled lymphocytes can be examined to estimate the entry and retention of migrating cells in particular tissues (4), but these approaches have been constrained to limited numbers of time intervals. We sought, therefore, a means to examine more extensive kinetic studies of lymphocyte accumulation in tissues.

2. Rat Studies

These were reported by Smith and Ford (1). Briefly, ^{51}Cr-labelled TDL were passaged from blood to lymph in primary recipients and their TDL collected within the first hour after transfer were transferred to secondary recipients. The TDL localization in these recipients was examined in the blood and tissues at 13 time intervals from 1 min to 24 h after transfer. The observations are the means from 4-5 animals per group and were kindly provided to us by Professor Ford prior to his untimely death. Further work in that laboratory has shown that passage of labelled TDL through an intermediate recipient enriches the TDL for CD4-bearing T cells (Dr. A. Ager, personal communication).

3. Mouse Studies

Mesenteric lymph node lymphocyte (MLN) suspensions were prepared from Balb/C mice and enriched for CD4 T cells using negative selection by panning on anti-immunoglobulin coated plates after treatment of the MLN with anti-CD8 antibodies as previously described (5). The CD4 enriched suspensions were labelled (^{51}Cr) and transferred to syngeneic recipients via a lateral tail vein (6). Measured samples of blood and tissues were obtained at 7 time intervals from 5 min to 200 min after cell transfer. The transferred cells were 95% (4% SD) homogeneous for the L3T4 determinant. The observations are the means from 6 animals per group.

TABLE 1. Parameter estimates for blood data

	Rat	Mouse
B_o	4.08 (0.15)	10.18 (0.98)
b	0.047 (0.006)	0.052 (0.007)
a	0.28 (0.01)	1.12 (0.24)
R^2	0.99	0.98

NOTE Detailed captions for Figure 1.

A: Observations in rat blood over 24h.

B: Same data as in A, but on expanded time scale to show early times more clearly.

C: Observations in rat spleen and Peyer's patches.

D: Same as C, but on expanded time scale.

E: Observations in mouse blood.

F: Observations in mouse spleen and Peyer's patches.

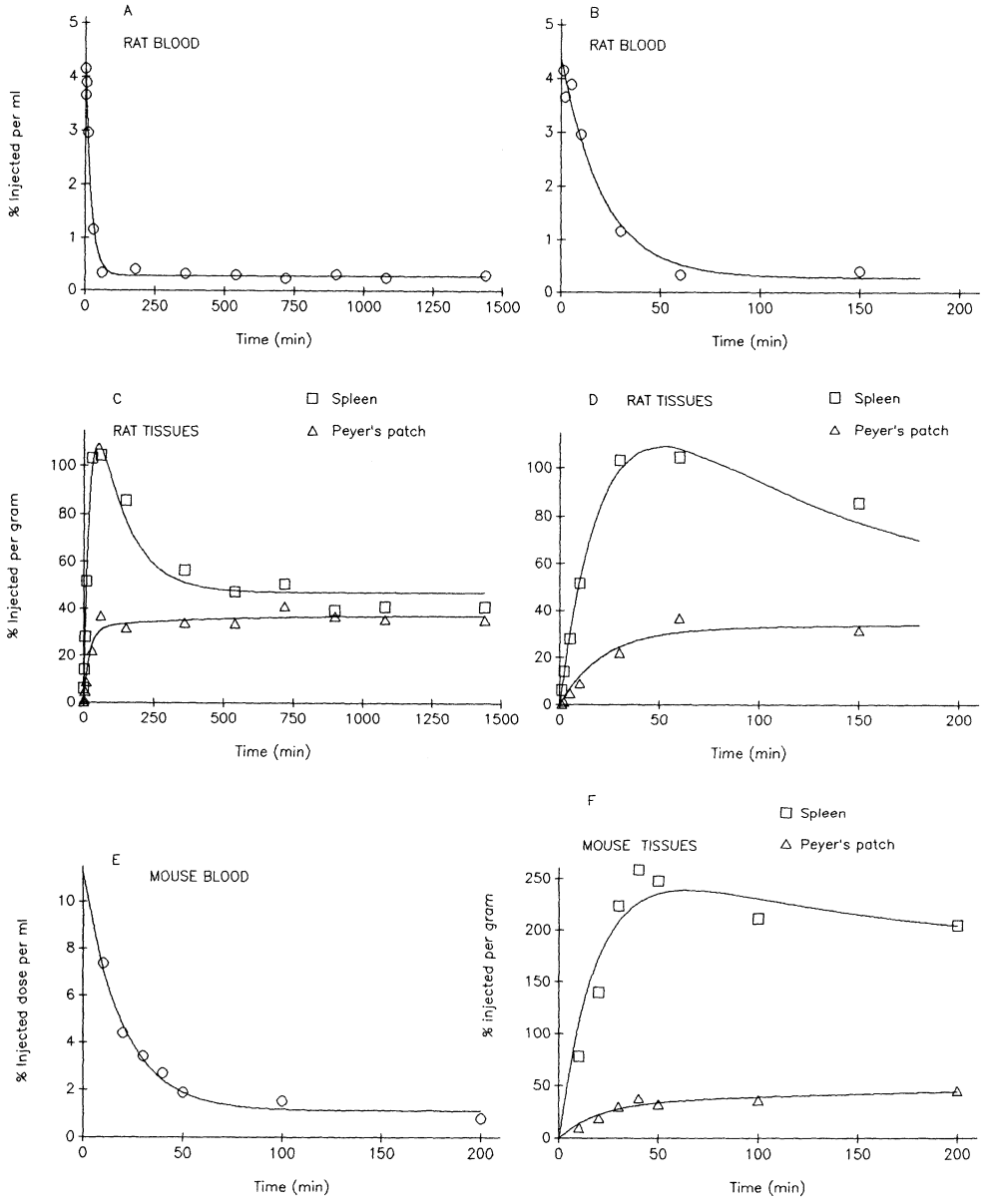

Figure 1. Time course of labelled lymphocytes in blood and tissues. In each panel the experimental data are indicated by symbols and the fitted curves obtained from the derived estimates are represented by solid lines. (See opposite page for detailed captions).

4. Analysis

The rate of accumulation of lymphocytes in a tissue can be written as

$$dP_i(t)/dt = [c_i P_b(t) - P_i(t)/m_i] \quad (1)$$

where: $P_i(t)$ is the percent of the injected dose of the cells in the tissue at a time t; $P_b(t)$ is the percent of the injected dose of cells per volume of blood; c_i is the rate at which the cells are cleared from the blood into the tissue; and m_i is the mean residency time or mean time to departure of the cells from the tissue. If m_i is in minutes and the distribution of cells is expressed per ml of blood or per g of tissue, then c_i has units of ml/g/min and is a measure of the product of the rate of delivery of cells to the tissue by the blood and the proportion of the delivered cells which are admitted (4). The first step was to examine the time course of the cells in the blood (Figure 1A,B,E). The observed values in the blood were fitted to the form

$$P_b(t) = B_o \text{ Exp } (-bt) + a \quad (2)$$

Nonlinear regression analysis was performed using Statgraphics (version 2.6, 1987, STSC Inc., Rockville MD, USA) and an XT compatible personal computer. The regression analysis program uses a Marquart algorithm (7) to estimate the parameters of the model from the timed observations, and permits analysis of variance to determine goodness of fit and error limits of the derived estimates. In both experiments, the time course of the transferred cells in the blood is satisfactorily described by equation (2) (Figure 1, Table 1). The proportion of the variation of the observations attributable to the regression model is expressed as the square of the multiple correlation coefficient (R^2). The significance of the regression estimates was assessed by the F-test (7).

Next, the time course of accumulation in tissues was examined. When the distribution of cells in the blood can be described by equation (2), the differential equation (1) can be integrated to yield

$$P_i(t) = \{c_i m_i B_o [\text{Exp } (-bt) - \text{Exp}(-t/m_i)] + c_i m_i a[1 - \text{Exp}(-t/m_i)]\} \quad (3)$$

Given B_o, b and a (Table 1), Equation 3 has two unknowns: c_i and m_i. Nonlinear regression analysis was again used to fit the tissue observations to equation (3). The results (Figure 1C,D,F and Table 2) suggest that the entire time course of the tissue observations can be satisfactorily described by this model. Although the maximal accumulation of labelled cells in the mouse spleen is approximately two-fold that observed in the rat studies (Figure 1), this is attributable to the increased availability of the transferred lymphocytes in the blood of the mice (Table 1). The values obtained for the clearance rate and the residency of the cells in the spleens of the two animals are quite comparable (Table 2). If the transferred TDL are taken to be predominantly CD4 cells, the dynamic behaviour of this T cell subset in the spleen and Peyer's patches appears to be similar in these species (Table 2).

TABLE 2. Parameter estimates for transfer of labelled lymphocytes into lymphoid tissues.

	c_i (ml/g/min)	m_i (h)	R^2
Spleen			
Rat	1.6 (0.1)	1.8 (0.1)	0.98
Mouse	1.3 (0.1)	2.0 (0.4)	0.84
Peyer's patch			
Rat	0.3 (0.02)	6.4 (0.5)	0.96
Mouse	0.2 (0.01)	5.4 (1.9)	0.90

5. Conclusion

Analysis of lymphocyte localization patterns as a function of time can provide useful estimates of kinetic aspects of lymphocyte migration in vivo. Our results suggest that it is feasible to analyze the time course of even quite complicated and extensive migration studies. The use of this approach in experiments directed at perturbing the contribution of defined molecular determinants should be useful in exploring the contribution of those determinants to the migration process. More quantitative evaluation of migration in vivo is likely to provide important insights into the ways in which migration is controlled in the intact animal.

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Characterisation of the lymphocyte-glandular mucosal tissue interaction using an *in vitro* adherence assay

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ABSTRACT. The in vitro adherence of lymphocytes to lacrimal gland (LG) tissue was compared to high endothelial venules (HEV) of cervical lymph node (CLN) tissue. Thoracic duct (TDL) and mesenteric lymph node (MLN) lymphocytes bound preferentially over peripheral lymph node (PLN) lymphocytes, while thymocytes (THY) did not bind. Lymphocyte binding to LG was specific for acinar cells (AC). Like CLN-HEV binding, TDL adherence to LG-AC requires viable, metabolically active cells and involves cellular cytoskeleton, surface protein, but not sialic acid. Unlike binding to CLN-HEV, TDL binding to LG-AC does not require divalent cations. The LG-AC interaction involves carbohydrate, but the sugar specificity differs. These findings suggest that the localization of circulating lymphocytes within one glandular tissue (LG) is mediated by a receptor-ligand interaction which differs from that involved in HEV interactions.

INTRODUCTION

Distinct organ-specific lymphocyte-endothelial cell recognition systems play a major role in lymphocyte traffic to organized peripheral and mucosal lymphoid organs (1-4). Further, such interactions are known to contribute to lymphocyte accumulation in both inflamed synovium (5) and skin (6,7). In general, these lymphocyte-endothelial interactions appear to be regulated by "homing receptors" on lymphocytes which interact with "vascular addressins" on HEV or other specialized endothelial cells (8). With respect to mucosal tissue, it is well documented that gut-derived lymphocytes pass through the MLN, enter the thoracic duct lymph, reach the blood, and selectively populate mucosal sites (9,10). While one mechanism accounting for the selective lodging of lymphocytes in non-glandular mucosal lymphoid and extralymphoid sites involves endothelial recognition (1,2,4), the mechanism accounting for the accumulation of lymphocyte populations in glandular mucosal tissue is less well defined. This study uses an in vitro adherence assay, widely employed to characterize lymphocyte-HEV interactions (11,12), to define the molecular interactions responsible for lymphocyte accumulation by LG in the rat model.

MATERIALS AND METHODS

Animals and Reagents. Fischer 344 rats (150g) were purchased from Charles River or bred in-house and used for all experiments. Glutaraldehyde and formaldehyde were purchased from Polysciences and Fisher, respectively. Na cacodylate, DL-lysine monohydrochloride, EDTA, cytochalasin B, trypsin, trypsin inhibitor, neuraminidase, D-mannose-6-phosphate, D-galactose-6-phosphate, L-fucose-1-phosphate and fucoidin were purchased from Sigma.

Cell Preparation. TDLs were collected from lymph 20-40 hrs after cannulation. Cells were harvested by centrifugation at 400xg for 10 min. Single cell suspensions were prepared from lymph nodes and thymus by mincing the tissues and gently pressing tissue fragments through 80 mesh stainless steel sieves. Following filtration through cotton wool, cells were washed 3x in RPMI 1640 and resuspended in RPMI with 1% FBS at $2x10^7$ cells/ml. Suspensions were kept at 4° and used within 1 hr. Viability, as determined by trypan blue exclusion, was at least 95%.

Section Preparation. Frozen CLN and LG were glutaraldehyde-fixed (3% in 0.1M Na cacodylate). Unreacted aldehyde groups were blocked by treatment with 0.2M DL-lysine monohydrochloride (10 min), slides washed in RPMI and then stored in RPMI with 1% FBS at 4° until use within 2 hrs.

Binding Assay. The assay was carried out as described previously by Stamper and Woodruff (11,12). Lymphocyte suspensions (0.2 ml) were placed over each section and the slides were rotated at 80 rpm for 30 min at 7°. Slides were washed by dipping repeatedly in cold PBS and fixed for 10 min in 3% glutaraldehyde. After washing with tap water, the sections were stained with methyl-green-thionin for 10 min at 37° and examined under the light microscope. Each experiment was performed using sections from single CLN or LG with 3-12 replicates. Data were expressed as mean cells/mm^2 $\pm$ SEM bound or as percent of control binding. Statistical analyses employed the Student's t-test.

RESULTS

Initially the binding of TDL, MLN, PLN and THY suspensions to CLN-HEV and LG-AC were compared (Fig. 1). Binding to HEV was 89.9, 79.4, 74.6, 62.3%; to AC 94.4, 87.2, 88.5, 74.7% for these populations, respectively. TDL displayed the highest binding for both CLN-HEV (153.5 $\pm$ 68.7) and LG-AC (104.9 $\pm$ 15.7). MLN populations bound better to both CLN-HEV (30.0 $\pm$ 4.4) and LG-AC (18.9 $\pm$ 2.9) than did PLN to CLN-HEV (23.5 $\pm$ 4.3) or LG-AC (13.2 $\pm$ 2.7). THY showed minimal binding to both CLN-HEV (2.2 $\pm$ 1.1) and LG-AC (0.3 $\pm$ 0.1). All differences were significant at $p < 0.05$.

In order to evaluate the parameters influencing LG-AC adherence, the binding of TDLs to both CLN-HEV and LG-AC was tested using a series of compounds with defined activity in the CLN-HEV system. Table 1 presents these data. TDL adherence to both CLN-HEV and LG-AC require viable (formaldehyde sensitive) and metabolically active (sodium azide sensitive) lymphocytes. TDL adherence to LG-AC does not require the divalent cations Ca^{++} and Mg^{++} (EDTA insensitive) unlike the binding to

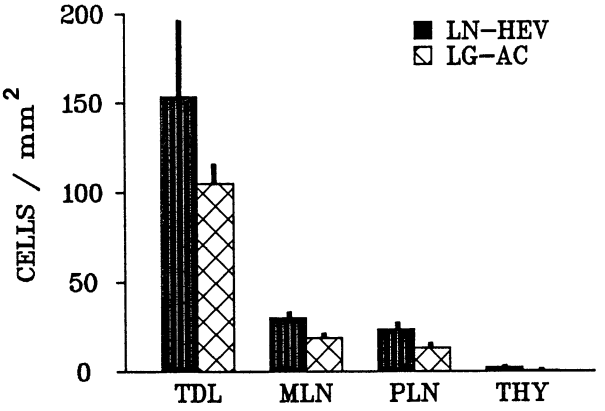

Figure 1. Comparative binding properties of TDL, MLN, PLN and THY suspensions to CLN-HEV and LG-AC (reflects binding to AC and AC basement membrane). Data are expressed as mean cell/mm^2 $\pm$ SEM.

CLN-HEV which is Ca^{++} dependant (EDTA sensitive; binding restored with Ca^{++}, but not Mg^{++}). It should be noted that EGTA treatment and Ca^{++} addition gave similar results (data not shown). Both types of interactions involve cytoskeleton (cytochalasin B sensitive). At the cytochalasin B concentration tested the adherence to CLN-HEV appeared more sensitive than that noted for LG-AC ($p < 0.05$), although no significant lasting effects were noted when TDLs were pretreated then washed prior to the assay. For both systems a TDL surface protein appears to be involved in the adherence (trypsin sensitivity). The specificity of the trypsin activity is documented by the lack of inhibitory effects on binding when a trypsin inhibitor was included. Further, TDL surface sialic acid is not involved in the adherence to LG-AC (neuraminidase insensitive). The LG-AC binding appears to involve carbohydrate recognition (fucoidin sensitive), as does CLN-HEV, although the LG-AC binding appears to be somewhat less sensitive ($p < 0.05$). The sugar specificity for the two systems differs with CLN-HEV showing greater inhibition of binding with mannose-6-P and fucose-1-P than LG-AC. The LG-AC binding shows inhibition with galactose-6-P.

DISCUSSION

The data show that: 1. lymphocyte populations display a differential adherence to LG tissue which parallels that noted for CLN-HEV binding; 2. lymphocyte adherence to LG tissue is specific for acinar epithelial cells; and 3. while many features of TDL adherence to CLN-HEV and LG-AC are shared, the receptor-ligand interaction involved in LG binding differs.

Preferential LG binding by populations enriched for mucosal lymphocytes (TDL and MLN) is consistant with the *in vivo* migratory patterns of such cells (9,10). Further studies are needed to determine whether the small, but significant increase in MLN binding over that by PLN is caused by their "mucosal nature" or is a function of other factors such as state of activation, lymphocyte subset distribution differences, etc. Increased binding by TDLs is expected since these are circulating

Table 1. Factors influencing TDL binding to cervical lymph node HEV and lacrimal gland AC.

Compounds Tested[a]		% of Control Binding	
		CLN-HEV	LG-AC
Metabolic Inhibitors			
Formaldehyde	1%	6.1 ± 0.3	21.6 ± 9.0
Sodium Azide	1mM	76.6	27.0
	10mM	10.2 ± 1.9	7.0 ± 2.5
	10mM(w)	>100	>100
Divalent Cations			
EDTA	1mM	6.2 ± 0.7	91.3 ± 4.6
EDTA + Ca^{++}	1mM + 2mM	78.0 ± 7.5	73.7 ± 13.4
EDTA + Mg^{++}	1mM + 2mM	15.7 ± 4.7	>100
Cytoskeleton			
Cytochalasin B	10µg/ml	10.2 ± 2.8	26.8 ± 12.1
Cytochalasin B	10µg/ml(w)	79.5 ± 34.7	60.5 ± 19.3
Surface Molecules			
Protein: Trypsin	10U/ml	43.9 ± 14.3	30.1 ± 15.2
Trypsin	100U/ml + inhibitor	98.1 ± 7.3	87.4 ± 18.0
Sialic Acid: Neuraminidase	2U/ml	ND	93.2 ± 28.9
Carbohybrates			
Mannose-6-Phosphate	10mM	31.1 ± 4.1	70.9 ± 27.7
Galactose-6-Phosphate	10mM	74.7 ± 22.2	25.1 ± 4.4
Fucose-1-Phosphate	10mM	30.8	66.5
Fucoidin	1µg/ml	5.6 ± 3.4	24.4 ± 8.3

[a]TDL adherence assays run in the presence of compounds at the concentrations noted. (w)=wash out of compound prior to assay. ND=not done.

cells, while lymph nodes contain a substantial sessile population. Finally, it will be of interest to determine the capacity of TDLs and other populations to bind additional glandular mucosal tissues.

The observation that lymphocyte adherence is specific for acinar epithelial cells suggests that selective lodging in mucosal tissues may be regulated by interactions with non-vascular elements after entry through the endothelium. For LG it has been postulated that entry is random and retention is regulated by the non-vascular microenvironment within the gland (13). In addition, studies with the thymus have suggested that adhesive interaction between cortical thymocytes and thymic epithelial cells direct the cellular composition of this organ by triggering prothymocyte maturation after immigration (14).

Table 2 presents a summary of the similarities and differences between LG adherence and lymphocyte interactions with CLN, Peyer's patch (PP, a mucosal lymphoid organ), inflamed skin and synovium. It is clear from our comparison with CLN-HEV and a compilation of investigations in rat, mouse and human systems (1-8) that lymphocyte adherence

Table 2. Comparison of properties of lymphocyte adherence to LG with CLN, PP, inflamed skin and synovial tissue.

Similarities:
- Viable, mature lymphocytes required (all systems)
- Lymphocyte surface protein involved, not sialic acid (CLN, PP, skin)
- Cytoskeletal involved (vs CLN; ? of degree)
- Fucoidin inhibition (vs CLN; ? of degree)
Differences:
- Epithelial target (vs HEV or specialized endothelial cell)
- No Ca^{++} requirement (Ca^{++} required in all other systems)
- G-6-P inhibition (M-6-P in CLN and skin)

to LG differs in its target cell specificity, requirement for Ca^{++} and sugar specificity. Further immunological and biochemical studies are needed to define the precise nature of the LG receptor-ligand interaction and its relation to other adhesion systems.

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In vivo adherence of lymphocytes to high endothelial venules of Peyer's patches and peripheral lymph nodes

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ABSTRACT. A simple fluorescence microscopy was established using carboxyfluorescein diacetate (CFD) for labeling mouse lymphocytes. By syngeneic transfer and subsequent examination of the adherence of lymphocytes to high endothelial venules (HEVs) of Peyer's patches (PPs) and peripheral lymph nodes (PLNs), the CFD-labeled lymphocytes were found to be superior to those labeled by tetramethylrhodamine isothiocyanate (TRITC) or by fluorescein isothiocyanate (FITC) in fluorescence intensity and longevity. PLN lymphocytes and T cells are superior to PP lymphocytes and T cells in adherence to HEVs of both PPs and PLNs. Both B cells of PPs and PLNs tend to adhere to HEVs of PPs better than to HEVs of PLNs.

1. INTRODUCTION

Lymphocytes re-circulate between blood and secondary lymphoid tissues [1]. The re-circulation is initiated by adherence to high endothelial venules (HEVs) and passage through the venule walls [2-3]. An in vitro test of adherence of lymphocytes to HEVs of peripheral lymph nodes (PLNs) has been established [4] where lymphocytes were incubated with frozen sections of lymph nodes and adherence to the HEV was then examined under a microscope. Using this method, studies in recent years have shown that lymphoid cells from PLNs tend to adhere to HEVs of PLNs better than those from Peyer's patches (PPs) [5]. However, this organ specificity remains to be confirmed definitively by tests of in vivo lymphocyte adherence to HEVs, where a rapid and accurate method of quantitation is required.

We developed a simple and accurate method for examining in vivo lymphocyte adherence to HEVs of PPs and PLNs. Lymphocytes were labeled with carboxyfluorescein diacetate (CFD) and syngeneically transferred; the adherence to HEVs of both PPs and PLNs were examined under a fluorescence microscope. PLN lymphocytes were found superior to PP lymphocytes in adhering to HEVs of both PPs and PLNs. No organ specificity was seen.

2. MATERIALS AND METHODS

Lymphocytes from PPs and PLNs of BALB/cJ mice were dispersed and labeled

with CFD by incubating at 37°C for 30 minutes in Hanks Medium which is in fact Ca^{++}- and Mg^{++}-free Hanks balanced salt solution with 2 mg/ml bovine serum albumin. CFD-labeled lymphocytes were syngeneically transferred and PPs and PLNs were examined in anesthetized recipients by video fluorescence microscopy or were removed at appropriate time intervals and were pressed flat thin between two microscope slides which were then examined under a fluorescence microscope; fluorescent lymphocytes present around HEVs of the PP or PLN were counted and averaged.

3. RESULTS

CFD was found to have a wide working range (10-80 ug/ml). The fluorescence intensity and longevity of CFD in lymphocytes adhering to HEVs were found to be much stronger and longer-lasting than TRITC and FITC. By video fluorescence microscopy, the CFD labeled cells were found adhering to HEVs of PPs and PLNs as soon as they were intravenously injected. Some of the CFD-labeled cells began to pass the venule wall within 10 minutes and as time passed they moved deeper and deeper into the lymphoid element of PPs and PLNs. These results suggest that CFD has essentially no effect on the migration property of lymphocytes.

Table 1. Differences between Peyer's patch and peripheral lymph node lymphocytes in adherence to high endothelial venules of Peyer's patches and peripheral lymph nodes.

	Labeled cells (number per node or patch) around HEV of	
Donor cell	PP	PLN
PP	59	93
PLN	221	445
PP-T	2	0
PLN-T	328	382
PP-B	31	0
PLN-B	59	19

*BALB/cJ lymphocytes were labeled with carboxyfluorescein diacetate and syngeneically transferred (2 x 10^5/mouse). PPs and PLNs were removed from the recipients 2 hours after transfer.

When lymphocytes from PPs and PLNs were compared (Table 1), PLN lymphocytes were found to be superior to PP lymphocytes in adherence to HEVs of both the PP and PLN. When lymphocytes were separated into T and B cells, PLN T cells were also found to be greatly superior to PP T cells in adhering to HEVs of both PPs and PLNs. Although PLN and PP B cells adhere better to HEVs of PPs than to HEVs of PLNs, both B cells were very inefficient in the adherence. The adherence to HEVs was not organ-specific

for lymphocytes from PPs and PLNs.

4. DISCUSSION

CFD is superior to TRITC and FITC in labeling lymphocytes for in vivo migration studies because CFD offers the advantages that: (1) it labels the cytoplasm of only live cells, (2) it does not react with any membrane and cytoplasmic components, which eliminates effects on adherence and motility of lymphocytes, (3) it has much stronger fluorescence intensity and greater longevity.

Unlike the results obtained using methods of in vitro lymphocyte adherence, the present method of in vivo lymphocyte adherence does not show organ specificity for lymphocytes from PPs and PLNs. This could be due to differences in the methodology. However, the in vivo method can provide direct information which the in vitro method generally fails to offer.

Because of the fact that PLN lymphocytes and T cells showed a comparable capability in adhering to HEVs of both the PP and PLN (Table 1), the inferiority of PP lymphocytes to adhere to HEVs of PPs and PLNs suggests that PP cells are an unique population. Their specific homing characteristic to mucosal tissues may be generated in PP through the very same or different process that generates IgA cells.

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In vivo migration and DTH responses by murine antigen-specific T cell lines

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Introduction

There is evidence to suggest that mucosal immunity may play a role in the pathogenesis of food-sensitive enteropathies. Such abnormalities can be induced in experimental animals by cell-mediated mechanisms [1, 2]. Most models of adoptive transfer of cell-mediated immunity have used freshly isolated cells [3]. However, a disadvantage of this approach is the variability and heterogeneity of the cell populations transferred. The aim of this study was to establish antigen-specific murine T cell lines for transfer and to investigate their involvement in the induction of mucosal inflammatory responses 'in vivo' after challenge with dietary antigens.

Materials and Methods

Gliadin was purified from a batch of "Avelon" wheat and ovalbumen (OVA) was obtained from Sigma. A colony of Balb/c mice was maintained through multiple generations on a cereal- and ovalbumen-free diet. 6-12 week old mice were used for all experiments.

ESTABLISHMENT OF LONG-TERM ANTIGEN-SPECIFIC T CELL LINES

Lymphocytes were obtained from draining lymph nodes after subcutaneous immunisation with antigen (gliadin or ovalbumen) and cultured according to a rest-stimulation protocol [4] for the establishment of antigen-specific T cell lines. Antigen-specific proliferation of T cell lines was tested using 0-1,000 μg/ml of test or control antigen in 4 day cultures. Cells were pulse-labelled with ^{3}H-Thymidine for the last 20 hours of culture. Phenotypic analysis of T cell lines was carried out at the end of rest phase by indirect immuno-fluorescence with rat anti-mouse Thy.1, Lyt.1, Lyt.2 and L3T4, and goat anti-rat fluorescein immunoconjugate.

DELAYED-TYPE HYPERSENSITIVITY (DTH) RESPONSES

i) Administration of T cells into footpad: OVA-specific T cells were harvested after rest phase and injected intradermally (I/D) into footpads ($1.25.10^{5}$ cells) with OVA or control antigen (BSA). Controls included cells or antigen only. Footpad thicknesses were measured before injection and after 24 and 48 hours. The mean of 10 measurements was taken for each footpad; results are quoted as mean ± SEM and compared by the Mann Whitney U-Test.
ii) Intravenous administration of T cells: OVA-specific T cell lines were harvested after a) resting phase or b) following antigenic stimulation. 8.10^{6} cells in 100μl RPMI 1640 were injected per mouse intravenously (I/V). 50μg antigen (OVA or BSA) or saline (25μl) were injected I/D into opposite footpads. Control animals were injected I/V with RPMI 1640

medium alone. Footpad thicknesses were measured as above.
iii) Oral challenge: Further groups of mice were fed daily with antigen for up to one month after I/V injection of cells as above. Animals were sacrificed at regular intervals, and the small bowel was assessed for enteropathic damage according to established criteria [1].

Results

Cell lines were Interleukin-2 (IL-2) independent, and antigen-specific after 4-5 cycles of rest/stimulation. Responses to the specific proteins were reproducible and dose-dependent. Phenotypic analysis of cell lines showed the cells to be mainly (>80%) Thy.1+, L3T4+, Lyt.1+/- and Lyt.2$^-$.

ADOPTIVE TRANSFER OF OVALBUMEN-SENSITIVE T CELLS

i) Following footpad injection of T cells and specific antigen, there was a significant increase in footpad thickness after 24 hours (0.479mm ± 0.138 versus 0.209mm ± 0.048, $P<0.027$) and 48 hours (0.297mm ± 0.06 versus 0.076mm ± 0.099, $P<0.003$), compared with controls.

ii) Resting T cells administered I/V mediated a significant increment in footpad thickness at 24 hours post-challenge (0.153mm ± 0.017 versus 0.05mm ± 0.014, $P<0.002$), compared with controls. However, when antigen pre-stimulated T "blasts" were used, the specific increment in footpad thickness was smaller and peaked at 48 hours post-challenge (0.088mm ± 0.016 versus 0.016mm ± 0.005, $P<0.033$).

iii) No significant changes in intestinal crypt depth, villous height, crypt cell production rate or intraepithelial lymphocyte density were observed at any time point in mice receiving oral antigen after I/V adoptive cell transfer.

Discussion and Conclusions

Murine T cell lines have been used previously in adoptive transfer experiments for the induction of antigen-specific DTH responses [5, 6]. We have demonstrated variations in the intensity and peak time of response in a footpad DTH response induced by resting versus stimulated T cell lines. The phenotype, biological activity and independence of exogenous IL-2 indicate that our cell lines are fully functional Th1 "inflammatory mediator" T helper cells. Therefore, their apparent inability to mediate mucosal hypersensitivity upon oral antigen challenge strongly suggests that inflammatory T effector cells alone are insufficient in antigen-mediated enteropathy. This contrasts markedly with results following adoptive transfer of cells freshly isolated from hyper-immune animals [1-3]. It therefore seems probable that other, additional effector cells are required for immune-mediated mucosal inflammatory responses.

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Direct transdiaphragmatic traffic of peritoneal macrophages to lung-associated lymphatic tissue

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ABSTRACT. Intraperitoneal inoculation (IP) is the most effective parenteral route for priming a respiratory immune response. We initiated tracer studies to define the distribution of both colloidal carbon and fluorescent microbeads injected IP. We observed, in addition to lymphatic drainage of the carbon, migration of macrophages containing carbon from the peritoneal cavity directly to the lung interstitium and bronchial associated lymphatic tissue (BALT) without passage in the blood. FACS analysis of migrant phagocytic peritoneal macrophages indicated that that they bear a phenotype ($Mac1^+$, Ia^+ and $L3T4^+$) consistent with antigen presenting cells. This suggests that peritoneal macrophages are a source of lung interstitial macrophages which could present antigen to immunocompetent mucosa-associated lymphoid cells in the lung following IP immunization. We propose that IP immunization results in simultaneous priming of both the systemic and mucosal immune response.

Introduction.

Respiratory immunity requires both local mucosal and systemic immunity. Secretory IgA is important in protecting the upper respiratory tract from inhaled foreign material and IgG prevents systemic spread of pulmonary infections from the lower respiratory tract (1). Generation of a protective antibody response is dependant on the route of immunization. We found that IP inoculation was the most effective immunization route for producing protective immunity against a lethal aerosol challenge of multitropic Rift Valley Fever (RVF) virus (2).

In order to determine if IP inoculation resulted in unique distribution of antigen or antigen presenting cells,

we traced the distribution of IP injected particulates to mucosal and peripheral lymphatic tissue.

Materials and Methods.

Carbon tracer studies: 0.1ml of a 1:2 dilution of Pelikan special black ink in PBS was given IP to Swiss Webster mice. Mice were killed at various time intervals. Abdominal, thoracic, and mediastinal viscera, including lymphatics and lymphatic tissues, were examined for free or cell-associated carbon. Cells from blood, pleural cavity and bronchial lavage were washed, counted, cytocentrifuged, stained with Diffquick (Am. Scient. Prod., M^{c}Graw Park, Il.) and the percentage of macrophages containing particles determined. Following bronchial lavage, mediastinal lymphatic tissues, lungs and diaphragms were fixed in 10% formaldehyde, dehydrated and cleared permanently in methylsalicylate. Sections of cleared tissue were then embedded in plastic and 1μm sections of areas containing carbon were stained and examined.
Cell phenotype studies: 0.1ml of a 1% suspension of phycoerythrin-labelled 0.8μm polystyrene beads (Pandex, Mundelein, Il.) was injected IP to Swiss Webster mice. Eighteen hours later mice were killed and pleural cavity lavaged using Ca^{++} and Mg^{++} free phosphate buffered saline (PBS) containing 3% foetal calf serum (FCS), 0.1% Sodium Azide and 10mM HEPES. Cell suspensions were labelled with either anti-Mac1 (M1/70), anti-Ia (M5/114) or anti-L3T4 and a second antibody goat anti-rat IgG fluorescein conjugate. The cells were analysed for 2 colour staining on a BD Facscan (Becton-Dickinson & Co., Mountain View, CA).

Results

Free and intracellular carbon was rapidly cleared into the diaphragmatic lymphatics. Both were observed in mediastinal and parasternal lymphatics and within caudal mediastinal and parathymic lymph nodes within 5 min of IP inoculation. Eighteen hours post IP introduction resident peritoneal mononuclear phagocytes that had ingested carbon demarcated the milky spots in the mesentery and omentum.

In addition, carbon was also observed in foci on the lung pleural surface. Light and electron microscopic examination of these discrete areas showed that the carbon was intracellular. These cells had the nuclear and cytoplasmic morphology of mononuclear phagocytes and delineated unique migration paths leading from the visceral pleura into the interstitium along alveolar septae. By 14 days post IP introduction labelled macrophages could be found in the area of the BALT and were still found in this area after several months. A carbon-laden macrophage was

rarely found in the alveolar spaces and no free carbon was observed in the lung.

Examination of cleared diaphragms showed free and intracellular carbon present in the intermuscular and subserosal lymphatic networks. In addition, macrophages with and without intracellular carbon were observed migrating across the diaphragm through perivascular channels between the muscle cells. Microscopic examination of pleural cavity cells revealed that within 5 min of IP inoculation, carbon was present in 1% of the pleural macrophages. This percentage increased to 88% at 24 hours and then remained constant at 72 hours. There was no difference in either the total number of cells or macrophages obtained from pleural lavages at any time studied. Twenty three % of pleural lavage cells contained polystyrene beads which had been injected IP 18 hours previously. Two colour FACS analysis of these cells showed that all the cells with intracellular beads were $Mac1^+$, 93% were Ia^+ and 78% $L3T4^+$. Eighty three percent of the non-phagocytic pleural cells also expressed Ia on their surface.

Discussion

The migration of peritoneal macrophages labelled *in vivo* was traced. We discovered a direct pathway for movement of macrophages from the peritoneum to the lung interstitium and BALT via the diaphragm and pleural cavity.

The majority of foreign material introduced IP, either free or intracellular, leaves the cavity via stomata situated in the mesothelium lining the diaphragm and enters lymphatic collecting ducts (3). The lymph then flows into the caudal mediastinal, parasternal and mediastinal lymphatics, through the parathymic lymph nodes and enters the venous circulation near the heart. Thus, antigen introduced IP is rapidly delivered to peripheral lymphoid tissue. In addition, using carbon and fluorescent polystyrene beads as tracers, we delineated a pathway of peritoneal macrophage migration to the mucosa-associated lymphoid tissue in the lung.

There is evidence in the literature that lung interstitial macrophages are efficient antigen presenting cells (APC)(4). These peritoneal macrophages that migrate directly into and remain within the lung interstitium may be a significant source of APC in the lung for respiratory immunity. The migrant population of peritoneal macrophages harvested from the pleural cavity are capable of involvement in antigen presentation as most are Ia^+ and $L3T4^+$. There is also a large population of Ia^+ non-phagocytic cells in this mobile population that could not be traced with the carbon.

Increased cytotoxic T cell activity in the lungs

following IP injection of tumour cells compared to SC or IV injection provides indirect evidence supporting the hypothesis that the peritoneal cavity may supply immunologically revelant cells to the lung (5). Pierce and Koster (6) found that the IP route primed for a mucosal antitoxic response to cholera toxin. Recent studies have shown IP is the most effective route for priming the mucosal immune response in the respiratory tract (2) and IP immunization followed by a local intranasal boost produces protective immunity against a lethal aerosol challenge of the multitropic RVF virus (unpubl. obs.).

Respiratory immunity to a multitropic foreign agent requires both mucosal and peripheral immune responses. Antigen introduced IP appears to result in priming both responses on account of the unique distribution of antigen to both mucosa-associated and peripheral lymphoid tissue. This antigen presentation to both mucosal and systemic lymphoid tissue results in committment of both IgA and IgG producing B-cells without the initiation of specific immunoregulated suppression of either mucosal or peripheral immune responses that frequently accompanies a mucosal (intraduodenal, oral) or parenteral (subcutaneous, intravenous, intramuscular) immunization route.

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SECTION F: MODULATION OF MUCOSAL RESPONSES

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Sympathetic neuromodulation of systemic anaphylaxis

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ABSTRACT. We investigated the influence of the sympathetic nervous system on inflammatory responses in rats sensitised with *Nippostrongylus brasiliensis.* Seven days following bilateral decentralisation of the superior cervical ganglia (sympathectomy) conducted 35 days after injection, rats were challenged intravenously with allergen to induce anaphylactic shock. Sympathectomy of sensitised rats minimised the impact of anaphylaxis and decreased the influx of neutrophils and leakage of plasma proteins in the respiratory tract. Moreover, localised sympathectomy reduced subcutaneous granuloma formation and the chemotactic behaviour of neutrophils towards fMLP was markedly attenuated. These results indicate that the interruption of cervical sympathetic pathways modifies systemic immunological events.

Introduction

Given that the sympathetic nervous system can significantly modify anaphylactic responses we have investigated the mechanisms of this control [1,2]. Infection of rats with *N. brasiliensis* followed by challenge 35-42 days later with worm allergen results in anaphylaxis and severe pulmonary inflammation. To explore the impact of the sympathetic nervous system on pulmonary inflammatory events following induction of anaphylaxis, we decentralised the superior cervical ganglia but left the remaining sympathetic output to the body intact. Our studies indicate that this form of sympathectomy markedly attenuated anaphylaxis in the rat and we show that the interruption of the cervical sympathetic pathway exerts widespread perturbations in inflammatory reactions.

Materials and Methods

Animals and induction of anaphylaxis: Male Sprague-Dawley rats (~250g) were purchased from Charles River Canada, Inc. Rats were infected with *N. brasiliensis*

as described previously [3] and challenged 35-42 days later with 0.1 ml i.v. of a soluble homogenate prepared from 150 worms in order to induce anaphylactic shock.

Surgery: Bilateral decentralisation of the superior cervical ganglia was performed on rats 35 days after infection. Briefly, a small incision was made in the cervical region of anaesthetised rats and the superior cervical ganglia were carefully separated from the vagus trunk and a 3mm section was removed between the superior and middle ganglia. Animals were allowed to rest for 7 days prior to allergen challenge. Sham-operated and unoperated rats were used as control groups.

Blood flow measurements: Blood flow was measured according to the methods described by Mathison *et al.*[4].

Collection of bronchoalveolar lavage fluid and immunoglobulin determination: Bronchoalveolar lavage fluid (BAL) was collected from rats as described previously [3]. Following separation of cells by centrifugation, total immunoglobulin levels in BAL fluids were determined by a double antibody ELISA [5].

Collection of neutrophils and chemotaxis: Peripheral blood neutrophils were harvested from a pool of five rats using Polyprep (Cedarlane, Canada) and labelled with ^{111} Indium - oxine (Amersham,Canada) for 30 min. A modified microchemotactic assay [6] using graded concentrations of formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma, U.S.A.) was employed and the chemotactic index of migration was calculated.

Granuloma formation: Subcutaneous inflammation was induced in infected sympathectomised and sham-operated rats by inserting a 5cm sterile string into their abdomen [7]. The strings were left for 1 wk to allow granuloma formation and then removed. The granulomatous tissue was dried at 180° for 2h and the net increase in dry weight was recorded.

Results:

To evaluate the effect of sympathectomy on pulmonary anaphylaxis, the leakage of serum-derived immunoglobulins in BAL was measured. While BAL fluids of sham-operated and unoperated control groups contained substantial concentrations of immunoglobulin following allergen challenge, the protein content in BAL fluids of sympathectomised rats was not increased (Table 1).

Since leakage of serum immunoglobulin into BAL fluids of rats challenged with allergen may have resulted from alterations in pulmonary haemodynamics, the blood flow to the bronchioles was determined during the course of anaphylaxis. The results (data not shown) indicate that pulmonary blood flows in sympathectomised and control rats were similar.

To characterise the impact of cervical sympathectomy on inflammatory responses, subcutaneous granulomas were induced in sympathectomised and sham-operated rats infected with *N. brasiliensis*. The results (Table 2) show that compared to control animals, sympathetic decentralisation of rats significantly diminished the develop ment of granuloma 1 week after implantation of string.

Given that both local and distant sites of sympathectomised rats displayed attenuated inflammatory responses following induction of anaphylaxis, it was suggested that inflammatory cells such as the neutrophil would be functionally impaired. The results (Table 3) show that while neutrophils obtained from sham-operated or unoperated rats exhibited a dose-dependent chemotactic response towards this peptide, the response of neutrophils from sympathectomised rats was significantly depressed.

TABLE 1. Immunoglobulin concentrations in BAL and serum of control and sympathectomised rats following allergen challenge.

Group	Immunoglobulin concentration[1] (mg) Serum	BAL
Unoperated	10.3 ± 0.1	0.78 ± 0.01
Sham-operated	10.1 ± 1.2	0.75 ± 0.05
Sympathectomised	9.10 ± 0.03	0.24 ± 0.08[2]

1. Values represent total mean of IgG, IgM and IgA from 11-25 rats ± SEM
2. Denotes $p < 0.01$ when compared to unoperated and sham groups given allergen

TABLE 2. Granuloma formation in rats infected with *N.brasiliensis*

Group	Granuloma size (g)[1]
Unoperated	0.43 ± 0.07
Sham-operated	0.63 ± 0.05
Sympathectomised	0.34 ± 0.12[2]

1. Values represent mean ± SEM for 4-5 rats
2. $p < 0.05$ compared with the sham-operated group.

Table 3. Effect of sympathectomy on neutrophil chemotaxis

Group	Chemotactic Index[1] Concentration of fMLP		
	10^{-9}	10^{-3}	Buffer control
Unoperated	5536±608	4409±153	2756±328
Sham-operated	6593±1444	4673±1086	ND
Sympathectomised	2373±708[2]	3349±59	1318±66

1. Values represent mean ± SEM for 5 rats.
2. $p < 0.05$ compared to two control groups

Discussion:

We have shown that anaphylaxis is attenuated by cervical sympathectomy, but without involving major disturbances in haemodynamic status [4, unpublished]. The present study establishes that peripheral immune reactions are modified by the decentralisation of sympathetic pathways leading to target tissues of the cervical and cranial regions. We propose that diminished granuloma formation and neutrophil chemotaxis are the result of perturbations in the output of inflammatory mediators and other cytokines induced by the sympathectomy. Indeed, others [8,9] have demonstrated marked changes in systemic immune reactivity following chemical and/or surgical sympathetic ablation. Characterisation of the cellular mechanisms associated with cervical sympathetic decentralisation will shed further light on the complex interaction between the nervous and immune systems.

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The induction of specific oral tolerance can be abrogated by exposure to certain unrelated antigens

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1. Introduction

It has been noted that clinical sensitisation to an inhalant or food antigen may occur during a reaction to another antigen or allergen. This suggests that there has been an interference with the normal mechanisms of homeostasis. One of the possible mechanisms of mucosal homeostasis is oral tolerance whereby mucosal (primarily gastrointestinal) exposure to an antigen at appropriate concentration leads to systemic hyporesponsiveness. It has been postulated that the primary function of oral tolerance is to minimise systemic reactions to antigens at mucosal surfaces, mainly the lungs and gut, which may be allergenic and damaging to the host. Approximately one ton of food is ingested per year by an adult and as much as 10^5 g of intact protein molecules may arrive in the proximal portion of the small bowel. It is possible therefore that the combination of secretory immunity and systemic tolerance which arises from such gut exposure to antigen may have an important role in immunological protection of the host. An understanding of the mechanisms whereby oral tolerance is induced and maintained is thus of importance. Conditions have been identified under which interference with oral tolerance may occur, which include a concurrent graft versus host reaction (Strobel *et al.*, 1985) or intestinal exposure to an unrelated antigen soon after gastric immunisation (Stokes *et al.*, 1983). In this study the aims were to determine in the murine model whether the induction of specific oral tolerance could be modulated by concurrent immune responses to an antigenically unrelated protein, and to determine whether size of antigen was an important parameter in any modulation.

2. Materials and Methods

Humoral or cellular oral tolerance of ovalbumin (OVA, MW 45,000), myoglobin (MYO, MW 18,500) or keyhole limpet haemocyanin (KLH, MW 83,000) was examined, and the effect of prior sensitisation with unrelated antigens compared. Three schedules were used: (a) feeding with two antigens and systemic challenge with one; (b) feeding with one and systemic challenge with two; (c) prior sensitisation to one antigen and oral challenge with two. Oral immunisation was performed by giving 10 mg of antigen in saline by intragastric intubation (Challacombe and Tomasi, 1980). Systemic immunisation for humoral responses consisted of intraperitoneal challenge

with 100 μg of antigen in complete adjuvant, and for cellular response sub-cutaneous challenge at the base of the tail with 100 μg of antigen in 100 μl of adjuvant. Humoral responses were assayed by an ELISA technique as described previously (Challacombe, 1986), and cellular responses by a lymphocyte proliferation assay (Challacombe and Tomasi, 1980).

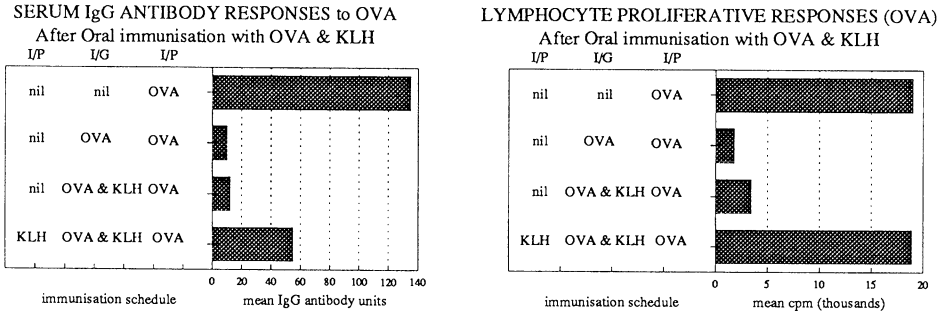
3. Results

Balb/c mice fed two unrelated antigens showed humoral and cellular oral tolerance to both which was equivalent to that seen with either alone (schedule a). Thus mice given a mixture of OVA and MYO orally showed oral tolerance to either or both of these antigens when challenged systematically. This was true of both humoral and cellular arms of the immune response. In addition oral tolerance to an antigen was not affected by systemic challenge with a mixture of antigens (schedule b). Thus when OVA was given intragastrically, and the mice were challenged with OVA and MYO, the animals were tolerant to OVA but showed normal responses to MYO.

In contrast, the induction of oral tolerance to an antigen given orally was profoundly affected if when the animal was immunised intragastrically, the antigen was given together with another unrelated antigen to which the animal was already sensitised. Thus when mice were given both OVA and KLH orally, and then challenged with OVA, oral tolerance to OVA resulted (Figure 1). If the oral immunisation with OVA and KLH was into animals already sensitised with KLH, then following the systemic challenge with OVA instead of tolerance, humoral responses were significantly greater at about 50% of those in controls (Figure 1). This applied to all three antibody isotypes. Oral tolerance of the cellular arm was completely abrogated by feeding OVA with KLH to mice already sensitised to KLH (Figure 2). In general, similar results were found whichever pair of antigens were used and no direct relationship with the size of the antigens was detected. In most instances abrogation of tolerance of the cellular responses was complete whereas humoral responses were partially abrogated.

4. Discussion

These results suggest that the induction of specific oral tolerance may be modulated by a concurrent immune response to an unrelated antigen. Neither oral immunisation with mixtures of antigens nor challenge with mixtures of antigens affected oral tolerance to individual antigens. The conditions under which tolerance was modulated were quite specific and occurred when oral tolerance was being induced with a mixture of proteins in an animal already sensitised to one of them. Under these conditions tolerance was not found to either protein even though they were antigenically unrelated. This observation suggests that inappropriate immune reactions can occur to one antigen during immune responses to another, and may be an explanation for the clinical reports of onset of allergy to an antigen whilst experiencing hay fever or



I/P = intraperitoneal, I/G = intragastric

other allergic reactions. The mechanism of such modulation is not clear, but it is possible that antigen-antibody complexes to one antigen may result in increased passage through the gut mucosa of a bystander antigen as reported *in vitro* (Brandtzaeg and Tolo, 1977). The normal mechanisms of tolerance induction would therefore be by-passed. A similar mechanism may explain the abrogation of the induction of oral tolerance when OVA is given orally in certain adjuvants (Strobel and Ferguson, 1986). The type of modulation of oral tolerance described in these experiments appears to differ from those described by Stokes *et al.*, (1983) who noted transient hypersensitivity to an antigen given orally during a defined period after oral immunisation to another antigen. Both these findings support the concept that allergy may result from inappropriate immune reactions to an allergen which are altered because of a concurrent response to another antigen, even though it is non cross-reacting.

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The translocation of immunoglobulin A and of albumin in the rat intestine is mediated by prostaglandins

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ABSTRACT. One of the functions of the peptide hormone cholecystokinin (CCK), is to promote secretion of solute and water in the small intestine. The hormone is secreted into the blood from the specialized endocrine cells lying among the intestinal epithelium when amino acids and fatty acids pass along the intestine. We have, already, shown that CCK also accelerates the transport of immunoglobulins A and G and of albumin into lumen of the intestine. We describe here the role played by prostaglandins in CCK-induced secretion of immunoglobulins. Hooded Lister rats were sensitized to ovalbumin. Twenty one days after the beginning of the sensitization schedule, they were anesthetized and a loop of intestine 10 cm long, 10 cm distal the pylorus was isolated and perfused with normal saline. The control group received an intravenous injection of CCK while the test group received the cyclo-oxygenase inhibitor, indomethacin in the perfusate before and during the injection of CCK. Both groups received an intravenous injection of ^{125}I-human serum albumin before the perfusion. Immunoglobulins were measured by an ELISA assay and ^{125}I human serum albumin in a ¢-counter. It was found that CCK causes a marked loss of albumin and IgA into the intestinal tract. This was prevented to a significant degree by the prior injection of indomethacin.

INTRODUCTION

Cholecystokin (CCK) is a peptide hormone present in the central nervous system as well as in the endocrine cells of the intestine [1]. Its main functions in the alimentary system are both motor, as well as secretory. The main secretory effects of CCK in the intestine are on the pancreas, where it stimulates the acinar cells to secrete the pancreatic enzymes. CCK also stimulates secretion of electrolytes and water into the lumen of the intestine [2]. We have, lately, further investigated the secretory effect of CCK in the intestine. We could show that i.v. injection of CCK to rats, previously sensitized with ovalbumin (OA) resulted in a rise of specific OA antibodies belonging to IgA and IgG [3] as well as IgM and IgE [4]. It is probable that CCK binds to presynaptic receptors of cholinergic nerves in the intestinal tract and augments the release of acetyl choline. There is circumstantial evidence that CCK receptors are also situated on

the basolateral membrane of the enterocyte. Of the intracellular events taking place in the enterocyte following stimulation with CCK, we have been able to establish that a rise of cytosolic calcium is part of the chain reaction initiated by CCK [4]. This was deduced from observations of the action of the calcium channel blocker, verapamil, in preventing the CCK-induced antibody release, on the one hand, and the effect of the calcium ionophore, A 23187 on the other [4]. The present investigation is aimed at showing that the production of prostaglandin (PG) is part of the secretory cascade required for the translocation of immunoglobulins. We did this by observing the effect of the prostaglandin inhibitor, indomethecin, on the CCK-induced release of antibodies.

METHODS.

Hooded Lister rats were used. They were immunized by an intraperitoneal injection of 250 ug of OA in 0.1 ml of 0.9% NaCl mixed with an equal volume of Freund's complete adjuvant (FCA). Fourteen days later a booster of 10 ug of OA in 0.1 ml of 0.9% NaCl mixed well with an equal volume of FCA. On the 21st day they were anesthetized with thiopentone and a loop of small intestine 10 cm distal to the pylorus and 10 cm long was severed and isolated in situ. This loop did not, therefore, receive any biliary or pancreatic secretions. The loop was perfused with 0.9% NaCl at the rate of 0.2 ml/min. They then received 0.5 Ci of ^{125}I-human serum albumin i.v. After a flushout period of 30 miunutes, specimens were collected every 2.5 minutes. After a baseline period of 10 minutes, 20 ng of CCK-octapeptide was injected i.v. (control group). In the test group, the PG inhibitor, indomethecin, 10 ug/ml was added to the perfusion fluid at the beginning i.e. 60 minutes before the injection of CCK-OP. IgA class antibodies to OA in the perfusate were measured by an ELISA assay [4] and albumin measured as cpm/min in a Packard, Prias model ¢-counter.

RESULTS.

Table 1. IgA antibodies to ovalbumin. Percent change in optical density readings in ELISA assay in control and test groups(X±SEM)

Time (mins.)	Control group (CCK only)	Test group (CCK+indomethacin)	p
	n=10	n=17	
2.5	121.8 ± 5.58	102.5 ± 13.4	<0.05
5	118.2 ± 3.2	99.8 ± 16.5	<0.01
7.5	112.1 ± 3.97	97.7 ± 13.3	<0.05
10	111.6 ± 4.75	95.9 ± 9.2	<0.05
12.5	113.7 ± 6.74	103.2 ±10.4	N.S.
15	106.9 ± 4.75	102.9 ± 9.4	N.S.

Table 2. Percentage change in levels of ^{125}I-HSA (measured as cpm/min) in lument of intestine following injection of CCK. (n=8) ($\bar{X}\pm$SEM).

Time (mins.)	Control group (CCK only)	Test group (CCK+indomethacin)	p
2.5	135.4 ± 7.0	104 ± 14.4	<0.001
5	122.5 ± 5.3	105 ± 21.4	N.S.
7.5	126 ± 9.0	111 ± 32/3	N.S.
10	114.4 ± 6.2	97 ± 12.7	<0.05

DISCUSSION.

The above experiments show that PG production is one step in the secretion of IgA and albumin into the intestinal lumen. This rise of PG is induced by the injection of CCK. The PG inhibitor indomethacin, prevents the secretory effect of CCK on albumin and IgA, showing that PGs are an essential step in furthering the transport of these proteins. We have previously shown that the CCK induced transport appears to be non-specific in the sense that it involves also electrolytes and water [4] in addition to the above proteins. Although we did not measure electrolyte loss in the above experiments, it seems likely that it, too, would have been inhibited by indomethacin. PGs in the gut are not produced within the epithelial cells. Rather, they originate within the lamina propria and the submucosa probably by a variety of cells, such as mast cells, fibroblasts, endothelial cells, vascular smooth muscle, smooth muscle and white blood cells [5]. Following secretion, they act on enterocytes. Within the enterocyte a number of intracellular steps appear to follow each other in rapid succession, resulting in the CCK-induced transport of solute and proteins. The observations concerning the action of PGs on intracellular mechanisms are still controversial. One group, represented, among others by Smith et al [6] have upheld the long known observation that PGE_2 stimulates epithelial cell adenylate cyclase and does this by a receptor mediated mechanism. On the other hand, Beubler et al [7] have found that physiological concentrations of PGs exert their secretory effect by facilitating the entry of calcium into the cell. This observation is more in accord with our own findings. Furthermore, it is believed that the secretion of serotonin, another intestinal secretagogue, precedes the production of PGs. The probable chain of events may, therefore, be that following the arrival of CCK at the region of the enterocyte, serotonin is released followed by the production of PGs. These open up Ca^{2+} channels and bring about a rise of cytosolic calcium. The end result is the active secretion of Na^+ and Cl^- at the apex of the cell or primarily, via the paracellular pathway [8]. PGE_1 and PGE_2 also decrease jejunal and colonic Na^+/K^+ ATPase activitiy [9]. Both actions of the PGs described above contribute to the loss of water into the lumen of the bowel. It appears from our observations that the transport of albumin and of IgA (and

probably also of IgG) is regulated by PGs and appears to take place simutaneously with the transport of electrolyte.

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Androgen regulation of the ocular secretory immune system

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INTRODUCTION

The principal tissue involved in secretory immune system of the eye is the lacrimal gland [1].This tissue, which is the origin of tear IgA and secretory component (SC) [2,3], possesses a high density of IgA plasma cells, a smaller population of IgG- and IgM-containing cells and a diverse array of T lymphocytes [4,5]. In addition, acinar and ductal cells produce SC [6,7], which appears to regulate the binding and transport of polymeric IgA into tears against an apparent concentration gradient [8,9].

Recently, research has shown that marked, gender-related differences exist in the ocular secretory immune system. The number of IgA-containing lymphocytes in the lacrimal gland [10,11], the production of IgA and SC by lacrimal tissue [2,12] and the concentration of IgA and SC in tears [9,12,13] are significantly higher in males, as compared to females. This sexual dimorphism seems to be due in part to the effect of androgens. These hormones, but not other steroids, increase the secretion of SC from the lacrimal gland [2] and elevate the tear levels of IgA and SC [12-14]. This androgen action, which is unique to the eye [14], requires the presence of an intact hypothalamic-pituitary axis [15,16].

To advance our understanding of the endocrine control of the secretory immune system of the eye, the present studies endeavored to: 1) determine whether androgen exposure modulates the transfer of IgA and IgG from lacrimal tissue to tears; 2) examine the influence of androgens on the accumulation of IgA- and IgG-containing cells in, and IgA production by, lacrimal tissue in hypophysectomized and/or orchiectomized rats; and 3) evaluate whether androgens directly regulate SC synthesis by lacrimal gland acinar cells in vitro.

MATERIALS AND METHODS

Male Sprague-Dawley rats (6-8 weeks old) were obtained from Zivic-Miller Laboratories, Inc., and maintained in constant temperature rooms with light/dark cycles of 12 hours duration. Orchiectomies, hypophysectomies, anterior pituitary ablations and sham-operations were performed by surgeons at Zivic-Miller on 6 week old rats. Animals were permitted to recover for 10 to 14 days before experimental use. To compensate for electrolyte imbalance in rats with complete or partial pituitary removal, animals were given an electrolye solution, as previously described [15]. Completeness of pituitary surgery was verified by measurement of serum thyroxine concentrations [15].

Tears were collected from the eyes of etherized rats and blood was obtained from the tail or heart by following reported procedures [13]. To allow determination of the IgA and IgG content in lacrimal glands, tissue vasculature was perfused in situ with 0.15 M saline to remove residual blood, then glands were processed for cytosol preparation according to published methods [9]. To

permit quantitation of IgA production by lacrimal tissue, gland segments were cultured for 21-24 hours and incubation media was analyzed for IgA levels [2]. For the establishment of acinar cell cultures, cells were isolated from male rat lacrimal glands by serial digestion of minced tissue with EDTA, followed by treatment with collagenase, hyaluronidase and DNase. Dispersed cells were passed through nylon filters, then centrifuged on Ficoll gradients to yield single acinar cell suspensions. Acinar cells were cultured for 3 to 12 days on reconstituted basement membranes (Matrigel) in modified Eagle's medium containing defined supplements and specified hormones or vehicle [7,17,18]. Tears, blood, cytosol and media samples were centrifuged at 10,000 x g and supernatants were stored at -20 C. To prepare lacrimal glands for the immunofluorescent analysis of IgA- and IgG-containing cells, tissues were transferred to St. Marie's fixative (19 parts 100 % ethanol: 5 parts glacial acetic acid) at 4 C. After fixation, glands were dehydrated using ethanol and xylene, embedded in paraffin and cut into 5 μm sections. Sections were placed on gelatin-coated slides, then deparaffinized immediately prior to staining [10,19].

Protocols for the measurement of rat IgA and rat SC (principally free form) by specific radioimmunoassays, IgG by enzyme-linked immunosorbent assay and total protein by the Hartree method have previously been described in detail [3,20]. The techniques for the immunofluorescent identification of IgA- and IgG-containing cells in lacrimal gland sections have also been reported [10,19]. For quantitative analysis of Ig-containing cells, 2 sections/tissue and approximately 15 microscopic fields (312.5 x magnification)/section were examined with a Zeiss Photoscope II fluorescence microscope. To determine the total number of IgA- and IgG-containing cells in lacrimal glands, the cell density/field was multiplied by gland weight (mg) and by a correction factor, which compensated for such variables as cell size, microscopic field volume and tissue density [19]. Statistical analysis of the data was performed with Student's t test.

Hormones used in these studies included testosterone, 17β-estradiol, progesterone, dexamethasone and aldosterone, which were purchased from Calbiochem-Behring, and dihydrotestosterone (Sigma). In addition, placebo (cholesterol, methyl cellulose and lactose)- and testosterone (50 mg)-containing pellets were obtained from Innovative Research of America; these pellets were designed for slow release of physiological amounts of testosterone over 3 weeks. For hormone treatment in vivo, testosterone was either solubilized in saline by glass-glass homogenization and injected subcutaneously or administered by subcutaneous pellet implantion in the subscapular space. Control animals, depending upon the experimental design, received either saline injections or placebo pellets. When utilized in acinar cell cultures, steroids were dissolved in ethanol and aliquots of steroid or ethanol (control) were evaporated prior to the preparation of media.

RESULTS

Effect of testosterone on the tear and lacrimal gland content of IgA and IgG [19]

To determine whether androgens regulate the tear level of both IgA and IgG, orchiectomized rats (n = 7-8/treatment group) were injected with saline (NS) or testosterone (T; 2 mg/day) for 4 days and tears were collected immediately before and 24 hours after hormone treatment. Testosterone administration induced a significant ($p < 0.01$) 3-fold rise in tear IgA content (3567 ± 675 ng), when related to values of saline-injected (1278 ± 154 ng) or pre-treatment (1158 ± 137 ng) controls. This increase was found irrespective of whether data was compared in terms of total amounts or after standardization to tear protein. In contrast, androgen exposure had no effect on tear IgG levels (NS = 9.42 ± 1.67 ng; T = 10.05 ± 1.94 ng). This differential response to testosterone administration was also observed following extended androgen treatment (n = 7-8/group; testosterone (50 mg) or placebo pellet implants) for 7, 11 or 17 days: testosterone significantly elevated tear IgA, but not IgG, amounts.

To evaluate whether androgens modulate the lacrimal gland content of IgA or IgG, orchiectomized rats (n = 6/treatment group) were implanted with placebo (P)- or testosterone (50 mg)-containing pellets; following 7 days of hormone exposure, tears and perfused lacrimal tissue were collected and glands were processed for the preparation of cytosol. Androgen administration caused a significant ($p < 0.05$), 84% increase in the IgA/protein ratio in lacrimal tissue of orchiectomized rats (Table 1). Moreoever, this androgen-induced rise in the cytosol IgA/protein

ratio was paralleled by a 300% elevation in the tear IgA/protein ratio, as compared to that in placebo-treated controls (Table 1). Consequently, testosterone action resulted in a significant ($p < 0.01$), 2-fold increase in the tissue to tear transfer ([tear IgA/protein]/[cytosol IgA/protein]) of IgA ($P = 2.16 \pm 0.46$; $T = 4.54 \pm 0.53$); this movement was against an apparent gradient. In contrast, androgen treatment had no influence on the IgG/protein ratios in lacrimal glands or tears (Table 1). In addition, transfer of IgG from lacrimal tissue to tears was down a 90-fold gradient.

Table 1. Impact of testosterone on the IgA and IgG content in lacrimal glands and tears

	IgA (µg)/Protein (µg) - %		IgG (µg)/Protein (µg) - %	
Treatment	Cytosol	Tears	Cytosol	Tears
Placebo	0.38 ± 0.06	0.89 ± 0.30	0.354 ± 0.046	0.004 ± 0.001
Testosterone	0.70 ± 0.13 *	2.93 ± 0.61 **	0.351 ± 0.063	0.008 ± 0.002

Orchiectomized rats (n = 6/group) were exposed to placebo- or testosterone (50mg)-pellets for 7 days, then tears and lacrimal glands were collected and tested for IgA, IgG and total protein content. Numbers equal the mean + SE. Significantly ($p < 0.05$) * or ($p < 0.01$) ** higher than placebo-value. Data from [19].

Influence of testosterone on the number of IgA- and IgG-containing cells in, and production of IgA by, lacrimal glands of hypophysectomized and/or orchiectomized rats [19]

To examine the influence of testosterone on the the number of IgA-, as well as IgG-, containing cells in lacrimal tissue, orchiectomized rats (5-9/treatment group) were administered injections (saline or 2 mg T/day for 4 days), or implanted with pellets (placebo or 50 mg T; for 7, 14 or 17 days), of vehicle or testosterone and lacrimal glands were obtained at the end of the experimental time course. Androgen treatment increased the density of IgA-containing cells by 18 to 49% in 5 out 8 separate studies (n = 141-263 microscopic fields/group). However, when densities were corrected for gland weight, no consistent differences were found between hormone and placebo-treated groups in the total number of IgA-positive lymphocytes. This lack of effect contrasted sharply with the androgen-induced 2.4- to 14-fold rise in the tear IgA concentrations of these same animals. As concerns IgG, testosterone exposure had no influence on either the density or total number of IgG-containing lymphocytes in lacrimal glands.

Studies were also performed to determine whether interruption of the hypothalamic-pituitary axis might decrease the number of IgA-containing cells in androgen-treated rats; this surgical procedure significantly reduces the effect of testosterone on tear IgA [15,16]. Animals (n = 7-10/treatment group) were orchiectomized and underwent hypophysectomy, anterior pituitary extirpation or sham-surgery. After a recovery period, rats were given testosterone for 4 (2 mg T/day) or 14 (50 mg T pellet) days. Ablation of the entire or anterior pituitary resulted in a 2.3- to 3-fold drop in the total number of IgA-containing cells in lacrimal glands, as compared to values in sham-operated controls. This decrease in the IgA-positive cell population coincided with a 2-fold decline in lacrimal gland weight. Analagous findings were observed in placebo-treated animals, wherein hypophysectomy or anterior pituitary removal significantly ($p < 0.05$) diminished the numbers of IgA-containing cells in lacrimal tissue.

To explore possible androgen control of IgA production by the lacrimal gland, orchiectomized rats (n = 5-8/treatment group) were treated with placebo- or testosterone (50 mg)-containing pellets for 7 or 14 days. After hormone exposure, lacrimal tissues were cultured overnight and media IgA, as well as SC, content was measured. In 4 out of 7 experiments, testosterone administration significantly ($p < 0.05$) increased the lacrimal gland output of IgA (e.g.: media IgA (ng)/tissue weight (mg): $P = 104 \pm 18$; $T = 252 \pm 47$). In contrast, androgens consistently stimulated a significantly enhanced release of SC; the extent of this androgen-associated rise in SC production, when compared to control, was always greater than, or equal to, that of IgA. These testosterone-induced elevations in IgA and SC output by lacrimal tissue could be significantly ($p < 0.05$) diminished by inclusion of cycloheximide (100 µg/ml) in the culture media.

A preliminary experiment also indicated that testosterone (50 mg pellet for 14 days) increases IgA and SC production by lacrimal tissues from rats, which had undergone orchiectomy and anterior pituitary ablation. Lacrimal glands from these animals (n = 8/treatment group) released significantly ($p < 0.01$) more IgA and SC during a 21 hour culture, than did tissues from placebo-treated controls. The magnitude of this hormone response, however, was approximately 2-fold less than observed with glands from rats with sham-pituitary surgery.

Impact of androgen exposure on SC synthesis by lacrimal gland acinar cells in vitro [17,18]

To determine whether androgens might directly stimulate the synthesis of SC by acinar cells from the lacrimal gland, cells were cultured on Matrigel in defined media for up to 12 days. Exposure of acinar cells to testosterone or dihydrotestosterone significantly increased cellular SC synthesis by a 2- to 4-fold factor, relative to that of control (C) cultures (Table 2). This response, which was elicited in the presence or absence of serum (10% fetal bovine serum) in the culture medium (Table 2), was both dose- and time-dependent, did not involve enzymatic conversion of testosterone to dihydrotestosterone and could not be accounted for by alterations in cell proliferation. In addition, the hormone action was specific for androgens: estradiol, progesterone, dexamethasone or aldosterone administration (10^{-6} M) had no effect on SC output.

DISCUSSION

The current investigation demonstrated that testosterone significantly enhances the level of IgA, but not IgG, in tears of orchiectomized rats. This androgen action coincided with an accumulation of IgA in the lacrimal gland, apparently through increased production by, rather than augmented recruitment of, IgA-containing cells. In parallel with the elevation in lacrimal gland IgA content, testosterone stimulated the movement of IgA against a gradient from lacrimal tissue to tears. This latter effect was most likely mediated through the androgen-induced synthesis and secretion of SC by acinar cells from the lacrimal gland.

These results extend our understanding of the endocrine regulation of the secretory immune system. At present, it is known that non-androgenic hormones and neuropeptides modulate multiple aspects of mucosal immunity in other sites, including the: 1) migration, tissue retention and proliferation of lymphocytes; 2) the transport and/or production of IgA and IgG antibodies; 3) synthesis and output of SC by epithelial cells and hepatocytes; and 4) mucosal defense against defined infectious agents [reviewed in 1]. The precise mechanisms underlying the site- and hormone/peptide-specific control of the secretory immune system remain to be determined.

Table 2. Androgen control of SC synthesis by acinar cells from the lacrimal gland

	Media SC (ng)	
Treatment	Serum-Free Media	Serum-Containing Media
Control	179 ± 81	739 ± 81
Testosterone	673 ± 38 **	1755 ± 257 *
Dihydrotestosterone	700 ± 42 **	1974 ± 335 *

Acinar cells (2×10^6 cells/well; n = 5/group) were plated on reconstituted basement membranes in defined serum-free or serum (10% fetal bovine)-containing media [18] with designated steroid hormones (10^{-6} M) or equivalent amount of ethanol (control). Numbers represent the mean $\pm$ SE of media SC synthesized during culture days 4 to 8. Significantly ($p < 0.01$)* or ($p < 0.0001$)** greater than control value. Data from [18].

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Modulation of the *in vivo* anti-rotavirus humoral immune response by neuroendocrines and neuropeptides

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ABSTRACT

Neuropeptides and neuroendocrine hormones (neurotransmitters) have been shown to modulate immune responses both *in vitro* and *in vivo*. Since reproduction and lactation are regulated by reproductive hormones, we investigated whether neurotransmitters could enhance anti-rotavirus immunity in milk. Rotavirus-free mice were immunized orally with killed bovine rotavirus (BRV) and boosted by giving BRV orally four weeks after the first immunization. Post-whelping, each dam (10 mice/group) was given a single injection of either prolactin (PRL) estrogen or testosterone. The effect of neuropeptides, substance P (SP) and somatostatin (SS) was also studied on serum and lactogenic anti-rotavirus humoral immune responses. The neuropeptides were administered using miniosmotic pumps. Control mice were treated at the same time with hormone or neuropeptide solvent alone. Immunized non-treated mice served as an additional control group. Blood and milk samples were collected over a period of 21 days and anti-rotavirus antibody titers were determined by an enzyme-linked immunosorbent assay (ELISA) and virus neutralization test. The results of this experiment revealed that in the groups given PRL or estrogen, there was enhancement of anti-rotavirus antibody titers both in serum and milk. In contrast, testosterone had a negative effect both on serum and milk antibody titers. The administration of neuropeptide SP also resulted in some enhancement of lactogenic antirotavirus antibody titer at day 9 post-whelping whereas an opposite effect was observed with administration of SS. A separate experiment revealed that a dose of 100 μg/mouse given on the first day post-whelping produce maximum enhancement of antibody both in serum and in milk. In order to investigate whether the enhancement of anti-rotavirus antibody was truly due to PRL and not by other factor(s), bromocriptine (BCR), a dopamine receptor agonist which inhibits the release of PRL at the level of the anterior pituitary, was used as a control. Mice given BCR exhibited a drastic reduction in anti-rotavirus antibody both in serum and milk samples measured by ELISA. The role of neurotransmitters in the modulation of lactogenic immune response and its significance in protection of neonates from enteric infections is discussed.

INTRODUCTION

Rotaviruses are the single most important causative agent of acute neonatal enteritis in most avian and mammalian species including humans. Rotavirus-induced enteritis

resulting in diarrhoea is most common during the first month of life in animals whereas children are more prone to rotavirus infection between 6 months and 2 years of age[6].

The economic and public health impacts have generated an impetus world-wide to develop an appropriate vaccine against these viruses[5,7].

Since enteric infection in animals takes place at a very early age, before they are able to mount an active immune response, it is of paramount significance that they passively acquire neutralizing antibodies from the mother. This can be achieved by hyperimmunizing mothers for passive transfer if protective antibodies in colostrum and milk to suckling neonates. Passive protection studies carried out in animals in our laboratory (Ijaz *et al.*, in preparation) and elsewhere[10] have shown that the continuous presence of rotavirus antibodies in the lumen of the intestine of neonates can prevent clinical disease associated with rotavirus infection. Since many mammalian species do not continue to secrete high levels of antibody in their milk for long periods of time, the antibody level in the intestinal lumen of the offspring drops rapidly, and the animals become fully susceptible even though they have acquired high levels of serum antibody[1].

It has been demonstrated both *in vivo* and *in vitro* that a number of neurotransmitters can influence the function of various lymphocyte populations probably by interacting with specific receptors on their lymphocyte[11,13]. It has been demonstrated *in vivo* that PRL, growth hormone and SP possess immunomodulatory properties whereas corticosteroids are immunosuppressive[2,12]. Sex-related differences have also been reported in different human infections indicating that males may be more susceptible to infection[8]. Lastly, there is mounting evidence to suggest that immune responses may regulate neuroendocrine functions which in turn could influence immune functions[11]. This gave us the impetus to study the effect of exogenous administration of neurotransmitters on the anti-rotavirus humoral immune response. In the present communication we report that exogenous administration of PRL or estrogen given on the day of whelping can significantly enhance anti-rotavirus antibodies in serum and milk of mice. In addition, we demonstrated that continuous administration of a neuropeptide, SP, results in enhancement of anti-rotavirus antibody in milk. Our data provide the first direct evidence that neurotransmitters can modulate the rotavirus specific antibody response *in vivo*.

MATERIALS AND METHODS

Animals

Rotavirus-free mice (CD-1) were purchased from the Charles River Breeding Laboratories (Wilmington, MA). Sera from the mice were tested for anti-rotavirus antibodies using ELISA assay and all mice were found to be seronegative. To maintain them rotavirus-free, the animals were housed in isolation throughout the experiment (20 °C and 60% relative humidity), in a room with cycled light system (light 12 h; dark 12 h) and with free access to sterile food and water.

Antigen Preparation and Immunization of Dams
Bovine rotavirus C–486 (UV-inactivated) was used for immunization of dams. The procedure for the preparation of virus and immunization of dams has been described previously[9].

Hormones and Neuropeptides
Estrogen (B-estradiol-3-benzoate, lot 95F-0166), testosterone (lot 65F-0054) and ovine PRL (20-50 international units/mg, lot 26F-00762) were obtained from Sigma Laboratories (St Louis, MO). Sterile olive oil was used as a vehicle for the lipid-soluble hormones (estrogen and testosterone) whereas PRL was dissolved in sterile phosphate buffered saline (PBS) (0.1M pH = 7.6). Neuropeptides SP (lot 124F-59205) and SS (lot 96F-0651) were also obtained from Sigma Laboratories (St. Louis, MO) and dissolved in PBS. A complimentary sample of BCR (lot 85111) was received from Dr Michele Beaulieu, Sandoz Canada Inc., Dorval, Quebec, Canada. It was dissolved in 70% ethanol in the presence of tartaric acid (1:10 w/w) and diluted in PBS to a concentration of 10 mg/ml.

Experimental Design
On the day of whelping, all vaccinated and control mice were divided into groups (10 mice per group) and each group (immunized and their corresponding control-non-immunized) received a single injection of one of the following hormones or combinations; estrogen (2 μg or 0.2), prolactin (100 μg or 10 μg), estrogen and prolactin (2 μg estrogen plus 100 μg prolactin or 0.2 μg estrogen plus 10 μg prolactin) or testosterone (5 mg or 500 μg). Neuropeptides SP and SS were administered continuously for two weeks using miniosmotic pumps (Alza Co., Palo Alto, CA) implanted subcutaneously. These pumps containing neuropeptides were installed 5 days after whelping. The rate of delivery of the pumps was 1 μl/hour (1 μg). Post-whelping, samples of blood and milk were taken on day 1, 5, 9, 14 and 21 and processed as described previously[9].

Enzyme-linked Immunosorbent Assay and Virus Neutralization (ELISA)
The levels of anti-rotavirus antibody in serum and milk samples were determined by a homologous ELISA and the ability of the samples to neutralize BRV was determined as described previously[9].

Western Blotting of Rotavirus Polypeptides
Antibodies to individual viral proteins were investigated by the Western-blotting technique described by Burnette[4]. Viral proteins solubilized in a non-reducing sample buffer and separated on a 10% polyacrylamide gel, were transferred to nitrocellulose paper (0.45 μm) (Bio-Rad Lab, Richmond, CA) by electroblotting at 250 mA 1 h at room temperature in a buffer containing 20 nM Tris – 190 mM glycine -

20% methanol v/v. After transfer, reaction of viral proteins with serum and milk samples were determined as described by Braun *et al* [3].

RESULTS

Influence of Neurotransmitters on Anti-Rotavirus Humoral Immune Response

To determine if exogenous administration of PRL or estrogen would modulate the rotavirus-specific antibody response in serum or milk, female mice were orally immunized with UV-inactivated BRV and boosted 3 weeks later. Their serum anti-rotavirus response was monitored by ELISA and VN. Animals were bred 6 weeks post-immunization. Post-whelping, each group of ten mice was treated according to one of the schedules reported in *Materials and Methods*. Control mice were treated at the same time with hormone (by injection) or neuropeptide solvent alone by mimiosmotic pumps. Immunized non-treated mice served as an additional control group. Samples of serum and milk were collected and analyzed for anti-rotavirus antibody titers (Figures 1 and 2).

The administration of high doses of PRL (100 μg/mouse) resulted in significant enhancement of anti-rotavirus antibody titers both in serum as well as in milk compared to immunized controls (Figure 1A). Mice injected with estrogen (0.2 μg/mouse) had increasd antibody responses in milk and serum collected 5 days post-whelping but no effect was seen in milk collected on day 9, 14 and 21 (data not shown), whereas testosterone administration reduced anti-rotavirus antibody titers most significantly in milk (data not shown). To determine that the enhancement of anti-rotavirus was truly due to PRL and not by other factor(s). BCR, a PRL inhibitor was used. Mice given BRC exhibited a drastic reduction in antirotavirus antibody both in serum and milk samples as measured by ELISA (Figure 1B). The group receiving SP showed an enhancement of anti-rotavirus antibody titers in milk collected on day 9 post-whelping, but no effect was observed in serum antibody titers as determined by ELISA and VN (Figure 2A). SS showed no effect on serum antibody levels, but there was a significant reduction in milk antibody titer on day 9 post-whelping (Figure 2B).

Effect of Administration of Neurotransmitters on the BRV-Polypeptide Specific Humoral Immune Response

Earlier studies carried out in our laboratory have clearly demonstrated that two outer capsid proteins (VP43 and VP7) and an inner capsid protein (VP6) of rotavirus independency induce neutralizing antibodies and may be important in neutralization of the virus *in vivo*. Following oral administration of mice with BRV the major response is against VP6 (data not shown). In order to investigate whether the weak humoral immune response to VP4 and VP7 could be potentiated by administration of neurotransmitters, serum and milk samples were tested in a Western blotting assay. Following administration of PRL, antibody responses to VP4 and VP7 in serum and milk were observed in addition to VP6. Similarly, following administration of SP

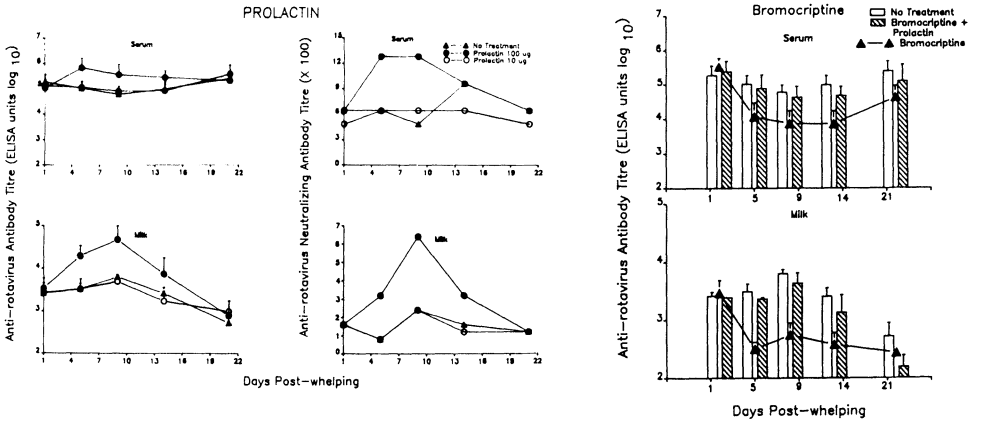

Figure 1A The effect of PRL on anti-rotavirus antibody response in lactating mice. A group of ten mice were immunized orally with inactivated BRV and boosted orally three weeks later. Animals were bred, and one day post-whelping, each animal received a single injection of PRL or a placebo as described in Materials and Methods. Serum and milk samples wre collected and anti-rotavirus antibody titers were determined by ELISA and VN tests. The results are expressed as the means ±SD of triplicate determinations. (B) The antagonistic effect of BCR administration and restoration by co-administration of PRL and BCR on the ant-rotavirus antibody titers in lactating mice. A group of ten mice were injected three times with BCR (250 μg/mouse) starting at day 1 post-whelping and every 2 days thereafter. Another group of ten mice were co-administered with PRL and BCR. The methods of sample collection and analysis were the same as described above.

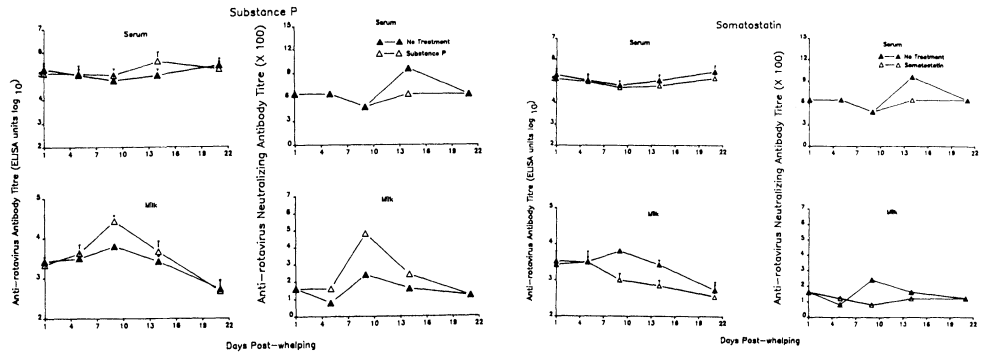

Figure 1B The effect of continuous administration of SP (A) and SS (B) on anti-rotavirus antibody response in lactating mice. A group of ten mice received SP(24 μg/day/mouse) continuously for 14 days by means of miniosmotic pumps. The minipumps containing SP were implanted subcutaneously on day 5 post-whelping. Serum and milk samples were collected and anti-rotavirus antibody titers were determined by ELISA and VN tests. The results are expressed as the means of ±SD of triplicate determinations.

additional responses to VP4 and VP7 were observed. Estrogen administration revealed an additional response to VP7 in serum and with no observed response in milk. In contrast, neurotransmitters which produced an adverse effect on immune responses reduced anti-rotavirus responses especially to VP6. BCR treatment also reduced the humoral immune response to VP6 as determined by Western blots (data not shown).

DISCUSSION

The immune process has generally been regarded as self-regulating, controlled by a number of mechanisms. Recent studies have indicated that neurotransmitters can also regulate mucosal immunity[2,12]. Since reproduction and lactation are regulated by neurohormones, we investigated whether neurotransmitters could potentiate anti-rotavirus antibodies in milk. Neuropeptides were selected for this study since their concentration in mucosal secretions have been found to be ten times higher than in blood. Furthermore, they have been reported to play an immunomodulatory role both *in vitro* and *in vivo* [12,13]. The results of these experiments demonstrate that the levels of anti-rotavirus antibodies in serum and milk can be enhanced by neurohormones (PRL and estrogen) and a neuropeptide (SP) if given the day after whelping. Furthermore, the increase in serum antibody levels was directed against all three major rotavirus proteins. In contrast, milk antibody responses were directed against all three proteins following PRL administration but only against VP6 following neuropeptide SP administration. Antibody responses to VP7 in milk are of significance because this protein has been shown to be involved in virus attachment to the host cell[7] and hence neutralizing antibodies to this protein prevent virus-host interactions required for infection. Therefore, the potentiation of a weak anti-rotavirus polypeptide specific antibody response by exogenous administration of PRL may be very important for passive protection of suckling neonates.

In addition to helping understand the role of neuroimmunomodulators in regulating lacteal immunity, the data obtained in the present investigation has clinical relevance. For example, it has been demonstrated that local antibody production by lymphocytes and transfer of immunoglobulin in uterine[15], lacteal[16] and lacrimal secretions[14] can be modulated by exogenous administration of PRL. If given at an appropriate dose, PRL can enhance anti-rotavirus antibody in lacteal secretions and help in providing sufficient antibodies during the period when suckling neonates are most susceptible to enteric infection and hence reduce these infections.

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Altered responsiveness to fed or injected protein in piglets given antigen at birth

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1. INTRODUCTION

We have previously demonstrated that piglets weaned onto a soya-based diet develop serum IgG antibody to major soya proteins. In addition, we have shown that oral administration of an acid precipitate of the soluble soya proteins to neonatal piglets can suppress this response, piglets given 100 or 1000 mg of protein having significantly reduced antibody levels. This reduction in antibody level also appears to be associated with a change in affinity.

Similar experiments in mice have suggested that oral administration of antigen to neonates results in priming for responses to systemic challenge (Hanson 1981). In contrast, older piglets appear to be capable of developing systemic tolerance to fed proteins (Newby et al, 1979; Bailey et al, in preparation). We attempted to determine whether antigen given to neonatal piglets results in the development of systemic tolerance as well as unresponsiveness to fed protein. This experiment would also indicate whether the serum antibody response to soya feeding was the product of a mucosal or systemic response.

2. MATERIALS AND METHODS

Farrowings were supervised and all piglets removed from the sow before being allowed to suckle. Piglets were given one gram of acid-precipitated soya protein in phosphate buffer by stomach tube and subsequently returned to the sow. Control piglets were given buffer only. At three weeks old half of the piglets were weaned onto a soya based diet and the remaining half were injected intraperitoneally with 2 milligrams of soya protein in saponin (Quil A).

All piglets were bled serially following injection or weaning and serum IgG anti-soya antibody was measured by indirect ELISA

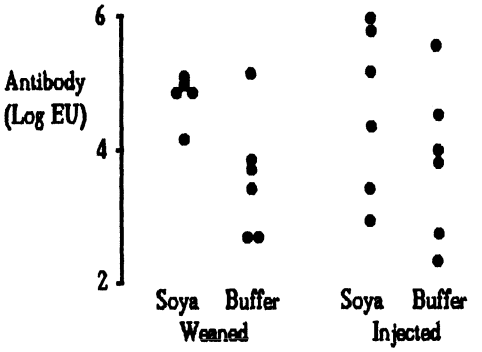
For statistical analysis, serum IgG antibody levels from day 11 after challenge were used for blocked analysis of variance.

3. RESULTS

All piglets developed detectable increases in serum IgG anti-soya antibody. In control piglets given buffer only at birth the increases following weaning or injection were comparable. Similarly, the mean responses to feeding and injection were both reduced in soya-stomach

tubed piglets.

Statistical analysis indicates a significant effect of neonatal exposure (soya or buffer) but no detectable effect of type of challenge (feeding or injection) nor interaction between neonatal exposure and challenge. The statistics therefore suggest that the feeding of soya to neonatal piglets has resulted in a similar suppression of the antibody response to systemic, as well as oral challenge.



However, an examination of the actual values for serum antibody 11 days postweaning shows a high degree of variation within groups (Figure 1). While the statistical analysis would take this into account, we feel that this result requires confirmation and the experiment is currently being repeated.

4. DISCUSSION

The results presented confirm that relatively small amounts of antigen given to neonatal piglets can affect the subsequent response to fed protein. The evidence also suggests that such piglets produce less antibody following subsequent injection. If this is the case then neonatal exposure to antigen may have resulted in a state of antibody tolerance mediated by a component common to the systemic and intestinal compartments. Alternatively, the antibody response following weaning may be a systemic response to absorbed antigen. That it is linked to a mucosal response is suggested by separate experiments in which we have shown that this normal antibody response to weaning is followed by an unresponsiveness to systemic antigen (similar to classical orally induced tolerance in mice) rather than by systemic priming. However, it may be that the induction of tolerance following weaning and the development of the antibody response occur in different compartments of the immune system.

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Effect of aging on induction of oral tolerance by intragastric administration of sheep red blood cells in mice

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ABSTRACT. Fed SRBC induced oral tolerance in young C3H mice but not in the aged. An injection of SRBC via portal vein did not tolerize C3H mice. Gut associated local immune system seems to play a key role in induction of oral tolerance and the tolerance inducing function declines with aging.
Mucosal immune system plays important roles in preventing invasion by viruses, bacteria, and other parasites. Oral injection of live virus generates local and systemic immunity (1). However, fed antigen often induces oral tolerance (2). Systemic immune system in mice shows age-associated resistance to tolerance induction by tolerogen via parenteral route (3). Here, we studied oral tolerance induction by SRBC in the systemic immune system of young and aged mice.

Materials and Methods

Mice. C3H/HeSlc and NZB/BlNJ mice were used.
Antigen injection. Mice received intragastric injections of $1x10^9$ or $8x10^9$ SRBC in 0.5ml saline or of 0.5ml saline with an intragastric tube every day for 7 or 14 days. All mice were immunized intraperitoneally with $2x10^8$ SRBC 7 days after the final oral administration and anti-SRBC antibody forming cells in spleens were enumerated by plaque forming cell (PFC) assay, 4 days later. Some mice were injected with $1x10^8$ SRBC in 0.2ml saline or with 0.2ml saline via portal vein under an anesthetic.
PFC assay. Antibody-forming cells were measured by direct and indirect methods of plaque assay in agarose gel.
Statistical analysis. Differences were considered significant if P was less than 0.05 by Mann-Whitney U-test.

Results

Orally injected 10^9 SRBC tolerized neither 2 mo old nor 8 mo old mice. Then, oral injection of $8x10^9$ SRBC for 14 days

induced a tolerance in the systemic immune system of 2 mo old mice. By contrast, the same treatment induced a strong IgG memory in the older animal (Table 1).

TABLE 1. Oral tolerance induction in C3H mice

Age in months	SRBC feeding	Anti-SRBC PFC/spleen ($\times 10^3$) IgM	IgG
2	-	160±24	0
2	8×10^9,14d	76±10	0
12	-	192±34	0
12	8×10^9,14d	15±6	86±15

The same SRBC treatment did not tolerize but immunize both 2 mo and 9 mo old NZB mice which show early resistance to parenteral tolerance induction (Table 2).

TABLE 2. NZB resist oral tolerance induction

Age in months	SRBC feeding	Anti-SRBC PFC/spleen ($\times 10^3$) IgM	IgG
2	-	398±32	17±17
2	8×10^9,14d	226±74	262±65
9	-	4±1	0
9	8×10^9,14d	192±93	8±8

Two mo and 21 mo old C3H mice were injected with 10^8 SRBC via portal vein. All mice were challenged and PFC assay was carried out as described. Both young and aged mice did not become tolerant but generated eminent IgG memory (Table 3).

TABLE 3. SRBC via portal vein immunize C3H mice

Age in months	SRBC injection	Anti-SRBC PFC/spleen ($\times 10^3$) IgM	IgG
2	-	133±11	0
2	10^8	9±2	191±93
21	-	14±1	0
21	10^8	13±2	100±10

Discussion

Tolerance induction by parenteral injection of antigens becomes increasingly difficult with age, especially, in autoimmune-prone strains. Here, we observed similar age-associated increase in resistance in the oral tolerance. It is not clear how fed antigens induce the oral tolerance in the systemic immune system. Liver may take an important part in it (4). However, results in Table 3 do not support the possibility. Mucosal immune system probably plays a key role in the induction of oral tolerance.

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The induction of oral tolerance in germ free and conventional Balb/c mice

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1. Introduction

Germ free animals are a useful model to study specific responses to antigens uncomplicated by a complex normal flora. Some aspects of immunity have been shown to be comparable in germ free and conventional animals (Tlaskalova et al 1983) whilst others have shown a reduced ability of germ free mice to express certain cell mediated reactions (Veda et al (1975). With regard to oral tolerance, some previous studies have suggested, using sheep red blood cells as an antigen, that oral tolerance cannot be induced in the germ free animal (Kiyono et al 1982, Wannemuehler et al 1982), whilst others using different antigens have shown that oral tolerance can be induced but not maintained (Moreau and Corthier 1988). The objectives of this study were to compare humoral and cellular responses in germ free and conventional mice, and to examine the effect of prior intragastric immunisation with soluble antigens on this response.

2. Materials and Methods

Conventional and germ free balb/c mice were used in groups of eight animals and matched for age and sex. All mice were between 8 and 14 weeks of age. Serum antibodies to ovalbumin were induced by intraperitoneal immunisations two weeks apart using the same antigen preparation in both series, using 100ug OVA in complete adjuvant. Serum was collected one week after the second immunisation. Antibodies of all three isotypes to OVA were assayed by ELISA as previously described (Challacombe et al 1987). Cellular immunity was induced by sub-cutaneous immunisation at the base of the tail by two injections two weeks apart and draining popliteal and inguinal lymph nodes were taken five days later. A lymphocyte proliferation assay was performed as described earlier (Challacombe and Tomasi 1980). The results of individual conventional animals were pooled and compared with those of the equivalent germ free group..

For the induction of oral tolerance, mice were given two daily intragastric injections of 10mg OVA in saline 7 days before immunisation for humoral or cellular responses as above.

3. Results.

IgG antibodies in germ free mice were approximately 35% of the levels found in conventional animals (mean 400 units compared with 1200 units). (Fig 1) Similarly serum IgA and IgM antibodies in germ free mice appeared to be about one third of those in conventional animals (fig 1). The relative affinity of the antibodies, assayed by the DEA dissociation technique of Devey and Steward (1988), appeared to be similar.

Cellular proliferative responses to OVA were also markedly reduced in germ free animals, being approximately 30% of those found with equivalent numbers of cells in conventional animals (fig 1. Mitogen responses (PHA, PWM and CON-A) however were similar in the two groups.

Prior intragastric immunisation with OVA reduced the humoral responses of all three isotypes in both germ free and conventional mice. When this reduction was expressed as a percentage of the respective control, then it could be seen that the reduction was by a similar percentage, even though the absolute counts were much lower in germ free animals (fig 2). Similarly cellular

responses were much lower in germ free animals but the reduction in those given prior oral immunisation, when expressed as a percentage of the positive control, was similar to that found in conventional mice (fig 2).

4. Discussion

Both humoral and cellular antigen specific responses are markedly reduced in germ free animals and in this respect our findings are consistent with those previously reported (Veda et al 1975, Moreau and Corthier 1988). This reduction in humoral responses seems to be a quantitative rather than a qualitative one since the relative affinity of antibodies appears to be similar. This appeared to be true of all three antibody isotypes. Presumably this reduction reflects not only the marked reduction in the total lymphoid tissue in these animals, but also the lack of the amplifying effect on immune responses of a normal gastrointestinal flora (Wannemuehler et al 1982). Interestingly, when equal amounts of lymphoid calls were cultured in the proliferative assay, antigen specific responses were reduced, but no diminution in the mitogen responses was found in germ free mice.

The results also showed unequivocally that in contrast to some reports oral tolerance to soluble antigens can be induced in the germ free mouse and is of a similar order to that of conventional animals. The germ free mouse could therefore be useful as a model for studies of oral tolerance.

fig 1

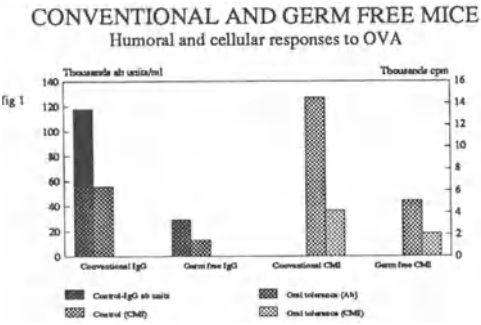
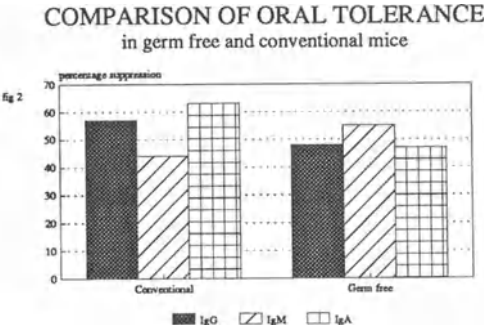


fig 2



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Prevention of collagen-induced arthritis by oral administration of encapsulated Type II collagen

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ABSTRACT. The oral administration of type II collagen (CII) either in solution or encapulated within stable lipid vesicles, before the conventional intradermal arthritogenic challenge with CII in Freund's Incomplete Adjuvant, lowered the incidence and reduced the severity of collagen-induced arthritis. The resistance to arthritis was accompanied by less severe delayed type hypersensitivity reactions to CII and lower titres of serum IgG2b anti-CII antibodies, when compared with arthritic rats. It is concluded that the tolerogenic properties of CII are retained upon encapsulation and that stable plurilamellar vesicles may be suitable vehicles for the oral administration of tolerogens.

Introduction

Type II collagen (CII) induces a polyarthritis in rats, when injected intradermally in Freund's Incomplete Adjuvant (FIA) [1]. CII is also a potent tolerogen, capable of preventing the induction of arthritis when administered intravenously [2]. Apart from the appropriate parenteral administration of CII we have shown that the pergastric administration of CII, by gavage, either before [3] or after [4] the conventional intradermal arthritogenic challenge with CII in FIA, lowers the incidence and delays the onset of collagen-induced arthritis. Although the phenomenon of orally-induced hyporesponsiveness or tolerance is well established it has only recently been exploitated in the regulation of autoimmune disease.

The antigen specificity [5], fate of the orally administered CII [6], immunological responses to the arthritogenic challenge and the cellular basis of the orally-induced hyporesponsiveness to CII [4] have been described. However, not every rat gavaged with CII is resistant to the arthritogenic challenge. This study aimed to increase the efficacy of the orally-induced tolerance to CII by its encapsulation in lipid vesicles. A hydroxyl-rich synthetic lipid was used that readily formed plurilamellar vesicles which were stable in 0.1M acetic acid and in the presence of bile salts. These vesicles had a high encapsulation efficiency for CII. The relative abilities of soluble CII and encapsulated CII to protect rats from a subsequent arthritogenic challenge were compared and found to be equivalent.

Materials and Methods

Preparation and encapsulation of CII. Soluble native CII was prepared from bovine nasal septa by papain digestion and differential salt precipitation and from the rat Swarm chondrosarcoma by differential salt precipitation [4]. The CII was encapsulated within stable plurilamellar vesicles (SPLV) by the addition of soluble CII to an ether solution of the desired lipid, the mixture was then emulsified in a water bath sonicator under a stream of nitrogen. The resulting suspension was finally shaken while being briefly warmed above the transition temperature (70°C) of the lipid. Efficient encapsulation of the CII within the vesicles was verified by electron microscopy. A 95% encapsulation efficiency was established by estimating the relative concentration of residual CII in an inhibition ELISA. The inhibition of the binding of a monoclonal anti-CII antibody to CII by the residual CII was compared to a standard curve.

Immunization of rats and assessment of arthritis. Inbred 12 week old male WA/KIR rats were obtained from the Kennedy Institute, Hammersmith, UK. 1mg of CII or encapsulated CII (splv/CII) was administered, pergastrically by gavage, daily for five consecutive days. The rats were challenged intradermally with 0.45mg CII in FIA 24 hours after the last gavage (day 0). Clinical symptoms of arthritis were recorded daily as previously described [4]. DTH reactions to CII were assessed by measuring the increase in ear thickness 48 hours after an intradermal challenge with 40ug of bovine CII. Serum antibody levels against both bovine CII and rat CII were determined by ELISA [3] and are expressed as mean $\log_{10}$ mid-point titres. Antibodies of different IgG subclasses were quantitated by ELISA utilizing mouse monoclonal anti-rat immunoglobulin subclass antibodies (Serotec) detected with a biotinylated rat anti-mouse IgG reagent (Jackson Laboratories) and streptavidin/HRP (Amersham International).

Results

Lipid vesicles have been reported to disintegrate in the presence of bile salts [7] thus it was important to chose a vesicle system that was stable both at a low pH and in the presence of bile salts. The choice of the lipid and the nature of the vesicle selected was based on studying the leakage of ^{14}C-inulin from multilamellar and plurilamellar vesicles formed by various natural and synthetic lipids in the presence of both 0.1M acetic acid (pH 2.7) and either 10mM sodium taurocholate or normal rat bile. Plurilamellar vesicles of the hydroxyl-rich synthetic lipid used in the studies described here had a high encapsulation efficiency and did not disintegrate in the presence of 0.1M acetic acid and 10mM sodium taurocholate, they retained 44% of their entrapped marker after 2 hours at 37°C in under these conditions.

The oral administration of such stable plurilamellar vesicles, containing a total dose of 5mg of CII, to rats prior to an arthritogenic challenge with CII in FIA, lowered the incidence and reduced the severity of the arthritis in an manner analogous to soluble CII (Table 1). Likewise both DTH reactions to CII and serum anti-CII antibody levels were decreased in rats gavaged with encapsulated CII (Table 1).

TABLE 1. A Comparison of the effects of orally administered CII and encapsulated CII on the incidence, time of disease onset, severity of arthritis and immune responses to the arthritogen (bovine CII).

Gavage days -5 to-1	Incidence day 35	Day of onset (mean ± sd)	Severity day 35 (% max poss)	DTH day 14 (mean ± sd)	IgG anti-CII day 20 (mean ± sd)
acetic acid	100% (11/11)	19.0 ± 5.74	37%	1.14 ± .395	3.470 ± .181
CII	57% (4/7)*	20.5 ± 9.45	30%	0.58 ± .600#	3.251 ± .412
SPLV/acetic acid	90% (9/10)	18.2 ± 6.69	40%	1.07 ± .275	3.521 ± .247
SPLV/CII	50% (6/12)*	21.1 ± 8.07	30%**	0.57 ± .362#	3.098 ± .263

*p<0.05 Fisher test; **p<0.02 Mann-Whitney U test; #p<0.05 Student's t-test

A detailed analysis of the immunoglobulin subclass and antigen specificity of the serum anti-CII antibodies 20 days after the arthritogenic challenge revealed that rats gavaged with either CII or encapsulated CII had significantly lower titres of anti-CII antibodies of the IgG2b subclass, against both the immunizing bovine CII and autologous rat CII (Table 2).

TABLE 2. Isotype and antigen specificity of anti-CII serum antibodies 20 days after the arthritogenic challenge.

	anti-bovine CII		anti-rat CII	
Gavage	IgG2a	IgG2b	IgG2a	IgG2b
acetic acid	4.062 ± .199	2.929 ± .134	3.466 ± .170	2.488 ± .111
CII	3.982 ± .458	2.026 ± .405*	3.698 ± .363	1.174 ± .381*
SPLV/acetic acid	4.027 ± .283	2.838 ± .314	3.646 ± .264	2.534 ± .375
SPLV/CII	3.844 ± .336	2.046 ± .443*	3.334 ± .375	1.648 ± .409*

*p<0.001 Student's t-test, all other combinations p>0.05

Discussion

When administered pergastrically by gavage, encapsulated CII was found to render WA/KIR rats refractory to an otherwise arthritogenic challenge of CII in FIA. The incidence of arthritis was lowered and those rats that did become arthritic had a less severe disease with a slightly delayed onset. Furthermore, rats gavaged with encapsulated CII had less severe DTH reactions to CII and lower levels of serum IgG2b anti-CII autoantibodies.

The encapsulation of CII did not lead to a loss of its original tolerogenic properties. That no distinction can be made between the effects of encapsulated CII and soluble CII shows that the efficacy of the orally-induced tolerance was not noticeably enhanced by the encapsulation of the CII. Furthermore, it is important that the encapsulating lipid did not exacerbate the arthritis. Not all lipids may be suitable for the oral administration of tolerogens since they may be potent adjuvants or arthritogenic in their own right as has been demonstrated for the synthetic lipoidal amine avridine [8].

The particular stable plurilamellar vesicle system designed and utilized in these studies will probably prove valuable in the induction of an orally-induced state of hyporesponsiveness to antigens or peptides that would odinarily be degraded in the gut. Indeed the use of vesicles to encapsulate myelin basic protein (MBP) may be an alternative to bicarbonate buffers and trypsin inhibitors that have been used in the pergastric administration of MBP to protect rats from the susequent induction of experimental allergic encephalomyelitis (EAE) [9]. A further advantage of the vesicles employed in this study is their stability at low pH and in the presence of bile salts which avoids stabilizing them by glutaraldehyde crosslinkage, a strategy known to improve the ability of parenterally administered vesicles containing MBP to prevent EAE [10].

This study highlights the therapeutic potential of pergastrically administered tolerogens by the demonstration that vesicles containing antigen are potent tolerogens, with no detrimental autoimmunogenic attributes, when administered pergastrically.

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Failure to induce oral tolerance to protein antigens by oral administration or by serum transfer of "adult gut processed" antigen in neonatal mice can partially be corrected by spleen cell transfer

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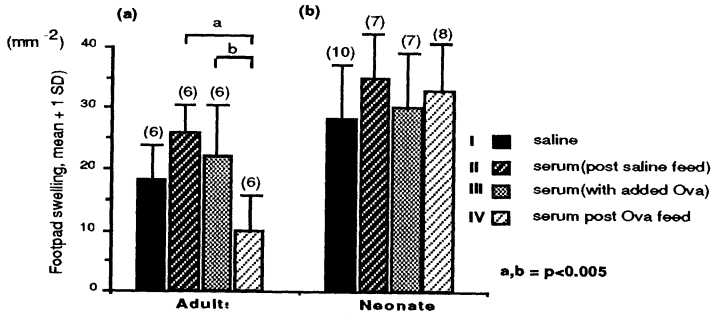
Abstract and Introduction. A regulatory defect in the induction of oral tolerance has been postulated in the pathogenesis of food hypersensitivity and it has been suggested that the early postnatal period is particularly vulnerable [1,2,3].
We have examined the underlying mechanisms which prevent the induction of oral tolerance to protein antigens in neonatal mice (BALB/c). Serum collected from adult mice 1 hour after feeding OVA was adoptively transferred to mice (1, 3 42 days). Whereas DTH was suppressed in *adult* recipients ($p<0.005$), no suppression of systemic DTH was found in *neonatal* recipients. Adult splenocytes were also transferred intraperitoneally 24 hours before a feed of OVA to neonates. Significant suppression ($p<0.001$) was observed *only for DTH* indicating that spleen cell transfer only partially confers adult-type reactivity.

The **experimental protocol** is outlined below:

SERUM TRANSFER:	**Neonate**	**Procedure**	**Adult**
(40µl/g body weight)	Day 0	i.p. injection	Day 0
	Day 28	Immunisation	Day 7
	Day 49	Test (Ab, DTH)	Day 28
SPLEEN CELL TRANSFER (10^8)	Day 1	i.p. spleen cells	
	Day 2	OVA,BSA (1mg/g) feed, than as above	

Figure 1: DTH responses after transfer of "gut processed" antigen



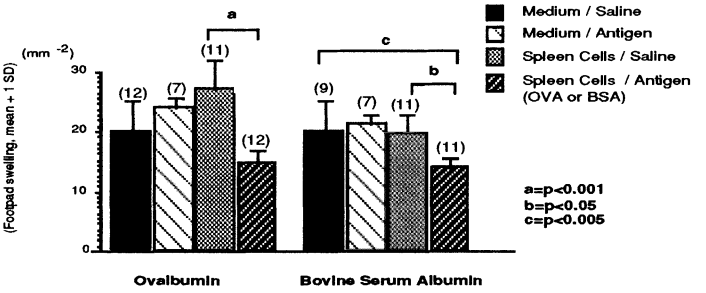
BALB/c mice (8-10 weeks) were fed an OVA and BSA free diet.The offsprings were treated within 24 hours of birth. OVA and BSA were fed by gavage at 1mg/g body weight. Serum was injected intraperitoneally (i.p.) at a dose of 40µl/g body weight [4,5].

Control animals received injections of either saline alone or pooled mouse serum "spiked" with ovalbumin (70ng/ml).

RESULTS: ***DTH suppression after serum transfer.*** Groups of neonatal or adult recipients were injected i.p. with either (i) saline, (ii) serum from saline-fed animals, (iii) serum "spiked" with OVA, or (iv) serum from OVA-fed animals.Serum anti-OVA IgG antibody responses did not differ significantly although *DTH responses* of adult recipients of 'active' serum (OVA-fed donors) were significantly *lower* than those of mice receiving serum from saline-fed donors or mice receiving serum "spiked" with OVA ($p<0.005$ for both comparisons; **(Figure 1)**. In contrast, there were no differences between the systemic DTH responses of the four **neonatal** experimental groups (panel b,$p>0.05$), suggesting that *adoptive transfer* of "gut- processed" OVA from adult mice to neonatal mice *does not confer tolerance* for systemic DTH.

Oral tolerance after adoptive spleen cell transfer.
Following the above protocol, there was no significant difference between specific IgG antibody levels to ovalbumin or BSA. In *contrast*, the groups of **neonatal** mice fed OVA and BSA on day 2 showed significant suppression of their DTH response ($p<0.001$- $p<0.02$; **Figure 2** for both antigens.

Figure 2:DTH responses after spleen cell transfer and an antigen feed



Discussion:

Previous studies (1,2,5) have shown that oral antigen administration to neonatal mice does not lead to oral tolerance and we have speculated that this might be due to the immaturity of the antigen processing capacity of the neonatal gut. Our studies demonstrate that failure of OT induction in neonatal mice is not due to a deficiency of local mucosal antigen processing capacity of the neonate, but due to an as yet unspecified (gut associated ?) immaturity of the neonatal immune system [see ref. 5].

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Oral tolerance mediated by dextrin-specific suppressor T cells, migrating from Peyer's patches to spleen

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Introduction

The excessive antigenic stimulation in GALT -feeding of soluble proteins or particulate T-cell dependent antigens or thymus independent II antigens such as dextran B1355S- leads to the induction of T helper cell dependent IgA responses at the mucosa together with the appearance of T cells which home to the systemic lymphoid tissues mediating oral tolerance [1, 2]. We have previously demonstated that rats which were protein deprived at weaning and subsequently casein refed, exhibited alterations of IgA B cell differentiation in GALT, accompanied by an abnormal increase in the $CD8^{+}$ T cell population [3]. Moreover a DTH response to dextrin could not be elicited suggesting that tolerance to a dietary antigen has developed. The aim of this study, was to further investigate this unresponsiveness.

Materials and Methods

MLN and Sp T cells were prepared according to Julius [4]. Antigen specific T-cells (dextrin-adherent) were obtained by slight modifications of the panning technique using dextrin-coated Petri dishes. DTH to dextrin, casein and ovoalbumin antigens was assessed by the degree of footpad swelling ellicited by challenge with specific antigen. The increase in footpad thickness was measured with a skin caliper SM 112 (Teclock, Japan). X±SE represents the mean value between footpad thickness before and 24h after the antigenic challenge.

Results

PP, MLN and Sp cells from donor rats -protein deprived at weaning and refed with a 20 % casein diet- when transferred i.v. into normal recipient rats inhibit the induction of the DTH response to dextrin comparable to that observed in control animals that had not recieved cells. Sp T cells as well as dextrin-adherent Sp T cells and a low number of MLN T cells between ($5.5x10^{5}$) when transferred into normal recipients were able to supress the DTH response to dextrin ($p<0.01$). But, a higher number of MLN-T cells ($1x10^{7}$) transferred into recipient rats enhanced the DTH response to dextrin ($p<0.05$) (Table 1)*.

TABLE 1.

Source of cells	Nº of cells	Dextrin	DTH±SE to Casein	Ovoalbumin
None (control)	- - -	0.42±0.06(13)	0.60±0.06(7)	0.64±0.14(6)
PP: normal	$0.3x10^6$	0.41±0.07(4)	0.60±0.06(5)	0.60±0.14(5)
PP: experimental	$1.2x10^6$	0.19±0.05(9)*	0.58±0.01(3)	0.59±0.09(3)
Sp: normal	$0.8x10^7$	0.47±0.03(5)	0.60±0.10(4)	0.61±0.10(4)
Sp: experimental	$3.5x10^7$	0.18±0.02(16)*	0.50±0.08(4)	0.58±0.10(4)
Sp-T: experimental	$1.5x10^7$	0.20±0.06(8)*	0.50±0.14(3)	0.52±0.17(6)
Sp-T: (dextrin-adherent)	$2.1x10^6$	0.20±0.04(6)*	- - -	- - -
Sp-T:(dex.non-adherent)	$2.4x10^7$	0.47±0.10(6)	- - -	- - -
MLN: normal	$0.7x10^7$	0.51±0.04(5)	0.61±0.31(4)	0.60±0.10(4)
MLN: experimental	$3.5x10^6$	0.23±0.03(18)*	0.61±0.31(4)	0.60±0.10(4)
MLN T: experimental	$5.5x10^5$	0.17±0.07(5)*	0.62±0.31(4)	0.68±0.02(5)
MLN T: experimental	$1.0x10^7$	0.64±0.09(4)	- - -	- - -

Phenotypic characterization of dextrin adherent Sp T cells indicated that 47% belongs to the CD8 subpopulation as compared to 18% found in the dextrin non adherent Sp T cells ($p<0.02$). Besides, MLN T cells could be isolated as Vicia Villosa$^+$ and Vicia Villosa$^-$ populations. The Vicia Villosa$^+$ poulation abrogated the hyporesponsiveness to dextrin in recipient rats and was mainly 0X22$^+$ (CD45R) when compared to Vicia Villosa$^-$ population ($p<0.005$) (Data not shown).

Discussion

Dextrin, due to its polysaccharide structure may behave like dextran (a type II T cell independent antigen). The above results seem to indicate that specific Ts cells induced by dextrin in the PP migrate to the Sp, via the MLN leading to oral tolerance. Besides the presence of Vicia Villosa$^+$ MLN T-cells bearing the CD45R phenotype (Tinf or TH population) is able to abrogate this oral tolerance. This seems to indicate the development of an intestinal DTH response, as reported by Mowat [1].

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Effect of breast feeding on the development of anti-idiotype antibody response to F glycoprotein of respiratory syncytial virus

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ABSTRACT. Groups of lactating BALB/C mice were immunized in the postpartum period with high doses of monoclonal antibody (Ab-1) to the F-glycoprotein (F.Gp) of respiratory syncytial virus (RSV). The immune response to F.Gp was studied in the breast feeding infant mice of such mothers at regular intervals before and after weaning. All infant mice exhibited anti-F.Gp activity in serum, which was detected until 6 wk of age. Splenic cells of such breast feeding infant mice collected after weaning exhibited in vitro synthesis of antibody against Ab-1, the antibody previously used for maternal immunization. Subsequent immunization with homologous purified RSV F.Gp resulted in a booster response for IgG, IgM, and plaque-neutralizing antibody to the immunizing RSV protein and to the whole virus in the infants primed via breast feeding. These data demonstrate induction of RSV-specific anti-idiotype antibody in the neonates via the process of breast feeding.

INTRODUCTION. Respiratory syncytial virus (RSV) is the most common cause of serious respiratory illness in young infants. Although several live attenuated and inactivated virus candidate vaccines have been tested to date, effective immunoprophylaxis against prevention of disease with this agent remains to be developed. In view of the difficulties encountered to date in developing effective RSV vaccine using conventional approach with whole virus to prevent serious disease in the infant population, we undertook studies to examine alternate approaches to induce active immunity to RSV in infants, using transfer of RSV-specific idiotypic immunity, without direct exposure to the whole virus or viral proteins.

MATERIALS AND METHODS. BALB/C mice were mated 15 to 25 week of age. On the 4th and 8th day after the delivery of the neonate, groups of lactating female mice and their suckling neonates were immunized according to the following protocol. Group a and b consisted of 30 to 40 lactating mice each, who were immunized with 500 or 0.25 μg of a monoclonal antibody to F glycoprotein (MAF) of RSV administered i.p. in

saline. As controls, additional lactating animals were immunized with 500 μg of monoclonal antibody to G glycoprotein (MAG) in saline (group C) or saline alone (group d). In group a and b, five to seven suckling mice were killed without additional treatment at regular intervals for 6 to 7 week after birth. Other infant mice were weaned at 3 week of age and 1 week later were challenged intraperitoneally with a single dose of 1.5 μg of F glycoprotein of RSV in a saline suspension. Group e consisted of suckling mice whose mothers had received no RSV immunization and they were inoculated with a polyclonal Ab-2 against MAF in a dose of 50 μg/ml protein administered intraperitoneally at 2 and 3 week of age. These infants were subsequently challenged with F glycoprotein of RSV as described above.

Specimen collection : Specimens of serum were obtained by retroorbital bleeding. Spleens were removed aseptically and sterile, single-cell suspensions were prepared by standard techniques. 10^7 spleen cells were cultured at 37°C in 2 ml of RPMl 1640 medium for 7 days and assayed for RSV antibody synthesis.

Ab-1 antibody : MAF (clone B-2-1) and MAG (clone C2) RSV antibody used in this study had been prepared in BALB/c mice in our laboratory and the characteristics were studied in detail previously (Tsutsumi et al. 1987). The Ig isotype of MAF was found to be IgG2a and possessed complement-independent neutralizing activity of 1 : 512 against live RSV.

Preparation of polyclonal Ab-2 against MAF : Coupling of MAF to KLH was done according to the method of Bona et al. (1979). Male DBA/2 mice were immunized by whole MAF conjugated with KLH and emulsified with complete Fluind Adjuvant and the serum collected was subjected to protein A column chromatography. The Ab-2 exhibited anti-Ab-1 titer of 1 : 16396 when tested against MAF in a cross-linked ELISA antibody assy. However, no reactivity could be detected between this Ab-2 and MAG. In competitive inhibition assay, the binding of biotin-labeled MAF to F glycoprotein was only inhibited by sera obtained from animals immunized with MAF but not with MAG. These result suggest that sera from immunized DBA/2 mice with MAF contained paratope-associated anti-idiotype which competed with antigen for the binding site on Ab-1.

Purification of F glycoprotein of RSV : The F glycoprotein derived from the long strain of RSV was prepared by affinity column chromatography, using MAF coupled sepharose 4B columns.

Detection of anti-F glycoprotein antibody response : The development of IgG and IgM anti-F glycoprotein antibody responses were assayed by ELISA.

Ab-2 determination : The induction of Ab-2 antibody response to MAF was measured by using an Ab-1 cross-linking ELISA assay with MAF $F(ab')_2$ preparation.

Detection of neutralizing antibody : Neutralizing antibody titers against RSV were determined by a plaque reduction assay in Hep-2 cell culture monolayer in 24-well microtiter plates using 100 PFU/μl RSV long strain.

RESULTS. After weaning at 21 days of age, no antibody activity was detected in the serum of those infants whose mothers had received 0.25 μg of MAF. However, ELISA IgG anti-F glycoprotein activity was regularly observed for up to 42 days of age and neutralizing activity persisted to 24 to 26 days of age in those infant mice of mothers who had received 500 μg of MAF post partum. Similarly, the spleen cell culture supernatants from the infants in group b or group d exhibited no Ab-2 activity against MAF. However, Ab-2 activity was consistently observed in the spleen cells of intants of mothers who had been immunized with 500 μg of MAF (group a). The Ab-2 antibody activity appeared to peak in the spleen cells collected at 4 week of age.

The effect of subsequent challenge immunization with purified F glycoprotein in the infants of mothers who had received MAF post-partum and in infants of control mothers who were inoculated with MAG or saline during the post-partum period are presented in Table. The anti-F glycoprotein antibody and plaque neutralizing antibody to the whole virus were significantly enhanced in the group a mice whose lactating mothers had received 500 μg MAF and the response appeared to be specific against the epitopes of F glycoprotein recognized by MAF. This is evidenced by significant inhibition of the binding of biotin-labeled MAF to the F glycoprotein by such post-immune infant sera, compared to the serum of non-immunized controls or infants whose mothers received 0.25 μg MAF or MAG or saline control post-partum.

The polyclonal Ab-2 against MAF was administered at 2 and 3 week of age to infant mice whose mothers had received no prior immunization. These infants were subsequently challenged with F glycoprotein at 4 week of age. A significant enhancement for IgM and IgG anti-F glycoprotein and of RSV specific neutralizing antibody activity was observed in the Ab-2 primed animals when compared to non-primed or placebo controls.

Development of RSV antibody response in infant mice after active immunization with F-gp of RSV at 4 wk of age

Immunization Group	Anti F-gp ELISA Antibody Titer (Mean±SE)*		RSV Neutralizing Antibody Titer (mean±SE)*	Inhibition of Binding of Ab-1 to F-gp (% Decrese in OD)**
	IgG	IgM		
a	197±9	1097±102	17±1	57±1
b	114±6	206±11	5±0.3	27±2
c	126±6	229±12	5±0.2	NT
d	104±4	208±8	5±0.2	28±1
e	NT***	103±6	NT	NT

* Expressed as reciprocal of dilution.
** Inhibition assay used a serial dilution of 1/100.
*** NT, not tested.

DISCUSSION. The observation of particular importance reported here is that high doses of maternal antibody transferred via breast feeding induced anti-idiotypic antibody, and subsequent immunization with the original viral protein (F glycoprotein) resulted in a specific priming effect for anti-F glycoprotein response, even in the presence of maternal antibody. It is suggested that transfer of protective idiotypes from the mother to the child could provide a dual effect to infants : priming the suckling neonate for a booster effect on subsequent exposure to antigen and at the same time, offer immediate passive protection against induced infection.

It must, however, be emphasized that fundamental differences exist between the human and the mouse suckling infants, in regard to the uptake and systemic transport of antigen, immunoglobulin or T cell products or other macromolecules present in the suckled milk or in the infants intestinal lumen. Essentially no uptake of milk immunoglobulin has been demonstrated during breast feeding in the human neonates, although trace amounts of milk IgA antibody may be detectable in the serum of infants under 18 h of age fed artificially, with milk containing high levels of IgA (Ogra et al. (1977)). However, the bulk of neonatal IgG and all its subclasses appear to be transmitted to the fetus transplacentally in latter parts of gestation (Leissring et al. (1962)). Therefore, the implications of breast feeding-associated transfer of anti-idiotypic immunity in man must be applicable at the level of mucosal-associated lymphoid tissue and the regulation of the immune response at the mucosal surface of intestine and possibly respiratory tracts. However, this model could also be applied to the transplacental idiotype transfer and priming for subsequent neonatal immunization.

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Effect of Peyer's patch excision on intestinal immune response to cholera toxin in rats

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ABSTRACT. Effect of Peyer's patch (PP) excision on immunocompetent cells was evaluated in rats in order to elucidate the role of Peyer's patch in intestinal immune response. The number of lymphocyte transported through intestinal lymph was significatly decreased after PP-deprivation. IgA containing cell number was remarkably decreased in intestinal mucosa, while other immunoglobulin classes or lymphocyte subpopulation was not significantly altered. When specific immne response against cholera toxin(CT) was examined, anti-CT immunoglobulin A secreting cells in intestinal lymph by ELISPOT assay and anti-CT antibody containing cells in intestinal mucosa were significantly suppressed after PP-deprivation.
We revealed that PP plays an important role in antigen specific immune response and lymphocyte transportation.

INTRODUCTION

Gut-associated lymphoid tissue(GALT) plays an important role in the intestinal immune defense against various antigens including bacteria, toxins and dietary antigens. PP are considered to be the sourse of IgA plasma cells. These will be necessary for adequate antigen presentation on mucosal site and for the homing properties of lymphoid cells. However, the precise role of PP is not fully understood. This study was designed to elucidate the role of PP in the intestinal immune response using experimental model of PP deprivation. The alteration of lymphocyte transport through intestinal lymph and immune response to CT were investigated.

MATERIALS AND METHOD

Male Wistar rats weighing 250g were used for the experiments. All visible PP were surgically removed from the serosal side. Sham operation was done in control rats. Two weeks after the operation, animals were used for the study. Intestinal lymphatics near cisterna chyli was cannulated according to the method of Bollman(1). Saline was infused into jugular vein at 2.4ml/hr. Flow volume and the number of lymphocytes were measured in intestinal lymph.

Lymphocyte subsets in peripheral blood and intestinal lymph were assessed by fluorescence activated cell sorter using monoclonal antibodies; W3/13-HLK(pan T), W3/25(helper/inducer T), OX8(suppressor/cytotoxic T) and OX6(Ia positive). The population of lymphocytes in the lamina propria of small intestine was determined by immunohistochemistry. Tissue sections were fixed in PLP solutions. After inhibition of endogenous peroxidase activities, T cell subsets, W3/13, W3/25, OX8 were stained by indirect immunoperoxidase method and immunoglobulin containing cells were stained by

direct method. Data were expressed as positive cells per 1000 mononuclear cells in the lamina propria.

Rabbits were immunized subcutaneously with purified CT and IgG fraction of sera was purified, then HRP was labeled by glutalaldehyde method. Two weeks after PP-deprivation or sham operation, purified CT (10μg/100gB.W.) was primed to rats intraduodenally according to the method of Pierce (2). Two weeks later CT was challenged intraduodenally and 5 days later experiments were done. To determine anti-CT antibody containing cells, small intestinal tissue sections fixed in PLP solution were used. After inhibition of endogenous peroxidase, tissues were incubated with CT(lμg/ml) for 1 hour and then reacted with peroxidase-labeled anti-CT antibody.

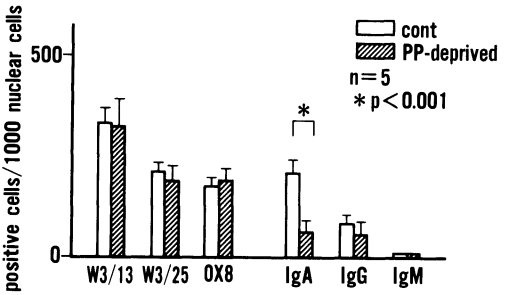
ELISPOT assay of Czerkinsky et al (3) was used for the specific antibody production against CT in intestinal lymph lymphocytes. Petri dishes were coated with purified CT(10μg/ml) and lymphocytes collected from intestinal lymph (10^6/ml with 5%FCS) were incubated for 6hrs at 37°C in 10% CO_2. Washing with PBS, HRP-labeled anti-rat IgA was added. After reation with paraphenylenediamine and H_2O_2 in agar in PBS, dark spots were counted.

RESULTS

There was no difference in intestinal lymph flow between PP-deprived and control rats, but the number of lymphocytes transported through lymphatics was significantly decreased in PP-deprived rats (control; (42.7±9.5) x 10^6 cells/hr vs PP-deprived ;(25.5±6.1) x 10^6cells/hr), suggesting the disturbed lymphocyte traffic through intestinal lymph. Lymphocyte subset of peripheral blood and intestinal lymph determined by FACS showed no significant difference between two groups. In small intestinal mucosa, T-cell subsets in lamina propria were not significantly different between two groups. However, it is noted that IgA containing cells in lamina propria was remarkably decreased in PP-deprived rats(Figure).

When specific immunological response against CT was observed in two groups, it is shown that anti-CT antibody containing cells in lamina propria of intestine and antibody secreting cells in intestinal lymph were significantly decreased in PP-deprived groups.

Anti-CT antibody containing cells in lamina propria by immunoperoxidase technique showed that the number of peroxidase-positive cells per 10000 mononuclear cells were 16±7 in PP-deprived groups and 37±10 in control groups. Anti-CT IgA secreting cells in intestinal lymph determined by ELISPOT assay were 66±15 cells per 10^6 cells in PP-deprived groups and 102±24 cells in control groups.



Mean±SD

FIGURE. Lymphocyte subsets and immunoglobulin containing cells in lamina propria of the small intestine.

DISCUSSION

Our present study revealed that Peyer's patches may have a crucial role in the lymphocyte transportation and in the antigen specific immune response in the intestinal mucosa.

There is now evidence that IgA secreting cells in the gut is maintained by the recirculation of lymphocytes which enter the blood by way of thoracic duct lymph and then migrate from blood into intestinal mucosa (4). With regards to lymphocyte migration, it is known that B-cells migrate from blood into lymph nodes and PP via the high endothelial venules (5).

Steer (6) described the output of lymphocytes from an area posessing a Peyer's patch to be 10 times greater than in lymph from an area lacking one, using a technique for the collection of lymph from the lacteals of the rat small intestine. In our study although PP was revealed to be important for the sourse of recirculating lymphocytes, there was no selective changes in certain lymphocyte subsets in intestical lymph or intestinal mucosa after PP deprivation.

There are paucity of data how PP are concerned with antigen specific immune response. We have shown that response to CT, which has been a most valuable antigen for the determination of mucosal immunoglobulin A response, was significantly inhibited in PP-deprived rats.

Deprivation of PP might cause the decrease of IgA precursor cells and regulatory T cell dysfunction because of the migration disturbances. On the other hands, an inadequate antigen presentation, for example, lacking of antigen uptake by M cells is supposed to be responsible for the decrease response to CT. However, it is also interesting that even all visible PP were excised, certain degree of antigen specific immune response still appeared to be remained. The failure of complete inhibition of producing anti-toxin antibody suggested the participation of mucosal site other than PP. In this respect intraepithelial lymphocytes and solitary lymphoid follicles might be responsible for the mucosal immune response after PP-deprivation.

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Rabbit ileal lamina propria mononuclear cells suppress the primary *in vitro* antibody response of spleen cells to KLH

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Intestinal lamina propria (LP) mononuclear cells have been found to suppress the peripheral T-cell response (human) and the spleen T-cell response (rabbit) to PHA. Rabbit spleen mononuclear cells have been shown to respond to primary in vitro immunization with KLH giving an IgM antibody response. The purpose of this study was to determine what effect rabbit ileal LP mononuclear cells would have on this primary in vitro antibody response to KLH.

1. MATERIALS AND METHODS

1.1.1 In Vitro Immunization. NZW rabbit spleen cells were used following RBC lysis. Spleen cells ($6x10^5$/ml) were cultured in DMEM (GIBCO, Grand Island, NY) with 10% heat inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 ug/ml) and gentamycin (40 ug/ml) at pH 7.4 in 0.2 ml volume in round bottom microculture plates (Costar #3799, Cambridge, MA) with 5% CO2 at 37° for 7, 9, 12 and 14 days. Autologous rabbit ileal lamina propria mononuclear cells (1) were placed into co-culture at either 0, $1.5x10^4$, $3x10^4$ or $6x10^4$/ml with the spleen cells in triplicate. KLH (Calbiochem, 374805) was added as an immunogen to each cell culture combination in triplicate in the following ng/ml doses: 0, 0.15, 0.5, 1.5, 5.0, 15, 50 and 150. The culture supernatant fluids were harvested and frozen at -70°C.

1.1.2 Enzyme-Linked Immunosorbent Assay (ELISA) for Specific Rabbit IgM Anti-KLH Antibody. Culture supernatants were added to KLH coated ELISA plates and incubated 18 hours at 4°C. Peroxidase conjugated affinity purified goat anti-rabbit IgM (Cappel, Melvern, PA) was added and incubated 60 minutes at 4°C. Substrate (2, 2^1-Azino-di-(3-ethyl benzthiazoline)sulfate), Solution A, and hydrogen peroxide, Solution B, (Kirkeguard and Perry, Gaithersburg, MD) were mixed and added then incubated one hour at room temperature. Optical density was read at 405 NM wave length.

1.1.3 Enzyme-Linked Immunosorbent (ELISA) for Total Rabbit IgM Immunoglobulin. Culture supernatants were added to affinity purified goat anti-rabbit IgM (Cappel, Melvern, PA) coated ELISA plates in multiple dilutions. Polyclonal affinity purified rabbit IgM (Cappel, Melvern, PA) added as a standard in duplicate wells using 8 dilutions for a standard curve then incubated for 18 hours at 4°C. Peroxidase conjugated affinity purified goat anti-rabbit

IgM antibody was added and incubated for 60 minutes at 4°C. Substrate was added and then incubated for one hour at room temperature. Optical density read at 405 NM wave length. Data processing done using a standardized reference curve and Immunosoft computer program (Dynatech, Alexandria, VA). Results are expressed in micrograms (mcg)/ml.

2. RESULTS

The higher KLH dose (5-150 ng/ml) response was suppressed by all three added LP cell concentrations as seen in a representative experiment in Figure 1. Total IgM levels in additional experiments were determined on supernatants from all cell combinations and found to be reduced by the addition of LP cells particularly at the 5% level as seen in Figure 2.

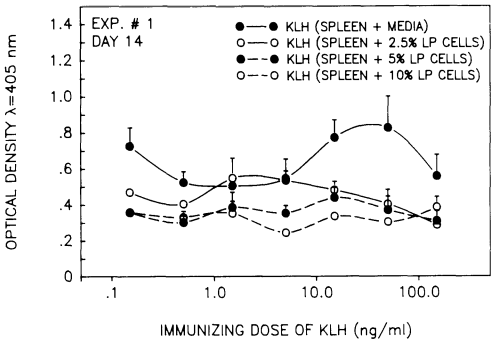

Figure 1. Suppression of spleen cell IgM anti-KLH response by LP cells.

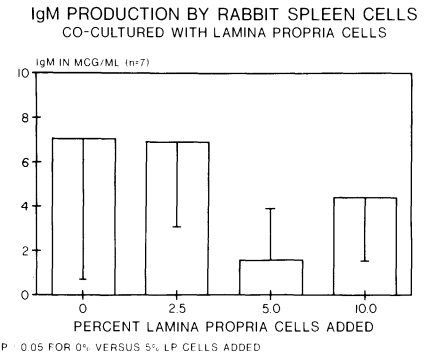

Figure 2. Suppression of spleen cell total IgM production by LP cells.

3. DISCUSSION

Rabbit ileal LP cells suppressed the spleen cell IgM specific anti-KLH response. This suppression could be directed toward the T-helper cell because of the known generalized suppression of spleen T-cells by LP cells. However, this suppression probably represents a generalized suppression of spleen B-cells rather than specific B or T cell suppression because of the suppression of IgM from unstimulated spleen cells. In conclusion, the rabbit ileal LP mononuclear cells suppress autologous spleen cell primary in vitro anti-KLH IgM response by a generalized suppression of spleen cell IgM production.

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Suppression of immune-mediated otitis media by mucosa-derived suppressor T cells

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1. Introduction

It has been established that an antigen antibody reaction or immune complex formation in the middle ear cavity, followed by complement activation, can be a pathogenic mechanism in otitis media with effusion (OME)[1]. On the other hand, the results of many studies support the idea that the tubotympanum is protected against microbial infections at the mucosal surfaces by the secretory immune system[2]. It is well known that there are important regulatory interactions between systemic humoral immunity and the mucosal immune system[3]. The oral administration of thymus-dependent antigens has been proven to induce the formation of regulatory T cells in Peyer's patches, which suppresses IgG/IgM production and enhance IgA production. Therefore, we investigated the effects of these mucosa derived suppressor T cells on the induction of IgG immune mediated in mice.

2. Materials and Methods

C_3H/HeN female mice bred in a specific pathogen free condition were used for experiments at 8 weeks of age. Crystallized ovalbumin (OVA) was used as a thymus-dependent antigen. For induction of immune-mediated otitis media, mice were preimmunized intraperitoneally with 500 ug of OVA emulsified in complete Freund's adjuvant (CFA). Twenty days after systemic immunization, 30 ug of OVA distilled in 0.8% hydroxypropyl cellulose (HPC) solution was inoculated into left tympanic cavity through the inferior bullae. The mice were killed 4 days after the inoculation of antigens. Induction of mucosa derived suppressor cells and their transfer were carried out according to the method of Richman[3]. Details were described previously[4]. To evaluate the extent of the inflammatory reaction in the middle ear, the tympanic membranes were observed before sacrifice under a surgical microscope. After sacrifice of the mice, the bullae were processed for the histological analysis. OVA-specific antibody titers in sera and middle ear effusions were measured by ELISA.

3. Results

The effect of mucosa derived suppressor T cells on the induction of IgG immune mediated OME was examined. Fractionated spleen cells were used as mucosa derived suppressor cells. Recipient mice were transferred with 2 x 10^7 fractionated spleen cells 1 day before systemic immunization. When splenic T cells from OVA fed mice were transferred, anti-OVA IgG antibody n production was suppressed. But it was not suppressed when splenic non-T cells were transferred (Figure 1). Otitis media was demonstrated in eight of the mice when splenic non-T cells from OVA fed mice were transferred as well as in nine mice

transferred with splenic T cells from saline fed donors. By contrast, otitis media was demonstrated in only one of ten mice when splenic T cells from OVA fed donors were transferred. These data suggest that IgG immune mediated OME can be suppressed to some extent by mucosa derived suppressor cells from antigen-fed mice.

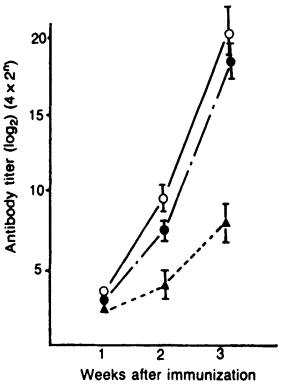

Figure 1. Kinetics of antiovalbumin IgG antibody in sera of mice transferred with splenic T cells from saline fed donors (○), splenic from ovalbumin fed donors (●), and splenic non-T cells form ovalbumin fed donors (▲).

4. Discussion

We attempted successfully to prevent immune mediated otitis media in mice by immunologic procedures with mucosa derived IgG specific suppressor T cells. Present results indicate that the suppression of otitis media in recipient mice can be attributed to the decreasing IgG antibody titer in the sera of mice that were transferred with mucosa derived suppressor T cells. These results strongly suggest that mucosal immunization can be effective in preventing OME because mucosa derived regulatory T cells are beneficial not only in augmenting mucosal immunity (IgA response) but also in preventing IgG immune mediated inflammatory reactions in the middle ear.

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SECTION G: MOLECULAR BIOLOGY OF THE IgA SYSTEM

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Molecular biology studies of human transmembrane secretory component: cDNA cloning and mRNA expression

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ABSTRACT. A 2.5 kilobase (kb) cDNA clone containing 92% of the coding region for human transmembrane secretory component (SC) was isolated from a mammary gland cDNA library. The cDNA clone encoded a protein of 693 amino acids which showed 54% similarity with the deduced amino acid sequence of rabbit transmembrane SC [1]. Northern blot analysis showed mRNA in human tissues in good agreement with SC protein expression, and secretory IgA (SIgA) transport [2].

1. Introduction

Human SC occurs in three molecular forms: as the transmembrane receptor protein (approx. 100 kD); as a stabilizing component chain (approx. 80 kD) of SIgA and SIgM (bound SC); and as free SC (approx. 80 kD) in exocrine secretions [2,3]. These unique properties of SC make it a very interesting protein. The structure of rabbit transmembrane SC has been deduced after cDNA cloning [1], whereas comparable structural information about human SC has been limited to classical protein sequencing of free SC [4]. Here we describe a partial cDNA clone including the transmembrane and cytoplasmic segments of human SC.

2. Experimental Procedures

cDNA cloning: A human mammary gland lambda gt11 cDNA library was screened [5] with a nick-translated cDNA probe for the rabbit transmembrane SC (kindly provided by Dr. Keith E. Mostov, Whitehead Institute for Biomedical Research, Cambridge, MA, USA).

Nucleotide sequencing: DNA sequencing was performed with the dideoxy chain termination method [6].

Northern blot analysis: Total RNA (20 μg) from various human tissues was isolated by the guanidinium isothiocyanate method [7], electrophoresed, blotted onto a nylon membrane, and hybridized with a nick-translated human transmembrane SC cDNA probe [5].

3. Results and Discussion

A cDNA clone (2.5 kb) containing 92% of the coding region for human transmembrane SC or poly-Ig receptor, was isolated from a mammary gland cDNA library [5]. This clone encoded a protein of 693 amino acids (Fig. 1) which showed 99% similarity with the primary amino acid sequence of human free SC as reported by Eiffert et al. in 1984 [4].

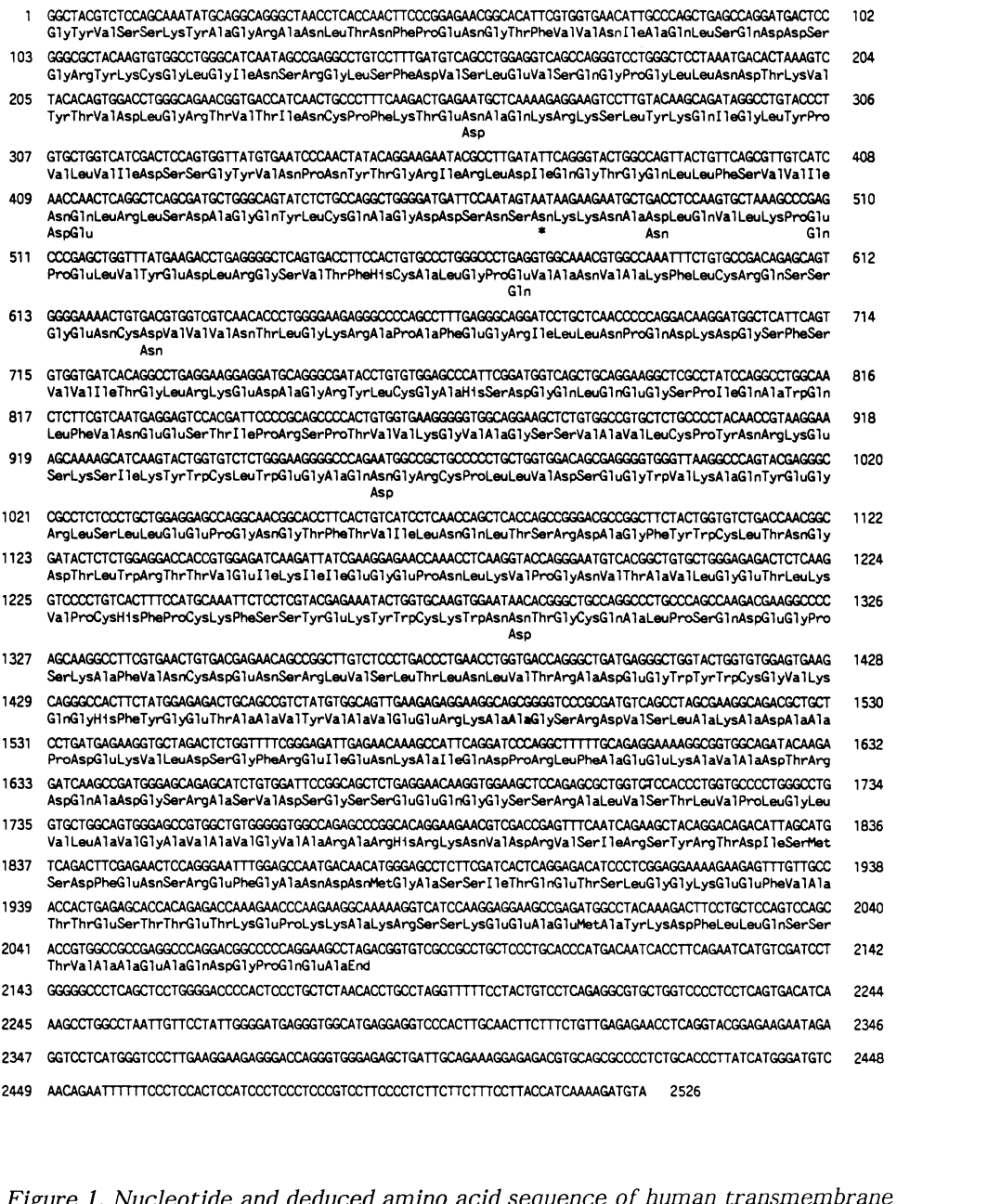

Figure 1. Nucleotide and deduced amino acid sequence of human transmembrane SC. The amino acids are shown in a three-letter code on the second line. The star on the third line (position 158) represents an additional aspartate whereas the indicated amino acids represent other discrepancies compared with the primary amino acid sequence of free SC as reported by Eiffert et al. [4].

The overall amino acid similarity with the sequence deduced for rabbit transmembrane SC was 54% [1]. The similarity was 50%, 74%, and 73% in the extracellular, membrane-spanning, and intracellular segments, respectively (Fig. 2). There was no apparent similarity in the transition between the fifth and the sixth domain postulated for rabbit SC [1]. In fact, there was a shortage of 15 amino acids in our deduced amino acid sequence. However, the second half of the postulated sixth domain was well conserved, especially within the proposed membrane-spanning segment. Determination of the exact site of physiological cleavage of free SC from the transmembrane receptor protein has been obscured due to a ragged C-terminus at the end of the fifth domain of free SC purified from human colostrum [4,8]. For rabbit SC, three putative cleavage sites have been proposed, located to the Ala-Glu dipeptides which occur three times upstream from the membrane-spanning segment [1,9]. Only one of these was found to be conserved in the deduced sequence of human SC, and may represent the actual cleavage site.

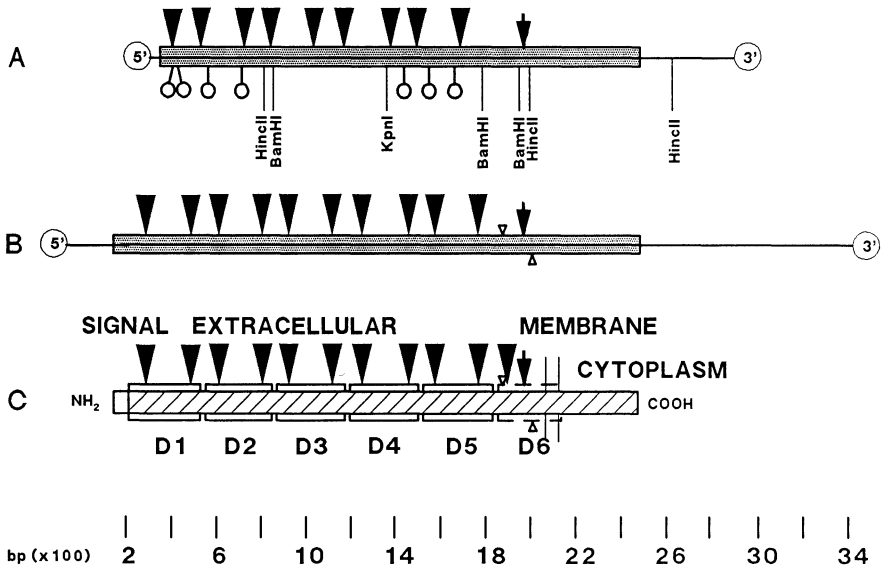

Figure 2. (A) Human SC cDNA from with some restriction sites indicated; (B) rabbit SC cDNA as reported by Mostov et al.; (C) Schematic depiction of putative domain (D1-D6) structure of rabbit transmembrane SC. Key: (►) Cysteine residues comprising the disulphide bonds. Carbohydrate-binding sites. (▷) Potential cleavage sites. (►) Conserved potential cleavage site in comparison with rabbit SC. (≡) Coding region. (—) Nontranslated region.

Northern blot analysis revealed one major SC mRNA form approx. 3.8 kb in size (Fig. 3). In human tissues strong hybridization signals were detected for duodenum, jejunum, colon and diseased kidney (uremic patient); weaker signals appeared for pancreas, lungs and endometrium. Conversely, SC mRNA was not detected in human liver, spleen, caput epididymis, prostate or myometrium. These results are in good agreement with our immunohistochemical studies of SC [2].

Furthermore, interferon- alone or in combination with tumor necrosis factor- α was found to upregulate SC mRNA expression in a human colonic cell line (HT-29), in agreement with our previous studies of SC protein synthesis [10].

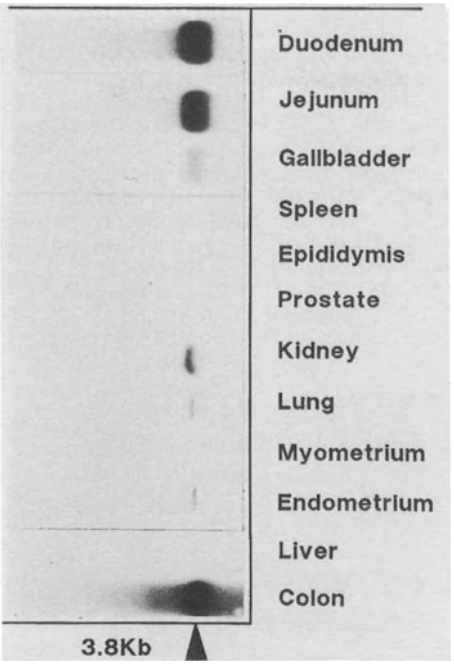

Figure 3. Northern blot analysis, adapted from Krajči et al. [5]. The hybridizing band, marked with an arrow, was estimated to be approx. 3.8 kb by comparison with RNA standards (Bethesda Research Laboratories).

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Functional expression of an Fc receptor cloned from neonatal rat small intestine

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ABSTRACT. Transmission of maternal IgG from milk across the intestinal epithelium of the suckling rat confers passive immunity. Transport is mediated by an epithelial cell Fc receptor. It is unidirectional because IgG can bind the receptor at the apical cell surface, exposed to the slightly acidic pH of the intestinal lumen, but not at the neutral pH of the serosal fluid at the basolateral membrane. We have cloned a cDNA encoding a putative Fc receptor from the small intestine of 11-day-old rats. Expression of this cDNA in rat and mouse fibroblast lines causes these cells to bind IgG with the pH dependence observed in the intestine of the neonate.

1. Introduction

Maternal IgG transmitted to fetal or neonatal mammals provides humoral immunity during the first weeks of independent life. In mice and rats there is some pre-natal transfer of IgG, by way of the uterine lumen and fetal yolk sac, but most is obtained from colostrum and milk. Receptors for the Fc region of IgG (FcRn, neonatal) mediate its internalization at the apical surface of intestinal epithelial cells of the suckling rat. The pH in the gut lumen is 6.0 - 6.5 (Rodewald 1976), which is optimal for binding of IgG to the FcR (Jones and Waldman 1972). IgG does not bind its receptor at the serosal fluid pH of 7.4 (Jones and Waldman 1972) and so dissociates when transcytosed to the basolateral membrane.

Fc receptors have been purified from intestinal epithelial cells of suckling rats in several laboratories (Rodewald and Kraehenbuhl 1984, Simister and Rees 1985, Jakoi, Cambier, and Saslow 1985). Detergent-solubilized intestinal FcR isolated by affinity chromatography on immobilized rat or human IgG is resolved by SDS-PAGE

under reducing conditions into components with M_rs of 45 - 53 (p51) and ~14 kd (p14). We identified the smaller component as β2-microglobulin (β2m) serologically and by partial amino acid sequence. Association of β2m with p51 was confirmed by cross-linking in intestinal epithelial cell brush borders by dithiobis(succinimidyl propionate) (Simister and Mostov 1989). Complementary DNAs were cloned that encode the Fc-binding subunit, p51: its predicted primary structure has three extracellular domains and a transmembrane region all homologous to the corresponding domains of class I MHC antigens (Simister and Mostov 1989). We report here that the expression of the p51 cDNA in rat and mouse fibroblast lines confers IgG binding, with the pH dependence observed *in vivo*.

2. Methods

2.1. EXPRESSION

Most of the 5' and 3' untranslated sequences were removed from the 1570 bp FcRn cDNA by digestion with MaeIII and the 5' overhangs were filled in using Klenow fragment. BamHI linkers were added. The resulting 1.2 kb construct was subcloned into the BamHI site of the retroviral expression vector pWE (R.C. Mulligan *et al.* unpublished.) and transfected into ψ2 or ψAM packaging cells (Cone and Mulligan 1984). Retroviral supernatants were used to infect murine 3T3 fibroblasts and rat1 fibroblasts. Clones resistant to G418 were selected and expanded (Korman et al. 1987).

2.2. METABOLIC LABELLING AND IMMUNOPRECIPITATION

The cell lines 3T3 and rat1 and their retrovirus-infected derivatives were maintained in DME with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin in 5% CO_2. For pulse-chase experiments, confluent or nearly confluent cell monolayers on 35 mm dishes were depleted of cysteine for 30 minutes in cysteine-free DME, supplemented with 5% FBS previously dialysed against 150 mM NaCl, and with 20 mM Na HEPES, pH 7.3. Cells were pulse-labelled for 30 minutes with 100 μCi/ml [^{35}S]-cysteine (Amersham, ~700 - 1100 Ci/mmol.) in the same medium. Labelled cells were harvested immediately (time zero), or after chasing the proteins labelled during synthesis through their maturation pathways for 1 - 4 hours in complete DME (which contains unlabelled cys).

To harvest the cells, we removed the labelling or chase medium and rinsed the dish with PBS. The cells were then scraped into 0.5 ml of SDS lysis buffer (0.5% SDS, 150 mM NaCl, 5 mM EDTA, 100 units/ml trasylol, 20 mM ethanolamine-HCl, pH 8.1), transferred to 1.5 ml microcentrifuge tubes and boiled for 5 minutes, cooled on ice and sonicated for 2 x 30 seconds (Branson sonicator with cup horn). 0.5 ml of 2.5% Triton dilution buffer (2.5% Triton X-100, 100 mM NaCl, 5 mM EDTA, 100 units/ml trasylol, 0.1% NaN_3, 50 mM ethanolamine-HCl, pH 8.6) was added to each lysate. Before immunoprecipitation, samples were pre-cleared with 2 μl of normal rabbit serum (1 hour at room temperature) and 100 μl of a 20% (v/v) slurry of protein A-sepharose (Sigma. 30 minutes at room temperature). The beads were spun down. The supernatants were transferred to new tubes and incubated with 2 μl of rabbit anti-p51 (Simister and Mostov 1989) (overnight, 4°C). Immune complexes were precipitated with protein A-sepharose as above. Beads were washed by brief centrifugation (10 seconds) and resuspension in 1 ml of wash buffer. Five washes in mixed micelle buffer (1% Triton X-100, 0.2% SDS, 150 mM NaCl, 5% w/v sucrose, 5 mM EDTA, 100 units/ml trasylol, 0.1% NaN_3, 20 mM ethanolamine-HCl, pH 8.6) and two in final wash buffer (15 mM NaCl, 5 mM EDTA, 100 units/ml trasylol, 0.1% NaN_3, 20 mM ethanolamine-HCl, pH 8.6) were done. Lastly, the beads were boiled in SDS-gel sample buffer. Eluted proteins were analysed on 10% polyacrylamide gels, which were then dehydrated in glacial acetic acid and fluorographed using 22% (w/v) PPO in acetic acid.

2.3. IgG BINDING AND INTERNALIZATION

Human IgG (ICN Immunochemicals) was labelled with Na[^{125}I] to a specific activity of 0.5 - 1 mCi/nmol using Iodogen (Pierce) according to the manufacturers instructions.

3T3 and rat1 fibroblasts and clones expressing FcRn were grown to confluence or near confluence on 35 mm dishes. The cells were first washed with PBS, 1 mM KI, pH 6.0 or 7.4, at 4°C. Then [^{125}I]-IgG (10^7 cpm/ml, ~10^{-8} M) in PBS, 1 mM KI, 1% ovalbumin, pH 6.0 or 7.4 with or without 0.5 mg/ml unlabelled IgG (3.3 x 10^{-6} M) was added to the dish. The cells were allowed to bind and internalize IgG at 37°C for 1 hour. Unbound ligand was removed with three washes of PBS, 1 mM KI, pH 6.0 or 7.4, at 4°C. Cells were dissolved in 2 x 0.5 ml of 0.1 M NaOH and transferred to vials. The bound radioligand was measured in a gamma counter. To correct the results for variations in the number of cells on dishes of different cell lines, binding data was normalized to total protein per dish. Protein in the cell solubilisates was determined by the BCA method (Pierce).

3. Results

3.1. EXPRESSION OF FcRn IN FIBROBLAST CELL LINES

Biosynthetic incorporation of [^{35}S]-cysteine into the products of retrovirus-infected 3T3 and rat1 cells shows that immunoprecipitable FcRn hc is made during a 30 minute labelling period as a 47 kD precursor that is converted during a 4 hour chase to a 52 - 60 kD protein (Figure 1), presumably by the maturation in the Golgi of high mannose N-linked oligosaccharide chains to complex type.

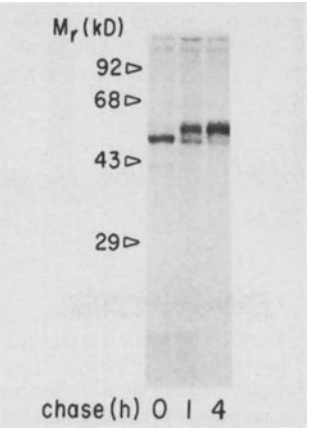

Figure 1. FcRn hc immunoprecipitated from lysates of murine 3T3 fibroblasts infected with a retroviral vector that contains the FcRn hc cDNA. Cells were pulse-labelled with [^{35}S]-cysteine and chased with unlabelled cysteine for the times indicated.

Clones expressing the most FcRn hc were selected on the basis of metabolic labelling and immunoprecipitation, normalized for protein as a measure of cell number. These were designated rfcr4 (rat1) and mfcr5 (3T3). Clones rfcr4 and mfcr5 specifically bound 20-fold and 7-fold more IgG (in 1 hour) than their respective parental lines at pH 6.0 (Figure 2). No specific binding was detected at pH 7.4.

4. Discussion

4.1 FUNCTIONAL EXPRESSION OF FcRn

Cloned p51, expressed in rat and mouse fibroblasts (rat1 and 3T3), is first detected in pulse-chase experiments as a 47 kd putative R.E.R. form. This molecular weight is consistent with the presence of high mannose oligosaccharide chains at the four potential sites of N-linked glycosylation of the 38.5 kd polypeptide the cDNA sequence predicts. Association with β2m with the heavy chains of class I transplantation antigens is, with few exceptions (Allen et al. 1986), obligatory for their export to the cell surface (Arce-Gomez et al. 1982). The precursor form of p51 undergoes oligosaccharide processing in the Golgi of both cell lines. These results show that murine β2m is sufficiently homologous to that of rat to associate with FcRn hc and allow its export from the endoplasmic reticulum.

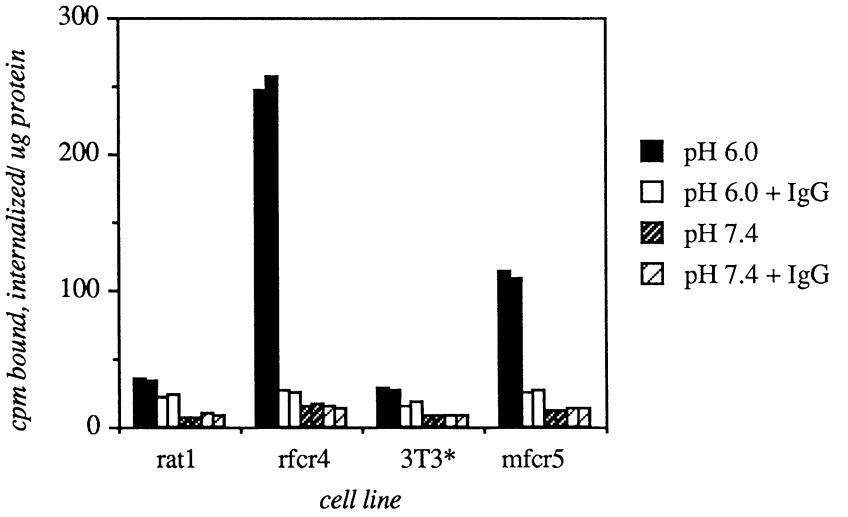

Figure 2. Binding and internalization of [^{125}I]-IgG by rat1 fibroblasts, rat1 cells infected with a retroviral vector that contains the FcRn hc cDNA (rfcr4), murine 3T3 fibroblasts infected with vector alone (3T3*) and 3T3 cells infected with a retroviral vector that contains the FcRn hc cDNA (mfcr5). Duplicate assays were done at pH 6.0 or pH 7.4 with or without competing unlabelled IgG. Counts were normalized for total protein to allow for variation in the number of cells on a plate.

The expression of p51 in fibroblast lines of rodent origin dramatically increased their specific binding and uptake of IgG at pH 6.0, but no specific binding could be measured at pH 7.4. This requirement for a slightly acidic pH for binding is characteristic of FcRn *in situ* in neonatal rat intestinal segments (Jones and Waldman 1972) and enterocyte brush borders (Wallace and Rees 1980) and was that used to purify solubilized p51 by affinity chromatography on immobilized IgG (Rodewald and Kraehenbuhl 1984, Simister and Rees 1985, Jakoi, Cambier, and Saslow 1985).

4.2. PREDICTING THE IgG BINDING SITE

Comparison with MHC class I antigens suggests three candidate regions for the IgG binding site: the binding sites for the T cell receptor and processed antigen and for CD8. Amino acids involved in TCR and antigen recognition in HLA-A2 are found on the upper and inner faces of the α-helices of the α1 and α2 domains. CD8 binds close to residues 227 and 245 in α3 (Connoly et al, 1988). The pH-dependence of IgG binding suggests the involvement of a charged group on the receptor or in Fc with a pKa between 6.5 and 7.5. Histidine residues ionize in this range, as may lysine and arginine if in a hydrophobic environment. Histidines 250 and 251 in α3 of FcRn lie close to the site of CD8 binding of classical class I molecules and the predicted α-helices of α1 and α2 are rich in basic amino acids. Ultimately, localization of the binding site must await mutagenesis studies.

5. Acknowledgements

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Characterizing the polymeric immunoglobulin binding region of human secretory component

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ABSTRACT

Secretory component (SC), an integral membrane receptor located on the basolateral face of secretory epithelial cells, transports polymeric immunoglobulins (PIg) into external secretions. The ectoplasmic portion of SC, which binds PIg, contains five Ig-like domains. We have chemically and enzymatically modified SC in order to determine the structural basis for PIg binding. Deglycosylated SC bound to PIg with an affinity similar to native SC, indicating that the N-linked carbohydrate chains on SC are not involved in PIg binding. Since denatured, reduced and alkylated SC did not bind to PIg, we concluded that the Ig-like tertiary structure of the polypeptide backbone of SC is essential for its function. Proteolytic fragments of SC were produced and tested for their ability to bind PIg. The only SC fragments which bound to PIg were those containing domain I of SC. Monoclonal antibody (MAb) recognition of the PIg:SC peptide complexes was restricted to those MAb which recognized the domain I containing fragments. This result indicates that the N- and C-terminal fragments of SC do not bind to one another under physiological conditions. However, one MAb (6G11), which reacts with domain I, did not react with the PIg:SC or PIg:SC peptide complexes. This same antibody was the only one that inhibited the interaction between PIg and SC. We propose that the initial specific binding of PIg to SC is depending on a tertiary polypeptide structure within the first domain of SC. Our MAb 6G11 may help to identify a critical region for this interaction.

INTRODUCTION

The major function of secretory component (SC), an integral membrane receptor located on the basolateral face of secretory epithelial cells, is to transport polymeric IgA and, to a lesser extent, polymeric IgM into external secretions [2,6]. Unlike other membrane receptors which are recycled or degraded after the transport process, a large segment of SC is released into the luminal secretions in association with its polymeric immunoglobulin ligand. The SC:polymeric immunoglobulin complex is referred to as secretory IgA or M (SIgA or SIgM). Secretory component can also be transported without bound ligand and released into the lumen as free SC. In this report we have used chemically and enzymatically modified forms of SC to examine some of the biochemical

requirements for the binding of PIgA to SC.

MATERIALS AND METHODS

ISOLATION AND MODIFICATION OF SC

Free SC (FSC) and reduced and alkylated FSC were prepared as described previously [7]. Deglycosylated FSC was obtained by digesting 1 mg of FSC, in PBS containing 50 mM EDTA, with 2 Units of Endoglycosidase F for 24 hr. The removal of carbohydrate was confirmed by assaying periodate treated FSC (native and deglycosylated) for reactivity with dansyl hydrazine [3]. Proteolytic fragments of FSC were produced by trypsin or V8 protease digestion at a 1:500 (w/w) enzyme:substrate ratio in PBS for 3 hr at 25°C. The proteolytic fragments were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), electroblotted onto aminopropyl derivatized glass fiber paper [1], or alternatively, Immobilon [5], and subjected to microsequence analysis using an Applied Biosystems Model 470A Protein/Peptide Sequencer.

BINDING OF NATIVE AND MODIFIED FSC TO PIgA

Round bottomed polystyrene microtitre plates were coated with 2μg/ml PIgA in 10 mM borate buffered saline, pH 8.2. After washing, the wells were challenged with 4μg/ml FSC, in its native or modified forms, in 10 mM sodium phosphate, pH 7.2, containing 0.05% Tween. To prevent the formation of disulfide linkages between SC and PIgA, all incubations were carried out at 4°C for 1 hr. Unbound FSC was washed away, and the bound material was subsequently eluted with non-reducing SDS-PAGE sample buffer (0.0625 M Tris HCl, pH 6.5, containing 10% glycerol and 2% SDS) diluted 45 fold in water, and analyzed by Western blot immunoassay. Alternatively, the FSC or proteolytic fragments of SC that bound to dIgA was detected in an ELISA assay utilizing monoclonal antibodies which bind to specific domains of SC. To determine relative affinity of the binding, varying amounts of the SC preparations were mixed with biotinolated FSC prior to incubation in the DIgA coated wells. The biotinolated SC bound to the plate was detected using an streptavidin-horse radish peroxidase conjugate and the results were expressed as the percent inhibition of FSC binding. The inhibition curves were plotted and compared to similar curves produced with native FSC.

MAb INHIBITION OF FSC FINDING TO PIgA

Ascites fluids containing MAb's were diluted in PBS containing 0.05% Tween (MAb) to achieve a concentration ranging from 10 to 0.1 times the ELISA titer against FSC. The diluted MAb's were incubated with 0.1 μg/ml SC for 5 hr at 4°C, then added to PIgA coated microtitre plates as described above. Specifically bound SC was detected by ELISA utilizing rabbit polyclonal anti-SC as a probe. Maximum binding of FSC to PIgA was measured in PIgA coated wells challenged with FSC only.

RESULTS

Table 1 lists the physical forms of SC tested for binding to DIgA in the Western blot and competition assays. FSC, deglycosylated FSC and selected tryptic fragments of FSC bound to PIgA, but reduced and alkylated FSC did not. The tryptic fragments which bound FSC were those which originated from the N- terminal portion of FSC; fragments which did not contain the first domain of FSC did not bind to PIgA. The relative affinity of the binding are also given in Table 1 . Deglycosylated FSC was consistently more potent and the mixture of proteolytic fragments less active than FSC in this assay. In our second series of binding assays, MAb's detecting epitopes on different structural regions on SC were tested for their ability to recognize complexes of PIg and SC, or PIg and SC peptides. Antibodies recognizing epitopes on the tryptic and V8 peptides which bind to PIg recognized the PIg:SC peptide complex, whereas MAb's recognizing C-terminal peptide structures that do not bind to PIg did not. The sole exception to this rule is MAb 6G11. The domain I epitope recognized by this antibody is accessible only on intact FSC and domain I containing peptides and becomes inaccessible when either of these is bound to PIg. We also tested the ability of MAb's to inhibit the binding of SC to PIgA. MAb 6G11 was the only one of 12 MAb's tested that inhibited the binding of SC to PIgA.

TABLE 1.

FSC Preparation	Binding to DIgA (Western blot analysis)	Relative Affinity
FSC	+	1.0
Deglycosylated FSC	+	7.5
Tryptic fragments of SC	50K, 42K	0.25
V8 fragments of SC	Not done	0.20
Reduced and alkylated SC	-	-

DISCUSSION

As a result of these studies on the interaction of modified forms of SC with PIgA we have concluded that a tertiary polypeptide structure within the first domain of SC mediates binding to PIgA. The findings that domain I of SC is important in PIg binding is consistent with results of studies of rabbit SC (4). Characterizing the nature of the SC:PIg interaction is necessary in order to understand the function of secretory component in transport and protection against proteolytic degradation of PIgA. Our studies on the interaction of SC with PIg may also serve as a general model for investigation of other immunoglobulin receptor:immunoglobulin interactions.

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Expression of heavy chain genes is restricted in Peyer's patch germinal center B cells

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ABSTRACT. We have studied Peyer's patches (PP) of conventional BALB/c mice to determine heavy chain (Igh) gene expression in PP germinal centers (GC). Using fluorescence activated cell sorting (FACS) analysis of surface immunoglobulin (sIg), we found that PP GC B cells expressed only sIgM or sIgA but not detectable sIgG1, sIgG3, or sIgE. In situ hybridization using isotype specific riboprobes on tissue sections or cytospots of purified GC B cells detected IgM and IgA message production within the GC B cell subset at heterogeneous levels between that expressed in small resting B cells and plasmablasts. Using Southern and Northern analyses, we further examined whether GC B cells delete their genomic DNA following a switch to a non-IgM/IgD isotype, and which mRNAα and mRNAμ species they express. Our results indicate that PP GC B cells appear to delete their DNA for Cμ and Cγ1 upon switching from IgM to IgA and that they express both secretory and membrane message for μ and α chains. From these studies we suggest that PP GC B cells are restricted in the Igh gene usage.

Introduction

Peyer's patches are regions of nonencapsulated secondary lymphoid tissue found in the wall of the small intestine (SI) of vertebrates. They are continually stimulated by new antigens taken from the lumen of the SI of conventionally reared mice and it is believed that this stimulation leads to the continual presence of GC in PP (1). GC contain large B blasts, many of which are undergoing division. Various molecular genetic events, such as Igh isotype switching and acquisition of point mutations in expressed V-genes in response to antigenic stimulation have been postulated to occur in GC.

Rose and coworkers showed that some species of GC B cells bound avidly and preferentially to a plant lectin, peanut agglutinin (PNA), thus providing a basis for isolating a pure population of these cells (2,3).

We undertook a molecular analysis of PP GC B cells to determine which Igh genes were expressed as either mRNA or protein products by FACS analysis of sIg, and in situ hybridization with Igh gene specific riboprobes. We further looked to determine whether deletion of Igh DNA

occurred following an Igh gene switch, and what species of mRNA was expressed in GC B cells.

Materials and Methods

Plasmids and subcloning: the plasmid pγ1/A5, an IgG1 specific cDNA sequence, was ligated into the plasmid pGEM 3Z; pABγ_{3-3}, an IgG3 specific cDNA clone and $C^{2}30$, an IgE specific cDNA clone were both ligated into the plasmid pGEM 4 blue. The IgM specific cDNA clone, pμ3741, and the IgA specific cDNA clone, pα J558, were ligated into pGEM 2.

Synthesis of ^{35}S-labeled ssRNA probes: Labeled RNA was synthesized from each of the plasmid constructs using either T7 (20 units) or SP6 (15 units) RNA polymerase in the presence of 2.5 mM GTP, ATP,CTP; 6 mM UTP and ^{35}S-UTP (New England Biolabs, Boston, MA) (0.125 mCi, 1200 Ci/mmol); 100 mM DTT; 32 units RNAsin ribonuclease inhibitor (BRL); 40 mM Tris-HCl, pH 7.5; 6 mM MgCl; 2 mM Spermidine; and 10 mM NaCl.

In situ hybridization: In situ hybridization was done according to the procedure of Sideras (4).

Results

Fluorescence Activated Cell Sorter (FACS) Analysis of Surface Immunglobulins: Using PNA labeled with Texas Red and a fluorescein labeled anti-κ as a B cell marker we were able to discern distinct populations of GC B cells that were PNA^{high} $sκ^{low}$ and PNA^{high} $sκ^{high}$. These populations average 1/5 of the total PP population. Using different sIg specific markers that were either labeled with fluorescein or biotinylated and then followed by a second step stain of fluorescein conjugated avidin, we found that about 30% of the GC PP B cells express sIgA, while greater than 60% express sIgM. Only about 10% of GC B cells within the PP expressed sIgD. We were unable to detect any sIgG1, sIgG3, or sIgE cells by FACS analysis. Thus, it appears that few if any B cells within the PP GC express non-IgM isotypes other than IgA.

We next were interested in discovering whether there were B cells within the PP that expressed both IgM and IgA simultaneously, since it is possible that these would be the cells, possibly within the GC, that were undergoing or had recently undergone a switch from IgM to IgA. FACS analysis of sIgM and sIgA expression showed that few if any B cells within the PP express sIgM and sIgA coincidentally, so it is unlikely that many cells undergoing an isotype switch within GC express both isotypes on their surface except very transiently.

In situ Hybridization to PP Tissue Sections from Conventionally Reared Mice: Riboprobes specific for mRNA transcribed from different Igh genes were tested on hybridomas or cell lines making known heavy chain isotypes and shown to be specific for only the appropriate isotype, and not to cross hybridize to cell lines expressing different Igh genes.

The μ specific riboprobe was hybridized to PP cryostat sections from conventional BALB/c mice. Cells expressing high levels of IgM specific message were found under the dome of the PP. The marginal zone, the area surrounding the GC that contains small B cells, express low, but detectable levels of IgM specific message. Within the GC there was a wide range of message expression. The heterogeneity in mRNAμ expression ranged from the low level seen in the marginal zone B cells to somewhat

focal areas of intermediate levels of mRNAμ to a few cells expressing the intense levels equivalent to that seen in cells of the dome. Few mRNAμ containing cells were detected in the lamina propria (LP).

The IgA specific riboprobe was hybridized to PP tissue sections to detect mRNAα containing cells. Cells expressing high levels of RNA were found under the dome as was seen with the IgM riboprobe and also in the LP. Few if any B cells in the marginal zone appeared to express detectable mRNAα. Within the GC, the B cells expressed α transcripts at an intermediate level, with a few cells expressing high levels of message.

In situ Hybridization to Cytospots Containing Purified PP GC B Cells: We purified the PNA^{high} $sκ^{low}$ GC B cell subset to determine what proportion of these cells expressed the different Igh transcripts. Table 1 shows that about 38% of the GC B cells express IgA specific message, while about 51% contain detectable IgM specific transcripts. We were unable to detect B cells within the GC B cell subset expressing either IgG1, IgG3, or IgE message by in situ hybridization. These findings lead us to believe that there may be some restriction in isotype potential of PP GC B cells to express only mRNAμ and mRNAα at easily detectable levels.

FACS purified $sIgA^+$ B cells or $sIgM^+$ B cells were hybridized with either IgA or IgM specific riboprobes. Table 1 shows that approximately 50% of the $sIgA^+$ B cells express detectable levels of mRNAα while <1% contain mRNAμ. Conversely, $sIgM^+$ B cells contain about 40% of cells expressing easily detectable μ gene transcripts, while <1% express mRNAα. While we have not probed the same cells for two Igh mRNAs it is likely that there are few B cells within the PP expressing easily detectable message for both μ and α-chain simultaneously.

Southern Blot Deletion Analysis of GC B Cells: Since Kraal et al. proposed that Igh gene switching may occur within the GC (5), we were interested in knowing whether deletion of heavy chain DNA accompanied the switch to expression of non-IgM/IgD isotypes or whether another mechanism was involved, such as differential splicing of a polycistronic message, or trans-splicing of sterile transcripts from non-μ C_H-genes onto a productive mRNAμ. Genomic DNA was isolated from a FACS purified population of PNA^{high} $sκ^{low}$ GC B cells and subjected to restriction enzyme digestion and Southern analysis with DNA probes specific for either IgM, IgG1, or IgA. Approximately 45% of GC B cells analyzed deleted their C_H gene for Cμ and Cγ1 if single allele deletion is assumed. The percentage of deletion for the Cμ and the Cγ1 alleles was approximately

TABLE 1. Proportion of PP cells expressing mRNA for (%):

	IgA	IgM
PNA^{high} $sκ^{low}$	38	51
$sIgA^+$	48	<1
$sIgM^+$	<1	40

Percent of total detectable cells positive in each B cell subset for either IgA or IgM regardless of level of mRNA expression.

the same, suggesting that the deletion of genomic DNA in PP GC B cells proceeds directly and not through another C_H gene.

Northern Analysis of PP GC B Cell RNA: Since we had determined that PP GC B cells express detectable levels of message for IgM and IgA we wanted to determine whether the RNA produced was for the secretory or membrane forms of message, or both. We found that the IgM specific probe detected both membrane and secretory message in GC B cells, whose sizes appeared to be approximately 2.1 and 2.4 kilobases (kb). The IgA probe detected three messages of approximately 1.7, 2.1, and 3.1 kb which correspond to the one secretory and two membrane messages previously shown for IgA^+ B cells.

Discussion

We have set out to determine the heavy chain isotypes expressed within PP GC. We found that the predominant non-IgM isotypes was IgA, with few if any IgG1, IgG3, or IgE B cells detected within the GC by either in situ hybridization or FACS analysis. This differs from what has been seen in the lymph node (LN) where sIgG1 appears to be the predominant non-IgM heavy chain isotype expressed, with few cells ever expressing sIgA (5). We have begun studies with stimulated LN GC B cells and have found that in situ hybridization detects LN GC B cells that express message for IgM and IgG3, but not IgA.

The reason PP GC B cells produce message for both secretory and membrane forms of IgM or IgA is unknown since it is generally believed that these cells do not secrete antibody in vivo. However, this may reflect development of some cells into preplasmablasts, as they prepare to leave the GC and go on to secrete antibody within the LP (see George et al., this volume).

Presently, we are using germ-free BALB/c mice to determine whether the preference within the PP to produce IgA as the sole non-IgM isotype is due to an inherent feature of the microenvironment of the PP GC, or the fact that PP are chronically stimulated.

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Production of alpha heavy chain mRNA in mucosal and lymphoid tissue of various strains of mice

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Introduction

C3H/HeJ mice, which are non-responsive to lipopolysaccharide (LPS), elicit higher IgA responses, including increased splenic indirect plaque-forming cell and serum antibody responses to orally administered antigen compared to the syngeneic LPS responsive C3H/HeN mice (1). These elevated IgA responses are thought to be due to induction of T helper cell activity in mucosal lymphoid tissue (2).

In this study, we compared levels of mRNA for the α heavy chain in various lymphoid tissues, lungs and gut of C3H/HeJ and C3H/HeN mice as well as three other mouse strains. The distribution and levels of mRNA from control mice were compared to levels following intraperitoneal (IP) immunization, a route that results in priming for protective respiratory immunity.

Materials and Methods

RNA was isolated by the method of Chirgwin et al (3) from tissues of both control mice and mice immunized IP 14 days previously with 0.5ml formalin inactivated Rift Valley Fever (RVF) virus NDBR-103. 10μg/lane were electrophoresed on 1.5% agarose gels containing 0.66M formaldehyde, 20mM MOPS, 5mM EDTA and 6,6μg ethidium bromide. The RNA gels were blotted onto nitrocellulose and probed with the cDNA probe α660 obtained from Cebra and Weinstein. The total IgA concentration in both sera and bronchial lavages were measured by ELISA.

Results

mRNA for the α heavy chain of IgA was present in all tissues studied. However, the distribution of α mRNA varied among tissues of the different strains. In addition, immunization had varying effects on the levels in the different tissues (Table 1)

Table 1. Effect of IP inoculation with RVF virus vaccine on tissue levels of α mRNA

Mouse strain	Intestine	Lung	Med.LN	Mes.LN	Per.LN	Spleen
C3H/HeJ	↓	↑	-	-	↓	↑
C3H/HeN	↓	-	↓	↓	-	↓
Balb/cByJ	↓	↓	↑	↑	↑	↓
BD/F1	↑	-	-	↓	↓	↑
Swiss Webster	↑	↓	↓	↓	-	↓

Comparison between control and immunized mice using densitometry tracings of Northern blots. Med.- Mediastinal, Mes.- Mesenteric, Per.- Peripheral, LN - Lymph node

The concentration of total IgA in serum increased from 73 μg/ml in control C3H/HeJ mice to 142 μg/ml in the immunized mice. The levels of IgA in serum decreased in all the strains investigated following immunization. The concentration of IgA in bronchial lavages also increased dramatically in the C3H/HeJ mice following immunization, from 71 ng/ml to 902 ng/ml. There was no significant change in the concentration of IgA in bronchial lavage from C3H/HeN mice and it decreased in all the other strains.

Discussion

We confirmed that there is increased splenic IgA responses following immunization in C3H/HeJ mice with increased α mRNA found in the spleen and elevated levels of IgA in the blood. Increased α mRNA expression in the lungs of C3H/HeJ mice correlates with vast increase in IgA concentrations found in the bronchial lavages of immunized mice. There was either no change or a decrease found in the concentration of IgA in the bronchial lavage of the other mouse strains which correlates with mRNA levels.

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T560: a murine lymphoma with receptors for IgA

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ABSTRACT. Tumor 560 is an Ig^-, Ia^+, $B220^+$ lymphoma that originated in the gut-associated lymphoid tissue (GALT) of a (B10 X B10.H2^a H-4^{b}pWts)F_1 hybrid mouse. One line of it (T560.1) bears IgA receptors on only a low percentage of its cells, while a second (T560.2), has a high percentage of IgA receptor-positive cells. These receptors differ, however, from those previously described on T cells in having a high affinity not only for IgA but also for IgM. Thus, in some aspects of its binding activity, the T560 receptor resembles the poly-Ig receptor which binds to both IgM and IgA and transports them across epithelial cells. The poly-Ig receptor is, however, normally restricted to glandular epithelial cells and hepatocytes and absent from lymphoid cells. Yet another kind of IgA receptor is present on the surface of cells contained in unseparated Peyer's patch (PP) cell populations; it differs from that of both T560 and T cell hybridomas in being highly inhibitable by IgG3 and poorly inhibitable by IgM. T560.2 presents antigen to antigen-reactive T cells and secretes IL-1- and IL-6-like activities.

1. Introduction

Specific receptors for various Ig isotypes have been implicated both in the biological activity of the cells that bear them and in the up- or down-regulation of B cells making the isotypes they recognize. Since the major Ig in GALT is IgA, we were intrigued by a chance observation that cells of T560, an Ig^- lymphoma of the B lineage that originated in mouse GALT, expressed receptors for IgA. One line of it, T560.1, was found to bear IgA receptors on only a very low percentage of cells, but a second, more adherent, more plasmacytoid-looking line, T560.2, has a high percentage of IgA receptor-positive cells. These receptors are of particular interest because they differ from IgA Fc receptors (FcαR) previously described on T cells in being 100% inhibitable not only by IgA but also by IgM and even by high concentrations of IgG2a and b. In the course of studying the T560 receptors, we made the additional observation that cells present in unseparated PP cell populations have an IgA receptor differing from that of both T560 and the T cell hybridomas described by others (1-9) in being highly inhibitable by IgG3.

2. Materials and Methods

2.1. MAINTENANCE AND PREPARATION OF CELLS

2.1.1. *T560 cell culture.* Both lines of T560 cells are maintained in Iscove's medium containing 0.1 u/ml insulin, 5×10^{-5} M 2 ME and 10% FCS (Iscove's I).

2.1.2. *Preparation of PP cells.* Normal, unseparated Peyer's patch (PP) cells are isolated by gently squashing the PP of 3 to 4 month old male or female BALB/c mice between the frosted ends of two sterile microscope slides in complete RPMI 1640 medium containing 10% FCS. Clumps are allowed to settle out and the single cell suspension is washed twice through neat FCS to remove bacteria that may be present.

2.1.3. *Maintenance of macrophage-like cell lines.* Two macrophage lines, FC1.9 and J774.BRB (J774) obtained from Dr. B. Diamond, Albert Einstein Medical College, have been used as positive controls for IgG2a and IgG2b rosette-forming cells (RFC). They are maintained in Dulbecco's modified Eagle's medium containing 10% FCS.

2.2. ROSETTE ASSAYS

2.2.1. *Preparation of IgA-coated erythrocytes:* Trinitrophenylated ox erythrocytes (TNP-ORBC) and uncoupled ox erythrocytes (ORBC), used as controls in the agglutination assay, are washed and resuspended at 10% vol/vol in Dulbecco's phosphate-buffered saline (DPBS). An $(NH_4)_2SO_4$-concentrated culture supernatant of MOPC-315 (IgAλ) myeloma cells is used to coat the TNP-ORBC. Agglutination assays are run at the time of each experiment to establish the highest concentration of myeloma protein that just fails to agglutinate the TNP-ORBC. Equal volumes of 1% TNP-ORBC in DPBS and the selected dilution of MOPC-315 myeloma protein are mixed and incubated at room temperature for 15 min, the cells are washed twice in DPBS and then resuspended at 1% in DPBS. Control, uncoated TNP-ORBC are subjected to the same procedures without the myeloma protein. The MOPC-315 myeloma protein used throughout these experiments was prepared from culture supernatant and could not be contaminated by a hypothetical natural anti-TNP antibody of another isotype such as might be contained in ascitic fluid.

2.2.2. *Preparation of IgG2a - and IgG2b - coated erythrocytes:* Sheep erythrocytes (SRBC) are washed, coated with mouse monoclonal IgG2a (UN2S1) and IgG2b (U188) anti-SRBC antibodies contained in the supernatant of cultured hybridoma cells and then washed and resuspended at 1% in DPBS as described above. In initial experiments, several concentrations of antibody-containing supernatants (which do not agglutinate) were tested and the concentration yielding the highest numbers of RFC selected for future experiments. This is rechecked from time to time.

2.2.4. *Rosette Assays:* The methods were adapted from those described in (10) and (11). Log phase-cultured or freshly prepared PP cells are washed twice and resuspended in DPBS at 5×10^6/ml. The reaction is carried out in 96 round-bottomed well tissue culture plates. To each well are added successively 20 µl FCS, 20 µl cells, 20 µl inhibitor solution or DPBS alone and, after a 10 min. incubation period at room temperature, 100 µl coated or uncoated control TNP-ORBC or SRBC (160 µl final volume). The plates are centrifuged for 5 min. at 4°C and incubated at 4°C for 1 hour. 50 µl volumes of the cells and of 0.1% toluidine blue in saline are then mixed and rosettes (nucleated cells to which 3 or more erythrocytes are bound) are counted in a hemacytometer. At least 200 cells are scored for each point. The % rosette-forming cells (RFC) scored with the control erythrocytes is subtracted from that scored in the presence of Ig. In practice, less than 0.5% of T560.1,

T560.2 or FC1.9 cells bind uncoated SRBC. With TNP-ORBC, low background numbers of rosettes are formed: T560.1, 1.6±0.4 (9 expts); T560.2, 3.8±0.4 (18 expts.); PP, 2.7±0.4 (9 expts.); FC1.9, 0.3±0.3 (3 expts).

2.2.5 *Inhibitors:* Inhibitors tested thus far include IgG3κ (FLOPC-21), IgG3λ (Y5606), IgG2bκ (MOPC-141), IgG2aκ (UPC-10), IgG1κ (MOPC-21), IgAκ (TEPC-15) and IgMκ (TEPC-183) (Sigma, St. Louis, MO). All were supplied at a nominal 1 mg protein/ml but Lowry assays done using a BGG standard showed several to contain considerably less. The data shown reflect the correct concentrations of inhibitors in the final assay volumes. One hundred mM concentrations of mannose, α-methyl D-mannoside, galactose, fucose, lactose and maltose and 3 mM concentrations of 3 dif- ferent galactans obtained from Dr. Michael Heidelberger have also been tested but none of them inhibited. The % inhibition of rosettes is calculated as

$$\% \text{ Inhibition} = 100\left(1 - \frac{\%\text{RFC in the presence of inhibitor}}{\%\text{RFC in the absence of inhibitor}}\right)$$

3. Results.

3.1. ORIGIN OF T560.

T560 is one of a series of tumors induced by Dr. G. Haughton, University of North Carolina at Chapel Hill (CH). It developed in the GALT of a (B10 X B10.H-2^a H-4^bWts)F_1 hybrid mouse undergoing a graft-versus-host reaction induced by B10.H-2^aH-4^b splenocytes. Some of its characteristics are unusual in comparison to those of the rest of Dr. Haughton's series of CH tumors. For example, although it is Ia^+, $B220^+$, Thy 1^-, Mac-1^-, Mac 2^- and non-specific esterase$^-$, and thus appears to be of the B-lineage, it is Ig^- and Ly 1^-; also, on the basis of MLR results, it was derived from the F_1 hybrid host of the $H2^aH4^b$ cells. Most of Dr. Haughton's other tumors bear surface Ig, are Ly 1^+ and were derived from the homozygous donor population of lymphoid cells (12). At its 6th *in vivo* passage in our hands it was adapted to tissue culture in Iscove's medium containing 5 X 10^{-5} M 2-ME, 0.1 u/ml insulin and 50% FCS; the FCS concentration was then gradually reduced to 10% and at that point the cultured cell line was frozen down in liquid nitrogen (T560.1). Line 2 was maintained in culture and has now been in culture (with periodic freezing down) for approximately 3 years. Expression of the receptors of both lines seems to be quite stable.

3.2. RECEPTORS FOR IGA, IGG2A AND IGG2B ON T560, PP AND FC 1.9 CELLS

Striking differences are found between the two lines of T560 and between T560, PP, FC1.9 and J774 cells (not shown) in their ability to bind erythrocytes coated with IgA, IgG2a and IgG2b (Table 1).

The frequencies of cells expressing receptors for IgA and for IgG2b in lines T560.1 and T560.2 are virtually reciprocal: T560.1 populations have low percentages of IgA and moderate percentages of IgG2b RFC whereas T560.2 populations have moderate percentages of IgA, low of IgG2b RFC. Both lines have low percentages of IgG2a RFC. Neither the FC1.9 nor J774 populations contain any IgA RFC but both these lines have the high percentages of IgG2a and IgG2b RFC published by others (13). Attempting to increase the amount of IgG2a or IgG2b on the surface of the SRBC by using higher concentrations of antibody to coat them does not increase the number of IgG2a or IgG2b RFC in

TABLE 1. Percentage of cells of the various types forming rosettes with erythrocytes coated with IgA, IgG2a or IgG2b.

Cells	IgA rosettes	IgG2a rosettes	IgG2b rosettes
T560.1	7.9 ± 1.1 (9)*	14.6 ± 2.8 (8)	34.8 ± 4.8 (8)
T560.2	34.0 ± 2.1 (18)	5.9 ± 0.7 (7)	7.0 ± 0.6 (7)
PP cells	11.9 ± 0.5 (9)	17.2 ± 1.5 (2)	18.0 ± 1.6 (2)
FC 1.9 cells	0.7 ± 0.4 (3)	84.5 ± 1.2 (6)	63.1 ± 2.6 (6)

* Mean ± S.E. (No. of Expts)

T560.1 populations. Similarly, increasing the amount of IgA on the indicator cells by increasing the degree of TNP-conjugation and/or using larger amounts of MOPC-315 does not reliably increase the number of IgA RFC in T560.2 populations although, to some extent, the number scored may depend on the particular preparations of IgA and TNP-ORBC used; e.g., we have occasionally seen T560.2 IgA RFC scores as high as 53% though they are more usually around 30 to 40%. In a few experiments we have examined T560.2 cells stained with biotin-conjugated IgA and FITC- avidin complexes. These preparations also yield scores of between 30 and 40% IgA-binding cells. Our data support the idea that less than 100% of T560.2 cells form rosettes with the coated erythrocytes because less than 100% of them have receptors of adequate avidity; i.e., the result reflects heterogeneity in the T560.2 cells rather than inadequate coating of the indicator cells. The heterogeneity does not appear to be genetic since clones derived from T560.2 give similar RFC scores. Expression of the IgA receptor and the IgG2b receptor (FcγRII) may be related to the cell cycle and/or the state of activation of the T560 cells. It is worth noting that cloned T hybridomas that express IgA Fc receptors are often considerably less than 100% receptor positive even after measures have been taken to up-regulate them (4,8); i.e., lack of 100% expression of this surface marker is not just a peculiarity of our lymphoma lines.

3.3. SPECIFICITY OF ROSETTE FORMATION AS DETERMINED BY ROSETTE INHIBITION.

To determine the isotype specificity of the RFC, rosette-formation was inhibited with a range of concentrations of various myeloma proteins (Table 2).

3.3.1 *Inhibition of IgA RFC.* Approximately 32% (31.9±2.6; 5 expts.) of T560.2 cells formed rosettes with IgA-coated TNP-ORBC. These proved highly inhibitable by IgA (TEPC-15) myeloma protein but even more so by IgM (TEPC183), the order of efficiency of inhibition by the isotypes being IgM>IgA>IgG2a>IgG2b>IgG1>IgG3. It is of interest that sufficiently high concentrations (between 50 and 100μg/ml) of both IgG2a and IgG2b can inhibit virtually 100% of T560.2 IgA RFC though the slopes of their inhibition curves indicate that they bind with much lower affinity than do IgA and IgM. Their ability to inhibit might depend on the presence of aggregates in the IgG2a and IgG2b preparations.

Approximately 12% of PP cells bear IgA receptors with quite different specificity from that of T560 receptors. The order of efficiency of inhibition of PP IgA RFC by myeloma protein was IgG3>IgG2a>IgA=IgG1>IgM=IgG2b, a result suggesting the possibility that we may be picking up a reactivity of the IgG3-binding murine FcγRIII (14) not hitherto described.

3.3.2. *Inhibition of IgG2a and IgG2b rosettes.* Because IgG2a and, to a lesser extent,

Table 2. Myeloma protein concentration (μg/ml) required for 50% inhibition of rosette formation.

Cells	Rosettes	Inhibitors						
		IgA	IgM	IgG2a	IgG2b	IgG1	IgG3*	IgG3**
T560.1	IgA	<3†	3.7	ND††	ND	ND	ND	ND
T560.2	IgA	<3	<3	16	47	15	31	45
PP	IgA	<10(9)°	22	11	20	5.8	1.9	2.1
T560.1	IgG2a	15.5	3.3	22	11	>100(128)	>100	ND
FC1.9	IgG2a	NI°°	NI	<17	>100(135)	NI	NI	ND
T560.1	IgG2b	<1.6	4.3	25	43	NI	37	ND
FC1.9	IgG2b	NI	56.5	>100(106)	4.4	>100(115)	NI	ND

* FLOPC-21.
** Y5606.
† Less or more than minimal or maximal amount tested required for 50% inhibition.
†† Not done.
• Numbers in parentheses represent the amount derived by extrapolation.
•• Not inhibitory at the maximal concentration tested.

IgG2b inhibited the IgA rosettes of T560 cells, we compared the IgG2a and IgG2b rosettes of T560.1 and FC1.9 cells, which bear bona fide receptors for IgG2a (FcγRI) and IgG2b (FcγRII), in their ability to be inhibited by the same myeloma proteins. Approximately 11% (11.2 ±1.9; 3 expts) of T560.1 cells formed rosettes with IgG2a-coated SRBC. The order of efficiency of inhibition of rosette formation was IgM>IgG2a=IgA=IgG2b>IgG1=IgG3. A much higher percentage, 33% (33.2±1.9; 3 expts.), of T560.1 cells formed rosettes with IgG2b-coated SRBC. The order of efficiency of inhibition of rosette formation was IgA=IgM>IgG2a>IgG3=IgG2b; IgG1 did not inhibit. The results with FC1.9 cells were completely different and quite consistent with published data (6). IgG2a rosettes (84.8± 1.8%; 3 expts.) were inhibitable only by IgG2a and, to a limited extent by IgG2b. Conversely, the IgG2b rosettes of FC1.9 cells (64.7± 4.4%; 3 expts.) were inhibitable only by IgG2b and to a lesser extent by IgG1 and IgM at their maximal concentrations. The results with T560.1 suggest that T560.1 may have some receptors for IgA and IgM but their number and/or avidity is inadequate for IgA rosette formation.

3.4. TRYPSIN-SENSITIVITY OF THE IGA RECEPTOR.

The IgA receptors on T560.1 and T560.2 cells, like those described on T2D4 cells by others (14), are sensitive to all preparations of trypsin tested, exposure to 0.1% trypsin for 30 minutes abolishing the ability of the cells to bind IgA-coated erythrocytes (Table 3). Paradoxically, however, exposing T560.1 cells to certain preparations of trypsin dramatically increases the expression not only of receptors for IgG2b, which are known to be trypsin resistant, but also of receptors for IgG2a, reported to be trypsin-sensitive at least on J774 and P388D1 cells (13,15). T560.2 FcγR, by contrast, are not increased by any trypsin treatment. Evidently, T560.2 cells are quite different from T560.1 cells not only in

Table 3. Effect of trypsinization on RFC binding IgA, IgG2a and IgG2b

Cells	Trypsin Treatment	% RFC binding erythrocytes coated with		
		IgA	IgG2a	IgG2b
T560.1	-	5.9	13.0	41.7
	+	0(100)	42.5(+226.9)	85.5(+105.0)
T560.2	-	43.4	3.9	6.1
	+	0(100)	2.9(25.6)	2.4(60.6)
FC1.9	-	0.5	81.3	58.1
	+	0(100)	82.3(+1.2)	73.2(+26.0)

Cells were incubated at 1 X 10^6/ml for 30 minutes at 37°C with 0.1% crystalline bovine trypsin (Worthington) in Hanks BSS and were then washed three times in medium containing 10% FCS to block the trypsin and once in PBS before assay. Numbers in parentheses represent the % inhibition or increase in RFC following trypsin treatment.

their quantitative but also in their qualitative expression of FcγR and receptors for IgA. Enhanced expression of FcγR has been previously documented on myeloma cells exposed to trypsin (16); apparently it also occurs on our lymphoma cells. The enhancing effect is not due to the presence of either chymotrypsin or magnesium sulphate in some of the trypsin preparations since adding diphenylcarbamyl chloride (which blocks chymotrypsin) to enhancing preparations or magnesium sulphate to non-enhancing preparations has no effect.

At the trypsin concentration used in this experiment, receptors for IgG2a on FC1.9 cells were unaffected. Subsequent experiments have shown that removal of the IgG2a (FcγRI) receptor from our lines of FC1.9 and J774 cells requires much longer exposure to higher concentrations of trypsin.

3.5. INHIBITION OF ROSETTE-FORMATION WITH 2.4G2 MONOCLONAL ANTIBODY.

The receptors of the three cell lines were tested for their sensitivity to inhibition with the FcγRII-specific mAb, 2.4G2 (16), contained in a culture supernatant. At a concentration adequate to block 100% of the activity of the IgG2b receptors on all three cell lines, there was no inhibition of the IgG2a receptor of FC1.9, but there was partial inhibition of the IgG2a receptors of both lines T560.1 and T560.2 and partial inhibition of the IgA receptors of T560.1 but not of T560.2. The experiment must, of course, be repeated with $F(ab')_2$ fragments of 2.4G2 to ensure that inhibition is mediated by recognition of the 2.4G2 epitope, rather than by blocking with the Fc portion of the rat IgG2b mAb. Normal rat IgG2b but not rat IgG2a completely blocks the FcγRII receptor but not theFcγRI of FC1.9 cells while it only partially blocks the receptor for IgG2b and has no effect on the receptor for IgG2a on T560.1 and T560.2 cells (data not shown). The partial inhibition by 2.4G2 mAb of the IgA receptor on T560.1 suggests interaction between the IgG2b receptor and the IgA receptor, raising the interesting possibility that, on the cell surface, two or more Ig receptor chains may be non-covalently associated together in binding Ig molecules.

Table 4. Effect of inhibition by 2.4G2 on RFC binding IgA, IgG2a and IgG2b

Cells	2.4G2	% RFC binding erythrocytes coated with IgA	IgG2a	IgG2b
T560.1	-	8.5	10.7	40.8
	+	6.3(25.9)*	6.5(39.3)	0(100)
T560.2	-	46.3	6.1	6.5
	+	47.3(+2.1)	1.5(75.4)	0(100)
FC1.9	-	0.4	86.9	52.6
	+	0.4	83.0(4.5)	0(100)

Inhibition of rosette formation by 20 µl undiluted 2.4G2 culture supernatant in a final volume of 160 µl (1:8 dilution).
* Numbers in parentheses represent the % inhibition.

3.6. NEGATIVE EFFECT OF PHORBOL MYRISTATE ACETATE (PMA) AND/OR IONOMYCIN ON EXPRESSION OF THE RECEPTORS.

Incubation of T560.1 or T560.2 cells with 1 ng/ml of PMA for as little as 1 hour markedly reduced expression of IgA, IgG2a and IgG2b receptors on both T560.1 and T560.2 cells. Ionomycin at 5 µg/ml had a similar but less profound effect except in the case of the IgG2a and IgG2b receptors of T560.1 which, at 24 hours, were slightly increased. Treatment with both ionomycin and PMA generally caused a reduction in receptor expression intermediate between that caused by either agent alone.

3.7. ABILITY OF T560 CELLS TO INTERACT WITH T CELLS (DATA NOT SHOWN).

One of the reasons for our interest in T560 cells stems from the fact that they not only have IgA receptors, but are also strongly Ia-positive and hence might be able to present IgA to IgA-reactive T cells. To evaluate their ability to interact with T cells, their capacity to induce a mixed lymphocyte reaction (MLR) by splenocytes of B10.A ($H\text{-}2^a$), B10 ($H\text{-}2^b$), BALB/c ($H\text{-}2^d$) and (B10 X B10.A)F_1 hybrid mice was tested. Both lines induced an MLR in both $H\text{-}2^a$ and $H\text{-}2^b$ as well as in $H\text{-}2^d$ but not in ($H\text{-}2^a$ X $H\text{-}2^b$) F1 hybrid cells indicating that the tumor originated in the F1 hybrid recipients of the $H\text{-}2^a$ 4^b cells injected prior to its induction. T560.2 cells were, however, about 4 times more capable of inducing the MLR on a cell-for-cell basis than T560.1 cells.

T560 cells also presented KLH to a line of KLH-immune (B10 X B10.A) F1 hybrid T cells as determined from the proliferative response of the T cells; T560.2 cells were highly effective, T560.1 cells less so at the ten-fold higher cell concentration tested.

Since the lines supported T cell proliferative responses to antigen, it seemed likely that they made IL-1 (18). That both lines constitutively secreted into the culture supernatant an IL-1-like activity was confirmed in an IL-1 assay using Con A-stimulated C3H/HeJ thymocytes (18). Both lines also produced an IL-6-like activity capable of supporting the growth of the IL-6-dependant cell line, B9 (19,20). T560.2 was the better producer of the two activities.

4. Discussion.

Receptors for IgA are normally present on low numbers (5-6%) of unseparated normal murine splenocytes (10) and on somewhat higher numbers of GALT cells but are up-regulated (1,2,21-23) by exposure to high concentrations of polymeric IgA which may account for the increased representation of IgA-receptor bearing cells in the IgA-rich environment of GALT.

IgA receptors described on normal, unseparated spleen cells are Fc-specific (10) and not inhibitable by mouse IgG2a or immune complexes containing rabbit IgG whereas those up-regulated by exposure to IgA *in vivo* (1) are slightly inhibitable by murine IgG2a and IgG2b. When T and B cell populations in the spleen are separated, the IgA receptors up-regulated on T cells are slightly inhibited by IgM but not at all by IgG (23), suggesting that IgG-inhibitable IgA receptors may be restricted to B cells. Although the IgA receptors on separated normal splenic B cells are somewhat less highly specific than those on T cells (1) they are not nearly 100% inhibitable by concentrations of IgG in the range of 100μg/ml, a concentration that inhibits virtually 100% of the IgA receptors of T560.2 cells. The ability of IgM and of high concentrations of IgG2a and IgG2b to inhibit the IgA receptors of T560.2 thus distinguishes them from the much more highly specific FcαR described on T and B cells (3-6, 9, 22). The overwhelming cross-inhibition of the T560 receptor by IgM might suggest the receptor to be similar or identical to the IgM receptor already described on normal B cells (24) except that the latter is reported to be not at all inhibitable by IgA.

Three features of the T560.2 cell line could affect the specificity of its IgA receptors as compared to those on populations of normal B cells: first, it is a malignant cell line whose IgA receptors may only superficially resemble those of normal B cells; second, it may represent an expansion of a rare cell present in the normal B cell population; third, its lack of cell surface Ig may allow it to assemble its IgA receptor differently from that on normal B cells.

In its cross-reactivity with IgM, T560 IgA receptor resembles the poly-Ig receptor responsible for transporting both IgM and IgA across glandular epithelial cells but this receptor is thought to be present only on epithelial cells and hepatocytes (in some species) and has not been described on lymphoid cells. It is conceivable, however, that the T560 IgA receptor could have an Ig-binding domain homologous to that on the poly-Ig receptor even if it does not share all the features of that receptor (24,25).

A second possibility is that the T560 IgA receptor is a murine homologue of the galactosyl transferase recently described on human epithelial and murine T cells (26,27). The T560 receptor is not inhibitable by galactose, but N-acetylglucosamine, which is the acceptor site for UDP-galactose and which, in the presence of α-lactalbumin, does inhibit binding of human IgA and IgM to this enzyme (26), has not yet been tested.

A third possibility is that the IgA receptors expressed on the T560 cell surface are non-covalently linked heterodimers or heteropolymers of known FcR polypeptide chains which if expressed alone or as homodimers would be monospecific. The idea that FcR might function on the cell surface as dimers or polymers even though they are usually isolated as single polypeptide chains (28) would be compatible with their membership in the Ig supergene family. The T560.1 IgA receptor, which is partially inhibitable with 2.4G2, could be made up of units containing FcαR and FcγRII chains, the PP receptor that cross-reacts with IgG3, of units containing FcαR and FcγRIII chains.

In its sensitivity to trypsin the T560 IgA receptor resembles the FcαR described on the T cell hybridoma, T2D4(3-6), whose FcαR and FcγR bear a similar reciprocal relationship after induction to that seen in T560.1 and T560.2. Whether the receptor is cleaved off the cell surface by the trypsin or whether the trypsin treatment results in activation of protein

kinase C, producing an effect similar to that produced by treating the cells with PMA is not yet clear.

The availability of two lines of T560 cells from the same tumor, one expressing high, the other low levels of the IgA receptor, should make it possible to prepare an antibody which can be used to isolate and purify the receptor. The ability of T560.2 cells to present antigen to T cells will allow us to test the proposition that T cells in GALT may recognize and respond to epitopes of IgA presented in the context of MHC Class II antigens. Such T cells may help to regulate the IgA response in GALT.

5. Acknowledgements

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6. References

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SECTION H: MUCOSAL VACCINES IN MAN AND THE HUMAN IgA RESPONSE

88

Induction of human secretory and serum immune responses: molecular properties of IgA antibodies

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ABSTRACT. The level and properties of antibodies (polymeric/monomeric forms and IgA subclasses) induced in various external secretions and in blood by systemic or oral immunizations with protein and/or polysaccharide antigens were examined in human volunteers. The purpose of these studies was to determine the influence of immunization routes and of antigen composition on the induction of IgA1 and IgA2 responses. Systemic immunization with polysaccharide and/or protein antigens induced a transient appearance of specific antibody-secreting cells of IgA, IgG, and IgM isotypes in the peripheral blood, followed by specific antibodies in serum and in saliva. While the protein antigens induced predominantly IgA1 responses, the polysaccharide-specific antibodies were mainly present in the IgA2 subclass. Most of the serum IgA specific for injected antigens occurred in a polymeric form. Although the response was weaker than after systemic immunization, oral immunization with influenza virus induced both secretory and to a lesser degree systemic antibodies. Previous exposure by mucosal or systemic routes influenced the quantity and quality of the immune response stimulated by oral or systemic immunizations.

1. Introduction

Induction of specific secretory IgA (S-IgA) antibodies in external secretions is desirable for the prevention of various systemic as well as mucosa-restricted infectious diseases. To achieve this goal, various immunization protocols have been considered including systemic and/or oral administration of antigens [Mestecky, 1987]. Although both systemic and secretory immune responses can be stimulated, the magnitude and Ig isotype distribution depends on the route of antigen administration. The purpose of our studies was to determine the molecular properties (polymeric and monomeric forms, IgA1 and IgA2 subclasses) of specific antibodies in sera and in external secretions, as well as of IgA secreted by mononuclear cells from peripheral blood of nonimmunized subjects or volunteers immunized systemically or orally with various types of microbial antigens.

2. IgA Subclass Of Naturally Occurring And Artificially Induced Antibodies

The distribution of serum IgA and S-IgA (in nasal wash, colostrum, and saliva) antibodies to food and microbial antigens (Table I) revealed that certain groups of antigens induce natural antibodies that are preferentially, or almost equally, associated with either IgA1 or IgA2 [Brown and Mestecky, 1985; Ladjeva *et al.*, 1989]. For example, antibodies to dietary proteins are mainly found in the IgA1 subclass in sera and in external secretions; serum antibodies to LPS are of IgA1 while in external secretions they are preferentially of IgA2 subclass [Mestecky and Russell, 1986; Russell *et al.*, 1986]. In contrast to naturally

occurring antibodies, the IgA subclass distribution is more pronounced after mucosal or systemic immunization. Although nasal wash antibodies to hemagglutinin (HA) of influenza virus were present in both IgA1 and IgA2 before intranasal immunization with live virus, only IgA1 anti-HA antibodies were induced in this fluid as well as in serum [Brown *et al.*, 1987]. In another study, systemic immunization with pneumococcal, meningococcal, or *Haemophilus influenzae* type b polysaccharides led to a preferential response in the IgA2 subclass, whereas vaccination with tetanus or diphtheria toxoid induced a modest IgA response which was virtually restricted to IgA1 [Lue *et al.*, 1988; Tarkowski *et al.*, 1989] (Fig. 1). Thus, depending on the type of antigen (protein vs polysaccharide), the ensuing immune response is accentuated in favor of either the IgA1 or IgA2 subclass. This type of subclass restriction also occurs in IgG isotypes: the anti-tetanus toxoid response is dominated by IgG1, and anti-polysaccharide responses by IgG2 [Hammarström and Smith, 1986]. Although the molecular mechanisms responsible for this phenomenon are not clear, it is likely that differences in processing of various types of antigens, as well as their T-cell dependence or independence, play a fundamental role.

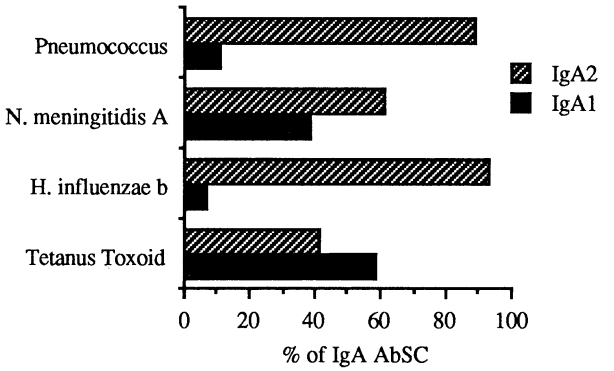

Figure 1. IgA subclass distribution of antigen-specific antibody-secreting cells (AbSC).

3. Polymeric/Monomeric Form Of IgA Antibodies After Immunization

Polymeric and monomeric forms of IgA (pIgA and mIgA) are characteristically distributed in human body fluids: at least 90 per cent of IgA in secretions is polymeric while in serum more than 90 per cent occurs in a monomeric form [Mestecky and McGhee, 1987]. The distribution of naturally occurring serum pIgA and mIgA *specific* to environmental antigens revealed that such antibodies are present in both forms [Russell *et al.*, 1986]. However, shortly after mucosal or systemic immunization with influenza virus or pneumococcal polysaccharide, most of the antibody activity was present in the pIgA as determined by gel filtration, presence of J chain, and the ability to bind SC [Brown *et al.*, 1987; Lue *et al.*, 1988]. Identical results were obtained in other independent studies in which IgA responses to various microbial antigens were analyzed with respect to pIgA and mIgA [for review see Russell and Mestecky, 1988]. Surprisingly, even after *systemic* immunization with nonreplicating microbial antigens, pIgA constituted the predominant form [Mascart-Lemone *et al.*, 1987]. This accord reached among several investigators using different antigens has prompted an important question concerning the cellular origin of antigen-specific pIgA in serum. To address this point, we have analyzed IgA with

respect to pIgA and mIgA in supernatants of cultured lymphoid cells from various organs and developed a method that allowed us to determine the secretion of pIgA at the single cell level [Lue *et al.*, 1988]. Results of these studies (Fig. 2) indicated that antigen-specific pIgA found in serum may originate from several sources, including lymphoblasts in peripheral blood, spleen, and lymph nodes, all of which contain cells capable of pIgA synthesis [Tarkowski *et al.*, submitted]. The low levels of total pIgA found in normal serum may be due to its prompt disappearance and faster catabolism when compared to mIgA, and the apparently short-lived pIgA response. In humans, circulating pIgA is not cleared by SC-mediated hepatobiliary transport [Delacroix *et al.*, 1982], so the functional significance of the early pIgA antibody response remains enigmatic. However, it appears that pIgA antibodies have enhanced avidity for antigen, and S-IgA antibodies have been reported to neutralize viruses by a different mechanism from mIgA antibodies [Taylor and Dimmock, 1985]. In other respects, pIgA is reported to be more effective than mIgA in binding cellular receptors [Mestecky and McGhee, 1987], but the consequences of the interactions remain to be explored.

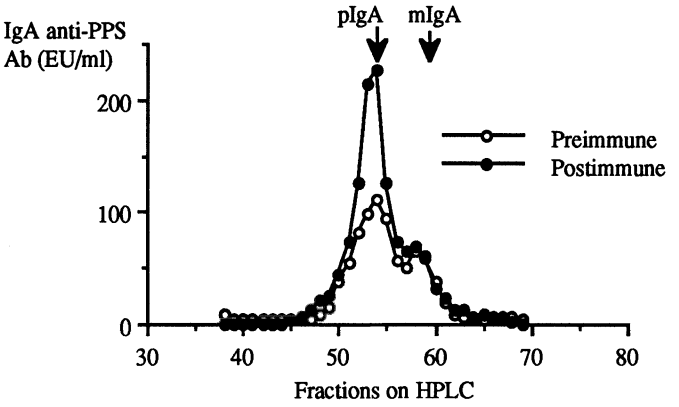

Figure 2. Molecular size profiles of IgA anti-pneumococcal polysaccharide antibodies determined by HPLC on serum samples obtained before and 2 wks after systemic immunization.

4. Induction Of The IgA Immune Response In External Secretions By Systemic Immunization

Ingestion of *Streptococcus mutans*, influenza virus, or various other microbial antigens leads to the appearance of S-IgA in external secretions (but usually not in serum) due to the dissemination of antigen-sensitized IgA-committed precursors of plasma cells from GALT to remote mucosal tissues [Czerkinsky *et al.*, 1987; Mestecky 1987]. In most cases, systemic immunization has been ineffective in inducing S-IgA responses [Mestecky 1987]. However, it has been shown that systemic immunization with an antigen that had been previously encountered through mucosal surfaces induces a specific S-IgA response in external secretions (Table 1) [Svennerholm *et al.*, 1980; for review see Mestecky 1987]. Using systemic immunization with polysaccharide antigens from bacteria (*S. pneumoniae Haemophilus influenzae* and *N. meningitidis*) that in the past almost certainly had colonized mucosal surfaces of young adult volunteers, we have demonstrated that both systemic and secretory IgA responses could be induced [Lue *et al.*, 1988]. However, the

TABLE 1. Serum and secretory antibody responses after systemic immunization

Antigen	Serum response	Secretory response	References
V. cholerae LPS	IgG	Secretory IgA in milk and saliva	Svennerholm, *et al.* 1988
Tetanus toxoid	IgG>IgA	Secretory IgA in saliva in 1 case	Mascart-Lemone, *et al.* 1987
Pneumococcal polysaccharides	IgA>IgG>IgM (IgA2>IgA1)	IgA in saliva, bile, and tears IgG and IgM in saliva	Lue, *et al.* 1988
H. influenzae type b	IgA>IgG>IgM (IgA2>IgA1)	IgA, IgG, IgM in saliva	Tarkowski, *et al.* 1989
Meningococcal types A, C, Y, W-134 polysaccharides	IgA>IgG>IgM (IgA2>IgA1)	IgA, IgG, IgM in saliva	Tarkowski, *et al.* 1989

magnitude of the secretory IgA response in saliva was considerably lower than the systemic IgA or IgG response. Similarly, systemic immunization with the influenza virus vaccine induced, in addition to systemic IgG and IgA responses, a modest increase in IgA antibodies in external secretions [Moldoveanu *et al.*, unpublished]. These data suggest that systemic immunization can be used, although with at best modest results, in the induction of specific IgA antibodies in secretions. This goal may be achieved with better results by oral immunization with novel antigen delivery systems [Mestecky and McGhee, 1989].

5. Regulation Of Human IgA Synthesis

Studies in experimental animals have suggested that the induction of B cells which become IgA-producing plasma cells is regulated by T cells and derived interleukins [McGhee *et al.*, 1989]. $CD4^+$ Th cells from GALT support antigen-specific and polyclonal IgA responses. Two interleukins, IL-5 and IL-6, are of most importance for IgA synthesis in mice, and it has been shown that IL-5 and IL-6 induce surface IgA^+ B cells to develop into IgA-secreting cells. Human IL-6 induces both antigen-specific and polyclonal IgA1 and IgA2 responses, and the subclass produced is dependent upon the tissue source of B cells. In this regard, higher frequencies of IgA1 B cells occur in spleen, while IgA2 B cell precursors, sensitive to IL-6, reside in the human appendix.

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Lymphoid cell populations dynamics and the mucosal immune response in humans

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1. Introduction

Investigations of the mucosal immune apparatus have for the most part concentrated on the function of animal and especially murine lymphocytes isolated from organized gut-associated lymphoreticular tissues (GALT) such as the Peyer's patches. There is convincing evidence in several animal species that memory cells, and in particular antigen-sensitized B cells arising from GALT, might disseminate from these sites to remote secretory tissues. It is generally believed that ingested antigens, once absorbed in immunologically sufficient quantities by a specialised epithelium covering Peyer's patches, can be processed and presented to underlying B and T lymphocytes. A fraction of locally activated B cells may leave the patches, transit through the mesenteric lymph nodes and thoracic duct, enter the circulation and while differentiating populate various secretory tissues including the gut, mammary and salivary glands, respiratory tract, where they further undergo terminal differentiation into plasma cells producing secretory antibodies, and especially in humans antibodies belonging predominantly to the IgA class (reviewed in [1]). These observations which have led to the concept of a "common or generalized mucosal immunological system" [2, 3] are especially important when considering strategies of vaccination against mucosal pathogens, since enteric delivery of immunogens is the most pratical and safe immunization route in humans and may soon constitute the most efficient mean to achieve immune protection against pathogens encountered at mucous membranes.

However, one should keep in mind that considerable species differences may exist with respect to mucosal immune reactivity to a given antigen, with respect to B cell traffic between different mucosal organs [4] and even within a given mucosal organ such as the intestine where a certain degree of anatomical specificity has been documented for the homing of IgA commited B cells which takes place preferentially at antigen sensitizing sites [5]. To date, most studies in humans have focused on the expression of specific B cell responses in secretions. While B cells and their products, which are typified by secretory IgA antibodies, have received a predominant attention by mucosal immunologists, little is known concerning the functional characteristics of other lymphoid cells populating mucosal tissues in the development of such immune responses. Such studies have largely been hampered by the presumed anatomical inaccessibility ot mucosal tissues in humans, the lack of appropriate methods for studying immune responses at mucosal sites in general, and by the paucity of safe and efficient, i.e.immunogenic and protective, mucosal vaccines in humans.Following the recent development of an oral cholera vaccine [6], a vaccine which has proven to be exceptionally immunogenic in humans and to evoke a protective secretory immune response in the intestine [7] (see also Holmgren, J., this volume), we have devoted extensive efforts to establish preparative and analytical methods permitting the study of antigen induced secretory immune responses at the clonal level in human volunteers orally immunized with cholera vaccine. In this respect, we have established a two-step enzymatic dispersion procedure for isolating highly viable and apparently functional lymphoid cells from small bioptic mucosal specimens in humans. The technique, which makes use of a combination of 2 enzymes, the cold acting enzyme thermolysin followed by collagenase, has proven suitable for isolating cells from minor salivary glands and from intestinal endoscopic specimens. A detailed description of the procedure will be presented elsewhere (see also

Nordström et al., this volume). A recent development of the ELISPOT technique using two immunoenzymatic visualization systems yielding distinct colour products has been established [8].The latter development together with improvements in sensitivity and a miniaturisation of the original technique have now permitted to reduce the number of cells required for a given assay by at least 10 fold.This consideration is of practical importance given the limited number of lymphoid cells available from dissociated human mucosal tissues. Reverse modifications of the ELISPOT technique have also been developed[9] and applied to detect individual cells secreting various lymphokines and accessory cytokines. Having developed suitable preparative and analytical techniques for studying cells isolated from human mucosal tissues, we took advantage of these developments to adress the following questions:

1) What is the cellular basis of a generalized secretory antibody response in humans ?
2) To which extent the circulatory pool of "activated" lymphoid cells arising from enteric immunization with cholera vaccine may reflect intestinal mucosal lymphoid cell reactivity in humans ?
3) What are the consequences of T cell activation at mucosal sites on the development of local and generalized secretory immune responses ?
4) Can selective abnormalities of the IgA system, such as selective IgA deficiency, be associated with functional changes in mucosal B-, T-, and/or accessory-cell reactivity ?

Peroral immunization with cholera vaccine induces specific antibody-secreting cells in peripheral blood and in salivary glands.

In order to adress the first question, we examined in 30 healthy adult Swedish volunteers whether immunization with oral cholera vaccine would elicit gut-derived antibody-producing cells in the blood and in distant mucosal tissues such as the labial minor salivary glands (MSG). The results of this study demonstrate that there was indeed a practically obligatory response with specific antibody-producing cells against the B subunit test antigen in both peripheral blood and MSG. The response in peripheral blood comprised both IgA and IgG antibody-forming cells in comparable frequencies and was already manifest one week after the first immunization in some volunteers (figure 1).This kinetically dramatic response with a brisk but transient elevation in antibody-secreting cell frequencies was then repeated and enlarged after each of two booster immunizations in more than 70 % of the volunteers.

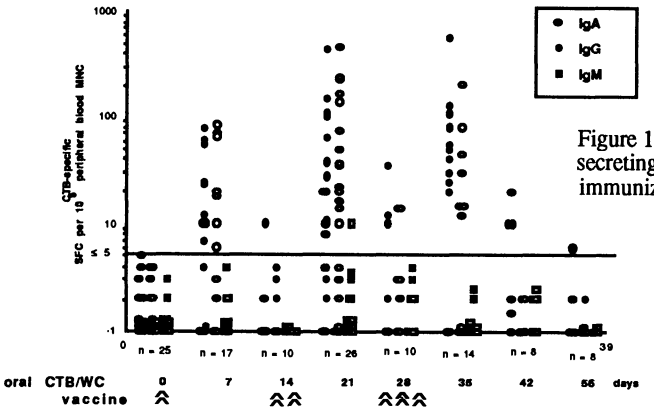


Figure 1. Human peripheral blood antibody-secreting cell responses after peroral immunization with cholera vaccine.

This blood response preceded the response in minor salivary glands (figure 2). Although no biopsies were taken after the first immunization, there was a substantial specific antitoxoid antibody-producing cell response in salivary glands. This response was essentially restricted to the IgA class. Furthermore, although biopsies were available from only a limited number of volunteers on each occasion (which explains the considerable variations in the magnitude of the responses monitored), most of the specimens taken after the first two immunizations contained specific IgA -secreting cells, in contrast to the control biopsies taken before immunization and which were completely devoid of such activity. It is also evident

after the third immunization that the response in salivary glands peaked at a later time than in the blood, that is at approximately two weeks after the booster immunization at which time the corresponding responses in the blood had essentially disappeared. That a certain degree of isotype selectivity in the salivary gland response to cholera B subunit had occured is suggested by the virtual absence of specific IgG response in these glands which contrasts with the presence of substantial numbers of IgG-secreting cells in peripheral blood.

Residence and expansion of cholera vaccine specific immunocompetent lymphocytes in the intestine of orally immunized healthy human volunteers

We have sought to gain an impression of the extent to which the circulatory pool of "activated" lymphoid cells arising from enteric immunization with cholera vaccine may reflect local intestinal lymphoid cell reactivity in humans. For this purpose, we examined the local B- and T- cell immune responses generated by cholera vaccine at local intestinal sites, in healthy human vaccinees following a standardized immunization/sampling protocol.

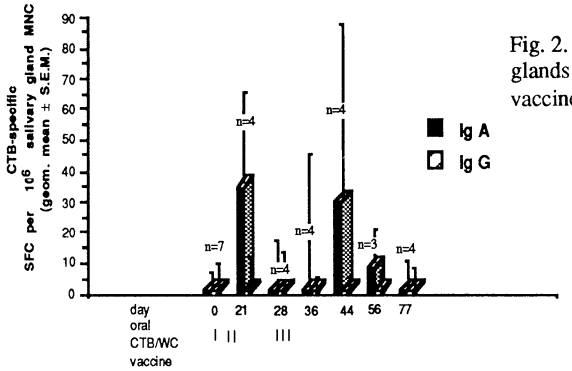


Fig. 2. Antibody-forming cells in minor salivary glands after peroral immunization with cholera vaccine.

The results of these studies demonstrate that:

1) intestinal B cell responses of considerable magnitude develop in the duodenal mucosa of these volunteers and are dominated by specific IgA ASC after a booster immunization (fig. 3). It also appears from these studies that an IgM response of appreciable magnitude develops after a single oral dose. The latter observation is worth mentioning in view of the virtual absence of specific IgM cells in the peripheral blood of essentially all volunteers whom we have so far examined (see fig. 1). In four of these volunteers, local B cell memory was documented by the effect of a single dose of cholera vaccine given 5 months after the initial immunization regimen; in all four individuals, marked IgA-antibody-secreting cell responses developed in the intestinal wall one week after oral administration of a third dose of cholera vaccine (figure 3).

2) activated T cells (and possibly NK cells) secreting gamma-interferon expand after booster immunization with oral cholera vaccine in the intestinal mucosa (figure 4). It is also noteworthy that the immunization regimen did not appear to influence the size of the circulating pool of gamma-interferon-secreting cells, at least at the times that we have examined (see also Quiding et al., this volume).

Thus cholera vaccine appears to be an extremely potent inducer of gamma-interferon, an observation that may have important implications concerning the role of this cytokine in immune protection at mucosal surfaces. Indeed, recent reports indicate that gamma-interferon may influence the efferent phase of a secretory intestinal antibody response by increasing epithelial cell surface expression of secretory component [10] and thus uptake and transport of potentially protective sIg, and may also directly regulate such basic physiological roles of epithelia as barrier function [11] and permeability to electrolytes [12]. Preliminary experiments indicate that the production of other cytokines by enzymatically dispersed intestinal cell suspensions may be influenced by oral immunization with cholera vaccine. In this respect, intestinal production of bioassayable quantities of IL-6, a cytokine involved in B cell differentiation and in accessory cell function, by short term (16 hrs) cultures of human duodenal cell suspensions appears to be increased after booster immunization with oral cholera vaccine (P. de Man et al., in preparation).

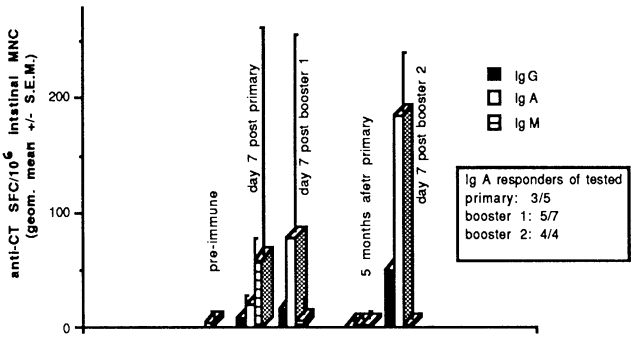
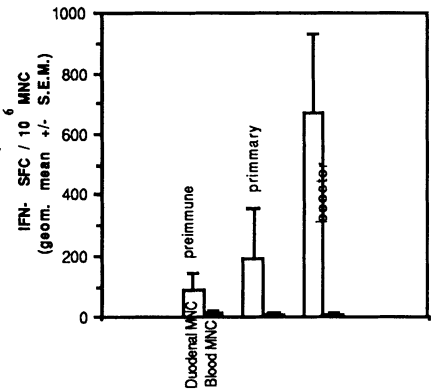


Fig. 3. Intestinal antibody-secreting cell responses after peroral immunization with cholera vaccine

Fig. 4. Intestinal gamma-interferon-producing cells in individuals perorally immunized with cholera vaccine



Intestinal and blood responses to oral cholera vaccine in individuals with selective IgA deficiency

Finally, the last question that we adressed was whether a selective abnormality of the IgA system such as selective IgA deficiency, could be associated with functional changes in mucosal B cell, T cell, and/or accessory cell reactivity? For this purpose, we used the same experimental protocol as outlined above, and examined the intestinal and circulating B-, and T- cell responses in 16 IgA deficient individuals comprising 11 individuals with known history of reccurent upper respiratory and/or enteric infections, and 5 asymptomatic individuals. As shown in fig. 5, CTB-specific circulating B cell responses developed in most IgA deficient individuals but were exclusively of the IgG isotype. Unexpectedly, CTB-specific IgM-secreting circulating B cells were not detected in any of IgA deficient individuals examined, despite increased frequencies of total IgM-secreting blood lymphocytes in the majority (8/15) of these individuals (data not shown). In contrast, large numbers of CTB specific IgM-secreting cells were detected in the intestinal mucosa of the same individuals after a single oral dose of cholera vaccine (figure 6). Furthermore, these intestinal IgM responses could be enlarged by a second dose of oral cholera vaccine, suggesting that compensatory IgM responses occur in most IgA deficient individuals but, at least in the case of oral cholera vaccine and its CTB component, are restricted to the intestinal mucosa. In addition, significantly larger numbers of CTB specific IgG-secreting cells were disclosed in the intestinal mucosa of IgA deficient individuals as compared to healthy volunteers.However, whereas there were no correlations between the

magnitude of CTB- specific IgM responses and the clinical status of IgA deficient individuals, significantly higher IgG responses were developed in infection-prone IgA deficient individuals as compared to "healthy" IgA deficient individuals and normal volunteers (Table I). Gamma-interferon and IL-6 production by intestinal mucosa and peripheral blood cell suspensions, when examined prior to and after immunization with cholera vaccine, were found unaffected indicating that abnormal production of either of these cytokine cannot account for defective IgA synthesis in these patients (data not shown).

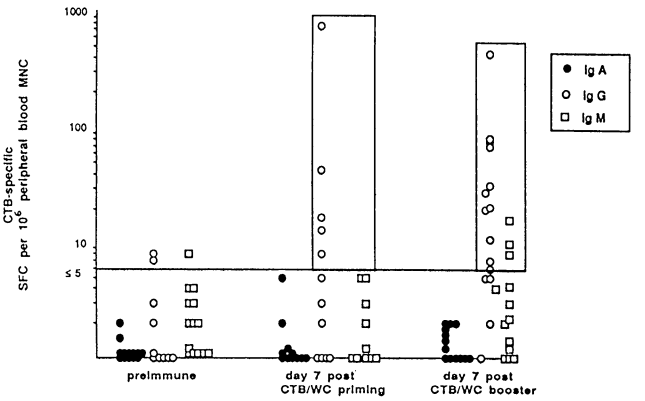


Fig. 5. Circulating antibody-producing cells after peroral immunization with cholera vaccine in IgA deficient individuals.

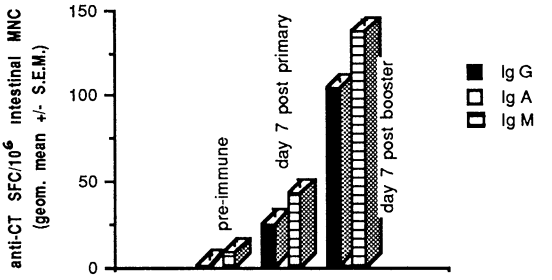


Figure 6. Intestinal antibody-secreting cells after oral immunization with cholera vaccine in IgA deficient individuals.

Summary

We have demonstrated the feasibility of functional analyses of subpopulations of lymphoid cells isolated from human mucosal tissues currently amenable to safe bioptic procedures. We have demonstrated that enteric immunization with a prototype immunogen, CTB/*Vibrio cholera* whole cell vaccine, induces the appearance of antigen specific antibody-producing cells not only in peripheral blood but also in minor salivary glands, that is at a remote site of initial antigen exposure; therefore this observation provides corroborative and direct support to the notion of a generalized mucosal immune network in humans [13, 14]. In addition, it provides additional support to our previous studies in animals indicating that cholera B subunit may be a useful carrier protein for preparing conjugate vaccines that through peroral administration could elicit immune responses against pathogens encountered at non-intestinal mucosae [15] .

The migration and tissue localisation of CTB-specific B cells activated at enteromucosal sites appears exquisitely restricted with respect to isotype commitment: gut IgA and IgG commited B cells can circulate

from the intestinal wall to the circulation, but only IgA commited B cells can seed into salivary glands. In contrast, IgM B cells activated in the gut do appear to have limited migratory potential.
The demonstration of large numbers of gamma-interferon secreting cells in the human intestinal mucosa indicates that this organ is the site of a pronounced and sustained state of T cell (and possibly NK cell) activation. That a boosterable increase in the frequency of gamma-interferon-secreting cells occurs in the intestinal wall after peroral immunization with cholera vaccine, supports the view that this cytokine may play a central role in the local defence of enteromucosal surfaces by acting not only on lymphomonocytic cells but also by affecting the functions of epithelial enterocytes.
Individuals with selective IgA deficiency appear to compensate their deficit not only with enhanced IgM production, but also with increased IgG production. However, increased intestinal IgG antibody production appears to be associated with increased susceptibility to chronic mucosal infections. In these individuals, intestinal gamma-interferon and interleukin 6 production appear unaffected, indicating that defective IgA synthesis in these subjects is not directly related to abnormalities in the production of these cytokines.

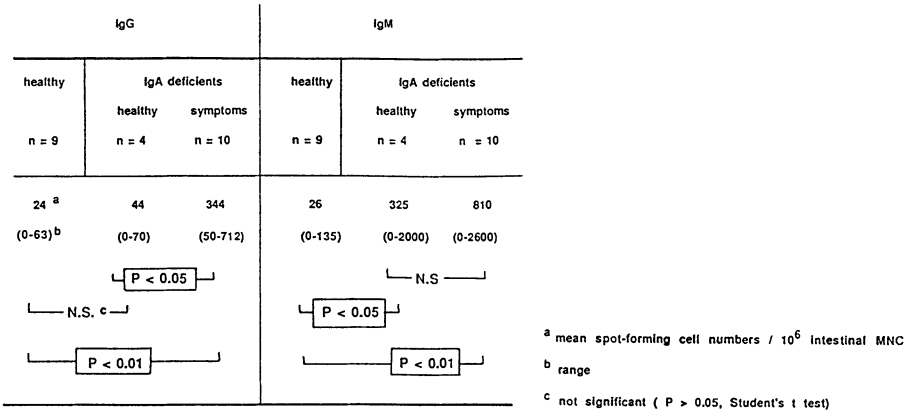


Table 1. Comparison of secondary intestinal antibody responses to oral cholera vaccine in normal and Ig A-deficient individuals

Aknowledgments: The studies summarized in this manuscript were supported by grants from the Swedish Medical Research Council.

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The current status of an oral vaccine for the prevention of recurrent episodes of acute bronchitis

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Immunisation at mucosal sites has often been considered a failure because on one hand the subtleties of in vivo antigen presentation and the effect of tight regulation loops have not been recognised, while on the other hand unrealistic expectations are made. The essential difference between systemic and mucosal immunisation is that sterilising durable immunity follows the former, while mucosal immunity often is limited to a less permanent stabilisation of a colonising process, which would otherwise cause clinical infection. Recognition that the common mucosal system was driven by the gut-associated lymphoid tissue (GALT), provided a framework for the manipulation of colonising processes at distant mucosal sites (1). We chose the human model of recurrent acute bronchitis to test the concept that a limited antigenic stimulus to GALT could modulate bacterial colonisation at the damaged bronchus mucosa to reduce the frequency of recurrent acute bronchitis in subjects with chronic obstructive lung disease (COLD). Colonisation of the damaged bronchus mucosa is polybacterial but non typable Haemophilus influenzae (HI) is commonly present and when present constitutes >90% of the total bacterial count. A trial of oral immunisation with HI reported a significant reduction in frequency of episodes of acute bronchitis in subjects with COLD who had taken three courses of an enteric coated preparation of HI. This presentation reviews the current status of oral immunisation with HI and in addition to confirming protection against acute bronchitis, provides evidence of a reduction in the level of colonising HI within the respiratory tract.

Clinical Trials

	Year	Diagnosis	No. in Trial	Duration (months)	Site
001	1983	Chronic bronchitis	50	3	Newcastle[1]
002	1986	Recurrent acute bronchitis	40	6	Newcastle[2]
003	1986	Chronic bronchitis	104	9	Newcastle[3]
004	1987	Chronic bronchitis	108	12	Newcastle[3]
005	1987-8	Chronic bronchitis	62	6	P.N.G.[4]
006	1988	Chronic bronchitis	64	6	Perth.[5]

[1] R. Clancy, A. Cripps, K. Murree-Allen
[2] R. Clancy, A. Cripps
[3] R. Clancy, A. Cripps, K. Murree-Allen, N. Saunders
[4] M. Tanden
[5] D. Lehmann, M. Alpers.

The format of each trial was similar, although only trials 003 and 004 included a 'run in' period to allow for correction due to different infection rates. Three courses of HI given at 0, 4 and 8 weeks (each course consisted of two tablets each morning for three consecutive mornings - each tablet contained 10^{11} HI). Each trial was a randomised, double blind, prospective study supervised by medical and nursing staff with monthly visits for clinical and microbiological evaluation. All infective episodes were documented by an 'infection questionnaire', with acute bronchitis defined as an episode of 'increased volume and purulence of sputum'.

Microbiology:

Trial 001	002	003	004	005	006
Quadrant score (trend observed)	Quadrant score - no winter colonisation in treatment group.	Reduced colony count (1 log - P=0.01)	Reduced colony count (50% - P=0.13)	Reduced colony count - 3 logs reduction	Carriage reduced by 50% in treatment group

The reduction in load of HI was seen in all trials at about 8-12 weeks; counts then returned towards placebo levels over 4-8 weeks, except in the PNG study, where even at 12 months the treatment group had less HI by 1 log. No similar reduction was seen in H. parainfluenzae where monitored though the level of specificity requires further study.

Clinical protection:

Trial	001	002	003	004	005	006
Colonisation %	70	39	6	11	80	29
Clinical Protection %	90	40	16	36	70	33
P = ( )	(0.001)	(0.16)	(0.13)	(0.03)	(0.045)	(0.024)

In all studies, protection was demonstrated especially in the period immediately following completion of the three courses. Protection varied with the level of colonisation in the study population ($r = 0.9$), and was most easily demonstrated at periods of high incidence of infection. In the PNG trial where a very high level of colonisation was noted, protection persisted for 12 months. Qualitative effects noted in one trial (006) using a visual analogue scale showed a global sense of improvement of symptoms in the treated group, possibly through a reduction in intrabronchial inflammatory response short of that required to give rise to the symptoms of acute bronchitis. In another study (002) a significant ($P > 0.03$) reduction in wheeze was noted in acute episodes, raising the possibility of an asthmatic response by inflamed bronchi to colonising bacteria in this group, which is reduced when colonisation with HI is suppressed.

DISCUSSION.

These six trials establish a clinical value for a killed HI oral vaccine in subjects prone to recurrent episodes of acute bronchitis with or without COLD. Protection varied with the prevalence of colonisation by HI. The duration of protection was at least six months, with a longer duration in the PNG study where a high prevalence of colonisation was noted. In each study a reduction in bacterial load was demonstrated, monitored by the incidence of throat colonisation and/or quantitative or semiquantitative bacteriology of sputum samples - this reduction lasted weeks to months in most studies, except for the PNG study where a reduction persisted through 12 months of observation.

The mechanism of oral immunisation is not entirely clear, but appears to involve activation of GALT T cells, Antibody studies in man and in a rat model do not correlate with protection. T cells can be detected in blood following oral immunisation, and they have been quantitated using limiting dilution technology (2) and in the rat model which measures clearance of HI from the respiratory tract (3) immunity could be transferred only with thoracic duct T lymphocytes following oral immunisation (4). Immunity in the rat model correlated with an enhanced recruitment of neutrophils into the bronchus lumen. This model also identified an important role for antigen stimulation of the bronchus, which possibly explains the variation in protection with incidence of colonisation, and the prolonged protection seen in populations with a high carriage rate.

This clinically valuable form of oral immunisation exploits the basic principles of mucosal immunity, demonstrating both the potential and the limitations for this approach to the prevention of mucosal infection.

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Local oral immunisation with synthetic peptides induces a dual mucosal IgG and salivary IgA antibody response and prevents colonisation of *Streptococcus mutans*

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Abstract

Synthetic peptides (SP) based on the amino terminal sequence of a 3.8kD streptococcal antigen have been produced and used in topical oral immunisation of the gingivo-mucosal epithelium of macaque monkeys. Linear or cyclised SP11 and random SP11 induced negligible or very low antibody levels and did not prevent colonisation of the teeth by *S. mutans*. Immunisation with SP17, SP21 and SP35 induced significant anti-SP as well as anti-native streptococcal antibodies in the gingival fluid and saliva, and either prevented or greatly reduced, colonisation by *S. mutans*. The results suggest that local immunisation with SP derived from the sequences of a *S. mutans* antigen may induce a dual gingival IgG and salivary IgA response against SP and native SA, which may be involved in preventing colonisation of *S. mutans* and the onset of dental caries.

Introduction

Oral immunisation with cells of *S. mutans* elicited predominantly secretory IgA antibodies in the saliva of rats (Michalek et al 1976) monkeys (Challacombe and Lehner 1979) and humans (Mesteky et al 1978a). Systemic immunisation with whole cells (Lehner et al 1975) and the 185kD cell-surface streptococcal antigen (Lehner et al 1981) or the 3.8kD streptococcal antigen induces predominantly serum IgG antibodies and CD4 T cell helper function (Lehner et al 1985). These responses significantly inhibit colonisation of *S. mutans* in the mouth and the development of dental caries. Previous attempts to localise the immune response to the oral cavity were successfully carried out using a 3.8kD streptococcal antigen in local gingivo-mucosal immunisation in rhesus monkeys (Lehner et al 1986). This antigen elicited both salivary IgA and gingival lgG antibodies to streptococcal antigen. This was associated with a significant reduction in colonisation by *S. mutans* and a decreased incidence of caries.

The 3.8kD streptococcal antigen has been sequenced and peptides of 11,17 and 21 residues were synthesised (Haron et al 1988). Both linear and cyclised forms of the peptides have been produced since linear peptides are often poorly immunogenic (Arnon et al 1971). The SP were non-immunogenic in macaque monkeys unless linked to the carrier tetanus toxoid. However, the dimer of SP17 was immunogenic without a carrier (Lehner et al 1989b).

In the present investigation we have attempted to induce a local immune response using defined peptides based on the amino terminal sequence of the 3.8kD streptococcal antigen (SA).

The SP were applied directly to the gingiva of 18 macaque monkeys and antibody to both 3.8kD SA and SP was detected using a solid phase radioimmuno assay (Lehner et al 1981). Some of the SP induced a dual gingival IgG and salivary IgA antibody response. The antibodies reacted with both SP and 3.8kD SA and were associated with the prevention of colonisatiom by *S.mutans*.

Materials and Methods

PEPTIDES

The linear structure of the peptides used in this experiment have been previously described (Lehner et al 1989b). Cyclised forms of SP11 and SP17 were prepared by the addition of cystein and incubation in ammonium chloride buffer. SP35 was produced as a dimer of the linear SP17 linked to SP21 peptide. An 11 residue random peptide (random 11) was also synthesised and used in sham-immunisation. All SP were purified by reverse phase HPLC.

IMMUNISATION

18 young macaque monkeys were used in this study and divided into 6 immunisation groups. 10μg of peptide (SP11 linear, SP11cyc, SP17, SP21 and SP35) in 150μl of sterile saline and 50% dimethyl sulphoxide was applied to the gingival sulcus on 6 separate occasions over a period of 16 weeks, as previously described (Lehner et al 1989a). The SP was retained in situ for 5 minutes by the use of silicone rubber impression trays to prevent washing away by saliva.

FLUID SAMPLES AND RADIOIMMUNOASSAY

Serum, gingival fluid washings, pilocarpine stimulated saliva and plaque were collected before and after each immunisation and thereafter at monthly intervals up to 30 weeks after the start of immunisation (Lehner et al 1986). A solid phase radioimmunoassay was used to detected antibodies using specific anti-monkey IgG and IgA reagents. The collection technique for gingival fluid results in about 100 fold dilution and can be considered only relative to the control values. The results were expressed as net counts per minute (c.p.m.) after subtraction of pre-immunisation values.

BACTERIOLOGY

Plaque was collected from the smooth surfaces of the maxillary central incisors and the fissures of the second deciduous molars before, during and after immunisation. The samples were enumerated as previously described (Caldwell et al 1977). The number of *S. mutans* recovered were expressed as a percentage of the total colony count on TYC medium.

Results

Sequential radioimmunoassay of gingival IgG and salivary IgA antibodies revealed a significant increase in both anti SP and anti SA antibodies after immunisation with SP17, SP21 and SP35 dimer (Fig.1). However the kinetics of the response differed in that salivary IgA was optimal 5 to 10 weeks after immunisation while gingival IgG appeared only 10 weeks after immunisation. Optimal levels of gingival IgG were not reached until week 26. The core peptide, (SP11), failed to induce an increase in either IgG or IgA, in its linear or cyclised form. Sham-immunisation with the random SP11 gave an initial increase in salivary IgA and some increased binding to 3.8kD SA by gingival IgG, but this was not maintained and may reflect a natural immune response to the colonisation by *S. mutans*. Overall the response to SP17, SP21 and SP35 was sustained throughout the course of the experiment. Salivary IgA antibodies elicited to SP21 showed a small increase in titre to 3.8kD SA and fluctuating titres were elicited by SP35. Small increases in serum IgG antibodies to the native SA and SP were detected but the antibody levels were negligible, compared with those induced by systemic immunisation. Comparable increases in serum antibodies against SA, SP17 or SP21 was also found by immunisation with random SP11.

Control animals immunised with random 11 showed increased colonisation by *S.mutans* on both smooth surfaces and fissures of the deciduous teeth. In contrast immunisation with SP17, SP21 and SP35 either completely prevented colonisation or decreased the proportion of *S. mutans* in those monkeys with pre-existing *S. mutans* on their teeth. Statistical analysis of the increase in colonisation at both sites showed a significant increase in the control group (immunised with random 11) and the groups immunised with SP17, SP21, and SP35 ($t=2.9159$ d.f. 18 $P < 0.01$)

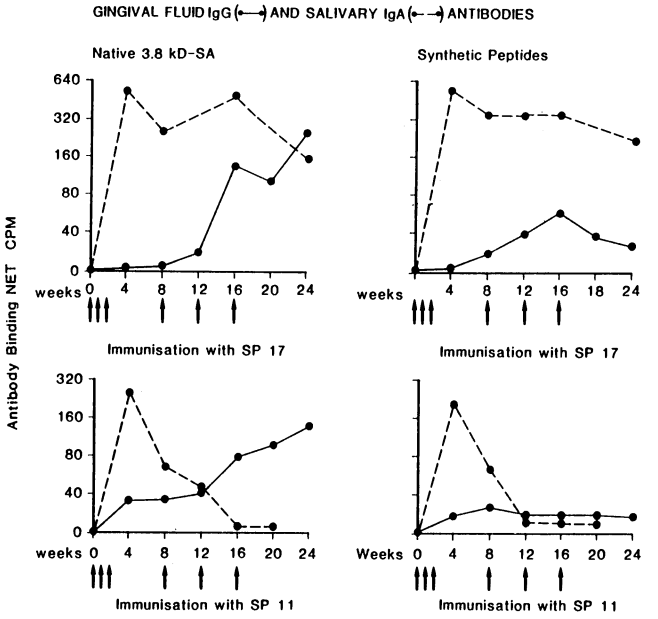

Fig 1. Sequential gingival fluid IgG and salivary IgA antibodies to 3.8kD-SA and synthetic peptides are given as net c.p.m. after local immunisation of monkeys with SP17 and random SP11.

Discussion

Synthetic peptides of 17 or 21 residues or a dimer of 35 residues induced an increase in gingival IgG and salivary IgA antibodies to the SP and native 3.8kD-SA. The kinetics of the response differed in that salivary IgA appeared earlier while gingival IgG appeared after 10 weeks. Low levels of serum IgG antibodies had the same kinetics as the gingival IgG. These negligible serum titres were consistent with those previously reported in local immunisation with 3.8kD SA (Lehner et al 1986) and could be due to either spillover into the circulation or a low level natural immune response to colonisation by *S. mutans* (Lehner et al 1981).

The mechanism of a dual antibody response is uncertain but the hypothesis is that IgG antibodies are produced by the gingival focus of lymphoid cells (Brandtzaeg and Tolo 1977, Page & Schroeder 1976) while salivary IgA antibodies may well be induced directly in the minor salivary glands scattered under the oral mucosa, especially the lips and cheeks (Nair & Schroeder 1983). We cannot discount the possible role of the gut associated lymphoid tissue (Mestecky et al 1978b) if some of the peptide is swallowed. However this seems remote given the small amount of SP administered (10μg).

The prevention or reduction in colonisation by *S.mutans* after local immunisation with SP is consistent with the results based on local immunisation with 3.8kD-SA (Lehner et al 1986). Gingival IgG and salivary IgA antibodies were also produced and led to decreased colonisation and reduced dental caries under the pressure of a carbohydrate rich human type diet.

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Oral *Streptococcus mutans* vaccines: diversity of approaches for the induction of mucosal responses

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ABSTRACT. In this paper, we have summarized our studies in humans and experimental animals concerned with the development of oral vaccines effective in inducing mucosal IgA responses. In our studies, we have used antigens of or anti-idiotypic antibodies that mimic *Streptococcus mutans*, the principal etiologic agent of dental caries, which has allowed an assessment of the induction of a mucosal immune response and a determination of the effectiveness of the response in protection against *S. mutans* infection. Evidence is provided indicating: 1) the usefulness of liposomes as carriers of purified antigen and as an adjuvant for the induction of mucosal responses to orally administered vaccine antigens, 2) the ability of anti-idiotypic antibodies to induce a protective mucosal response and 3) the induction of a primary and secondary salivary IgA anti-*S. mutans* serotype carbohydrate (CHO) immune response in humans after swallowing capsules containing *S. mutans* CHO in liposomes.

INTRODUCTION

Immunoglobulin A is the principal immunoglobulin isotype in human external secretions and is considered to be the first line of defense against infections which involve mucosal surfaces [1,2]. Studies in both humans and experimental animals have shown that oral administration of antigen results in the appearance of IgA antibodies in external secretions, such as saliva, via the common mucosal immune system [1,2]. Thus, the development of vaccine approaches which are effective in inducing mucosal IgA responses would be of global importance towards establishing ways of protecting societies against a variety of infectious diseases.

One area of our research for over a decade has been primarily concerned with investigating characteristics of oral vaccines important in the induction of a mucosal, especially salivary, immune response protective against the infectious oral disease dental caries, in which the principal etiologic agent is *Streptococcus mutans*. In our studies, we have investigated several types of oral vaccines for their ability to induce caries immunity which will be reviewed here. In terms of antigens, these include whole cells and purified antigens of *S. mutans*, anti-idiotypic antibody and cloned *S. mutans* gene products. Adjuvants or potentiators of immune responses which we have studied for their use in oral vaccines include liposomes (artificial membrane vesicles), muramyl dipeptide and gram-negative bacterial vectors. Early studies in experimental rats have shown that oral administration of *S. mutans* whole cells results in a salivary IgA immune response which

correlated with protection against disease [3,4]. Purified antigens were shown to be less immunogenic than particulate forms when used as oral vaccines; however, the oral administration of purified *S. mutans* antigen in liposomes and/or with muramyl dipeptide to rats has been shown to induce (potentiate) salivary IgA immune responses [4]. In humans, it has been shown that oral administration of *S. mutans* whole cells results in the induction of a primary and secondary salivary IgA immune response [5]. Furthermore, the demonstration of antigen-specific, IgA antibody-forming cells to *S. mutans* antigens in the circulation of orally immunized human volunteers provided additional evidence for a common mucosal immune system in humans [6]. Here we have briefly reviewed the evidence supporting the potential usefulness of liposomes as adjuvant/carriers of purified antigens and of anti-idiotypic antibody for oral vaccine development.

MATERIALS AND METHODS

Animals

Germfree Fischer rats used in these studies were derived from a breeding colony of animals and maintained in Trexler plastic isolators at the University of Alabama at Birmingham. The experimental procedures, immunization regimen and assays used were the same as described elsewhere [3,4].

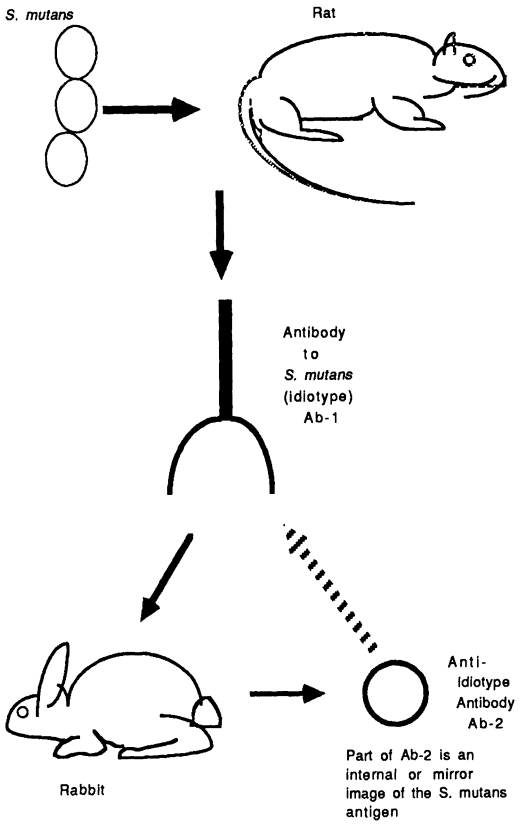
Human Volunteers

Five subjects used in this study were asked to swallow enteric-coated capsules containing 500 μg of purified serotype carbohydrate antigen of *S. mutans* (CHO) in liposomes (CHO-L) daily for seven days [prepared as described elsewhere in this book; Childers *et al.*]. Three individuals underwent a second immunization which involved swallowing capsules daily for an additional seven days. Unstimulated parotid saliva samples were collected from each individual prior to, during and after each immunization period and assessed for antibody activity by ELISA [see Childers *et al.*, this book].

Oral Vaccines

The *S. mutans* whole cell antigens and purified antigens used in these studies were purified as described previously [3,4 and Childers *et al.*, this book]. Oral vaccines composed of antigen in liposomes were produced by hydrating a monolayer of phosphatidylcholine, cholesterol, and dicetylphosphate (total lipid = 2 mg/ml) with aqueous antigen followed by microemulsification (inlet pressure; 80 psi, 10 min, M110, MediControl, Newton, MA). Liposomes were characterized for size and homogeneity using flow cytometry [7].

The anti-idiotypic antibodies used as the oral vaccine were generated as illustrated below. Briefly, rats were systemically immunized with *S. mutans* whole cells and the serum antibody was purified and used to immunize rabbits. Rabbit IgG from immunized (anti-Id) or control (NRIgG) rabbits was purified and then incorporated into liposomes and given to germfree rats by gastric intubation as indicated above.



RESULTS

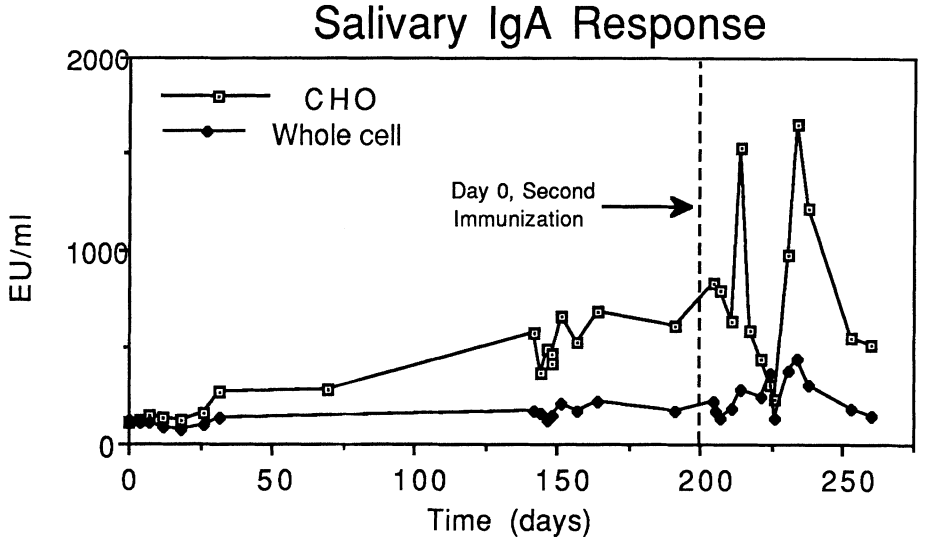
Studies in our experimental rat caries model have been designed to establish the effectiveness of oral *S. mutans* vaccines for inducing salivary IgA immune responses and the effectiveness of the response in protection against *S. mutans* infection. As indicated in the table below, rats given *S. mutans* whole cells by gastric intubation exhibited a salivary IgA response and a 40% reduction in caries activity. When rats were given *S. mutans* CHO, essentially no response was noted. However, when the CHO was incorporated into liposomes and given orally to rats, a good salivary IgA response and reduction in caries activity were seen.

In a separate study, rats were given by gastric intubation rabbit anti-idiotypic antibodies (anti-Id) or normal rabbit IgG (NRIgG) in liposomes. As shown in the table below, rats given the anti-Id vaccine showed a good salivary immune response and a 20% reduction in caries activity.

Oral vaccine	Salivary IgA anti-*S. mutans* activity	*S. mutans* in plaque (% reduction)	Caries activity (% reduction)
Whole cell	42*	54	40
CHO	6*	8	6
CHO/liposome	30*	53	38
Anti-Id/liposome	53**	53	20
NRIgG/liposome	28**	0	0
None	<5*	0	0

*ELISA units.
**O.D.414 x 100 of a 1/80 dilution.

Based on the studies in animals, it was of interest to determine if the CHO-L vaccine could induce a mucosal response in humans. The figure below is representative of the salivary IgA responses obtained in the subjects. Following the primary immunization, a peak response was seen between 21-32 day, whereas, following the second immunization, an earlier salivary IgA response to the CHO was seen.



DISCUSSION

The results of this study provide evidence indicating: 1) the usefulness of liposomes as carriers of purified antigens and as an adjuvant for the induction of mucosal responses to orally administered vaccines, 2) the ability of anti-idiotypic antibodies to induce a protective mucosal response, and 3) the induction of a primary and secondary salivary IgA immune response in humans after swallowing capsules containing *S. mutans* serotype carbohydrate in liposomes.

ACKNOWLEDGEMENTS

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Human mucosal responses to oral liposome-*Streptococcus mutans* carbohydrate vaccine

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Streptococcus mutans (a principle etiologic agent of dental caries)whole cell vaccine, when given orally to humans, has been shown to induce both primary and secondary mucosal immune responses [1]. Oral administration of purified *S. mutans* antigens to rats results in low responses, however, when the antigen is administerred with adjuvants, improved immune responses occur [2]. Therefore, one approach that is being investigated for oral vaccine development involves using liposomes (artificial membrane vesicles) as a vehicle for antigen delivery and evidence has been provided for the induction of a salivary immune response in rats given a liposomal-antigen vaccine orally [2,3]. Therefore, the purpose of the following series of investigations was to initiate human clinical trials to study the induction of immune responses to purified serotype carbohydrate antigen of *S. mutans* (CHO) when incorporated in liposomes and given by the oral route.

CHO was prepared by nitrous acid extraction [4] using washed *S. mutans* whole cells. The crude extract was purified by ethanol precipitation followed by size exclusion and ion exchange chromatography. Purity was established by assessing the CHO preparation for protein and nucleic acid content.

CHO liposomes (CHO-L) were produced by hydrating a monolayer of phosphatidylcholine, cholesterol, and dicetylphosphate (total lipid = 2 mg/ml) with aqueous CHO followed by microemulsification (inlet pressure; 80 psi, 10 min, M110, MediControl, Newton, MA). The liposome preparations were characterized for size and homogeneity as previously reported [5].

Five subjects injested an enteric coated capsule containing 0.5 or 1.0 mg CHO-L daily for seven days. In addition, after a period of time, 3 individuals underwent a second immunization, while one also underwent a third immuniztion. Unstimulated parotid saliva was obtained prior to, during, and following the immunization period using Schaefer cups.

An ELISA was used to analyze saliva antibodies to *S. mutans* CHO. Briefly, duplicates of four 2-fold dilutions of saliva or six dilutions of a serum pool were added to wells of antigen coated and fetal calf serum (10%) blocked 96-well microtiter plates. Following sample incubation, biotin-conjugated goat antiserum to human IgA, IgM, or IgG (Tago, Burlingame, CA) was added and then developed with streptavidin-alkaline phosphatase conjugate (0.4 μg/ml; Southern Biotechnology, Birmingham, AL) and substrate (Sigma Chemical Co.). A log-logit curve fitting program (Assayzap, Biosoft, Elsevier, Amsterdam) was used to construct a standard curve from O.D. readings from the serum pool assigned arbitrary ELISA Units (EU). Samples were compared to this curve and multiplied by the dilution factor to determine the EU/ml of CHO specific antibody.

Protein and nucleic acid determinations showed less than 1 μg/mg of CHO contamination (<1 %). Analysis of L-CHOs indicated that the preparations were composed of uniform, small (~100 nm), unilamellar vesicles (data not shown).

ELISA analysis indicated that salivary IgA (no IgM or IgG) responses to CHO were seen in 4/5 subjects upon primary immunization (see Table). Responses, in general, were modal and peaked between 21-32 days after immunization. After the second immunization, an earlier salivary IgA response to CHO occurred in 2/3 subjects (14 days). Upon a third immunization of one individual, a salivary IgA response was seen 10 days after immunization.

Table: Salivary IgA Immune Response to CHO

Subject	1° Response	2°Response	3°Response
#1	128 (32)*	288 (14)	205 (10)
#2	146 (32)	116 (14)	ND
#3	0	ND**	ND
#4	138 (21)	0	ND
#5	44 (21)	ND	ND

* Values represent the % increase in EU/ml of saliva IgA over baseline; (day of peak response)

** ND = not done

These results show that orally administration of a CHO-L vaccine to human volunteers results in mucosal immune responses. Also, an anamnestic salivary IgA response to the CHO was seen after re-immunization. Further studies are needed to establish the dose of antigen and characteristics of liposomes for induction of optimal immune responses.

This study supported by USPHS grants DE00155, DE08182, and DE04217.

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Specific immune response in the human respiratory tract following oral immunization with live typhoid vaccine

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ABSTRACT. In this paper the ability of a live, oral typhoid vaccine *Salmonella typhi* Ty21a to stimulate specific antibody responses in the lower respiratory tract of non-smoking human subjects was evaluated. Significantly elevated post-vaccination anti-typhoid immune responses were detected in the bronchoalveolar lavage fluid (BAL) of orally vaccinated subjects compared with parenterally vaccinated or unvaccinated control subjects. The response was in the IgG class with no difference in anti-typhoid IgA antibody response. It appeared that the IgG antibody detected was a transudate from serum. Salivary IgA antibody responses not significantly elevated following oral vaccination, confirming salivary antibody determination as a poor indicator of respiratory or intestinal immunity. It is concluded that it is possible to induce a specific immune response in the lower respiratory tract following oral immunization with *S. typhi* Ty21a.

Introduction

Stimulation of a local antibody response through local immunization represents one effective means of providing protection against disease caused by mucosal pathogens. The development of the concept of the common mucosal immune system, whereby the local presentation of an antigen at one mucosal surface can stimulate an immune response at distant mucosal surfaces (1) has led to the examination of oral vaccination as a means of stimulating an effective immune response in the respiratory tract. This paper examined the human immune response, especially of the respiratory tract, to the live oral typhoid vaccine *Salmonella typhi* Ty21a. This vaccine organism has been considered as a potential carrier of the "protective" antigens of other pathogens and the demonstration that Ty21a is able to stimulate a respiratory immune response following oral administration might enable consideration of this organism as a carrier of protective antigens of respiratory pathogens.

Subjects and Methods

Informed consent was obtained from 17 non-smoking healthy adults (aged 18-34 years) with no previous or current history of exposure to typhoid nor symptoms of gastrointestinal or respiratory disease. All subjects were physically screened and laboratory tests of respiratory function were performed.

The subjects were allocated to three groups: *Group A* - 5 control subjects each having fibreoptic bronchoscopy (FOB) with bronchoalveolar lavage (BAL) only; *Group B* - 6 subjects orally vaccinated with 10^{11} live *S. typhi* Ty21a, jejunal fluid collected pre- and post-vaccine by intestinal intubation, salivary and serum collections, and post-vaccination FOB & BAL; *Group C* - 6 subjects subcutaneously vaccinated with two doses of $5x10^8$ commercial killed typhoid vaccine organisms, serum, jejunal fluid, and BAL also obtained as for Group B.

The first 50 ml washout of the BAL fluid, was assayed for antibody and total immunoglobulin separately from the subsequent 100 ml washout. The results are entered as X1 and X2, where X represents the immunoglobulin class and the number denotes the first 50 ml or subsequent 100 ml of BAL.

Class-specific anti-typhoid antibodies in serum and secretions were quantified using a previously described ELISA (2), the results being adjusted for total class-specific immunoglobulin content by a single radial immunodiffusion method, and expressed as units of specific antibody/mg of total class-specific immunoglobulin.

Results

Four-fold or greater jejunal anti-typhoid IgA antibody responses were observed in 5/5 and 1/6 subjects of Groups B & C respectively (p=0.000074), specific IgG responses being undetectable. Significant four-fold or greater anti-typhoid serum IgA responses were also detected in 6/6 and 3/6 subjects in Groups B & C respectively. All subjects in Group B had a four-fold or greater rise in specific serum IgG, whereas in the Group C responses were only measurable in the same 3 subjects with similar specific IgA responses. A significant salivary IgA anti-typhoid LPS antibody response was not observed in Group B (p=0.057). Salivary specific IgA antibody responses correlated poorly with intestinal specific IgA responses (r=0.23, p=0.71).

There was no difference in the anti-typhoid IgA response in BAL fluid obtained from vaccinated or control subjects. The specific IgG antibody level of in BAL G1 of Group B was significantly elevated above Group A (p=0.0.0043) and Group C (p=0.015). However, there was no significant difference in the BAL G2 specific IgG levels between the groups (p=0.065).

The Group B adjusted serum IgG anti-typhoid antibody titres were significantly elevated above the specific IgG of either of the two adjusted BAL fluid samples G1 and G2 (vs BAL G1, p=0.028; vs BAl G2 p=0.021) of the same day. In no subject did the specific serum IgG titre not exceed the response of the BAL fluid samples; a linear correlation existed between the specific serum IgG and BAL IgG antibody responses (p=0.0026 & 0.0082 vs BAL G1 & G2), suggesting that the specific IgG response detected in the lower respiratory tract is a transudation of specific serum IgG, not as a result of local production.

Discussion

In conclusion, with this study we have shown that an antigen-specific immune response to an orally administered vaccine *S. typhi* Ty21a in the lower respiratory tract occurs and is primarily in the IgG antibody class. This appears likely to be a transudate from serum and not through local production, in keeping with the reported observations of others (3). This is supported by studies of the human nasal mucosal immune response following the local administration of Influenza A vaccine, where the specific mucosal IgG response was likely to have also been a serum transudate (4). In addition we have confirmed that the salivary IgA response is a poor indicator of both intestinal and lower respiratory tract immune responses.

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Expansion of gamma-interferon producing cell populations in the intestinal mucosa after peroral immunisation in humans

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ABSTRACT. We have examined the frequency of cells spontaneously secreting gamma-interferon (IFN-γ) in enzymatically dispersed human duodenal biopsies, following primary and booster immunizations with a protective oral cholera vaccine.In most volunteers, booster immunization gave rise to increased frequency of intestinal IFN-γ-secreting cells (mean increase 7 times). In contrast, such an increase in the frequency of IFN-γ-secreting cells was not observed with peripheral blood or tonsillar cell suspensions. Our findings lend further support to the notion that IFN-γ may have a critical role in the defence of intestinal surfaces against potentially harmful microorganisms.(Supported by the Swedish Medical Research Council).

1. Introduction

Immune or gamma-interferon (IFN-γ), a cytokine produced by certain subsets of T-lymphocytes and large granular lymphocytes, has numerous effects on cells of the immune system (reviewed in 1). That IFN-γ may also control intestinal epithelial cell functions has received considerable support from the results of recent in vitro experiments. Thus, IFN-γ modulates major histocompatibility complex expression by epithelial enterocytes (2) and may thus promote local accessory cell function; moreover, IFN-γ has the capacity to increase expression of secretory component by epithelial enterocytes and thereby to control the transport of potentially protective secretory immunoglobulins (3). That IFN-γ might have a direct role in the protection of epithelial surfaces has been suggested by its ability to modify the permeability of epithelial enterocytes (4) and of tight junctions connecting them (5). Direct bacteriostatic activity of IFN-γ has even been documented against certain microorganisms (6).
We have recently developed an assay to detect individual IFN-γ-secreting cells, and a novel enzymatic dispersion procedure to isolate viable and apparently functional lymphoid cells from human mucosal tissues. These developments have prompted us to examine the frequency of IFN-γ-producing cells in the human intestinal wall, before and after active immunization with a protective enteric vaccine.

2. Materials and methods

2.1 IMMUNIZATION REGIMEN

Seven healthy adult volunteers received two doses of an oral cholera B-subunit/whole cell vaccine (CTB/WC) (7), 3 weeks apart. Duodenal biopsies and peripheral blood were collected before the initial immunization and 7 days after each vaccination.For comparison, 5 adult patients undergoing tonsillectomy were immunized in the same way. Tonsillar tissue and peripheral blood were obtained 7 days after the booster immunization.

2.2 CELL ISOLATION

Intestinal cells were isolated by sequential enzymatic dispersion of duodenal tissue with thermolysin and collagenase/dispase (see Nordström, I. et al, this volume). The representativity of isolated cells was documented by conventional cytological, immunofluorescence, and FACS analyses of isolated cells and by immunoperoxidase staining of frozen sections. Mononuclear cells (MNC) from peripheral blood and from mechanically dispersed tonsillar tissue were obtained by standard gradient centrifugation.

2.3 ENUMERATION OF IFN-γ-SECRETING CELLS

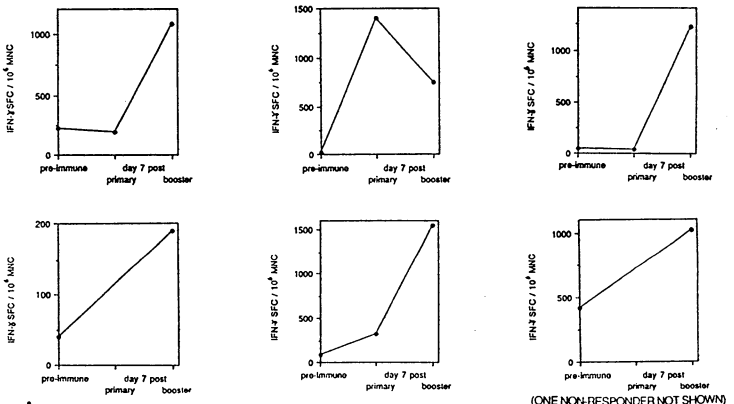
Cell suspensions were assayed for numbers of IFN-γ-secreting cells by a two-site reverse ELISPOT technique using epitope specific monoclonal anti-IFN-γ antibodies (Ab) as capture and developing reagents (8) (see also Quiding et al, this volume).

3. Results

High frequencies of cells spontaneously secreting IFN-γ were disclosed in cell suspensions obtained from specimens of "normal" (not intentionally immunized) intestinal mucosa, as compared to other tissues (geom. mean = 97 spot-forming cells/10^6 MNC, range 20-420) (See also Quiding et al, this volume). Exposure of cell suspensions to cycloheximide (2mM) during cell plating inhibited by up to 90% IFN-γ mediated spot formation. In addition, the amount of IFN-γ produced in short-term (20h) cultures of intestinal cells was high, since as few as $8x10^4$ unfractionated duodenal MNC could sometimes produce quantities of IFN- immunoassayable (i.e.> 2,5 i.u /10^6 MNC) by conventional ELISA.

The number of intestinal cells spontaneously secreting IFN-γ rised after a single primary immunization with oral CTB/WC vaccine in 2 out of 7 volunteers. However, a booster immunization gave rise to increased frequencies of IFN-γ-secreting cells in all but one volunteers, ranging from 2 to 37 times pre-immune values (mean increase 7 times) (Fig. 1). In contrast, such a boosterable increase in the number of IFN-γ-secreting cells was not observed in peripheral blood nor in tonsillar suspensions, at any of the time intervals examined.

Fig. 1 INTESTINAL INTERFERON-GAMMA PRODUCING CELL RESPONSES AFTER PERORAL IMMUNIZATION WITH CHOLERA VACCINE IN HEALTHY HUMAN VOLUNTEERS; INDIVIDUAL DATA FOR THE SIX RESPONDING INDIVIDUALS



4. Conclusions

The human intestinal mucosa is a major source of IFN-γ. The presence of such large numbers of IFN-γ-secreting cells in this tissue probably reflects constant stimulation of the intestinal mucosal surface by environmental antigens.

Furthermore, the frequency of IFN-γ-secreting cells in the human duodenal mucosa can be substantially increased by oral immunization with a prototype enteric immunogen, i.e. cholera vaccine. This observation provides further support to the hyphothesis that IFN-γ has an important role in the defence of mucosal surfaces against enteropathogenic microorganims.

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An oral bacterial lysate for the prevention of recurrent infections of the respiratory tract. Results of a double-blind placebo controlled 6 months study in 342 patients

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A preparation of 7 bacteria, playing a dominant role in bronchial infections, has been developed as the oral immune modulator LW 50020 for the prevention and therapy of recurrent infections of the upper and lower respiratory tract.

The reported clinical study PIROL (Prevention of Infection Recidivations of the respiratory tract) has been carried out to show the safety and efficacy of LW 50020.

Composition and administration: One capsule LW 50020 contains a lysate of at least 1 x 10^9 bacteria of each of the following strains: Staphylococcus aureus, Streptococcus mitis, Streptococcus pyogenes, Streptococcus pneumoniae, Branhamella catarrhalis, Haemophilus influenzae, Klebsiella pneumonia.
The capsule contains 3 mg of lysate and is filled with mannit to a total amount of 100 mg.

The therapy was as follows: One capsule once daily in the morning for 28 days, followed by a 28 day break and a booster-application of one capsule per day for further 28 days. This two months application scheme was repeated three times during the clinical study (see application chart).

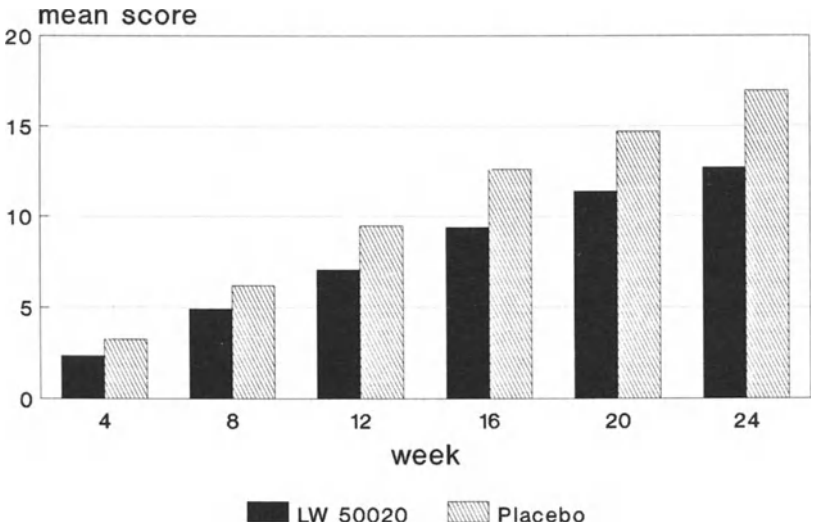
Method: Design: Randomized, prospective, placebocontrolled, multicentre, double-blind study (parallel group comparison).

Study population (inclusion criteria): Adult patients affected with recurrent infections of the respiratory tract. Patients of either sex ranging in age from 18 to 50 years and consulting a physician because of at least four complete recurrences of respiratory tract infections per year (rhinitis, sinusitis, otitis, pharyngitis, laryngitis, bronchitis and mixed forms).

Conduct of the study: The check-ups have been carried out at four-week intervals, blood specimen have been taken at the beginning and after the 4th and 24th week.

Patients: A total of 342 patients entered the study at 42 centres. The protocol was followed in 303 cases (protocol correct) over the period of 6 months (key point available) by 34 centres.

In the registration and analysis of adverse reactions no patients were considered as drop outs or withdrawals (intent to treat analysis).
Baseline values: The treatment groups were in all aspects well matched including the laboratory test at the beginning of the trial.
Clinical results - efficacy: The severity of clinical symptoms (main criterium) showed statistically significant ($p < 0,02$) differences between the verum and placebo treated groups when evaluated at the keypoint, i. e. after a 6 months observation period.



Maximum severity of clinical symptoms (product of the number of infections and the maximum severity of five symptoms). Cumulative data of 150 patients verum and 153 patients placebo ($p < 0.02$).

Clinical results - safety: 8 patients of the LW 50020 group compared with 4 patients of the placebo group reported adverse reactions (AR). Most of the ARs were minor and transient and only 4 patients had to terminate the medication before the end point. The comparison of all laboratory results at the 4th week and the 24th week with the values before treatment showed no differences between the LW 50020 group and the placebo group.

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Antibody-secreting cell responses after vaccination with parenteral killed, oral killed or oral live vaccine

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1. Introduction

The enumeration of antibody-secreting cells (ASC) in the peripheral blood has recently been introduced as a method for assessing the humoral immune response of the mucosa (Kantele et al. 1986, 1988), where IgA is the dominating immunoglobulin class. This approach is based on investigation of blood lymphocytes, believed to originate from the mucosa and now to be on their way back there (Bienenstock et al. 1980). An IgA dominated ASC response is seen after oral vaccination (Kantele et al. 1986, Czerkinsky et al. 1987), whereas after parenteral vaccination, the response has usually been dominated by IgG. However, these studies have been performed with non-identical antigens. Hence, it is not known how the administration route, nature of the antigen, or the use of live or killed bacteria contribute to the ASC response. In order to answer these questions we set out to study the ASC response to an identical bacterial vaccine given parenterally or orally, live or killed.

2. Study design and methods

42 healthy volunteers were vaccinated with Salmonella typhi Ty21a given as a killed parenteral vaccine (8 group KP; subjects; the vaccine was prepared from Ty21a bacteria isolated from the live vaccine (Vivotif) and killed by heat and phenol), as a killed oral vaccine (group KO; 14 subjects) or as a live oral vaccine (group LO; 20 subjects). The parenteral vaccine was given as one dose of $0.5x10^9$ bacteria, and the oral vaccines as three doses (on days 0,2 and 4) of $0.4x10^{12}$ (KO) or $1-3x10^9$ (LO) bacteria each. It should be noted that live bacteria (LO) undergo limited multiplication in the gut, thus increasing the number of bacteria acting in immunization.

Immune responses of the vaccinees were followed by enumerating specific ASC from their peripheral blood with the enzyme

linked immunospot assay (ELISPOT). The antigen used in this assays was bacterial strain SL 2404, which shares the O-9,12-antigen with the vaccine strain.

3. Results and discussion

No specific antibody-secreting cells were seen in any of the groups before vaccination; thereafter a rise was seen in all groups with a peak generally on day 7 and a fall in subsequent days. The responses were highest in group LO followed by groups KO and KP in this order (geometric means of the peaks of the responses 4.0, 24.8 and 178.1 ASC/10^6 cells, respectively). In the orally vaccinated subjects the response was dominated in most cases by IgA, in the parenterally vaccinated mostly by IgM.
The number of bacteria used for vaccination was considerably smaller in the parenterally vaccinated than in the orally vaccinated subjects, which could, at least partly explain the very low responses in the former group (the dose could not be increased because of the pyrogenicity of parenteral whole cell typhoid vaccines). The significantly higher responses in group LO compared to those in group KO- although the dose of the latter was very large - suggests the importance of properties of living bacteria in stimulating the immune system. Live Salmonellae are known to penetrate into epithelial cells, which would e.g. allow them closer contact with macrophages and lymphocytes in the Payer's patches. The immunoglobulin class distribution seemed to depend on the administration route, so that only oral immunization gave an IgA-dominated response. This conclusion must, however, be taken with caution because the numbers of ASC in the parenterally immunized (KP) group were so small. By contrast, both live and killed bacteria in the oral vaccines gave the same Ig-class distribution in the ASC response.

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Antibody secreting cells after oral vaccination with live *Salmonella typhi* vaccines

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ABSTRACT. 60 volunteers were vaccinated with different dosage schedules of oral live Salmonella typhi Ty21a vaccine. Their immune responses were followed by enumerating specific antibody-secreting cells (ASC) in their peripheral blood, and by measuring serum antibodies. The former assay gives information on the mucosal immune response, whereas the latter assay measures systemic immune response. Three groups of 20 persons were vaccinated with either three, two or one dose of vaccine in suspension formulation. ASC-responses were seen in all of the groups; both the response rate and magnitude inreased with increasing number of doses. Serum antibody responses were seen in all groups, but less frequently than ASC-responses. We conclude that the ASC assay is a sensitive method that can be used as a measure of the immunogenicity of an oral vaccine.

1. Introduction

Local immune system on mucosal surfaces acts as the first line of specific defence against invading microbes. Until recently, there has been a general lack of methods for studying local immune responses e.g. in the gut. Immunogenicity of each new oral vaccine candidate or dosage has therefore been tested in field trials or evaluated on the basis of the serum antibody response; the latter is, however, not believed to give information on the mucosal immune system. A new method for studying local immune responses, based on the cell circulation theory, has been lately introduced (Kantele et al. 1986, 1988, Forrest 1988). The elaboration of secretory antibodies dominated by IgA class is preceded by a maturation circulation of the antigen-specific cells: after contact with the antigen, activated Peyer's patch lymphocytes travel via local lymph nodes, lymphatics and blood back to the mucosa to secrete antibodies (Bienenstock et al. 1980, Tomasi TB Jr. 1983). These homing lym-

phocytes can be isolated from the blood and their antibody synthesis followed in vitro. This method is evaluated in the present study by measuring the antibody-secreting cell (ASC) response to the well-known live oral Salmonella typhi Ty21a vaccine. This vaccine has been tested in large-scale field trials (Wahdan et al. 1982, Levine MM et al. 1986, 1987), which enables us to compare the results from this study to the protective ability of the vaccine given at different dosage schedules.

2. Materials and methods

2.1 Study design. The study vaccine was oral live Salmonella typhi Ty21a in suspension formulation. 60 healthy volunteers, previously not vaccinated against typhoid fever, were divided randomly in three groups of 20 persons. The first group got one, the second two and the third three doses of vaccine. The interval between doses was 2 days. Their immune responses were followed by enumerating antibody-secreting cells in their blood at intervals after vaccination and by measuring serum antibodies.

2.2 ASC assay. Mononuclear cells were obtained by centrifugation on Ficoll-Paque. Their antibody synthesis was assayed with solid-phase enzyme-linked immunospot (ELISPOT) method (Czerkinsky et al. 1983, Sedgwick et al. 1983). The antigen used in the assay was bacterial strain SL 2404, which shares the O-9,12-antigen with the vaccine strain.

2.3 Serum antibody assay.Serum antibodies were quantitated with enzyme immuno assay (EIA) with SL 2404 as coating antigen. A twofold rise in titer was regarded significant.

3. Results and discussion

No specific ASC were seen in any of the volunteers before vaccination. After vaccination specific ASC appeared in the blood of most vaccinees; the response rate increased from 16/20 to 19/20 and 20/20 with increasing number of vaccine doses (1, 2 or 3). The number of ASC peaked on day 5 or 7 and decreased thereafter (Fig.). Also the number of ASC increased with increasing number of doses. In most cases the responses were dominated by immunoglobulin class A.
Serum antibody responses were seen in all Ig classes (IgA, IgG and IgM) but in a smaller fraction of the vaccinees. Again, the response rate (from 1/20 to 8/20 to 16/20) increased with increasing number of vaccine doses.

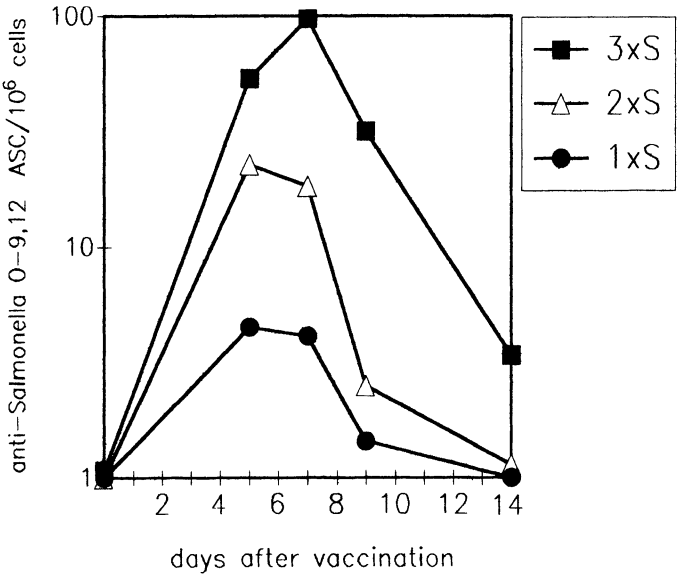

Figure. Specific antibody-secreting cells (ASC) after oral vaccination with Salmonella typhi Ty21a given as one, two or three doses as indicated alongside the curves. Each curve represents the goemetric mean of 20 persons and the sum of IgA-, IgG- and IgM-ASC.

It is not clear why the 3-dose-schedule was so much better; it could be due to a larger immunizing dose or to longer exposure of the mucosa to the immunogen. The increasing immunogenicity with increasing number of vaccine doses parallels the greater protective ability with increasing number of doses as observed in field trials (Levine MM et al. 1986).
The data from this study indicates that the ASC assay can be used as a sensitive laboratory measure on the immunogenicity of an oral vaccine.

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SECTION I: MUCOSAL VACCINES – ANIMAL MODELS

99

Protection of rat gut against cholera toxin obtained with only biliary or intestinal secretory IgA antibodies

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ABSTRACT. Repeated intrajejunal (IJ) immunization of rats with CT significantly decreases the CT-induced fluid accumulation in jejunal ligated loops (JLL). This protection is thought to be mediated by anti-CT IgA Abs produced by gut lamina propria plasmacytes and directly secreted locally. In rats, however, bile is also an important indirect source of gut sIgA. After bile duct ligation (BDL), protection of IJ-immunized rats was only slightly decreased. On the other hand, passive transfer during 24 h of bile from IJ-immunized, but not normal, rats into the gut of normal animals, significantly protected JLL made in such recipients. After fractionation of immune bile on a CT-immunosorbent, only the anti-CT sIgA Ab-fraction, premixed in vitro with CT, neutralized its effect in JLL. The data indicate that the intestine of IJ-immunized rats can be protected by each of both pathways, direct and indirect, of anti-CT sIgA secretion into the gut.

1. Introduction

The polymeric IgA Abs secreted by rat gut lamina propria plasmacytes can reach the gut lumen by two pathways: a direct one, through the intestinal epithelium, or an indirect one via the mesenteric and thoracic duct lymph, the plasma, the hepatocytes, and the bile (1). Several authors have outlined the important role of sIgA Abs for intestinal protection against enteric disease, such as CT-induced diarrhea (2-4). Experiments were performed to establish if both direct and indirect pathways of anti-CT sIgA secretion into rat gut were required for its protection against a CT-challenge, or if each pathway could be protective on its own.

2. Materials and Methods

2.1. IMMUNIZATIONS.

Male OFA rats (weighing ± 250 g) were intrajejunally (IJ)-immunized three times, at two weeks interval, with 25 µg of CT (kind gift of the Institut Mérieux, Lyon, France) in 0.5 ml of phosphate-buffered saline, pH 7.2, containing 0.02% gelatin.

2.2. COLLECTION OF BILE AND BILE DUCT LIGATION.

Bile was obtained by cannulation of the bile duct as previously described (5). The immunized animals were cannulated at 4 days after the last IJ boost. Bile duct ligation (BDL) was carried out by applying two 0.5 cm-distant ligatures on the bile duct as reported elsewhere (1).

2.3. CHOLERA TOXIN NEUTRALIZATION TEST.

This assay was performed in jejunal ligated loops (JLL) as described earlier (6), and expressed as mg/cm. The JLL were considered as "protected" if the fluid accumulation was smaller than 50 mg/cm (7).

2.4. IN VIVO BILE TRANSFER FROM NORMAL AND CT-IMMUNE RATS TO NORMAL RATS.

The bile of 10 IJ-immunized rats was cannulated on day 3 after the last IJ boost of CT. One day later, the free end of the cannula was passed through the abdominal wall of an unimmunized rat and , after BDL of the recipient, inserted into his duodenum. Twenty four hours later, JLL were constructed in each of the 10 recipients and challenged with 1 μg of CT. The same procedure was applied to 6 donor-recipient pairs of normal unimmunized rats.

2.5. ISOLATION OF ANTI-CT sIgA-ANTIBODIES.

Six one ml-samples of fresh bile collected from six IJ-immunized rats at 4 days after the last IJ boost were pooled and passed through a CT-Sepharose immunoabsorbent column prepared as described earlier (7). The anti-CT Abs were eluted with acid buffer, brought to neutral pH and, in parallel with the unretained bile proteins, concentrated back to the initial volume of bile as reported (7). Anti-CT ELISA IgA and IgG Ab-titers were measured on CT-coated plates using affinity-purified peroxydase-labellled rabbit anti-rat-IgG and -IgA Abs prepared in our laboratory.

2.6. STATISTICAL ANALYSIS.

The significance of the results was analyzed by the unpaired Student's t-test.

3. Results

As shown in Table 1, withdrawal of the bile supply by BDL during 1 to 4 days only gave rise to a slightly decreased potency of CT in JLL prepared in such unimmunized animals. Not a single "protected" JLL was observed. The decrease in potency of CT was never significant even after 4 days of BDL ($P = 0.097$).

TABLE 1. Influence of BDL on CT-induced fluid secretion in JLL constructed in normal and IJ CT-immunized rats.

Duration of BDL	Normal rats	No. of protected JLL[a]	Immune rats	No. of protected JLL
Not BDL	305 ± 38[b,c,d,e,f]	0/5	11 ± 26[e,g]	5/5
1 day	294 ± 65	0/5	N.D.[h]	N.D.
2 days	272 ± 31[c]	0/5	N.D.	N.D.
3 days	284 ± 72	0/5	N.D.	N.D.
4 days	254 ± 47[d,i]	0/5	101 ± 159[f,g,i]	4/5

[a] Protected = JLL weight at 4 h after CT-challenge is < 50 mg/cm. [b] All figures are mean JLL weights (mg/cm) ± SD, n = 5. [c] $P = 0.172$. [d] $P = 0.097$. [e] $P < 0.001$. [f] $P = 0.023$. [g] $P = 0.251$. [h] N.D. = not done. [i] $P = 0.071$.

In rats IJ-immunized with CT, 4 out of 5 JLL were still "protected" at four days after BDL. The mean loop weight was, nevertheless, not significantly different ($P = 0.251$) from that of non-BDL immunized rats. If compared, however, to the mean loop weight of normal rats after 4 days of BDL, the difference was just below significance ($P = 0.071$), despite the fact that none of the 5 loops were "protected" in the non immunized group, in contrast with 4 out of 5 loops in the immunized group.

After surgical transfer of the physiological bile flow from immune rats to the gut of normal animals, JLL constructed in the recipients were "protected" against a 1 μg CT-challenge in 9 out of 10 cases, and the mean loop weight (33.9 mg/cm ± 21.3) was significantly ($P < 0.001$) smaller than that obtained in recipients of normal bile (287 mg/cm ± 66) in whom not a single loop out of 6 was "protected".

None of the 6 JLL challenged with 1 μg of CT, premixed with 0.5 ml of the immune bile proteins unretained on the CT-immunosorbent, were "protected". In contrast, all 6 JLL challenged with 1 μg of CT, premixed with 0.5 ml of the CT-immunosorbent-retained and acid-eluted immune bile proteins, were "protected". Their mean loop weight (11 mg/cm ± 10) was significantly smaller ($P < 0.001$) than that of JLL challenged with CT premixed with the unretained immune bile proteins (242 mg/cm ± 81).

The reciprocals of the IgA anti-CT Ab titer, tested by ELISA, in the native immune bile, its immunosorbent-unretained, and -retained fractions were, respectively, 800, 0, and 600. Corresponding anti-CT IgG titers were 100, 0, and 60. "Protection" of JLL against CT-challenge thus appeared to be associated with the fraction of immune bile proteins containing specific anti-CT Abs, most of which were of the IgA class.

4. Discussion

There have been several reports showing that animals orally or IJ-immunized with CT were protected against the fluid-accumulating effect of a subsequent CT-challenge in their JLL (3,4). Our group (6) and others (8) also observed that various amounts of fresh immune rat bile, premixed with CT in vitro, neutralized its toxic effect in JLL constructed in normal rats.

Our CT-immunoabsorbent experiments clearly demonstrated that the protective factors in immune bile are anti-CT Abs, most of which are sIgA, with a small contribution of IgG. Others (9,10) have proposed that the bile (and milk) of rats having received CT repetitively by the oral route contained an antisecretory factor or hormone (a protein of ± 40-60 kD) which would be the protective substance against CT-induced fluid accumulation in the gut. Our data do not exclude a minor contribution of this still poorly characterized factor to the protection observed, but they also show that the role of Abs, and particularly of sIgA Abs, cannot be ignored.

In this communication, we also showed that the gut from an unimmunized rat could be passively protected against a CT-challenge by receiving during 24 h the physiological bile supply from an IJ-immunized rat. In such recipients, "protection" of the JLL was due essentially to IgA biliary Abs provided to the gut by the indirect IgA pathway. However, in the experiments with a 4-day BDL in CT-immunized rats, this indirect pathway was unavailable, and yet the JLL constructed in such animals were "protected" against the CT-challenge. We suggest that this was due to IgA Abs provided to the gut only by the direct pathway, i.e. direct transport through the intestinal epithelium.

In conclusion, it appears then that the intestinal mucosa of rats IJ-immunized with CT is sort of "doubly protected" against a CT-challenge. Both the biliary and the intestinal anti-CT sIgA Abs, each on their own, can protect the intestinal mucosa against CT, further confirming our initial proposal (1) that the indirect biliary pathway of sIgA Abs in the rat was reinforcing its direct local gut humoral defenses.

5. Acknowledgements

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Synergy between B subunit and whole cholera toxin in their action as an adjuvant for the mucosal immune response of mice to keyhole limpet haemocynanin

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1. Introduction

Cholera toxin (CT) is a potent stimulator of an immune response when fed to mice, as little as 10 ug of CT induces both an IgG and an IgA response in serum and intestinal secretions [1]. As well as being a potent antigen CT can also act as an adjuvant ,promoting strong antibody responses to unrelated antigens fed simultaneously [2,3,4,] The mechanism by which CT acts is unknown, However the failure of parenteral injections of CT to influence the response to fed proteins and the need for CT and the second protein to be fed simultaneously indicate that the site of action is in the intestine [3]. There is not a clear correlation between the antibody response to CT and that to the second protein .This can be interpreted as showing that CT acts as a switch , activating the pathway to an IgA response with the final outcome being dependeent on the immune system's ability to respond to the second protein, which may involve several factors such as antigen uptake, genetic restriction and number of epitopes [4]. The optimal dose of CT as an antigen is 10ug [3], this dose is also the optimum as an adjuvant for 5mg of keyhole limpet haemocyanin [KLH]. The 500 fold higher dose of KLH required to give the same level of response as CT may be a reflection of enhanced uptake due to the latters ability to bind to the intestinal mucosa . The B-subunit of CT has been shown to have no adjuvant effect on the IgA response to fed KLH [3],suggesting that the toxic action of the A-subunit is responsible for the effect.[3]. When 10ug of B-subunit is fed to mice there is only a poor response, but this can be increased by as little as 100ng of whole CT to the level obtained by 10ug of CT [3]. The greater efficacy of CT as an adjuvant for B-subunit compared to KLH could be related to the ability of B-subunit to bind to the intestinal mucosa in close proximity to the CT. This can explain why 500 times less B-subunit is required to stimulate a response compared to KLH. However if CT does act like a switch it does not explain why 100 times less CT is required to activate the switch for B-subunit compared to KLH. The simplest explanation for these conflicting results is that the ability of the B-subunits to bind to GM1 and the activity of the A-subunit both have an important functional role in activating the IgA response and behave synergisticaly . If this is so one would predict that a mixture of CT and B-subunit would also act as an adjuvant for an unrelated protein such as KLH. We therefore tested this hypothesis experimentally.

2 Materials and methods

2.1 Immunisation of mice

Groups of C57/Bl6 mice were fed 5mg of KLH with either 10ug CT, 0.5ug CT, 10ug B-subunit or a mixture of 10ug B-subunit + 0.5ug CT. A group fed 5mg KLH and unfed controls were also included. The mice were fed on three occasions 10 days apart and antibody responses were recorded eight days after the last dose.

2.2 Serum antibody

Serum antibody was measured by ELISA as previously described, the results were compared to a standard and expressed in arbitrary units .The number of units in each standard is defined by its reciprocal end point titre.[4]

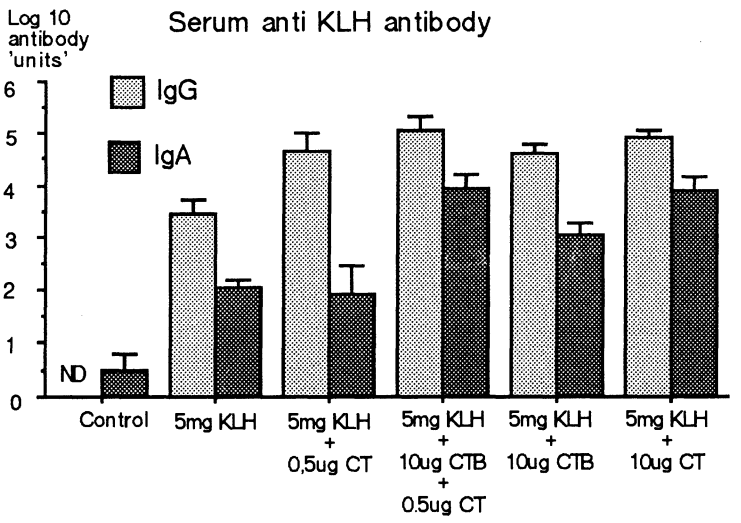
2.3 Intestinal antibody producing cells.

Lamina propria cells were isolated as described [5] the number of cells producing specific antibody of IgG or IgA isotype measured by a spot forming assay[6]

2.4 Statistics

Antibody and spot forming cell results were analysed using analysis of variance,. Dfferences between individual groups were assessed using the least significant difference.

Figure 1



3 Results

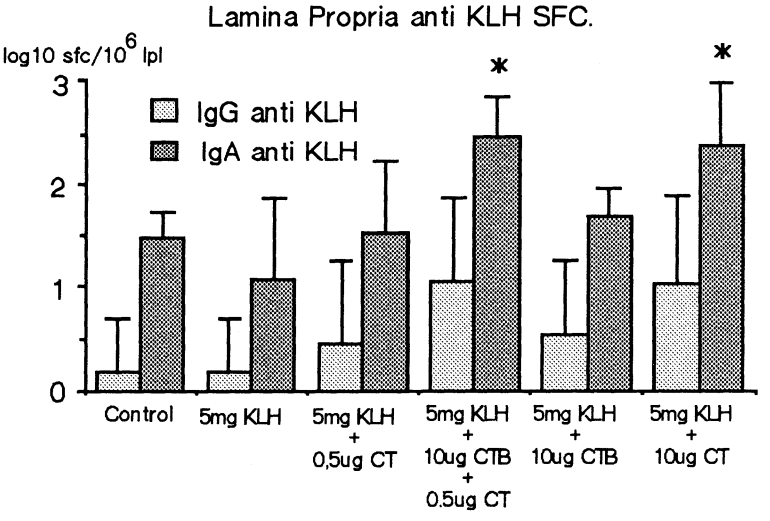
2.1 Serum antibody to KLH

The results of the serum antibody ELISA are shown in Figure 1, This shows that there is a significant IgG response to KLH fed on its own and that any of the combinations of CT and B-subunit fed on their own or together resulted in significant increases in the IgG response to KLH. There were no further differences between the different doses of CT, B-subunit and the combination of both in their ability to enhance the IgG response.The IgA anti KLH response was quite different, 5mg of KLH did induce a slight serum response ,this was unaffected by 0,5ug of CT. There was a statistically significant tenfold increase in this response if 10ug of B-subunit was fed with the KLH.The feeding of 10ug of CT gave a 100 fold increase over that obtained with KLH alone this response was significantly greater than that obtained with B-subunit.The feeding of 10ug of b-subunit plus 0.5 ugCT enhanced the anti KLH response by as much as 10ug whole toxin ,giving a significantly greater result than either component on its own.

2.2 Lamina propria anti KLH spot forming cells

The number of IgG anti KLH spot forming cells in the lamina propria was small and although there were slight increases in the CT and synergy fed groups these were not statistically significant.There were higher background numbers of IgA anti -KLH spot forming cells in all groups including the control However both the group fed 10ug CT and the group fed 10ug B-subunit + 0.5ug CT had responses which were significantly above background Fig 2.

Figure 2



4 Discussion

The results of this experiment confirm our hypothesis that both the GM1 binding of B-subunit and the toxic action of whole CT are important factors in the adjuvant action of CT for IgA responses. The ability of either B-subunit or 0.5 ug of CT on their own to enhance IgG anti -KLH but not IgA anti -KLH emphasises the separate nature of the different isotype responses. The exact way in

which CT and B-subunit act is still not clear but recent results in other laboratories indicate that an effect on B-cell isotype switching is involved [7,8] However the results of this experiment open the possibility that there are two or more sites of action involving CT or B-subunit..

Another point which these results tend to indicate is that uptake of antigen is not a critical factor in an IgA response as there are circumstances in which IgG but not IgA is induced. Clearly ,if there is enough antigen for an IgG response there ought to be enough for an IgA response unless there is some unusual requirement for high levels of antigen for an IgA response. The model of an IgA switch seems to be more consistent with the available evidence.

The importance of binding *per se* as a factor in IgA responses merits more attention . There are reports of elevated mucosal antibody responses to adherent antigens [9] and it would be interesting to see if CT can alter the levels of response to such antigens .In particular whether a small (0.5ug) dose of CT would be effective or if B-subunit and whole CT would be required.

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Anti-cholera toxin secreting cells, counted by ELISPOT, in various gut-associated lymphoid tissues and spleen of rats immunized several times with CT by three different routes

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1. INTRODUCTION

Secretory IgA (SIgA), the main antibody (Ab) along mucosal surfaces, can protect the host's gut against cholera toxin (CT). Intestinal responses against CT were mostly studied by means of immunohistology of gut sections (1), titration of Ab in intestinal secretions (2) or in supernatants of cultured gut immunocytes (3). More recently, anti-CT Ab-secreting cells have been counted using plaque-forming (PFC) (4) or spot-forming cell (SFC or ELISPOT) (5) methods. Here, we used the ELISPOT-assay for enumeration of IgG-, IgA- and IgM-anti-CT secreting-cells isolated from the intestinal lamina propria (LP), Peyer's patches (PP), mesenteric lymph nodes (MLN), and spleen (SP) of rats repeatedly immunized by various routes with CT.

2. MATERIALS AND METHODS

OFA rats were immunized at 12 days interval with 25 µg of CT (gift of Institut Mérieux, Lyon) with a last boost of 50 µg. Immunizations were intraduodenal (ID) 1 to 6 times, intraperitoneal (IP) and intravenous (IV), each 2 and 5 times. Rats were killed 5 days after the last boost. LP lymphocytes were isolated (viability >98%) (6), as well as those from PP (7), MLN and SP (8). Cells were suspended in ice cold supplemented RPMI 1640 medium, and counted. For the ELISPOT-assay, cells diluted in RPMI were plated in 96-well CT-coated culture plates, and incubated overnight at 37°C. Then, after removing the cells, anti-class affinity-purified Abs (rabbit anti-rat) were added, followed by biotinylated goat anti-rabbit IgG and finally extravidin-alkaline phosphatase, using inter-step washings with PBS-0.05% Tween 20. After addition of the substrate (9), macroscopic blue spots were counted using an inverted microscope. Controls included a coat with bovine serum albumin, cells from normal rats, and the use of normal rabbit IgG. Results (means of duplicates) were expressed in SFC-number /10^7 immunocytes.

3. RESULTS (Figure 1)

On day 5 after a single ID injection, no SFC could be found in any of the 4 lymphoïd tissues investigated. However, after 2 to 4 ID injections, significant increasing responses occurred in all tissues. ID injections stimulated SFC mainly in LP, with LP >>> MLN > SP >> PP. Anti-CT-SFC, primarily in LP and to a lesser extent in PP, were mainly IgA (>90% of total SFC in LP), whereas in MLN and SP, SFC were IgG >> IgA and IgM. The SFC number peaked after 4 ID immunizations in all tissues. The maximal response was reached in LP, where as much as 7.5% of plated cells were SFC (99% IgA). After 5 and particularly 6 ID injections, a weaker response occurred in gut as well as extra-intestinal lymphoid tissues, with the strongest decline in LP. The rare IgM-SFC occurred mostly in MLN and SP. The importance of the ID route to induce a gut response was outlined by the virtual lack of any anti-CT SFC in LP and PP after 2 and even 5

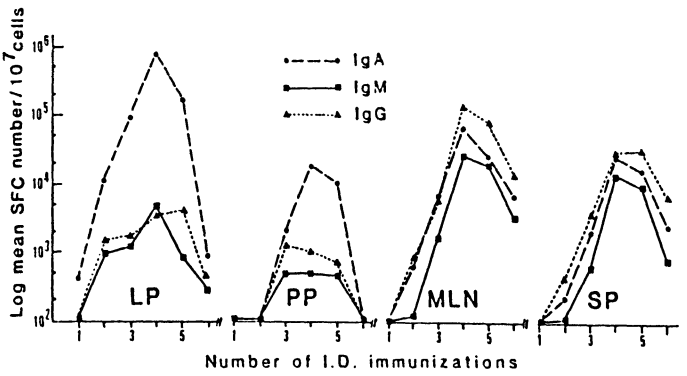

Figure 1. Anti-CT SFC of IgA, IgM and IgG class per 10^7 mononuclear cells in lamina propria (LP), Peyer's patches (PP), mesenteric nodes (MLN) and spleen (SP) of rats ID-immunized 1-6 times with CT.

IP or IV immunizations, although there were numerous SFC of the 3 classes in MLN and SP.

4. DISCUSSION

To our knowledge, no previous study measured IgA-, IgG and IgM anti-CT responses by the ELISPOT-assay alltogether in LP, PP, MLN, and SP in rats immunized repeatedly by 3 different routes. Quantitatively, like others (3,10), we observed that enteral immunizations with CT elicited the strongest response in LP, confirming the homing of CT-specific cells in the gut LP after ID delivery of CT. The large increase in SFC observed in LP between 2 and 4 ID immunizations indicates that repeated enteral exposure to CT generates immunological memory in the gut SIgA system (10). The predominance of the intestinal anti-CT IgA response after oral immunization had been shown with several methods (1-4). However, the discrepancy between IgA and IgG was more obvious with ELISPOT than with Ab-ELISA in intestinal washings. The fact that MLN and SP contained SFC expressing IgG, IgA and IgM confirms the suggestion (4) that PP contain precursors for IgA, IgG, and IgM cells. A previous study in mice (5) showed a weaker increase in SFC after 4 and 5 oral immunizations, but a decrease by further immunizations was not established. Our decreasing numbers of anti-CT-SFC in MLN and SP after repeated oral immunizations confirm similar reports in mice (4,11). However, the decreased response was also seen in PP, in contrast with (4) and in LP, in contrast with (5). As shown in mice (11), five IV immunizations gave no detectable anti-CT SFC in rat LP and PP. Thus, systemic parenteral delivery (IP and IV) of CT does not induce any significant intestinal Ab-secreting-cells.

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Enhancement of enteric mucosal immune responses by cholera toxin or cholera toxin B-subunit

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INTRODUCTION

Cholera toxin (CT) and cholera toxin B subunit (CTB) are used as carrier-proteins for the induction of mucosal immune responses (1,2). Both CT and CTB adhere to the intestinal epithelium (GM1 molecules) and cause immunestimulation by enhanced antigen-uptake by the epithelium. Next to the enhanced antigen-uptake CT has adjuvant effects on the immune response. We determined the stimulatory effect of coupling CT and CTB to the antigen ovalbumin on the intestinal immune response against ovalbumin after intraduodenal booster immunization. Intestinal responses were measured by quantitating the OA-specific antibody secreting cells (ASC) in the lamina propria of the small intestine.

METHODS

OA and CT or CTB were coupled by glutaraldehyd in a molar ratio of 5 to 1. This molar ratio between OVA and CT or CTB gave the highest binding to GM1 as determined by a GM1-ELISA procedure. C3H/He mice were primed intraperitoneally with 100 ug OVA in water-in-oil emulsion. Four weeks later the mice were given booster immunizations intraduodenally (i.d.) with 150 ug OVA or OVA-CT(B) conjugates. OVA-specific ASC were isolated from the lamina propria of the small intestine (3) and quantitated by an ELISA-spot assay.

RESULTS

After ip priming with OVA in a water-in-oil emulsion, mice were given i.d. booster immunizations with OVA, OVA-CT or OVA-CTB. OA-specific IgA ASC were quantitated in the small intestine of immunized mice. Both CT and CTB enhanced the response significantly (Fig. 1). CT stimulated the response about 3 times, CTB stimulated the response about 2 times. Coupling of CTB to the antigen was essential to obtain the stimulatory effect, while CT gave a weaker but significant stimulation when administered together with the antigen (results not shown).

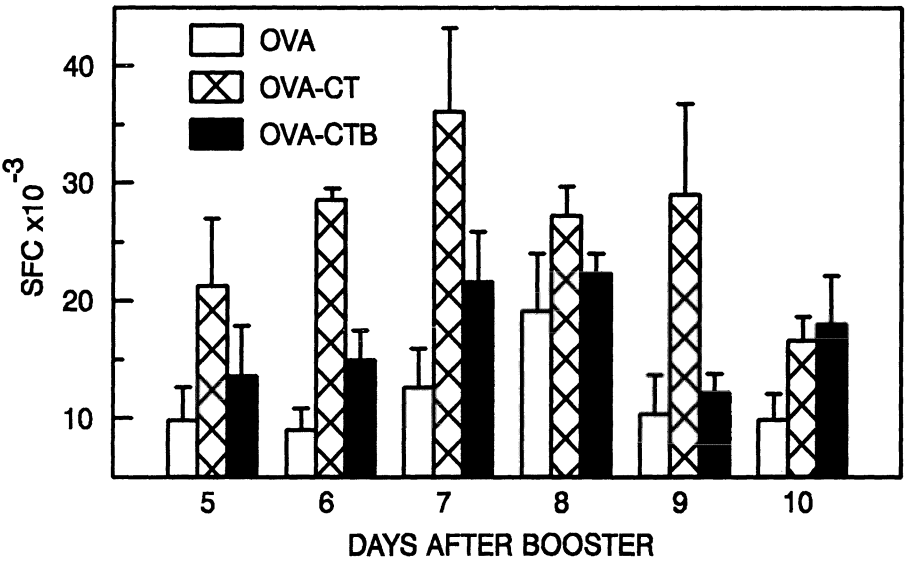

Fig. 1 Number of spot forming cells (SFC) per small intestine of mice boostered id with OVA, OVA-CT or OVA-CTB. Results are expressed as the mean (n=5) with the S.E.

DISCUSSION

CT and CTB can stimulate mucosal immune responses when coupled to the antigen. For CTB this covalent coupling is essential. CTB can stimulate the response only through binding to GM1 ganglioside of the epithelial cells, that results in enhanced antigen uptake by the intestinal epithelium. For that reason we determined the optimal molar ratio between antigen and carrier by adherence of conjugates to GM1. We showed that there exists a claer relationship between the capacity of the conjugate to bind to GM1, demonstrated by ELISA, and the potential to stimulate the intestinal anti-ovalbumin response (results not shown). The stimulatory effect of OVA-CT and OVA-CTB conjugates, coupled in the optimal molar ratios, was further studied by isolating lamina propria lymphocytes and quantitating the OVA-specific IgA ASC. The stimulatory effect of CT and CTB on the i.p.-i.d. immunization regime used in this study is not very high, but still demonstrates that both CT and CTB are effective as protein carriers. This indicates that CTB can be used as carrier in immunization regimes in which the antigen is presented only by the mucosal route.

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Neutralisation of cholera toxin (CT) in jejunal ligated loops (JLL) of rats immunized with a mouse monoclonal (MC) auto-anti-idiotypic (ID) antibody (Ab) against anti-CT ABS

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1. Introduction

The potential use of anti-ID Abs as surrogate antigens in the vaccination field has recently received much interest (1). This study reports on the use of a mouse MC auto-anti-ID Ab, obtained from the spleen cells of mice immunized with CT, as a CT-mimicking antigen injected intra-peritoneally (IP) into rats in order to obtain Abs against CT as well as in vivo protection against a CT-challenge into JLL prepared in such rats.

2. Materials and Methods

BALB/c mice were immunized 3x intrajejunally at 15 days interval with 30 μg of CT. After a month rest, they were boosted IP with 100 μg of CT and their spleen cells collected 4 days later, fused with NSO cells and plated in selective hybridoma growth medium (2). Anti-CT ID-clones were detected by ELISA, and the auto-anti-ID clone by particle counting immunoassay (3) using agglutination of latex coated with mouse anti-CT IgG (or normal IgG as control). Subcloned (limiting dilution) hybridomas were injected IP into BALB/c-NMRI hybrid mice to obtain ascites from which the MC anti-ID Ab, an IgG, was isolated. OFA rats were immunized with the anti-ID: group A (n=7) received 5, and group B (n = 10) 4 IP doses, spaced 15 days, of 50 μg of anti-ID in (in)complete Freund's aduvant. Five days after the last dose, serum and bile (4) were collected for anti-CT ELISA, and JLL (two 5 cm-long loops / rat) were prepared (5) in immunized and control rats for intra-JLL challenge with 0.5 ml of buffer (PBS with 0.2% gelatin) or CT (1 μg in PBS-gelatin). Bile collection lasted for the 4 h during which the JLL was challenged. After 4 h, JLL were carefully dissected and weighed, and fluid accumulation was expressed as mg/cm (5). Anti-CT Ab was titrated in sera and cannulated bile by ELISA using plates coated with CT (4 μg/ml), saturation with 1% bovine serum albumin (BSA), dilution of reagents in PBS-0.1% Tween-1% BSA, revelation with peroxidase-labelled pure rabbit anti-rat-IgA or -IgG Abs and O-phenylene diamine. Positive controls were standard anti-CT rat serum or bile (5). Sera and bile of unimmunized rats served as negative controls.

2. Results

The purified MC anti-ID strongly agglutinated latex particles coated with mouse MC or polyclonal (PC) anti-CT Abs, but not latex coated with normal mouse IgG or non-anti-CT MC Abs. The results of the CT- or buffer-challenges into the JLL of the 2 groups of anti-ID immunized rats are listed in Fig. 1. In group A, 3 rats completely neutralized CT and 3 others only partially, resulting in a JLL weight (mean mg/cm ± SD) of 159.2 ± 57.1, still statistically (unpaired t-test) larger than the buffer control (96.8 ± 16.8; n = 9; P = 0.013). CT-JLL weight in control rats was much larger (464.3 ± 49.4; n = 3; $P < 0.001$). In group B, CT elicited uniformly small JLL weights (109.4 ± 28.0; n = 9), not significantly larger than the buffer control

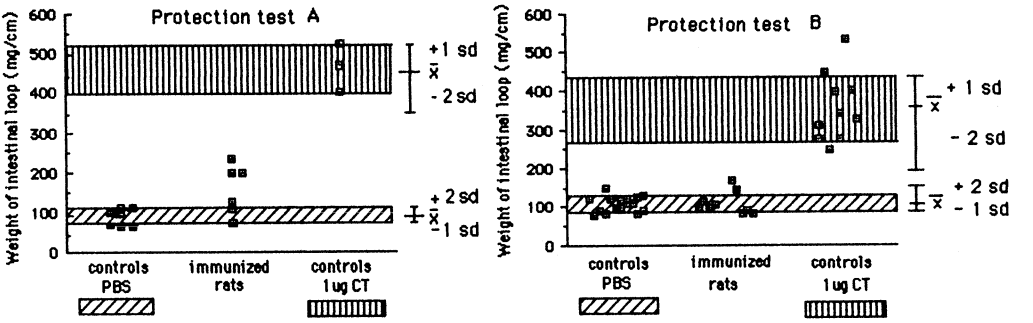

Figure 1. Fluid accumulation (mg/cm) in JLL, prepared in control rats or in the 2 groups (A and B) of anti-ID immunized rats, and challenged with 0.5 ml of PBS-gelatin or with CT (1 μg).

(107.2 ± 18.8; n = 17; $P = 0.811$). CT-challenges in 9 control rats yielded JLL weights of 355.1 ± 84.8, very significantly ($P < 0.001$) larger than both preceding values.

4. Discussion

A MC anti-ID Ab-secreting clone was obtained together with several MC anti-CT ID-secreting clones. This saved much of the time required to inject animals for obtaining anti-IDs. It was surprizing to obtain gut protection after IP immunizations. However, our data indicate that not only serum IgG, but also biliary IgA anti-CT Abs had been obtained; the latter could play a major role in the gut protection observed. So far, there was no correlation between gut protection and IgG or IgA Ab titers in both groups. Soluble CT inhibited the binding of the rat IgG and IgA anti-anti-ID to CT-coated wells. Our data demonstrate that repeated IP anti-ID immunization induced high levels of anti-CT Abs in serum and bile, and protection against a CT-challenge in JLL, further supporting anti-ID vaccination efforts. Our MC auto-anti-ID Ab will be studied for its "internal image" quality and possible interaction with Gm1-ganglioside, the CT-receptor. Attempts are in progress to immunize rats and mice by injecting various mouse MC anti-IDs Abs, obtained by immunizing mice with MC anti-CT ID Abs, some of which no longer react with Gm1-bound CT (6). Different protocols, using various adjuvants, as well as oral administration of the MC anti-IDs are planned.

5. Acknowledgements

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Disseminated mucosal anti-toxin antibody responses induced through oral or intrathecal immunisation with toxoid-containing biodegradable microspheres

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ABSTRACT. The utility of biodegradable and biocompatible microspheres as a vaccine delivery system for the induction of systemic and disseminated mucosal antibody responses was investigated. Following intraperitoneal (IP) injection into mice, microspheres constructed of the copolymer poly(DL-lactide-co-glycolide) (DL-PLG) which contained a formalinized toxoid vaccine of staphylococcal enterotoxin B (SEB) were 500-fold more effective in the stimulation of circulating IgG anti-toxin antibodies than was the free toxoid. However, neither free nor microencapsulated SEB toxoid induced a detectable mucosal IgA anti-toxin response following IP administration. In contrast, three peroral immunizations with toxoid-microspheres stimulated circulating IgM, IgG and IgA anti-toxin antibodies and a concurrent mucosal IgA response in saliva, gut washings and lung washings. IP immunization effectively primed for the induction of a disseminated mucosal IgA response by a subsequent single oral dose of toxoid-microspheres. Booster immunization of IP primed mice via intratracheal instillation (IT) of toxoid-microspheres was the most effective means of inducing mucosal anti-toxin antibodies, and raised specific IgA titers in excess of 2,500 in both gut and bronchial washings. Soluble SEB toxoid was ineffective in stimulating antibody responses when administered to either naive or primed mice via the oral or IT routes. These results indicate that biodegradable and biocompatible microspheres represent an adjuvant system with potentially widespread application in the induction of both circulating and mucosal immunity.

Introduction

The mucosal surfaces of the body represent a tremendous surface area; over 400 m^2 in man. The absorptive function of the wet mucosal surfaces such as the gut and respiratory tract dictate that they are semipermeable. These imperfect barriers constitute the major paths through which antigenic materials enter the body, and the site of replication or the portal of entry for a large number of pathogenic organisms. Unlike the blood, the major antibody isotype associated with the mucus secretions is IgA and it is predominantly present in a dimer form in association with J chain and secretory component. The daily synthesis of secretory IgA (sIgA) exceeds that of the other isotypes combined; illustrating the importance of sIgA in protection. However, sIgA antibodies are efficiently induced only through immunization of mucosal tissues, and not by the commonly employed parenteral routes of vaccination. Numerous approaches to mucosal immunization have been investigated, with most centering on oral ingestion or intranasal instillation to immunize the gut-associated or bronchial-associated lymphoid tissues, respectively. Although varying degrees of success have been achieved, the application of these approaches to purified vaccine antigens would benefit from an improvement in vaccine delivery to IgA inductive sites, retention of vaccine integrity, and an appropriate adjuvant.

Our own work in this area has involved the investigation of 1-10 um biodegradable and biocompatible microspheres as an oral vaccine delivery system. These microspheres are constructed of poly(DL-lactide-co-glycolide) (DL-PLG) with the vaccine trapped within. Following oral administration, the microspheres protect the vaccine from degradation in the gut, and are absorbed only by the M cells of the Peyer's patches, and then passed to macrophages in the underlying lymphoid area (1). This absorption is restricted to particles which are $\leq$10 um in diameter (1,2), and is enhanced by increased hydrophobicity (2). Ingestion by macrophages leads to accelerated degradation of the ester linkages in the DL-PLG, and results in rapid expression of the antigen on the surface of these accessory cells. Importantly, antigen delivery by this mechanism results in a strong adjuvant effect, and antibody responses are potentiated up to 500-fold (1-3). Three spaced oral immunizations with microspheres containing a toxoid vaccine of staphylococcal enterotoxin B induced the appearance of both circulating and disseminated mucosal anti-toxin antibodies. In the studies reported here, we have examined the effect of prior parenteral priming on the immune response induced by subsequent oral immunization. Further, the potential of microspheres for vaccine delivery through inhalation has been addressed.

Materials and Methods

Specific pathogen free BALB/c mice of mixed sexes were used throughout these experiments. They were bred and maintained in our barrier facilities at the University of Alabama at Birmingham. Except as specified in the text they were allowed food and water ad libitum, and were entered into experimental protocols at 8-12 weeks of age.

A formalinized vaccine of staphylococcal enterotoxin B (SEB) was prepared as described by Warren et al. (4). Monolithic microspheres containing enterotoxoid were made using a solvent-evaporation process by previously described methods (5). Size distributions of the microsphere batches were obtained by measuring the diameters of 500 microspheres on scanning electron micrographs. The diameters were then used to calculate the size distribution as a function of internal volume.

Groups of mice were orally-administered microencapsulated enterotoxoid in 0.5 ml of 8 parts tap water : 2 parts 7.5% sodium bicarbonate via a blunt-tipped feeding needle. Microspheres were suspended in PBS for IP and IT immunizations and delivered in 0.5 and 0.05 ml, respectively.

Blood was collected from a puncture of the retroorbital plexus in calibrated, heparinized capillary pipettes and the plasma harvested following centrifugation. Saliva and gut wash samples were collected as described by Elson et al. (6). Lung wash fluids were obtained by total lung lavage with 1 ml BSS.

Radioimmunometric assays were performed in Immulon strips (Dynatech) coated with enterotoxin at 1 ug/ml in BBS, pH 8.4, overnight at 4^0C. Affinity-purified IgG goat anti-mouse IgM, IgG or IgA heavy chain specific antibody was obtained from Southern Biotechnology Associates. The results are presented as the reciprocal of the sample dilution producing a signal significantly different from that of the group-matched prebleed at the same dilution as determined by nonoverlapping SD of the means from the triplicate determinations (end-point titration).

Results and Discussion

Three peroral immunizations at 30 day intervals with 100 ug doses of SEB Toxoid-microspheres effectively induced the appearance of concurrent circulating and disseminated mucosal anti-toxin antibodies 20 days following the tertiary administration (Fig. 1). The plasma anti-toxin response included IgM and IgA components at titers of 2,560 and a specific IgG titer in excess of 40,000. Each of the tested mucosal secretions, saliva, gut wash fluid and lung wash fluid, contained significant levels of toxin-specific IgA, and the washings from the gut and lungs additionally contained specific IgG. Parallel oral immunizations with doses of nonencapsulated toxoid ranging from 1 to 100 ug were ineffective at inducing a detectable immune response in any of the tested fluids. In addition, doses of nonencapsulated toxoid above 50 ug per immunization induced oral

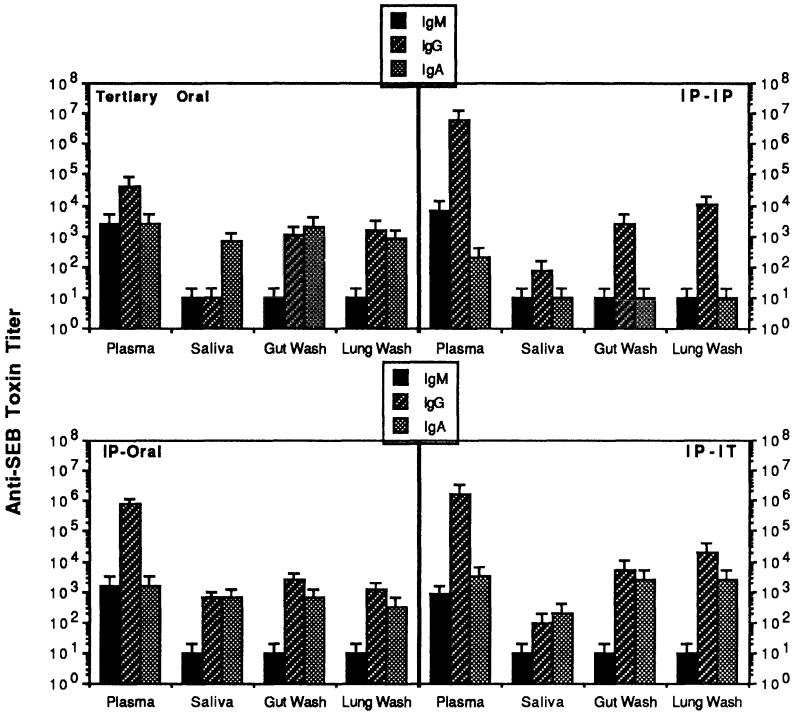

Figure 1. Anti-toxin titers of the IgM, IgG and IgA isotypes in the plasma and mucosal secretions of mice immunized with microencapsulated toxoid.

tolerance as demonstrated by suppressed circulating antibody levels to systemic challenge (data not shown).

In contrast to oral immunization, two IP immunizations with 100 ug doses of microencapsulated toxoid, separated by 30 days, induced a plasma IgG anti-toxin response at a titer of 6,553,600 on day 20 after boosting (Fig. 1). Despite the fact that this circulating IgG response was 64-fold higher than that induced through oral immunization with microencapsulated toxoid and over 500-fold that induced by systemic immunization with nonencapsulated toxoid, no significant IgA anti-toxin antibodies were detected in the plasma or mucosal secretions. This hyperimmunization was, however, capable of resulting in the appearance of IgG antibodies in the samples of each of the secretions.

The relationship between the systemic and mucosal immune systems was investigated by determining the isotype and distribution of antibodies induced through oral or IT boosting of IP primed mice. As in the other groups of mice, 100 ug of encapsulated toxoid was used at each administration and the plasma and secretion samples were collected 20 days after secondary immunization (Fig. 1). Oral immunization following systemic priming boosted the level of serum IgG anti-toxin titer to 819,200, and resulted in the appearance of circulating IgA antibodies which were not induced by systemic boosting. In addition, the IP-Oral protocol was nearly as effective in the induction of mucosal IgA anti-toxin antibodies as was tertiary oral immunization. Similar, but more dramatic, results were obtained when the boosting was performed by IT instillation of the microencapsulated toxoid (Fig. 1). The level of circulating IgG antibodies was boosted, and plasma IgA anti-toxin was detected to a titer of 3,200. In addition, each of the mucosal secretions contained significant levels of specific IgG and IgA. This immunization method was particularly effective at inducing both IgG and IgA antibodies in the lung fluids, with IgG and IgA anti-toxin titers reaching 20,480 and 2,560, respectively.

These results illustrate the utility of microencapsulation of vaccines for the potentiation of immune responses. The greatest advantage to this approach is in the ability to directly immunize mucosal tissues with antigens which are otherwise ineffective mucosal immunogens. In particular, the finding that IT instillation of microencapsulated antigens is highly effective in the induction of pulmonary antibody responses suggests that this system may find application in immunization against upper respiratory pathogens either through aerosol or intranasal use.

Acknowledgements This work was supported by USPHS grants AI 24772, AI 21774 and Contract DAMD17-86-6160 from the U.S. Army Medical Research Acquisition Activity.

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Efficient intranasal vaccination of mice against human respiratory syncytial virus with an experimental iscoms subunit vaccine

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ABSTRACT. The efficiency of vaccination via the mucosal route of an experimental human respiratory syncytial (RS) virus ISCOMS subunit vaccine based on the fusion protein of RS was evaluated. The circulating neutralizing antibody response was monitored when vaccine was inoculated intranasally in comparison with a parenteral route (intramusculaly); 10^5 infectious units of RS Long strain virus were used as live virus control. Results indicate the feasibility of intranasal vaccination with the ISCOMS.

INTRODUCTION. RS is an important pathogen of infants and children that causes severe lower respiratory tract infection and for which no vaccine is presently available. Both live and subunit vaccines are presently under development, and subunits of the fusion protein have been shown to induce protection and are expected to form the basis of an efficient vaccine (reviewed by Murphy et al.,1988).

Vaccination via the mucosal route is not a new concept since immunization via the respiratory or gastrointestinal tracts have been proven effective. Live poliovirus vaccine administered by the oral route has been available for use in humans since 1960. Live rubella virus vaccine, which has been administered by subcutaneous route was also found to be effective by the mucosal route. Intranasal immunization with envelope glycoproteins of Sendaï virus (Ito and Matumoto, 1986) and human parainfluenza 3 (Ray et al., 1988) have been reported to protect from challenge. Other strategies such as nasal vaccination with influenza virus hemagglutinin in combination with cholera toxin B, which is known to be an excellent mucosal self-adjuvant, have shown promise (Tamura et al., 1988).

In the present study, we report on the efficiency of an ISCOMs vaccine (Morein et al., 1984) made from the fusion protein and nucleoprotein of human respiratory syncytial virus (Long strain), in inducing circulating neutralizing antibodies when given by the mucosal intranasal route.

MATERIAL AND METHODS. The Long strain of human RS was used throughout the study. Virus was grown on a continuous cell line of ovine kidney and the RS experimental ISCOMS vaccine was prepared as described before (Trudel et al., 1989).ISCOMS showed a characteristic honeycomb structure, 30-35 nm in diameter (Fig. 1) and shown by immunoblotting to be composed of the fusion glycoprotein and the nucleoprotein. Aliquots of 100 µg of ISCOMS were free of residual infectious virus.

For vaccination assays, groups of three BALB/c mice were injected either intranasally or intramuscularly with three doses of 50 µg of either RS ISCOMS or free glycoproteins mixed with 1% (w/v) Quil A, 21 days apart. Live virus vaccine, containing 10^5 infectious units, were also given to control groups. Serum neutralization assays were carried out against 20 PFU. of RS virus and titers were expressed as the last dilution which allowed 50% residual infectivity.

RESULTS. ISCOMS (Table 1) were more efficient in inducing circulating neutralizing antibodies than the subunits mixed with Quil A and the live virus vaccine; the latter two induced a comparable response. The intranasal immunization route was as efficient as the intramuscular route for vaccination with ISCOMS. Controls remained negative. Moreover, intranasal vaccination with ISCOMS induced 8-fold higher neutralizing antibody titers than with both subunits plus adjuvant and live virus vaccines

TABLE 1.NEUTRALIZING TITERS IN MICE AFTER VACCINATION

	RS ISCOMS	RS SUBUNITS + QUIL A	LIVE RS VIRUS	CONTROL
Instillation (intranasal)	1/256	1/32	1/32	neg
Injection (intramuscular)	1/256	1/64	1/64	neg

DISCUSSION AND CONCLUSION. The neutralizing antibody titers reached confirm our previous work on the immunogenicity of ISCOMS injected intramuscularly into guinea pigs (Trudel et al., 1989). Moreover, the ISCOMS were more efficient in inducing neutralizing antibodies in mice (4-fold increase compared to guinea pigs). Although neutralizing antibodies are not the only markers of protection against RS, the fact that passive immunization with neutralizing monoclonal antibodies protects mice from infection justifies the value of monitoring neutralizing activity when measuring efficiency of vaccination.

Subunit vaccines in the form of ISCOMS permit an adequate configuration of the antigenic proteins which might mimic its natural tridimensional structure that restores immunogenicity. This added to the fact that Quil A is a better adjuvant than aluminium phosphate or hydroxyde (Bomford, 1987) could prove very important in vaccine development.

ACKNOWLEDGEMENT. This work was supported by the Medical Research Council of Canada (MT-6682)

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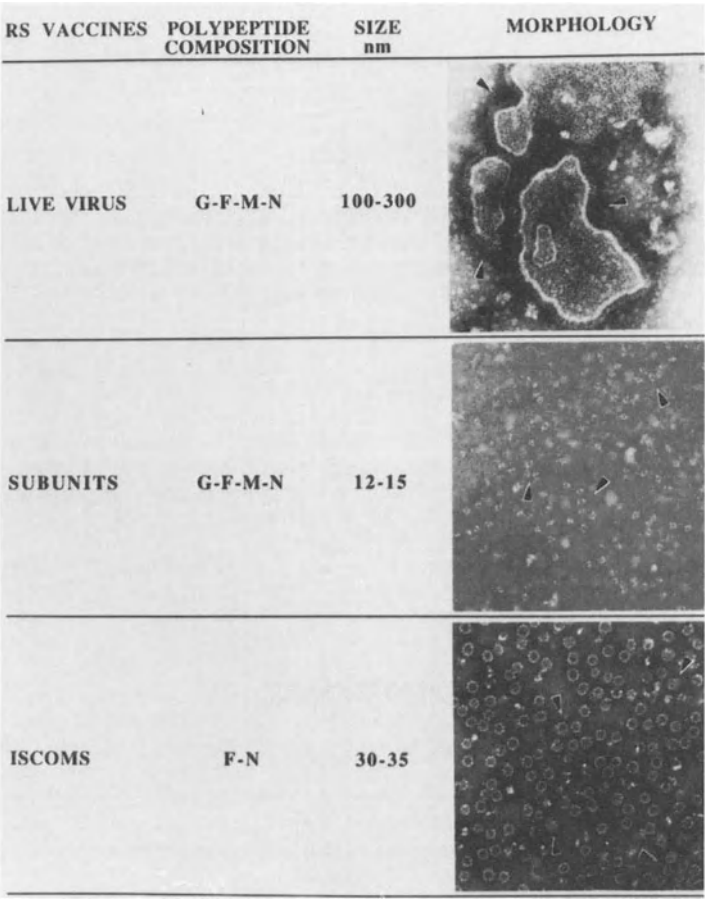

Figure 1. Physical appearance of the experimental RS vaccine upon electron microscopy examination of negatively stained preparations. G: major glycoprotein; F: fusion glycoprotein; M: membrane protein; N: nucleoprotein

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Peyer's patches immunization with *Streptococcus mutans* in rats induces predominantly IgG antibodies in saliva

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ABSTRACT. Only IgG antibodies against antigen I/II (Ag I/II) from Streptococcus mutans were found in the saliva of rats immunized twice in the Peyer's patches with that bacteria. IgA anti-Ag I/II were on the other hand found in the bile and in some milk samples from the rats. It is suggested that the Peyer's patches may not contribute to the induction of salivary IgA antibodies against the *S. mutans* antigen and that the homing of blast cells from the Peyer's patches is influenced by the nature of the antigen.

Introduction

Several attempts have been made to direct the efferent arm of the mucosal immune network against *Streptococcus mutans* vulnerable colonization sites in order to inhibit the development of dental decay in the host. This is exemplified by different immunogens from *S. mutans* and given orally with the hope that they should stimulate the Peyer's patches (PP). The PP are considered as major inductive sites of generalized secretory antibody responses.
In the present study we have examined the levels and isotype distribution of salivary antibodies against *S. mutans* cell wall associated protein antigen I/II (Ag I/II), a major protective antigen, in rats immunized in the PP with *S. mutans* .

Methods

Lactating Sprague-Dawley rats were immunized twice, two lactation periods apart (6-10 weeks), one day after delivery at both occasions, by injection of heat-killed *S. mutans* (10^9 bacteria/0.1 ml/animal) whole cells into the PP.
Samples of pilocarpine stimulated whole saliva were collected before, and eight days after each immunization, and were assayed by ELISA for activity of IgA, IgG and IgM antibodies against Ag I/II of *S. mutans*. For comparison, samples of milk, bile, and serum, were analyzed.

Results

The salivary anti-Ag I/II antibody activity eight days after the second injection of heat killed *S. mutans* into the Peyer's patches is shown in figure 1. After the booster dose all rats had salivary IgG antibodies but no IgM or IgA antibodies. In the bile on the other hand 9/10 rats had IgA and/or IgG anti-antigen I/II antibodies after the booster dose and one rat had a low level of IgM antibodies.
All rats had positive titers of IgG and IgM antibodies in serum. In milk four rats responded with IgA antibodies and all rats with IgG and IgM antibodies against the *S. mutans* antigen.

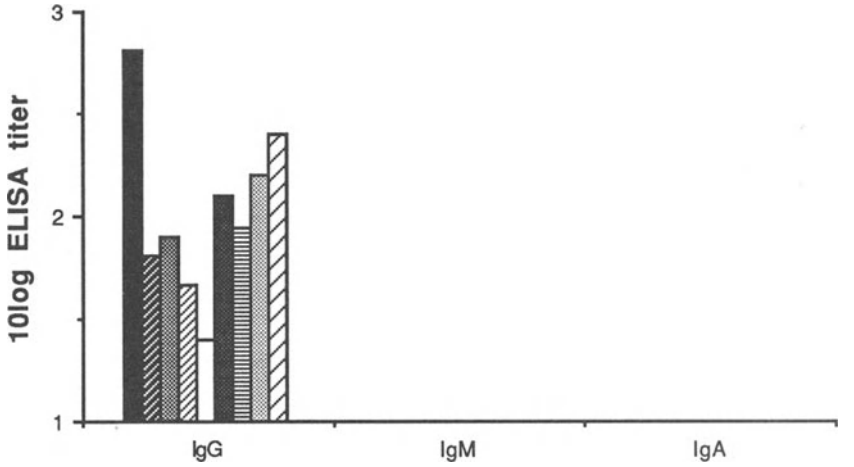

Fig. 1. The salivary anti-antigen I/II antibody activity eight days after the second injection of heat killed *S. mutans* into the Peyer's patches. Each bar represents one rat.

Discussion and conclusion

The results of this study indicate that the PP may not contribute to the induction of salivary secretory IgA immunity to *S. mutans* in rat. Conversely injection of ferritin into the PP induces salivary IgA antibodies, thus the homing of blast cells from the PP to the salivary glands might be influenced by the nature of the antigen similar to the divergent antibody response in milk and bile after immunization in the PP with Escherichia coli. The presence of IgA anti-Ag I/II in the bile and in some milk samples in the present animals gives substance to this hypothesis
The presence of high levels of IgG antibodies against Ag I/II in saliva and also in milk may be explained by passive transfer from serum or local production by the salivary and the mammary glands. We are currently adressing these questions.

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Induction of mucosal immune response against bacterial pilus antigen in chickens

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1. Introduction

To analyse local antibody production an in vitro culture system was developed based on the method of Svennerholm and Holmgren (1). Here characteristics of this culture system are presented.

2. Materials And Methods

2.1. Chickens

Female white leghorn chickens were vaccinated at the age of 4-6 weeks.

2.2. Antigen and Vaccination

Animals received 20 µg i.p. on day 0 and 1000 µg intracloacally (i.cl.) on day 14 of E.coli K99 on 1.8% $AlPO_4$. Section was performed on day 21 unless indicated otherwise.

2.3. Antibody determination

Isotype-specific antibody determination was performed by indirect ELISA.

2.4. Culture method

Intestinal tissue was flushed and washed with icecold Hanks' Balanced Solution containing 500 IU/500 µg Pen/Strep per ml. 0.9 g of tissue was suspended in icecold RPMI 1640 (4 ml) with 200 IU/200 µg Pen/Strep per ml. Samples from tissue-culture medium were taken directly and after 16 h of culture. The increase of absorption in the ELISA was used as a measure for antibody release.

3. Results

After an i.p. priming vaccination and an i.cl. booster vaccination with K99 it was possible to demonstrate in vitro antigen-specific antibody production. Antibody of IgM and IgA isotype was present in culture supernatant of vaccinated animals after 16 h of culture. IgG antibodies were released very quickly and were present in significant amounts at the onset of the experiment.

Antibody production of the IgM and the IgA isotype was strongly inhibited by various protein synthesis inhibitors (e.g. Cycloheximide, Cytochalasin B, Emetine or Puromycin) and by incubation at 4°C. Release of IgG was hardly inhibited.

Using this culture system in combination with i.p./i.cl. vaccination it was demonstrated that an interval of 7 days between boosting and culture was optimal for the demonstration of IgM and IgA production while IgG production was not influenced to a large extent by the lenght of this interval.

The influence of number and routes of vaccinations on the observed IgA production was determined in the last experiment. Best results were obtained by a combination of double i.p. followed by single i.cl. administration of antigen. IgA production in that case could be inhibited 48% by incubation at 4°C.

4. Discussion

The presented experiments clearly demonstrate that in chicken intestinal tissue antibody production is taking place. Antibodies produced are mainly of the IgA and IgM isotype as can be concluded from the following properties. Both IgA and IgM production is inhibited by incubation at 4°C or in the presence of protein-synthesis inhibitors. IgG on the other hand is present in significantly increased amounts already at t=0 h, indicating extremely rapid release of antibodies. This release is not or only to a small extent inhibited by incubation at 4°C. Finally IgA and IgM production is greatly influenced by the timing of the local (i.cl.) antigen administration. This in contrast to IgG production which is rather independent of the local administration of antigen.

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Colloidal carriers as antigen delivery systems for oral immunisation

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Introduction. Over the years, there have been many attempts to exploit colloidal carrier systems, e.g. emulsions, nanoparticles and liposomes, for the oral delivery of macromolecules, with limited success, reviewed by O'Hagan et al. (1988). However, recent advances in novel drug delivery systems have highlighted the potential of polymeric colloidal particulates for site-specific delivery by parenteral, nasal and oral routes (Davis et al. 1984). Such polymeric particles may prove to be efficient antigen delivery systems following oral administration and may lead to the development of more effective oral vaccines for non-replicating antigens.

Here we describe preliminary studies in experimental rats involving oral administration of a model antigen ovalbumin (OVA) both as a soluble dose and associated with colloidal carriers.

Materials and Methods. Poly(butyl-2-cyanoacrylate) particles [p(BCA)] (100nm), which are biocompatible, biodegradable and strongly adsorb proteins and drugs, were prepared according to the method described by Douglas et al. (1987) and after preparation, OVA was adsorbed to the particles at a concentration of 4 mg/ml.

Polyacrylamide microparticles (2.55μm) with entrapped OVA were prepared according to the method of Ekman and Sjoholm (1978), the particles contained 100μg OVA/mg dry weight (amino acid analysis).

In both studies, male Wistar rats (200g) were gastrically intubated with 1mg OVA, either associated with a colloidal carrier or as a soluble dose, on 4 consecutive days, commencing 2 weeks after a priming dose of 5mg OVA by intraperitoneal (i.p.) injection. Fourteen days after the initial intubation (study day 14), saliva was collected from all the animals following stimulation by i.p. pilocarpine (100μg/100g body weight).

About 2 months later (study day 50 or 65), saliva samples were again collected from the animals 4 days after they had all received a booster dose of 1 mg soluble OVA by gastric intubation.

Results. The ELISA A_{405nm} values for the groups receiving OVA associated with the carrier systems were compared in unpaired student "t" tests, with the values for the groups receiving soluble

OVA at the same time intervals.

TABLE 1. Salivary IgA antibody response (A_{405nm}) to orally administered OVA adsorbed to 100nm p(BCA) particles (mean ±SE n=8).

	Study day 14	Study day 50
Soluble OVA	0.489 ±0.041	0.578 ± 0.036
p(BCA) OVA	0.654 ±0.037*	0.708 ± 0.041**
1/5 dilutions of saliva		

TABLE 2. Serum IgG antibody response (A_{405nm}) to orally administered OVA adsorbed to 100nm p(BCA) particles (mean ±SE n=8).

	Study day 14	Study day 50
Soluble OVA	0.416 ±0.040	0.359 ± 0.057
p(BCA) OVA	0.681 ±0.018***	0.426 ± 0.053
1/10 dilutions of sera		

TABLE 3. Salivary IgA antibody response (A_{405nm}) to orally administered OVA incorporated in microparticles (mean ± SE n=8).

	Study day 14	Study day 65
Soluble OVA	0.404 ±0.026	0.434 ±0.029
Micropart. OVA	0.329 ±0.026	0.593 ±0.031*
1/8 dilutions of saliva		

* significant at $p<0.01$ ** significant at $p<0.05$

***significant at $p<0.001$

Discussion. The results demonstrate that the humoral immune responses to a soluble antigen administered orally can be enhanced by the association of the antigen with a particulate carrier system. The significance of these results for the possible induction of protective immune responses in the secretions against pathogens which normally infect mucosal sites is as yet unknown. The model formulations used here were unsophisticated and various modifications are possible to improve the delivery system.

Acknowledgements. This work was performed with the financial assistance of Beechams, Great Burgh, Surrey and the S.E.R.C.

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Safety, immunogenicity and efficacy testing of an oral vaccine strain based on the expression of an *Escherichia coli* adhesin in laboratory *Escherichia coli* strain HB101

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1. Introduction

Expression of relevant bacterial antigens on live, avirulent vaccine strains is one approach to vaccine development for enteric infections. Successful live oral vaccines of this type must be based on appropriate choices of both antigen and vector strain to stimulate protective immunity without causing disease. It is known that luminal antibody to adhesins of E.coli can protect against challenge with these enteropathogens. To test whether expression of the pilus adhesin (AF/R1) from the rabbit diarrheal pathogen RDEC-1 on the standard laboratory E.coli strain HB101 (frequently used as a recipient for cloned DNA) is an appropriate oral vaccine construct, we fed this construct to rabbits, then determined its safety, immunogenicity and efficacy.

2. Materials and Methods

2.1. BACTERIAL STRAINS.

Vaccine candidate strain M5 was produced by transfer of the 86Md AF/R1 virulence plasmid from RDEC-1 to HB101, and AF/R1 expression confirmed by adherence to brush borders. RDEC-1 for challenge studies were grown overnight in Pennassay broth (conditions that promote AF/R1 expression).

2.2. RABBIT IMMUNIZATION AND CHALLENGE.

For immunogenicity studies, strain M5 (doses: 10^2, 10^5, or 10^9) was administered by oro-gastric tube with $NaHCO_3$ during weeks 1, 2, and 4 to rabbits (N=12/group). Serum was obtained before inoculation, and serum and bile were obtained at the time of sacrifice (week 5). Safety was assessed by monitoring for signs of disease (wet tail, weight loss, unformed pellets, or death), and colonization was assessed by

culturing rectal swabs for M5. For efficacy studies, 10^9 M5 or $NaHCO_3$ were administered (N=10/group) as before, with a challenge dose of 10^7 RDEC-1 given during week 5. RDEC-1 colony counts from cecal contents were performed at 3 and 11 days after challenge with RDEC-1.

2.3. ASSESSMENT OF IMMUNOGENICITY.

Paired pre- and post- inoculation sera, and bile obtained at the time of sacrifice were assayed for anti-AF/R1 antibody by ELISA's. Antibody to M5 antigens was sought in bile by the immunoblot method. Heat-saline extracts of M5 were electrophoresed in polyacrylamide gels, electroblotted to nitrocellulose, and then reacted with bile samples to detect antibody to M5. Rectal mucosal biopsies were maintained in supplemented CMRL media in 95% O_2, on a rocker for 24 hours. Anti-pilus antibody activity was measured in media by ELISA.

3. Results

Safety of M5 was demonstrated, since no animal developed disease and only 1/36 was colonized by M5 (determined by culture of rectal swabs) following oral inoculation. A definite serum immune response (two-fold rise in ELISA O.D.) to the AF/R1 pilus occurred in 7/21 paired samples available for study, but a relationship between inoculum size and ELISA O.D. was not seen. Anti-pilus antibody was detected in 19/20 bile samples, with highest ELISA O.D.'s in bile of animals given 10^9 M5. Reactivity with the pilus subunit was confirmed by immunoblots. Anti-pilus activity was not detected in organ culture media.

Mean cecal RDEC-1 counts in animals fed M5 before challenge with RDEC-1 tended to be lower than in unvaccinated animals at 11 and 3 days, but differences were not significant ($p<.21$, $p<.12$).

4. Discussion

The recipient E.coli strain HB101 has the desirable quality as a vector strain of readily accepting and stably reproducing foreign genetic material. Its known property of poor replication in the intestine, could confer great safety advantage, might be reversed by expression of an adhesin, or might render the strain inefficient in stimulating mucosal immunity. In this study, expression of the rabbit pilus adhesin AF/R1 did not compromise the safety of HB101 as a vector, since none of the animals fed M5 developed disease. In vivo immunogenicity of the pilus antigen was demonstrated by seroconversion of some animals and by the presence of anti-pilus antibody in bile. However, this immunogenicity was insufficient to prevent colonization by the pathogen following challenge. Immunogenicity and efficacy of future vaccine strains may be improved by utilizing carrier strains better suited than HB101 to survive and multiply in the GI tract.

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C3H mouse model of genetic predisposition to vaccine induced allergy

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ABSTRACT. Allergic reactions in recipients of Rift Valley fever vaccine (RVFV) are extremely rare. When vaccine was given by various routes to selected mouse strains, intraperitoneal (IP) priming of C3H/HeJ mice with RVFV resulted in acute respiratory arrest of all mice upon secondary IP inoculation on day 14. All C3H/HeJ mice given intranasal (IN) boost had distress but only 67% died. In contrast, no C3H/HeN mice died following re-exposure to RVFV vaccine. C3H/HeJ mice have high splenic IgA responses as shown by Kiyono et al. [1]. We examined commitment of local B-cells to IgA and IgE expression by Northern blot analysis of extracted RNA from tissues of control and RVFV immunized mice. Using the α660 cDNA probe we found increased α mRNA in lungs and spleens of C3H/HeJ, while there was decreased α mRNA in the same organs of C3H/HeN mice 14 days after IP immunization. Northern blots were also probed for ε mRNA with the cDNA chimera, $C^{2}30$. Low levels of ε mRNA were found in all tissues of control C3H/HeN mice. Immunization resulted in differences in ε mRNA levels consistent with association of locally-produced IgE with respiratory allergy in C3H/HeJ mice.

INTRODUCTION.

The formalin-inactivated Rift Valley fever vaccine has been tested in over 2173 human subjects with only one individual exhibiting an allergic reaction. Such low frequency of allergy is consistent with host genetic predisposition rather than intrinsic reactogenicity of the vaccine. This vaccine has been evaluated in experimental animals for efficacy against exposure to virulent virus (ZH-501, [2]). Routes of vaccination, doses of vaccine or challenge virus, and routes of challenge were studied in mouse models of Rift Valley fever infection [3]. Earlier studies were primarily conducted in C3H/HeN, Balb/c and A/J mice and vaccination by either subcutaneous (SC) or intraduodenal (ID) routes followed by SC or IN boost did not induce acute hypersensitivity reactions. Because of mucosal and parenchymal tropisms of the virus, isolated mucosal or peripheral priming were not sufficient to yield complete protection from either SC or aerosol challenge [3]. C3H/HeJ mice were added to these protocols because of

the observation of Kiyono et al. [1] that C3H/HeJ mice had enhanced IgA responses in Jerne plaque assays of spleen cells. We attempted to boost IP primed C3H/HeJ mice by IP or IN inoculation and encountered dramatic acute hypersensitivity reactions that had not been seen with C3H/HeN and other mouse strains. The present study is concerned with the analysis of the model and genetic elements in vaccine induced allergy. The results show that formalin inactivation of viral antigen, IP priming and differences in organ-specific expression of ε mRNA underlie the allergic predisposition of C3H/HeJ mice.

MATERIALS AND METHODS.

Animals. Adult female C3H/HeJ, C3H/HeN (OuJ), Balb/c ByJ, BDF_1, A/J and Swiss Webster mice were obtained from Charles River and the Jackson Laboratory. Vaccine inoculations were given IP and SC via 30 gauge needle and tuberculin syringe. IN inoculations were given via micropipet dispenser. Euthanasia was via CO_2 exposure. Blood IgA and IgE levels were determined by ELISA.
Vaccine. Rift Valley fever virus vaccine (IND 365) was prepared from Entebbe strain virus grown on fetal rhesus lung cells and formalin inactivated (0.5%). The formalin was inactivated with sodium bisulfite. The vaccine also contains Eagles basal medium, 0.5% (W/V) human serum albumin, Neomycin sulfate equivalent 50 µg/ml and Streptomycin 50 µg/ml.
RNA isolation - mouse tissues. Peripheral, mediastinal and mesenteric lymph nodes (LN), spleens, intestines and lungs were removed and immediately frozen in liquid nitrogen (organs from each group were pooled). RNA was isolated from frozen mouse tissue by the method of Chirgwin et al. [4]. Purified RNA was stored in sterile water at -70°C. RNA concentrations were adjusted to 10 µg per lane. Samples were heated to 65°C for 2 min and loaded onto 1.5% agarose gels containing 0.66 M formaldehyde, 20 mM MOPS, 5 mM EDTA, 6.6 µg ethidium bromide. Gels were elctrophoresed at 18-20V for 18 hr.
cDNA probes. The chimeric plasmid $C^2$30 containing 1300 bp cDNA for the mouse IgE heavy chain. This plasmid was the kind gift of Dr. Fu-Tong Liu [5]. The α 660 plasmid [6] was the kind gift of Dr. John Cebra and Peter Weinstein. This construct is a Gemini plasmid (Promega, Madison, WI) containing α 660 bp cDNA for mouse IgA.
Northern Blots. RNA gels were blotted overnight onto nitrocellulose. Filters were baked under vacuum at 80°C for 2 hr. Prehybridization was carried out for 20 min at 47°C in 40% formamide, 4X SSC, 1X Denhardt's solution, 7mM Tris pH 7.4 and 25 µg Salmon sperm DNA. Hybridization was carried out in the same buffer at 47°C for 20 hours. Radiolabeled probe was added to a final concentration of $1x10^8$ dpm. Post hybridization washes were done for 30 min each in 2x,1x,.1x SSC, 0,1%SDS and for 5 min at 68°C.

RESULTS AND DISCUSSION.

CeH/HeJ mice that died within 30 min of IP or IN secondary

inoculation 14 days after intraperitoneal immunization with RVF vaccine exhibited signs of acute respiratory distress. The lungs were hyperinflated, remaining inflated when the thoracic cavity was opened. There was congestion of the abdominal viscera and hyperperistalsis. Formalin-inactivated RVFV virions in the vaccine triggered this phenomenon. C3H/HeJ that were primed IP with whole vaccine did not show hypersensitivity or acute death after IP challenge with human serum albumin (HSA), Eagles basal medium with Neomycin sulfate and Streptomycin. Exposure to live RVF virus or β-propriolactone inactivated virions did not induce acute hypersensitivity. C3H/HeN, Balb/cByJ, BDF_1 and A/J mice were completely resistant to this phenomenon, while 20% of Swiss Webster mice died. C3H/HeN and C3H/HeJ mice were studied for acute hypersensitivity after SC, ID, or IP primary inoculation followed 14 days later by SC, IN or IP secondary inoculation with RVF vaccine. Of 100 C3H/HeN and 106 C3H/HeJ mice studied the only deaths were among C3H/HeJ mice that were IP primed and IP (100%) or IN (67%) boosted.

The above data indicated that IP priming and formalin modified RVFV antigen(s) initiated the allergic phenomenon but there was a very strong genetic component. Although mouse models of hypersensitivity are exceedingly complex [7,8], with networks of immunoregulatory cells, cytokines (IL-4 vs γ-IF), differences in mediator responsiveness [9], levels of IgE Fc receptor expression, and IgE binding factors. We examined messenger RNA expression for ε and α heavy chain in total RNA extracted from lymph nodes, spleen, lung and intestines of untreated and IP vaccinated mice (Table 1).

TABLE 1. Effect of IP vaccination with RVFV on ε and α mRNA levels

Mouse strain	Intestine		Lung		Med. LN		Mes. LN		Per. LN		Spleen	
	E	A	E	A	E	A	E	A	E	A	E	A
C3H/HeJ	D	D	I	I	I	=	I	=	D	D	I	I
C3H/HeN	D	D	D	=	D	D	D	D	D	=	I	I
Balb/cByJ	D	D	=	D	D	I	I	I	D	I	D	D
BDF_1	I	I	D	=	D	=	I	D	D	D	=	D
Swiss Webster	D	I	I	D	I	D	I	D	D	=	I	D

Comparison between control and immunized mice using densitometry of Northern blots. E = ε mRNA, A = α mRNA, D = decreased, I = increased, Med. = mediastinal, Mes. = mesenteric, Per. = peripheral, and LN = lymph node.

Low levels of ε mRNA were found in all tissues of control C3H/HeJ, BDF_1 and Swiss Webster mice and higher levels in the C3H/HeN and Balb/c mice. However, we found increased ε and α mRNA levels in lungs and spleen of C3H/HeJ, while there was decreased or unchanged ε and α mRNA levels all organs except the spleens of C3H/HeN mice 14 days after immunization with RVFV. The ε mRNA changes for BDF_1 resembled that of C3H/HeJ.

There were no significant differences among total serum IgE levels (ng/ml) of control or immunized C3H/HeJ (9.8 ± 1.4) and C3H/HeN (5.1 ± 1.2) mice. The highest control levels of IgE were found in Balb/cByJ (22.7 ± 4.8) and BDF_1 (15.6 ± 11). All strains showed at least double the control IgE levels after immunization. The IgA levels are reported in the meeting paper by Pitt et al.

We have shown that substrains of the C3H mouse have potential as a model of vaccine-induced acute hypersensitivity. Although this strain is intermediary between SJL and Balb/c mice with regard to IgE responses [7], there are organ-specific differences in IgA and IgE commitment following IP immunization that correlate with differences in expression of mucosal immunity and/or allergy in the mouse strains tested. C3H/HeJ and A/J mice are susceptible and resistant to antigen-triggered bronchospasm, respectively. They hold opposite relationships with regard to acetylcholine mediated hyperreactivity [9]. Could there be upregulation of IgE receptors to compensate for alterations in neurotransmitter binding? In the case of the C3H/HeJ and C3H/HeN mice the complexity of converging etiologies [8,9] should be minimized because there are limited genetic differences. We have not determined what role the genes controlling IL-4 and γ interferon play in this system.

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Only microbial antigens are efficient mucosal immunogens

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ABSTRACT. The antibody response to bacterial versus dietary antigens was studied in germfree rats colonized with E. coli and fed pellets containing egg and milk proteins. The antibody response against the bacterial antigens (O6 LPS, type 1 fimbriae, and ß-galactosidase) was stronger, and mostly appeared earlier than that against the food antigens (ovalbumin and ß-lactoglobulin), although the latter were present in larger quantities and were not less well absorbed. A similar difference in immunogenicity was seen if whole bacteria versus ovalbumin were injected directly into Peyer's patches, while ovalbumin was a good systemic antigen. The results suggest that bacteria stimulate antigen-pesenting cells in the GALT, and thereby confer strong immunogenicity to all substances carried in/on the bacteria, whereas soluble proteins do not possess this stimulating capacity.

1. INTRODUCTION

The gut-associated lymphoid tissue is confronted with antigens ranging from microorganisms to dietary macromolecules that pass undegraded over the gut epithelium (1, 2). There are indications that the GALT is more reactive towards microbes than soluble proteins (3). To test this hypothesis, germfree rats were colonized with E. coli and fed pellets based on egg and milk whey powder. The antibody response against three E. coli antigens (O6 LPS, type 1 fimbriae, and ß-galactosidase) and two dietary antigens (ovalbumin and ß-lactoglobulin) was followed in serum, bile and milk. The results will be reported in detail elsewhere (4).

2. MATERIALS AND METHODS

Germfree rats of the AGUS strain were monocolonized with a type 1-fimbriated E. coli strain of serotype O6K13H1, and conventionalized after nine weeks. They were fed pellets in which half of the protein sources of standard pellets were exchanged against equal amounts of egg and milk whey powder. The intake of pure ovalbumin and ß-lactoglobulin in pregnant or lactating rats was calculated to be 1 g/d of each.

Serum and bile were obtained after 1, 2, 3, 5 and 16 weeks, and milk after 5 (1st lactation period) and 16 weeks (2nd lactation period), and assayed using ELISA. The antibody activity was expressed as the log titre giving an absorbance of 0.1 over background.

Three sterile rats were given 100 mg ovalbumin and 1.5 mg crude endotoxin by tube feeding, and the blood concentration of ovalbumin and endotoxin was assayed by ELISA or a Limulus assay.

Antibodies in bile were assayed 8 days after immunization in Peyer's patches with 10^9 formalin-killed E. coli O6K13H1, or 10 mg ovalbumin in PBS. Serum IgG antibodies were assayed 21 days after a subcutaneous immunization with 10^9 E. coli, or 1 mg ovalbumin, in Freund's incomplete adjuvant.

3. RESULTS

3.1. Antibody response against antigens on a colonizing E. coli strain versus dietary antigens.

Within a week after colonization antibodies against O6 LPS appeared in serum and bile. A few weeks later antibodies against type 1 fimbriae followed, while the response against E. coli ß-galactosidase was not seen before 16 weeks. The response against food antigens was weak, and these antibodies mainly appeared in milk (Table 1).

3.2. Uptake of undegraded ovalbumin versus endotoxin.

The serum concentration of ovalbumin 1 hr after intragastric administration was 23 ng/ml, while no rat had an endotoxin activity in serum above 20 pg/ml at any time. Therefore, ovalbumin was at least 15 times more efficiently absorbed than endotoxin.

3.3. Antibody response against whole bacteria versus ovalbumin given systemically or into the Peyer's patches.

Bacteria were much more immunogenic than ovalbumin, also when injected directly into the Peyer's patches (Table 2). In contrast, both ovalbumin and ß-lactoglobulin were good antigens when given systemically.

4. DISCUSSION

The above results indicate that the GALT, in contrast to systemic lymphoid tissue, distinguishes between antigens of bacterial origin and other soluble macromolecules. A strong response was seen not only against O6 LPS and type 1 fimbriae, but also against the intracellular protein ß-galactosidase, which cannot be expected to differ very much in physicochemical properties from dietary proteins like ovalbumin and ß-lactoglobulin. This suggests that the presentation of any substance to GALT within a "bacterial context" renders it strongly antigenic. The discrimination must, in consequence, be performed by cells that encounter

TABLE 1. Antibody response against bacterial and dietary antigens in serum, bile and milk.

	Median log ELISA antibody titre								
	Serum			Bile			Milk		
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
E. coli antigens:									
O6 LPS	5.0 (16)	4.7 (1)	2.1 (16)	2.8 (5)	1.8 (1)	2.8 (5)	1.6 (5)	1.8 (5)	<1
Type 1 fimbriae	3.0 (16)	1.6 (1)	1.4 (16)	1.8 (5)	1.0 (2)	1.9 (16)	2.1 (16)	1.3 (16)	1.9 (16)
ß-galactosidase	1.6 (16)	<1	<1	n.d.	n.d.	n.d.	2.9 (16)	3.6 (16)	3.0 (16)
Dietary antigens:									
ovalbumin	<1	1.3 (16)	<1	<1	<1	<1	<1	1.4 (16)	1.7 (16)
ß-lactoglobulin	<1	<1	<1	<1	<1	<1	<1	<1	<1

The antibody titre of a sample was defined as the reciprocal of the highest dilution giving an absorbance of 0.1 over background. The figures within brackets denote the time (in weeks) of the peak antibody response. n.d = Not determined.

TABLE 2. Antibody response against bacteria and ovalbumin given systemically, or into the Peyer's patches.

		Median log antibody titre	
Immunization ag	Detection ag	Peyer's patch immunization	Subcutaneous immunization
Ovalbumin	Ovalbumin	<1[a]	3.7[b]
Whole *E. coli*	O6 LPS	3.5[a]	2.0[b]

a) Bile IgA antibodies 8 days after immunization in Peyer's patches
b) Serum IgG antibodies 21 days after subcutaneous immunization

intact bacteria, presumably antigen-presenting phagocytes in the GALT (5). These cells could be stimulated to produce interleukins by bacterial adjuvant substances like LPS, and then present antigens in an immunogenic fashion (6). Soluble proteins, whether originally of bacterial or dietary origin may not stimulate interleukin production and may therefore instead be presented in a tolerogenic manner.

Although O6 LPS and type 1 fimbriae were present on the same bacterial cells, antibodies against the former were of highest titres in bile, but against the latter in the milk. Such differences have earlier been reported after immunization into the Peyer's pathces, indicating that a differential homing, or proliferation at local sites, exists for cells with different antigen specificities (7).

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The effect of dietary glutamine on the mucosal immunoglobulin response

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INTRODUCTION

Total parenteral nutrition (TPN) is an important therapeutic modality in patients with dysfunctional gastrointestinal physiology. A recognized sequelae of TPN is intestinal mucosal atrophy which presumably occurs from disuse. We have previously established that the use of TPN results in a marked reduction in biliary secretory IgA (s-IgA). The purpose of the present study was to asses the effect of glutamine enhanced TPN solution on biliary IgA and IgM concentration and on bacterial translocation to MLN.

METHODS

36 female Fisher rats (100-150 gm) were randomly divided to 3 groups of 12 animals each. Group I (Control) was fed rat chow and H2O ad.lib. Central venous catheters were placed in animals in Groups II (TPN) and III (TPN-GLN) and they were fed isonitrogenous, isocaloric standard hyperalimentation solutions except the Group III solution contained 2% glutamine. Following 2 weeks of feeding, bile was collected for S-IgA and IgM assay and mesenteric lymph nodes were harvested and cultured to determine bacterial translocation from the gut. S-IgA and IgM were measured using an ELISA technique.

RESULTS

Group	S-IgA ug/ml±SD	IgM ug/ml±SD	Incidence Culture+MLN
I (CONTROL)	858 ± 61	208 ± 88	0/12
II (TPN)	494 ± 109*	385 ± 207*	7/12*
III (TPN-GLN)	739 ± 148#	1376 ± 1316*	1/12

*p < .05 versus I, #p < .01 versus II, Mann-Whitney and Chi square.

Results indicate that a standard solution of TPN results in a decrease in biliary s-IgA and significant bacterial translocation to mesenteric lymph nodes. The most common bacteria cultured were E. Coli and Proteus Vulgaris. This fall in biliary IgA was accompanied by

a statistically significant rise in IgM. Biliary concentration of IgA was maintained at control levels when glutamine was added to the solution and this was associated with a significant reduction in the bacterial translocation rate.

DISCUSSION

Bacterial translocation from the gut is emerging as an important pathogenic event in many disease states. Confining the indigenous intestinal microflora to the gut lumen is a complex process. The involvement of s-IgA in this process has not been directly examined. S-IgA functions by preventing the attachment of bacteria to the enterocyte. This process may be the initiating event in bacterial translocation from the gut. The present study demonstrates that animals fed a standard solution of TPN develop a significant reduction in biliary s-IgA and bacterial translocation to mesenteric lymph nodes. TPN induced gut atrophy does not appear to spare the GALT and therefore results in an intestinal immunodeficiency state such that bacteria are no longer confined to the intestinal lumen. Glutamine has been shown to be trophic to the intestinal mucosa when added to the TPN solution resulting in increased mucosal cellularity and nitrogen. Glutamine in this model is immunostimulatory to both IgA and IgM synthesis. Because glutamine is the principle fuel for intestinal cells, GALT atrophy may be prevented with its use. The rise in biliary immunoglobulin concentration in the glutamine supplemented group was associated with a decrease in bacterial translocation to control levels. These data suggest that maintenance of biliary IgA levels may prevent bacterial translocation to MLN. Further work in this area is necessary.

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Effect of TGE coronavirus strains on numbers and Ig-isotope expression of specific antibody-secreting cells from mesenteric lymph node after oral administration

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Transmissible gastroenteritis (TGE) is lethal for piglets under two weeks of age but usually results in a transient diarrhea in older pigs (Hooper et al.(1966)). Caused by a coronavirus which preliminary infects intestinal epithelial cells, this swine disease can be used as model for studies on the regulation of the secretory IgA response. It is well established that the majority of IgA antibody-secreting cells (ASC) of the intestinal mucosa, before homing back to the lamina propria, transit through the mesenteric lymph node (MLN) where they undergo a maturation step (Mc Williams et al.(1977); Roux et al.(1981)). Mesenteric cells were isolated from adult pigs 3, 11 or 30 days after oral immunization with either a virulent (GEP-II) or two attenuated (Purdue-115, Nouzilly) strains of coronavirus and were co-cultured with the virus for one week. Responses to TGE virus were measured by enumeration of specific ASC according to the Ig-isotype (ELISASPOT assay) from these mesenteric leukocyte cultures (Berthon et al.(manuscript in preparation)).

Results showed that GEP-II strain, which induced diarrhea in these pigs, induced an antibody response increasing from day 3 to day 30 with a predominance of IgA ASC. In contrast, attenuated strains (no diarrhea after the viral ingestion) generated a short antibody response, decreasing from the 3rd day after immunization and mainly represented by IgG ASC. These data support the view that the induction and the maintenance of IgA synthesis are correlated with the magnitude of the gut antigenic stimulation, with the possibility of emigration of the attenuated virus strains through the gut resulting in the stimulation of the systemic immune system.

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Mice having different H-2 haplotypes respond differently to priming with a mucosal antigen

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INTRODUCTION

Inbred mice possessing different H-2 haplotypes respond differently to a secondary challenge with a mucosal antigen (MA) extracted from mice small intestine mucosa. BALB/c mice synthetize detectable levels of anti-MA antibodies whereas C57BL/6 mice do so at marginal levels (1). Our objective was to investigate if these high and low responder mice behave similarly at a cellular level, to priming with MA at early periods when no anti-MA antibodies are detected in serum.

MATERIALS AND METHODS

MA purification: mice small intestine scrapings were prepared as described (2). After chromatography, the first peak exiting after the void volume was used as MA immunogen.
Immunization schedule: male BALB/c and C57BL/6 mice 2 months old received a single dose of 1 mg N of MA emulsified in FCA. Mice of the same strains received FCA or saline alone and served as controls. All animals were sacrificed 15 or 30 days after.
ELISPOT assay: single cell suspension of spleen B-cells was made in RPMI 1640 + FCS. Different concentrations were added in triplicate to Millititer HA plates previously coated with MA (3). After incubation and washings, optimal dilutions of HRP-conjugated anti-mouse IgG or IgM were added. Following overnight incubation, a HRP chromogen was added. Individual wells were examined for the presence of red spots and enumerated at low magnification with the help of a stereomicroscope.
Statistical analysis: Student t test was used to determine significance levels between mean values of the number of spots recorded.

RESULTS

As shown in Figure 1, 15 or 30 days after immunization, the number of SFC-IgM (spot-forming cells secreting IgM) was significantly higher in MA-immunized BALB/c than in C57BL/6 or control BALB/c mice ($p < 0.0001$).

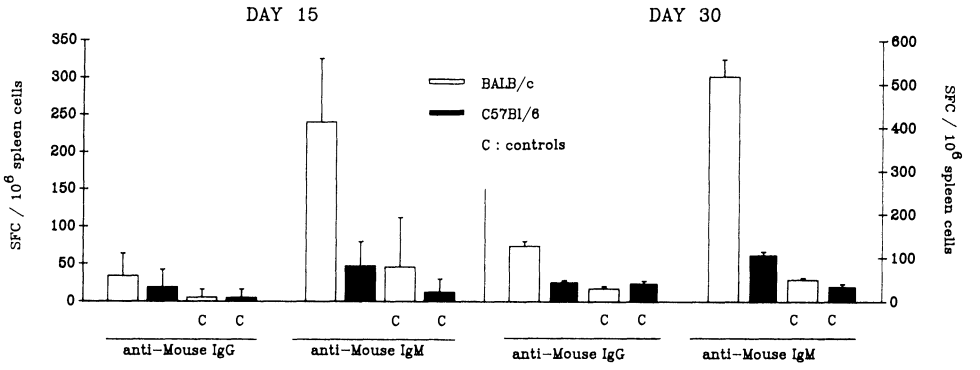

Figure 1. Number of spots obtained at different times after injection.

At the same periods, the number of IgG-SFC in immunized BALB/c was significantly higher than in C57BL/6 mice, although at later periods the p values were much smaller (day 15, $p<0.01$; day 30, $p<0.0001$). However, when comparing these numbers with those obtained in control animals, the degree of significance was high ($p<0.0001$).

DISCUSSION

The unresponsiveness of mouse to natural antigens is under genetic control(4). The susceptibility to the development of autoimmune diseases is also under similar control, maping to the major histocompatibility locus in autoimmune thyroiditis (5) and orchitis (6). In experimental encephalomyelitis, the control is excerced by genes located outside the major histocompatibility complex (7). The results presented here clearly showed that BALB/c mice (H2-d) is a high responder to a primary challenge with MA at the cellular level, whereas C57BL/6 mice(H2-b) is a low responder to the same antigen. At that time, no MA antibodies can be detected in sera using a sensitive ELISA assay (1). These obsevations reenforce our hypothesis that the response to MA and the subsequent development of autoimmune enterocolitis (1) is under genetic control.

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Activation of defense mechanisms in the lung mucosa following oral immunization

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1. Introduction

The stimulation of local lymphocyte populations in the Peyer's Patches leads to the local IgA immune responses in the mucosa including the lung. The same lymphocytes have contact via M-cells with the antigen and produce slgA in other mucosa areas after traffic via the blood (1,2,3,4). For prophylaxis in acute respiratory infections it is necessary to stimulate not only slgA in the lung but also phagocytosis and interferon production or activation of lysosomal enzymes. Activation of alveolar macrophages after oral immunization was demonstrated here.

2. Materials and Methods

2.1.ANIMALS
Female NMR1-mice, 8 - 10 weeks old, weighing 25-30g and male Wistar rats, weighing 250-300g were uses.

2.2.VACCINE
Polyvalent bacterial vaccine (Infectvac), an inactivated extract of 7 species of ARI-relevant bacteria.

2.3.CHEMICALS
Sugars and lectins were obtained from Serva GmbH.

2.4.BACTERIA
Streptococcus pneumoniae ATCC 6301 was cultivated in mucin-blood agar concentrated to 10 bacteria ml PBS. For infection, 40 ul/mouse were applied intranasally in lightly anaesthetized animals.

2.5.VIRUS
Mice pathogenic influenza virus (A/PR/8/34,H1N1) was used. For experimental infection, mice were exposed once for 5 min to the influenza virus (lethal doses) in aerosol form. For the oral immunization, an intraoesophageal tube was used. Mice were immunized 10 times within 14 days with 0.2ml vaccine/dose. The infection was performed 4 days after the last immunization dose.

Bronchoalveoloar lavage (BAL): Bronchoalevolar lavage was performed in animals by inserting a steel tube into the exposed trachea through high transverse incision between tracheal rings.

Phagocytosis: The activity of the isolated alveolar macrophages (10 cells/ml) in the phagocytosis (endocytosis) and killing procedure was estimated with the help of a combined radiometric method.

Interferon: Measurement of cytopathogenic effects on mice-L929 cells after incubation with

Enzyme activities: N-acetylglucosaminidase and B-glucuronidase were estimated spectrophotometrically using synthetic substrates (p-nitrophenyl-N-acetyl B-D-glucosaminide, respectively.

3. Results

a) We could demonstrate with S. pneumoniae infection model, using simple lectin-blocking sugars in vivo the active role of the bacterial lectin for infection (3). In this paper we have found that the administration of 200 ug N-ace tyglucosamine (1 h before infection) led to survival of 13 out of 20 animals. Galactose or other non bacterial lectin relevant sugars did not show this effect (survival 4 out of 20 animals). More practical evidence for vaccination against acute respiratory infections (ARI) would involve the "unspecific" immune stimulation of the mucosa.

b) We could demonstrate the activation of alveolar macrophages after oral stimulation (there is no traffic of macrophages from the gut to the lung (3). Most effective was the combination of polyvalent bacterial vaccine, containing an immunomodulator (WGA) for oral immunization. The lectin alone and in combination with the vaccine (3.5 x 10 bacteria + 10 ug WGA) led to an increase of defense mechanism in the lung. A strong activation of phagocytosis and phagocytosis killing in the lung, an activation of interferon production in the lung and in the serum and increase of lysosomal enzyme activity of alveolar macrophages, respectively was found.

4. Discussion

Recent results have demonstrated the ability of oral administered lectins to elicit serum and/or secretory antibodies (1) via interactions of lectins and lymphocytes in the blood. We have found a strong activation of macrophages in the lung after oral administration. We believe neither the ability of the traffice of lectin molecules or of macrophages from the gut to the lung (via blood) nor previous exposure of the lung to the lectin could explain our findings. Rather, lectin-activated lymphococytes (via lectin receptor) could transport the information from Peyer's patches to activate macorphages in the lung.

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SECTION J: MUCOSAL MAST CELLS

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Mast cell heterogeneity: protein composition and synthesis in rat intestinal mucosal and peritoneal mast cells

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ABSTRACT. Rat intestinal mucosal mast cells (IMMC) differ from peritoneal mast cells (PMC) in many characteristics. We employed two-dimensional gel electrophoresis (2-D gel) to characterize proteins of these mast cell subtypes, and developed procedures to study protein synthesis. Marked differences were identified in the protein compositions of IMMC and PMC. Most apparent were differences in the constituents of the cytoplasmic granules especially among proteins with a basic pI. Certain basic proteins in both mast cell subtypes bound the serine protease inhibitor ^{3}H-diisopropyl fluorophosphate (DFP), and in immunoblotting experiments with specific antibodies to rat mast cell protease (RMCP) I and II, RMCP I in PMC was identified as a single entity of 26 kDa, pI >9.0, whereas RMCP II in IMMC appeared as four isoforms of 24 kDa, 8< pI <9. In vitro translation of mRNA from PMC and IMMC uncovered that the two mast cell subtypes differ markedly in the spectrum of polypeptides synthesized. These approaches will help uncover the molecular bases of mast cell pleomorphism.

Introduction

Heterogeneity of mast cells [1] has been recognized in rodents, nonhuman primates, ruminants and humans. In the rat two subtypes of mast cells can be isolated from their in vivo environments and purified [2]. Rat intestinal mucosal mast cells (IMMC) differ from peritoneal mast cells (PMC) in their distribution, structure, mediator content and responsiveness to certain secretagogues and antiallergic drugs [1]. Because no systematic analysis of the protein content or synthetic capabilities of these mast cell subtypes was available, we isolated and purified the two subtypes of mast cells from rats and employed 2-D gel to characterize cellular proteins, and developed procedures to study protein synthesis.

Materials and Methods

ANIMALS: Outbred Sprague Dawley rats were infected s.c. with 3,000 larvae of Nippostrongylus brasiliensis to induce hyperplasia of IMMC and facilitate their isolation and purification.

ISOLATION OF MAST CELLS: Peritoneal cells collected from rats were subjected to a two-step discontinuous gradient (30% and 80%) of Percoll to get PMC of 97-99% purity [2]. To isolate and purify IMMC, the small intestine without Peyer's patches and mesentery was cut into small pieces and incubated with 1.3 x 10^{-4} M EDTA containing buffer to remove mucus and epithelial cells. The tissues were then incubated with 25 U/ml of collagenase. Resultant lamina propria cells were collected, passed through a nylon wool column and semi-purified with 30% and then 80% Percoll. Enriched mast cells were further purified by unit gravity velocity sedimentation. Final purity of IMMC was 86-93%.

TWO-DIMENSIONAL GEL ELECTROPHORESIS: Purified PMC and IMMC were washed three times with protein-free buffer and suspended in IEF-sample buffer (0.5% L-α-phosphatidyl choline, 9.6 urea, 2% ampholytes, 5% 2-ME, pH 2.8), which is suitable for solubilizing basic proteins [5], with protease inhibitors, 2mM PMSF and 100 kallikrein inhibitor U/ml Aprotinin. The cell suspension was allowed to stand at room temperature for 1h and then centrifuged at 140,000 g for 1h at 20°C. The aqueous supernatant separated from a lipid layer was used for IEF.

2-D gel was performed according to the method of O'Farrell [3] and O'Farrell et al. [4]. Non equilibrium pH gradient electrophoresis (NEPHGE) or isoelectric focusing (IEF) in a tube gel was used in the first dimension and SDS-PAGE with 10% polyacrylamide slab gel in the second dimension. Gels were usually stained with silver. Radiolabelled proteins in some gels were detected by fluorography.

mRNA ISOLATION AND IN VITRO TRANSLATION: Mast cell RNA isolated using the methods of Chomczynski and Sacchi [6] was used for in vitro translation using both rabbit reticulocyte lysate and wheat germ systems. Polypeptides translated from 2.4 μg of total RNA in the presence of ^{35}S methionine were characterized by 2-D electrophoresis and fluorography.

Results

Whole cell proteins of PMC and IMMC were separated by 2-D gel using NEPHGE in the first dimension (Fig. 1). PMC had seven dominant basic proteins (PB2-PB8) of 26 to 37 kDa (Table 1) and, although NEPHGE cannot establish the exact pI, using pH readings we estimated the pI to be approximately 9 to 9.5. For PMC we detected at least 80 to 90 neutral or acidic proteins, most of which were 20 to 100 kDa and pI 6 to 7.5. The 45 kDa protein (NA8) is the most prominent among the

proteins (IB1-IB9; Table 1), whereas the pattern of proteins in the neutral-acidic range was similar to that of PMC. The four most prominent basic proteins in IMMC (IB6-IB9) had the same molecular mass of 24 kDa and approximate pI range of 8.5 to 9.

Cytoplasmic granules isolated from mast cells [7] were submitted to 2-D gel and showed that most of the basic proteins (PB2-PB8) were granule-associated. This was true also in IMMC. Therefore, the most prominent difference between PMC and IMMC are in the basic proteins associated with granules.

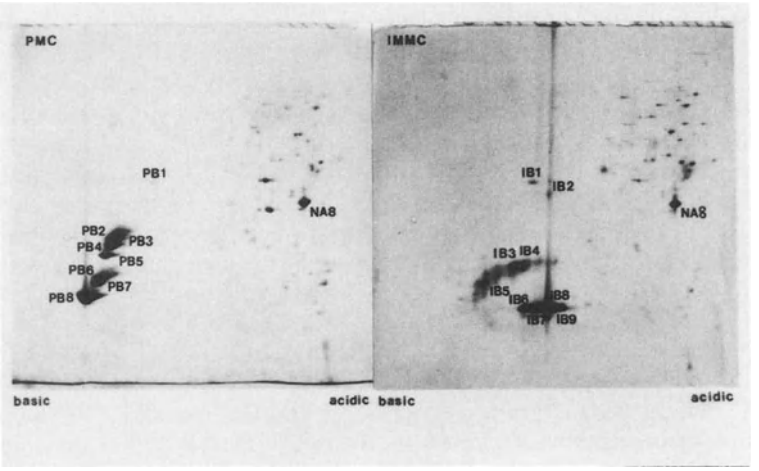

Figure 1. Spectrum of proteins visualized by 2D gel analyses of peritoneal (PMC) and intestinal mucosal mast cells (IMMC).

TABLE 1. Molecular mass of mast cell basic proteins

PMC	kDa	IMMC	kDa
PB1	52	IB1	52
PB2	37	IB2	46
PB3	36	IB3	33
PB4	35	IB4	33
PB5	33	IB5	29
PB6	29	IB6	24
PB7	28	IB7	24
PB8	26	IB8	24
		IB9	24

Immunoblotting using specific antibodies against RMCP I or RMCP II showed that in PMC only PB8 (26 kDa) was positive for anti-RMCP I antibody, whereas IB6, IB7, IB8 and IB9 (24 kDa), and IB2 (46 kDa) were reactive with anti-RMCP II antibody in IMMC.

We then investigated the binding of specific serine protease inhibitor, ^{3}H-DFP, to the mast cell proteins. Three major spots of 29 kDa, 28 kDa and 26 kDa corresponding to PB6, PB7 and PB8, respectively, were positively detected by fluorography in PMC, suggesting that in addition to RMCP I (PB8), PB6 and PB7 are also serine proteases. By contrast, IMMC had four ^{3}H-DFP binding proteins of 24 kDa corresponding to IB6, IB7, IB8 and IB9, and another ^{3}H-DFP binding protein of 46 kDa corresponding to IB2, all of which were reactive with antibodies to RMCP II.

The similarity of neutral-acidic proteins in the two mast cell subtypes was further shown by 2-D gel with equilibrated IEF using a mixed Bio Lyte 3/10 and 5/7 (1:4) in the first dimension (data not shown).

Polypeptides translated in vitro from mRNA of PMC and IMMC exhibited a broad range of pI and Mr. Abundant basic polypeptides appeared similar to those in whole cells, but specific identities remain to be confirmed. PMC and IMMC patterns of translated polypeptides could be easily distinguished.

Discussion

Our 2-D gels demonstrate that marked differences exist between PMC and IMMC in their granule-associated basic proteins. We established that the prominent basic protein in PMC, PB8 (26 kDa) is RMCP I. PB6 (29 kDa) and PB7 (28 kDa) are ^{3}H-DFP-binding serine proteases like RMCP I (PB8). PB6 and PB7 may be subunits of tryptase, another serine protease in mast cells, reportedly having two subunits of 33 kDa and 34 kDa in humans [8] and of about 35 kDa in dogs [9]. The other major basic proteins in PMC granules (PB2-PB5) of 37 to 33 kDa did not bind ^{3}H-DFP. At least one of these proteins may be carboxypeptidase, which is not a serine protease, but associated with granules and reportedly is of 35 kDa [10]. The most dominant neutral-acidic protein is 45 kDa and pI 6-6.5 (NA8) and may be actin [11].

For IMMC five spots were detected with anti-RMCP II. The four major basic proteins (IB6-IB9) of 24 kDa are likely different pI isoforms of RMCP II. RMCP II reportedly consists of a single polypeptide chain of approximately 25 kDa and is somewhat basic at neutral pH, but not as basic as RMCP I based on the amino acid composition [9]. Another minor spot reacting with anti-RMCP II, IB2 (46 kDa), may be a contaminating protein identified with the polyclonal antibodies. IMMC may have no tryptase-like enzyme in adequate abundance for detection in our assays.

In vitro translation of mRNA from PMC and IMMC confirmed and extended protein distinctions between these rat mast cell subtypes. The identification of translated polypeptides has not been established to date, but such studies will clearly facilitate understanding the molecular bases of mast cell pleomorphisms.

Acknowledgements

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Activation of mucosal mast cells by antigen or psychoneural factors. Effects on intestinal epithelial function

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1. Introduction

Mucosal mast cells (MMC) are a major component of the mucosal immune system. Significant numbers of MMC are present in the gut under normal circumstances and hyperplasia occurs in several clinical and experimental disease conditions. However, the role that these cells play in altering physiology in the gut has only recently begun to be appreciated.

Most of the current information regarding the effects of MMC on the epithelium has come from experimental studies of intestinal anaphylaxis. We have shown that in vivo antigen challenge in the lumen of the small intestine of sensitized rats results in the accumulation of water and electrolytes [1]. Isolated intestinal preparations from sensitized rats also respond to antigen with active ion secretion, and the movement of charge (short-circuit current, Isc) can be monitored continuously via a chart recorder [2]. Thus, this preparation can serve as a model system to examine physiological responses to MMC activation. Here we describe the use of pharmacological manipulations to examine the mechanisms by which such responses occur.

Morphological studies have demonstrated an intimate association between MMC and nerves in gut mucosa [3]. The importance of such interactions has been shown in the skin where injection of neuropeptides such as substance P (SP) causes a wheal and flare reaction which can be blocked by anti-histamines [4]. SP also is a potent stimulator of isolated MMC [5]. We now have functional evidence for interaction between MMC and SP nerves in the gut. Recently, the role of the central nervous system in the regulation of immune responses has become the focus for a number of investigations. In this study we provide evidence that MMC can be activated in the absence of antigen by psychoneural factors following Pavlovian conditioning and that physiological changes occur as a consequence.

2. Methods

2.1. ANIMALS

Male Sprague-Dawley rats weighing approximately 300g were sensitized to egg albumin (EA) using alum and pertussis adjuvants [6]. Rats were studied 12-15 days later. To examine the role of substance P-containing nerves, neonatal rats were treated with capsaicin (50 mg/kg); controls were untreated littermates. These rats were then sensitized at 3 months of age. In the conditioning studies, mast cell hyperplasia was induced by infecting sensitized rats with 3000 larvae of Nippostrongylus brasiliensis at day 14. Conditioning training was begun 3 weeks later, following worm expulsion.

2.2. CONDITIONING

Experimental rats were placed in an isolation chamber and exposed to an audiovisual cue (a flashing light and humming noise), and then removed and injected subcutaneously with 300 μg EA. An unpaired negative control group was exposed to both cue and antigen, but separated by 24 h so that no association developed. This training was repeated weekly for 3 weeks. In the fourth week, experimental rats were divided into 2 groups: positive control rats were challenged with cue and EA as before; paired rats received cue plus saline injection. Unpaired rats (negative controls) received cue plus saline. Blood was sampled and segments of jejunum removed for in vitro studies 60 min later. Elevation of rat mast cell protease II (RMCP II) in serum was used as an indication of activation of MMC.

2.3. USSING CHAMBER STUDIES

Proximal jejunum was stripped of external muscle (leaving the submucosal plexus intact), opened and mounted between two chamber halves as described [8]. The chamber included stimulating electrodes which were used for electrical transmural stimulation of nerves in the preparation. The spontaneous potential difference (PD) across the tissue was determined and the current required to eliminate the PD was introduced by an automatic voltage clamp. This short-circuit current (Isc) was recorded continuously. Using gut segments from sensitized rats, we had previously shown that the Isc increase in response to antigen was due to net chloride secretion and was inhibited by doxantrazole, a stabilizer of MMC [2].

3. Results

3.1. IN VITRO RESPONSE TO ANTIGEN

A significant increase in Isc occurred in intestine from sensitized but not control rats within 1-2 min after adding EA (100 μg/ml). The response involved an initial peak and a sustained elevation. This pattern of response appeared to be due to the mediators released since the initial

peak was inhibited by the 5-hydroxytryptamine ($5\text{-}HT_2$) antagonist, ketancerin (KET), and the sustained rise by the cyclooxygenase inhibitor, piroxicam (PIR). Both phases were inhibited by the H_1 antihistamine, diphenhydramine (DPH). Almost complete inhibition occurred in the presence of both PIR and DPH (Table 1).

3.2. ROLE OF MUCOSAL NERVES

The role of mucosal nerves was examined by pre-incubating tissues with the neurotoxin, tetrodotoxin (TTX), before adding antigen. This treatment completely abolished the response to electrical transmural stimulation. TTX inhibited both phases of the response to EA. Atropine was without effect, suggesting that non-cholinergic nerves were involved. Therefore, we used capsaicin (CAP) treatment to examine a possible role for substance P-containing nerves. CAP treatment resulted in > 50% inhibition of the early peak and sustained components of the response (Table 1).

Table 1. Effect of inhibitors on the Isc response to EA

Inhibitor	Inhibition of Isc Response (% of original)	
	Peak	Sustained
KET (10^{-5}M)	59.8*	14.0
PIR (10^{-5}M)	39.2	79.5**
DPH (10^{-5}M)	49.1*	32.4*
DPH+PIR (10^{-5}M)	82.2**	85.0**
TTX (5×10^{-6}M)	49.3*	23.5*
CAP (50 mg/kg)	58.5*	55.5*

* $p < 0.05$, ** $p < 0.001$ compared with original

3.3. EFFECT OF CONDITIONING ON MMC

Nematode infection of EA-sensitized rats resulted in ~10 fold increase in MMC. Thus, the system was set up to optimally demonstrate differences in serum concentrations of RMCP II. Baseline concentrations (from blood obtained 24h before challenge) were subtracted from concentrations in blood 60 min after challenge. Unpaired rats had a minimal rise in RMCP II after challenge. Positive control rats had a dramatic rise, significantly increased compared with unpaired rats (Table 2). Paired rats demonstrated an increase of similar magnitude as that in positive controls. Therefore, these findings indicate that MMC can be activated in the absence of antigen by psychoneural signals from the central nervous system.

3.4. EFFECT OF CONDITIONING ON GUT FUNCTION

Baseline Isc was used as an indication of the secretory tone of the intestinal tissues. In isolated jejunum from positive controls, Isc was consistently elevated compared with unpaired rats regardless of whether the studies were performed 30 or 60 min post challenge. In paired rats, no change was evident at 30 min but Isc was increased at 60 min (Table 2). In addition, the Isc exhibited cyclical oscillations which were eliminated

by TTX and when the tissue was bathed in chloride-free buffer. These results suggests that activation of mucosal nerves during the conditioning challenge contributed to intestinal secretion.

Finally, in vitro responses to EA were measured in these tissues. Tissues from unpaired rats had larger responses to EA than positive controls. Surprisingly, tissues from paired rats had supramaximal responses, significantly increased over those in unpaired rats.

TABLE 2. Effects of conditioning on mast cells and Isc

Group	RMCP II (μg/ml)	Basal Isc ($\mu A/cm^2$)	Δ Isc to EA ($\mu A/cm^2$)
Unpaired	2.1±0.4	69±3	37±6
Positive	7.9±1.1*	82±3*	19±4*
Paired	8.8±1.4*	89±3*	55±7*

* $p < 0.05$ compared with unpaired rats

4. Discussion

Our studies indicate that MMCs can be activated by immune or by nervous pathways following conditioning. Activation of MMC results in net ion secretion in the small intestine which appears to be due to a combination of histamine, 5HT and prostaglandin (or other cyclooxygenase products of arachidonate metabolism) acting on the epithelium. The effects of these mediators occur at least in part via mucosal nerves. Our findings also suggest that secretory responses to antigen can be augmented by psychological factors.

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Distribution and characterisation of oral mucosal mast cells

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1. Introduction

Mast cells (MC) are normal components of healthy tissues (1, 2). So far, two distinct subsets have been characterized mainly in the rat: mucosal MC, identified in the intestinal mucosa and in MC cultures from the bone marrow, and connective tissue MC, present in the skin and in the peritoneum (2). These two subpopulations differ not only in their location but also in their sensitivity to formalin fixatives (2), their histochemical characteristics (3) and their functional properties (4). In the present study, we investigated the histochemical and functional characteristics of murine oral MC and we compared them with those of the skin, peritoneum and intestine. Moreover, we studied the distribution of MC in all of these tissues.

2. Material and methods

CD-1 male mice, 17 to 20 weeks of age, were killed by exsanguination. The oral mucosa (cheeks and soft palate), the tongue, a piece of small intestine (duodenum) and shaved skin from the back of the animal were excised and fixed. Two samples of each tissue were fixed in periodate-lysine-paraformaldehyde (PLP) (5, 6) at 4°C for 3 to 4 hours or in absolute ethanol (7) at 4°C for 20 hours. Two groups of eight mice were submitted to peritoneal washing and the cells recovered from each group were pelletted and fixed in either one or the other fixative, and then embedded in paraffin.

Between 50 to 100 serial paraffin sections, 5 µm thick, were cut from each block. All of these sections were stained with either alcian blue/safranin (3,8), astra blue/safranin (3), azure A (3), berberine sulfate (9), toluidine blue 0.5% and 0.005% (3), Giemsa (Sigma, St-Louis, MI, U.S.A.) or avidin -FITC(10).

To visualize the effect of compound 48/80, mice were injected intradermally (i.d.) with 0.5ml of sterile compound 48/80 (0.18 mg/ml in PBS) or with sterile PBS as a control. Two mice received compound 48/80 on one side of the back (shaved) and PBS on the other side. Two other mice received compound 48/80 in one cheek and PBS in the other cheek. After i.d. injections, each mouse received 0.1ml of sterile Evans blue (0.5% in PBS) intraveneously. Vasodilation was visualized by the rapid formation of blue spots either on the skin or on the cheek.

3. Results

FITC-labelled avidin, astra blue, azure A, alcian blue, Giemsa and toluidine blue 0.5% stain all MC populations whereas safranin and berberine sulfate do not. Only 0.005% toluidine blue showed a differential staining pattern, reacting positively with peritoneal, cutaneous and oral MC and negatively with MC of the intestinal mucosa.

MC are most abundant in the skin, peritoneum and certain areas of the oral mucosa, namely the soft palate and the cheek-labial junction. However, in the intestinal mucosa, only occasional MC are seen. As for MC in the tongue and the rest of the cheeks, their density is somewhat intermediate between that of cutaneous and intestinal MC. In all of the tissues studied, MC are observed surrounding blood vessels, nerve endings and glands, whether salivary or intestinal. Only in the tongue are there a few MC present in the interacinar connective tissue. MC are never found in the epithelium. They are mainly present in the connective tissue but are also found in between muscle fibres.

4. Discussion

Oral MC appear to constitute a homogenous population with the histochemical and functional characteristics of cutaneous and peritoneal MC. They stain positively with astra blue, alcian blue, FITC-labelled avidin, azure A, Giemsa and 0.5% and 0.005% toluidine blue whereas none stain with either safranin or berberine sulfate. In addition, *in vivo*, these MC populations release vasoactive mediators in response to compound 48/80. Intestinal MC differ from cutaneous, peritoneal and oral MC in the absence of reactivity to compound 48/80 (4) and of staining with 0.005% toluidine blue. From present observations, two subpopulations of murine MC can thus be distinguished: a first one analoguous to rat connective tissue MC (8) and a second analoguous to rat mucosal MC (8).

In this study, skin, intestinal and oral tissues MC were found mainly in the connective tissue, but also in between muscle fibres. They are most abundant around blood vessels, nerve endings and ducts.

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Catabolism of IgE by rat mucosal and connective tissue mast cell chymases

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ABSTRACT. Because IgE is internalized and detected immunohistochemically within mucosal mast cells of parasitized rats, the ability of the soluble mucosal mast cell chymase rat mast cell protease II (RMCPII) to degrade IgE was analysed and compared with that of the connective tissue mast cell chymase RMCPI. Both enzymes degraded IgE, producing distinctive peptides, but catabolism by RMCPII was much more rapid.

1. INTRODUCTION

During infection with either the intestinal nematode Nippostrongylus brasiliensis or the liver cestode Taenia taeniaformis mucosal mast cells (MMC) of the rat lung and intestinal lamina propria contain intracellular IgE (Mayrhofer, Gowans and Bazin, 1976) Mayrhofer et al (1976) noted that IgE was restricted to the surface of CTMC which, like a number of basophil cell lines, may either not internalise membrane-bound IgE (Isersky et al, 1979), or, alternatively, CTMC may internalise IgE and, if degraded rapidly, it might be undetectable.

Despite the 75% amino acid sequence homology between the relatively insoluble connective tissue mast cell chymase, rat mast cell protease I (RMCPI) and the highly soluble mucosal mast cell chymase RMCPII they express distinct chymotryptic activities (Woodbury et. al. 1989). However, the in vivo functions of these 2 chymases are still poorly defined and further comparative studies of their activities against native substrates are required. Because of the differential distribution of IgE in mast cell subsets, the capacities of RMCP I and II, to cleave purified rat IgE were assessed.

2. MATERIALS AND METHODS

Serum collected from rats 1 week after secondary infection with N. brasiliensis was used as a source of IgE. which was isolated by affinity purification using cross absorbed, monospecific goat anti IgE coupled to sepharose 4B.

RMCPI and II were purified according to the method of Gibson and Miller (1986). Specific enzyme activities were quantified spectrophotometrically by cleavage of a commercial substrate, CBZ-tyrosine-NPE. Values were calculated in nanokatals/milligram of protein. Protein concentrations were calculated using the Pierce protein assay.

Substrates and enzymes were diluted to the appropriate protein concentration in PBS, 1M NaCl and 0.01% sodium azide, and mixed in the ratio of 1 nanokatal of enzyme to 3.4ugs of substrate protein. 74 nanokatals of RMCPI (132ugs) and II (25ugs) were each added to 252ugs IgE and incubated at 37^{o}C. After incubation for 0.5, 5, 10, 30, 60 minutes or for 24 hours, 2 aliquots of 15ugs and 5ugs of immunoglobulin were removed and diluted in reducing and non-reducing sample buffers respectively. An additional 27ug sample of IgE was removed after 0.5 and 60 minutes and after 24 hours. The samples were diluted 1:1, in normal rat serum to inhibit chymotryptic activity.

For control purposes IgE and IgG were treated in a similar manner, in the absence of chymases. Times of sampling were 0.5 and 60 minutes, and 24 hours, and subsequent treatment was as described above. Results were assessed by SDS-PAGE and Western blotting using ^{125}-labelled mouse monoclonal anti rat IgE.

2.2 Biological activity of IgE by PCA

Control and enzyme treated IgE preparations were tested for biological activity by PCA, using N. brasiliensis adult worm homogenate as antigen.

3. RESULTS

3.1 Purity of IgE

By SDS-PAGE purified IgE, consisted of a major 66,000 MW band and minor bands bands of 74,000, 46,000 and 23,000 MW. The 66,000 MW band together with a 46,000 and 38,000 MW bands were detected on Western blots using iodinated murine monoclonal anti-IgE. The weak band of 74,000 MW. was not identified on Western blot by anti-rat-IgE nor was the 23,000 MW band which is presumably light chain.

3.2 Digestion of IgE by RMCPI

Partial degradation of the Epsilon chain was observed within 30 seconds of addition of RMCP I, and seventeen bands with molecular weights between 64,000, to 18,000. were demonstrable by SDS-PAGE. After 24 hours, only the epsilon chain and four additional products (47,000, 43,000 28,000, 26,000 and 20,000 MW) were still detectable. Control samples incubated in the absence of RMCPI remained intact. throughout the test.

By Western blotting the epsilon chain was not detectable in any of the enzyme treated preparations. However, four antigens of 43,000, 36,000, 21,000 and 19,000 MW were demonstrable with monoclonal

anti-rat IgE after 30 seconds. A sequential loss of the 43,000 and 21,000 MW bands occurred after 60 minutes and 24 hours respectively and antigens of 36,000 and 19,000 MW were still detectable at 24 hours.

Two antigens with molecular weights of 42,000 and 36,000 were observed, together with the intact Epsilon heavy chain in Western blots from control preparations and remained unaltered over the 24 hour period of inoculation.

3.3 Digestion of IgE by RMCP II

Addition of RMCPII to purified IgE resulted in almost instantaneous cleavage of the epsilon heavy chain into 14 bands with MWs ranging from 65,000, to 13,000. A major band of 21,000 MW was particularly abundant and was still present after 24 hours incubation. In contrast, the other degradation products were barely detectable after 24 hours and intact Epsilon chain was no longer demonstrable.

By Western blotting, the degradation of the 66,000 MW epsilon heavy chain was virtually complete after 30 seconds and at least 4 other antigens with molecular weights between 43,000 and 36,000 were identified as were peptides of 30,000 and 19,000 MW. After 24 hours, only the 43,000 and 19,000 MW antigens were still present.

It is likely that the 21,000 MW product on SDS-PAGE and the 19,000 MW antigen detected in the blot are the same. Small differences in the mobility of the markers or of the digestion products may account for the small difference in molecular weight.

3.4 Functional Assessment of IgE Degradation Products by PCA

The functional activity *in vivo* of affinity purified IgE was assessed by PCA. Treatment of IgE with RMCPI or II caused immediate reductions in PCA reactivity. After digestion of IgE for 60 minutes PCA reactions were weak or undetectable. Control incubation of IgE in the absence of chymases was without effect.

4. DISCUSSION

Both CTMC and MMC bear high affinity receptors (Fc Rl) for IgE but the role of mast cells in the catabolism of this immunoglobulin has not been determined. It is possible that IgE-antigen complexes are either stored within or catabolized by the secretory granules. To test this theory the abilities of RMCP I and II to cleave rat Epsilon heavy chains were compared.

Both RMCP I and II were able to cleave rat IgE but, surprisingly, in view of the fact that intracellular IgE is detected in MMC (Mayrhofer et al 1976), RMCPII cleaved IgE heavy chain more rapidly than RMCPI. Dramatic depletion of Epsilon heavy chain occurred within 30 seconds of the initiation of the reaction. There were fourteen products of RMCPII digestion, one of which, with a molecular weight of 19,000 was abundant early in the reaction and was

still demonstrable though in reduced quantities, after 24 hours. This peptide was also demonstrable by Western blotting.

Partial proteolysis of IgE heavy chain by RMCPI occurred within 60 minutes producing seventeen epsilon chain products some of which were recognized on Western blot. The continued presence of the intact epsilon chain, though at reduced concentrations, may reflect a resistance to degradation in some IgE heavy chain polypeptides.

This study emphasises the differences in substrate specifity of RMCPI and II (Woodbury, et.al. 1989) but does not provide an answer for the observed differences in IgE staining between mucosal and connective tissue mast cells. Further studies are needed to clarify the role of the rat mast cell proteases in IgE degradation.

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Ultrastructural organization of the murine oral mucosa in health and candidosis

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1. Introduction

The oral mucosa is continuously exposed to microbial challenge including, in humans, opportunistic pathogens like *Candida albicans*. Candidal colonization may result in the development of a saprophytic association with the host or may cause the appearance of superficial localized oral infections [1]. In order to cause an infection, *C. albicans* must overcome both bacterial antagonism and natural immunity at the mucosal surface. T-cell and macrophage-mediated immunity are of central importance in host-defense mechanisms against a number of mycoses, including candidosis [2]. The acute inflammatory reaction is also a major factor in limitating the spread of infection by opportunistic fungi [3]. In the mouse, the keratinized mucosal surface acts as an important physical barrier to most microbial invasion. A diffuse immune system with two major compartments (the oral epithelium and underlying connective tissue, and the minor salivary gland network) is responsible for immune surveillance in the deeper layers [4]. In order to investigate the general features of host-parasite interactions in the development of oral mucosal infections, we compared the ultrastructural organization of the oral mucosa in the healthy mice and in mice with a candidal infection, with special emphasis on the oral epithelium and underlying lamina propria.

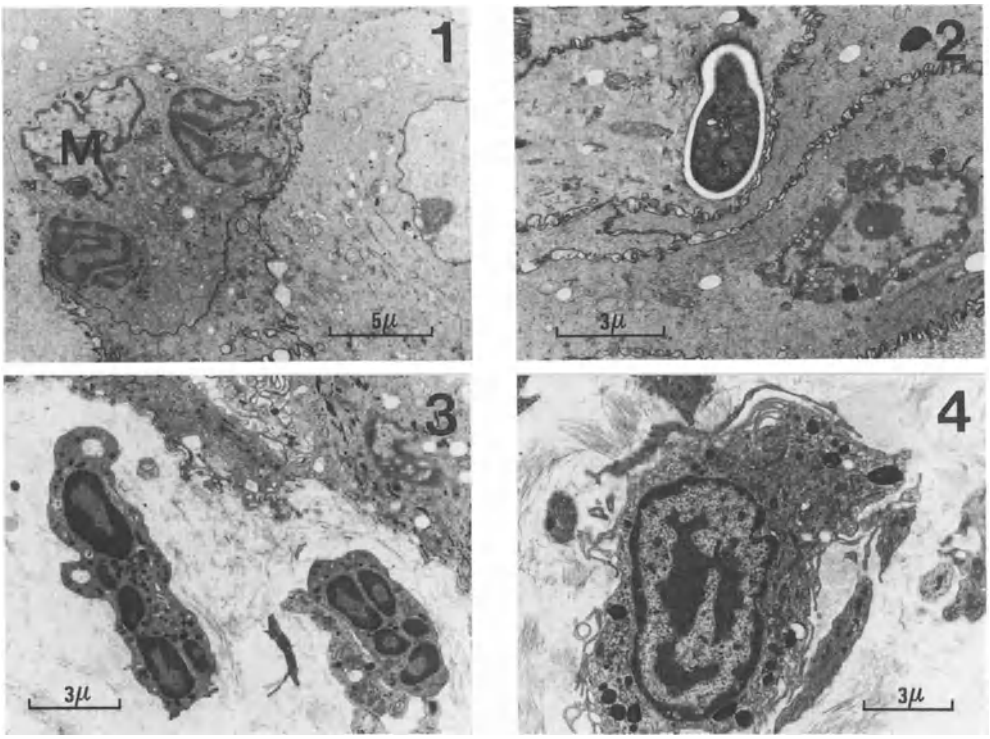
2. Materials and methods

Candidal infections were induced in normal CD-1 mice by topical application of 10^8 *C. albicans* blastospores. Normal and infected oral tissues were either excised, fixed and embedded in paraffin for light microscopy, or fixed by vascular perfusion, excised and processed for electron microscopy.

3. Results and discussion

On the basis of immunocyte frequency and distribution, the oral epithelium represents the first strategic line of defense. In the healthy mucosa, Langerhans cells and interepithelial lymphocytes were regularly associated with the basal epithelium, whereas the lamina propria normally displayed few immunocytes with a predominance of mast cells over lymphocytes and macrophages in glandular areas and in well vascularized and innervated regions. Shortly after infection, *C. albicans* was seen in association with indigenous bacteria on the epithelial surface and the number of immunocytes dramatically increased both in the oral epithelium and the lamina propria. Distinct profiles of the inflammatory reaction could be related to the evolutionary stages of infectious foci: sites of mycelial proliferation resulted in a massive recruitment of PMNs in the superficial epithelial layers, while mononuclear cells predominated when few mycelial elements were present. Ultrastructurally, neutrophils forming small microabscesses (sometimes including macrophages).

(Fig. 1: M=macrophage) made up an important portion of epithelial inflammatory lesions when candidal hyphae were observed penetrating superficial layers. *C. albicans* elements were seen in an intracellular location (Fig. 2), some of which were in a degenerated state, although no direct contact with inflammatory cells was seen. Signs of intercellular epithelial edema were observed in the stratum spinosum. Inflammatory infiltrates in the lamina propria comprised neutrophils (Fig. 3), macrophages (Fig. 4), eosinophils and lymphocytes coming from blood vessels by diapedesis. Mast cells were also seen in association with groups of neutrophils and eosinophils in the sub-epithelial connective tissue. In conclusion, present data suggest that, following invasion of the superficial epithelial cells to establish an intracellular habitat, *C. albicans* induces an inflammatory response in the underlying tissues. This would likely be a critical event for *C. albicans* -induced immune reactions to occur, especially by alteration of lymphocyte and macrophage trafficking. This experimental model of oral candidosis in the mouse should provide new insights into the development and stimulation of oral mucosal immunity as well as local interactions of opportunistic pathogens with host defense mechanisms.



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Early events in irradiation-mediated depletion of rat gut mucosal mast cells

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INTRODUCTION

Mast cell heterogeneity is now well recognised in the rat (1,2). Mast cells contain potent mediators of the inflammatory response which work either by direct enzymatic action on the surrounding tissues or by recruiting other effector cells. Sudden release of these mediators, as occurs in intestinal anaphylaxis leads to structural and functional damage to the gut and subsequent depletion of mast cells (3). Radiation causes inflammatory gut damage which is in some respects similar to that following mast cell degranulation, e.g. increased vascular permeability and tissue eosinophilia. Whereas connective tissue mast cells (CTMC) appear to be radio-resistant; marked radiosensitivity of rat gut mucosal mast cells (MMC) has been demonstrated 7 days after 4.5 Gy whole body irradiation during experiments in Graft-versus-host reaction (4). Our hypothesis was that radiation stimulated degranulation of MMC and the released mediators contributed to post-irradiation gut damage.

The aim of the present experiments was to characterise the radiation sensitivity of rat gut MMC.

METHODS

A series of experiments has been carried out using male Wistar rats (250-400 g). Tissues for MMC counts were fixed in Carnoy's solution and 5 um sections stained with toluidine blue. Light microscopy with a fixed field technique using a calibrated square graticule was used for cell counts; results were expressed as cells per mm^2. Samples of gut for quantitation of tissue rat mast cell protease type II (RMCPII) were homogenised and analysed using a radial immuno-diffusion technique. Serum RMCPII was measured by an ELISA technique. Single dose whole body irradiation was performed on anaesthetised animals using a fixed head source which delivered a total dose of 4.5 Gy.

An expanded MMC population was produced by giving s.c. injections of 4000 L3 Nippostrongylus brasiliensis larvae 5 weeks and 1 week before definitive experiments.

Anaphylaxis was induced in previously Nippo infected rats by an

i.v. injection of 500 w.e. of worm antigen.

EXPERIMENTS AND RESULTS

1. The timing of gut MMC depletion after irradiation

Naive animals were anaesthetised and irradiated with 4.5 Gy. Pairs were killed with ether at intervals from 1 hour to 1 week after irradiation. Tissue samples for histology and assay of RMCPII were taken from jejunum, ileum, colon and rectum. Blood samples were separated by centrifugation and serum stored at -20°C for measurement of serum RMCPII.

MMC counts, amount of tissue RMCPII in jejunum, and serum RMCPII levels are shown in table 1. These show a depletion of MMC starting 8 hours after irradiation with virtual disappearance of MMC at 7 days. The fall in tissue and serum RMCPII levels parallel that in MMC counts. Amounts of tissue RMCPII and MMC counts of the areas of gut sampled behaved in a similar way.

A similar definitive experiment confirmed these findings. Serum RMCPII was used as an index of the MMC population and tissue measurements were made on sacrifice of the animals. Groups of 6 naive and nippo primed animals were irradiated or sham-irradiated. Blood was taken on days 0, 2, 4, 6, and 7. In naive animals the mean serum RMCPII fell from 438 (+/- SEM 30) ng/ml on day 0 to 31.5 (+/- 3.35) on day 2 and this low level was sustained until day 7. However there was no similar change in serum RMCPII in control animals, 472.5 (+/- 37.1) ng/ml on day 0 and 361.3 (+/- 27.4) ng/ml on day 7. The MMC counts and levels of tissue RMCPII (see table 2) support the serum findings on day 7. A similar pattern was found in those with an expanded MMC population. The basal level of serum RMCPII was higher 2429 (+/- 218) ng/ml and 243.8 (+/- 20.8) ng/ml on day 4. These low levels persisted up to day 7. Whereas no change occurred in the primed control animals - 1594 (+/- 169) ng/ml at the start of the experiment and 1437 (+/- 490) ng/ml on day 7. The MMC counts and tissue RMCPII measurements once again reflect the serum findings (table 2).

2. Does irradiation cause sudden systemic release of RMCPII?

Groups of 6 naive and nippo primed animals were exposed to irradiation or challenge with i.v. worm antigen. Blood for serum RMCPII estimation was taken at 0. 10, 30, 60 and 90 minute intervals. The results show that whereas anaphylaxis leads to a 10-fold increase in serum RMCPII, resting levels 1672 (+/- 191) ng/ml to 19,038 (+/- 3168) ng/ml by 30 min after worm challenge, there is no corresponding increase in the primed or naive animals even up to 90 min after irradiation.

DISCUSSION

The functional and structural changes in the gut following intestinal anaphylaxis have in part been attributed to the sudden release of RMCPII, which is known to attack basement membrane collagen (5). Mast cells contain many other chemical mediators of inflammation which may also contribute to the damage. We have shown that there is no sudden systemic release of RMCPII analagous to anaphylaxis following

irradiation even in animals with an expanded MMC population. It seems unlikely, therefore, that MMC products cause the mucosal damage and functional disturbances in intestine damaged by irradiation.

The process leading to disappearance of the MMC in our experiments following irradiation is not clear. We assume that they die rather than migrate. Two distinct forms of death of nucleated eukaryotic cells are recognised by pathologists - necrosis and apoptosis (6). We speculate that the MMC undergo apoptosis - involving chromatin cleavage, nucleolar and cytoplasmic fragmentation to form apoptotic bodies. Macrophages and epithelial cells quickly recognise these cell fragments, phagocytose and digest them. In contrast to necrosis, the lysosymal enzymes of the cell dying by apoptosis are not activated. This mode of cell death has been observed in many cell types, including thymocytes after treatment with glucocorticoids (7) and irradiation (8).

Though our hypothesis has been disproved, our finding of the loss of MMC, an important effector cell in the mucosal immune system may have relevance, via a local immunodeficiency, to the pathogenesis of the acute and chronic radiation injuries seen in the gut.

ACKNOWLEDGEMENTS

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TABLE 1. Cell counts and levels of RMCPII in jejunum and serum after 4.5 Gy irradiation

Tissue after irradiation (hours)	Jejunum		Serum
	MMC ^{2} (no/mm^2)	Tissue RMCPII (μg/g)	RMCPII (ng/ml)
Control	138	91	760
1	147	105	862
4	128	114	757
8	90	74	651
24	19	10	115
72	8	4	49
168	2	<3	17

TABLE 2. Cell counts and levels of RMCPII in jejunum 7 days after 4.5 Gy irradiation

Group	Tissue RMCPII (μg/g)		MMC ^{2} (per mm^2)	
Naive control	134	(6)	212	(4)
Naive + irradiation	Undetectable		1.7	(1)*
Primed control	1587	(126)	837	(25)
Primed + irradiation	116	(7)*	153	(8)*

Numbers in ( ) = standard error of mean *$p<0.01$

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Role of mucosal mast cells in T lymphocyte mediated enteropathy

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Introduction

Activated mucosal T lymphocytes are believed to cause the enteropathy found in disorders like coeliac disease and several parasite infections. Nevertheless, it is not known whether the local T cells act directly on the gut or if their role is to recruit other non-specific inflammatory cells such as mucosal mast cells whose numbers are also increased (1).

Intestinal graft-versus-host reactions present a useful experimental model of clinical enteropathies due to T lymphocytes. Intestinal GvHR not only reproduces most of the pathological features, such as villus atrophy, crypt hyperplasia and increased numbers of mucosal T cells, but is also associated with recruitment of MMC (2,3). In this study, we have used two models of intestinal GvHR in mice to examine whether MMC are required for the development of T cell mediated enteropathy.

Materials and Methods

Graft-versus-Host Reactions: Adult $(CBAxBALB/c)F_1$ mice were injected with $6x10^7$ CBA spleen cells intraperitoneally, while BDF_1 W/W^v mice and their +/+ littermates were given 10^8 C57Bl/6 parental spleen cells intravenously. The progress of systemic GvHR was assessed by measurements of body weight and splenomegaly.

Measurement of Mouse Intestinal Mucosal Mast Cell Protease: MIMCP was measured in samples of serum and homogenised segments of jejunum using the ELISA described in detail elsewhere (4). This assay is specific for MIMCP and shows little or no cross-reaction with the related protease derived from connective tissue mast cells.

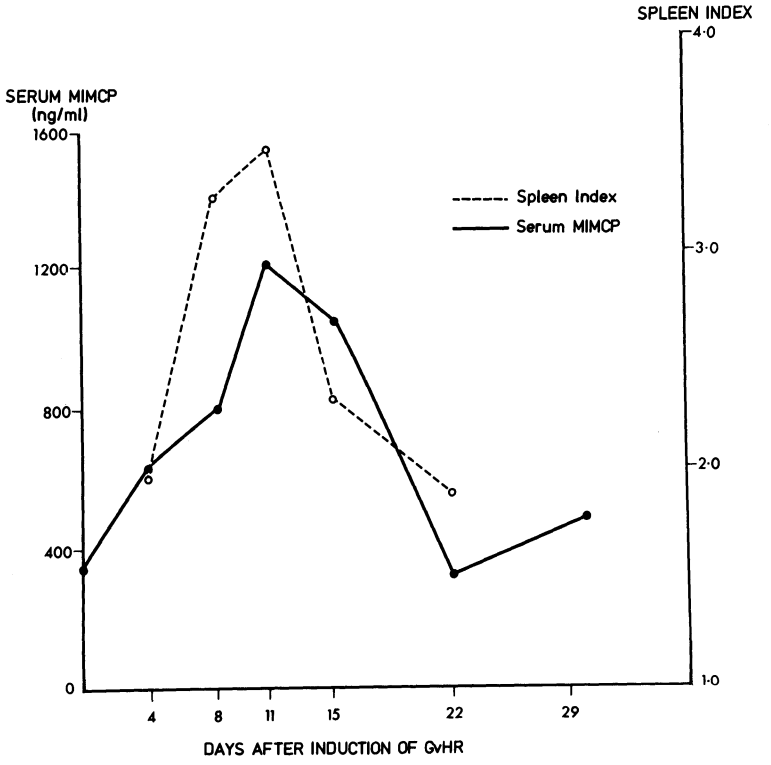
Measurement of Intestinal Pathology: Villus and crypt lengths and crypt cell production rates (CCPR) were measured in pieces of jejunum taken from mice given colchicine, using microdissection and metaphase arrest analysis as described previously (2). MMC were counted on adjacent segments of jejunum fixed in Carnoy's solution and stained

with Astra Blue pH 0.3.

Statistics: Villus and crypt lengths are expressed as means + standard deviations and were compared by Student's t test, while CCPR were compared by covariance analysis. MIMCP levels are expressed as geometric means and were compared by Wilcoxon's Rank Sum test.

Results

Mucosal Mast Cell Numbers in Intestinal GvHR: Initial studies indicated that the numbers of MMC rose sharply during the mild GvHR which occurs in 1 week old (CBAxBALB/c)F_1 mice given parental spleen cells (2). This was confirmed in adult hosts of the same strain, indicating that MMC hyperplasia was not merely a feature of the immature intestine. This model of GvHR only causes crypt hyperplasia in the intestine, but further work showed that MMC also increased during the more severe GvHR in BDF_1 hosts, which produces villus atrophy as well as crypt hyperplasia. In general, the numbers of MMC correlated with other aspects of intestinal pathology and systemic disease, but this was not invariably the case. In view of the possible errors of enumerating MMC which might arise due to e.g. degranulation, we thought it important to make a functional assessment of MMC in GvHR.



Summary and Conclusions

Our experiments confirm and extend previous work by showing that the numbers and activity of MMC are increased in experimental animals with GvHR. Earlier work had shown increased numbers of MMC in neonatal mice with GvHR (2), but it has only become possible recently to measure MMC-specific products in the mouse. Using this technique, we now show that this hyperplasia is associated with functional activation, as measured by local and systemic release of the MMC-specific protease, MIMCP. This occurred in two separate models of intestinal GvHR, one associated with mild, proliferative changes and the other which causes intense crypt hyperplasia and villus atrophy. Furthermore, MIMCP levels correlated closely with the progress of systemic GvHR and with the evolution of intestinal pathology. These findings are supported by other work which reported a similar increase in MMC and the MMC-specific protease, RMCPII, during a GvHR in rats (5). Therefore, our results support the idea that MIMCP is the murine equivalent of RMCPII and confirm that activation of mucosal T lymphocytes is a potent signal for the recruitment and activation of MMC in both species. Furthermore, it seems probable that this mechanism also accounts for the MMC hyperplasia found in other enteropathies with a pathology similar to that of GvHR, including parasite infections and coeliac disease (1).

In view of this parallel, we thought it important to determine the role of MMC in the intestinal pathology of GvHR. Although the close association between the activity of MMC and the development of disease suggested that MMC were important effector cells in enteropathy, this was not supported by our work using W/W^{V} mice. These animals had no MMC and showed virtually no MIMCP response to GvHR. Nevertheless, they developed more severe systemic and intestinal GvHR than normal littermates. Together with the fact that irradiation increases the severity of intestinal GvHR in rats, but depletes MMC (5), our results suggest that MMC may play a repair-role in T cell-mediated enteropathy.

Legends to Figures

Figure 1: Parallel rise in splenomegaly and serum MIMCP levels during GvHR in (CBAxBALB/c)F_1 mice.

Figure 2: Intestinal pathology during GvHR in W/W^{V} and normal +/+ BDF_1 mice (Arrows represent CCPR)

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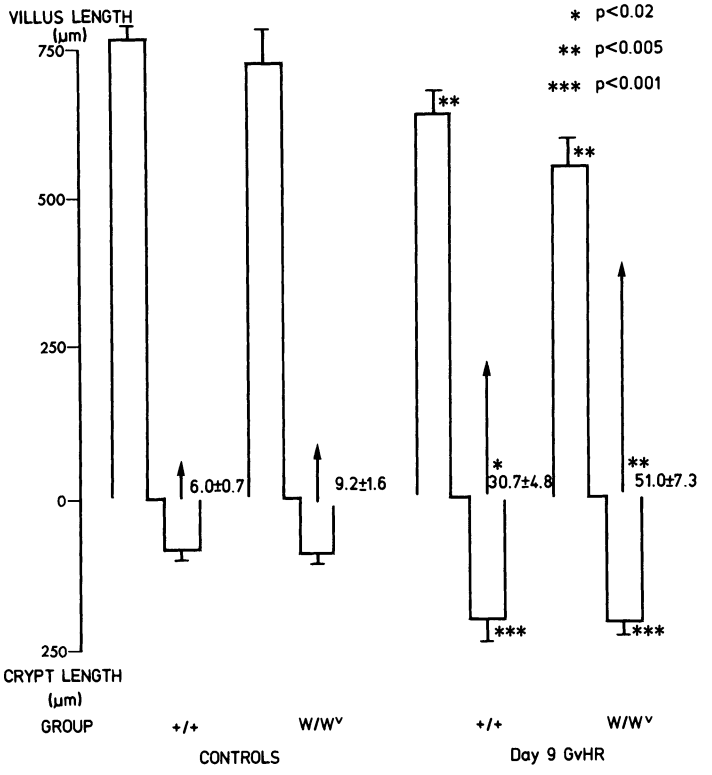
MIMCP Levels in Mice with GvHR: Evidence of MMC protease release was sought by analysing MIMCP activity in serum and jejunal homogenates by a sensitive and specific ELISA. MIMCP activity rose rapidly in the serum of adult (CBAxBALB/c)F_1 mice with GvHR and its level correlated exactly with the degree of splenomegaly (Fig.1) and with other aspects of enteropathy, such as crypt hyperplasia. Jejunal MIMCP activity also rose with the same pattern and both returned to normal as the GvHR waned. Serum and jejunal MIMCP activity was also increased during the severe GvHR in BDF_1 +/+ hosts and again correlated with the degree of intestinal damage, with the highest levels being found when villus atrophy was most severe.

Progress of Intestinal GvHR in MMC-Deficient Hosts: These results confirmed that an increase in the number and activity of MMC is a characteristic feature of GvHR. Therefore, our final experiments examined whether an absence of MMC would alter the development of enteropathy in GvHR. To do this, a GvHR was induced in mast cell-deficient W/W^v mice. In comparison with normal congenic BDF_1 hosts, W/W^v mice had virtually no MIMCP activity in serum or jejunum, either normally or during GvHR. They also developed more severe weight loss in GvHR and some died during the experiment, while no BDF_1 +/+ hosts died of GvHR. Finally, W/W^v mice had much more severe enteropathy in GvHR, with significantly greater villus atrophy and crypt hyperplasia (Figure 2).



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Isolation of mast cells from guinea pig and human conjunctiva

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Introduction

The development of methods to isolate, purify and culture mast cells from different tissues has enabled detailed analyses of their structural and functional properties [1,2]. Comparative studies of mammalian mast cells have revealed subpopulations which are characterized by distinct phenotypes [3,4]. Mast cell subsets differ in the production of inflammatory mediators and in their pattern of activation.

Most of the information concerning the biology of ocular mast cells has been obtained from *in situ* histochemical studies which have characterized the distribution and morphological features of the cells. A role for conjunctival mast cells in ocular allergies has been suggested by evidence of increased degranulation in experimental models of ocular anaphylaxis [5]. Detailed information about the biochemical and functional properties of conjunctival mast cells, which is fundamental to understanding the role of the mast cell in the pathogenesis of anaphylactic reactions of the eye, requires isolated preparations of these cells. Consequently, to begin our cellular and molecular studies of ocular mast cells, we have adapted techniques to partially purify mast cells from enzymatically dispersed guinea pig and human conjunctival tissue.

Materials and Methods

Guinea pigs (Charles River Breeding Labs, Wilmingtom, MA) were anesthetized with CO_2 and exsanguinated from cervical vessels. Immediately after sacrifice, orbits were exenterated and total conjunctival tissue was isolated. Human conjunctiva was obtained by biopsy from volunteers and from autopsy specimens 2-6h post-mortem. Tissues were minced in Dulbecco's Modified Eagle's Medium (DMEM from GIBCO, Grand Isaland, NY) containing 0.1mg/ml soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO) and washed twice with Ca- and Mg-free Hank's balanced salt solution ($HBSS^=$ from GIBCO). Single cell suspensions were prepared by consecutive incubation of tissue fragments with: 1) 1.8mM EDTA (GIBCO); 2) 275u/ml collagenase (Worthington Biochemical, Freehold, NJ)-698u/ml hyaluronidase (Sigma)-10u/ml elastase (U.S. Biochemical Corp., Cleveland, OH)-10u/ml DNase I (Calbiochem Corp., La Jolla, CA); 3) 550u/ml collagenase-1396u/ml hyaluronidase-20u/ml elastase-10u/ml DNase I; 4) 1.8mM EDTA; and 5) 825u/ml collagenase-10u/ml DNase I. Separated cells were removed from undigested tissue after each step by filtration through 150μm nylon membranes (Tetko, Elmsford, NY) and reserved at 4°C in DMEM containing 20% fetal bovine serum (GIBCO), 10u/ml DNase I, and penicillin[10,000u/ml]-streptomycin[10,000μg/ml]-amphotericin B[25μg/ml] (GIBCO).

Mast cells were recognized in suspension and in Carnoy's-fixed smears by Alcian blue staining and in glutaraldehyde-fixed sections stained with alkaline Giemsa. In addition, human mast cells were detected in methanol-fixed smears of conjunctival cells by indirect immunofluorescence using a monoclonal anti-tryptase antibody (G3, generously provided by Lawrence Schwartz, M.D., Ph.D., Medical College of Virginia, Richmond, VA) and rhodamine-conjugated goat anti-mouse antibody (Organon Teknika-Cappel, Malvern, PA) [6].

Following initial dispersion of conjunctival cells, the cell suspensions were enriched for mast cells by centrifugal elutriation using a Beckman JE-6B rotor (Palo Alto, CA) and a Cole-Parmer peristaltic pump (Chicago, IL) [1]. Enzymatically dispersed conjunctival cells released during all digestion steps were pooled, resuspended and processed in $HBSS^=$ supplemented with 7.5g/L bovine serum albumin, 30mg/L $NaHCO_3$, 12mg/L DNase I, and buffered with 20mM HEPES (all from Sigma). Initially, fractions were collected at a rotor speed of ≈1820 rpm and countercurrent flow rates from 11-40 ml/min; subsequently, the rotor speed was reduced to ≈1400 rpm and fractions were obtained at flow rates from 19-29 ml/min. Nucleated cell viability was determined by trypan blue exclusion.

For short-term cultures, unseparated, dispersed conjunctival cell suspensions and mast cell-enriched fractions were plated at 0.5-2.0 x 10^6 cells/ml in DMEM containing 10% fetal bovine serum, 10u/ml DNase I, and penicillin[10,000u/ml]-streptomycin[10,000μg/ml]-amphotericin B[25μg/ml]. Cultures were incubated at 37°C in a fully humidified atmosphere containing 5% CO_2.

All solutions used for the isolation and culture of conjunctival cells were adjusted to pH 7.2-7.4 and 260mOsm and 300mOsm for samplesfrom guinea pig and human sources, respectively.

Results

Conjunctival tissues were dispersed effectively by incubation with chelating agents and enzymes. This method yielded ≈42 x 10^6 and ≈117 x 10^6 nucleated cells/g wet weight for guinea pig and human samples, respectively. Dispersed cell suspensions from both species were characterized by viabilities ≥88%.

In cell suspensions prepared from 52 guinea pig ocular samples, mast cells represented 1.5±0.5% of the nucleated cells. Histological examination of the residual, undigested conjunctival tissue revealed well-preserved cells of which only 0.1% were mast cells. Examination of fixed tissue sections of conjunctiva from 9 guinea pigs showed that mast cells represented ≈1.4% of the cells. In experiments assessing the yield of mast cells released by the different enzymatic digestion steps from conjunctiva isolated from 16 guinea pig eyes, it was observed that ≈86% of the mast cells were released during the fifth and final incubation step.

In 2 experiments in which conjunctiva from 9 normal volunteers was dispersed, mast cells comprised 2.4% (n=7) and 0.4% (n=2) of total nucleated cells. No mast cells were detected in residual, undigested tissue. Similarly, in 5 experiments using conjunctiva obtained at autopsy from 8 donors, mast cells constituted 0.5% of the conjunctival cells. Additionally, all mast cells were released during the final 3 dispersion steps. In experiments in which methanol-fixed smears of dispersed human conjunctival cells were stained by immunofluorescence with anti-tryptase monoclonal antibody G3, a prominent speckled pattern was observed throughout the cytoplasm, suggestive of the granular distribution of tryptase.

Following initial dispersion of guinea pig conjunctival cells, mast cells were enriched by centrifugal elutriation. Maximal purity of guinea pig mast cells in a single fraction was 29%, which represented a 10.9-fold enrichment and corresponded to 360,000 mast cells.

We wished to evaluate the ability of conjunctival mast cells to survive in short-term culture without the presence of an exogenous feeder layer or T-cell growth factors to determine: 1) the feasibility of allowing dispersed conjunctival cells to recover prior to further purification and/or analyses; and 2) the kinetic parameters for our efforts to immortalize these cells. In 6 experiments in which enzymically released guinea pig conjunctival cells were placed in culture, 11-77% of the mast cells survived for 24h. By comparison,only 18-39% of total nucleated cells persisted in the same cultures.

Discussion

We have adapted techniques to isolate and partially purify mast cells from guinea pig and human conjunctival tissues. Recovery of mast cells from human and guinea pig conjunctiva was less than the recovery from other tissues; some of the difference may arise because mast cells are increased in number in the chronically inflamed tissue used as a source in other studies.[1,2]. We have demonstrated the feasibility of isolating conjunctival mast cells in sufficient numbers to allow further purification and *in vitro* analyses. It is anticipated that studies of isolated conjunctival mast cells will contribute to a better understanding of the role of the mast cell and its products in the pathogenesis of ocular anaphylaxis.

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SECTION K: EFFECTOR FUNCTIONS OF IgA

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Properties of the IgA receptor from human neutrophils

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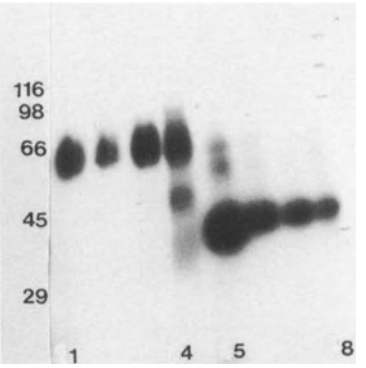
Introduction

Neutrophils are the major phagocytic cells in the body. IgG and complement, which have receptors on the surface of neutrophils, opsonise invading organisms thereby initiating phagocytosis. IgG and complement receptors have been well characterised (1,). We have recently purified a protein with an apparent MW of about 60K by affinity chromatography on IgA- Sepharose, which retains ligand binding activity. The specificity and functions of this receptor have now been studied.

Results and Discussion.

We have purified an IgA binding protein with a MW of about 60K from human neutrophils by affinity chromatography on an IgA-Sepharose column. IgG FcR11 was also purified as a control on an IgG-Sepharose column. Human neutrophils were first surface radiolabelled with ^{125}-I and then solubilsed with NP-40 in the presence or absence of protease inhibitors. The NP-40 soluble fractions were incubated with either IgA-Sepharose or IgG-Sepharose. The bound material was eluted with 0.5 N acetic acid and analysed by autoradiography on an SDS gel. Similar amounts of radioactivity were obtained for both IgA receptor and IgG receptor suggesting similar number of receptor sites on the cell surface. The purified IgA binding protein rebinds to IgA-Sepharose but not to IgG-Sepharose. This binding is inhibited by IgA but not by IgG. The IgA binding protein appears to be considerably resistant to proteolysis under different conditions of neutrophil membrane solubilisation. The figure below shows IgA binding proteins (lanes 1- 4) and IgG receptors (lanes 5- 8). In lanes 1 and 5 no protease inhibitor was used whereas phenyl methionyl-sulphonyl fluoride [PMSF] (lanes 2 and 6), DFP and PMSF (lanes 3 and 7) or DFP, PMSF and iodoacetamide (lanes 4 and 8) were used as protease inhibitors. Across this range of conditions there appears to be a change in the molecular weight of the IgA receptor from 55K where no protease inhibitors were added, to the 60K region where one or more protease inhibitors were added. Similarly, the IgG receptor also moves from 31K (lane 5) to 34K (lanes 5- 8) as previously reported (2).



We have also shown that IgA (25ug), aggregated by heat or chemical cross-linking with DSS, causes the degranulation of neutrophils, releasing lysozyme and B-glucuronidase. This occured as efficiently as with IgG (25ug) aggregated by the same methods. The heat aggregated IgA or IgG (50ug), induced a much greater respiratory burst, measured by chemiluminescence, than the same amount of the proteins aggregated by DSS. No degranulation or respiratory burst was observed when neutrophils were stimulated with IgA and IgG aggregated by cross-linking with carbodiimide or glutaraldehyde. IgA-Sepharose and IgG-Sepharose both induced a respiratory burst of similar magnitude in neutrophils, the IgA response being more rapid than the IgG. This would appear to suggest that IgA containing immune complexes can be as efficient in stimulating the release of inflammatory agents from neutrophils as IgG complexes.

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Locomotor response of rat PMN induced by IgA

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INTRODUCTION

Immunoglobulins or immune complexes are considered to induce PMN(polymorphonuclear leukocytes) migration chiefly through the activation of complement, with subsequent liberation of the chemotactic peptide, C5a. However, the effect of immunoglobulin molecules on PMN migration is controversial. Recently, our group(1,2) observed that immunoglobulin per se induced locomotor response of PMN. By using rat IgA monoclonal antibodies (3), the effect of monomeric(m-) and polymeric(p-) IgA, and IgA immune complex(IC) on the migration of PMN was investigated.

MATERIALS AND METHODS

Separation of rat PMN. PMN were isolated from the peripheral blood of a Wistar male rat by centrifugation on a high density(S.G.1.095) Ficoll-Hypaque gradient, as described(4), at 200g for 50 minutes.

Rat IgA and IgA IC. Rat monoclonal IgA antibodies to dinitrophenyl(DNP) hapten were made and purified as reported elsewhere(3). The monoclonal p-IgA antibody(0.1mg/ml) was mixed with DNP_8-bovine serum albumin(BSA) (0.13mg/ml).At this ratio,soluble IgA IC was formed in antigen excess(3).

Chemotaxis. Locomotor response of PMN was tested with a modified Boyden chamber(48-well microchemotactic chamber;Neuroprobe)(5),using polycarbonate filter sheet(pore size:3μm,Nucleopore). After incubation at 37°C for 20 minutes, PMN on the lower surface of the filter was stained and counted in high power microscopic field (H.P.F.; 500X).

RESULTS AND DISCUSSION

Influence of IgA on locomotion of PMN. Monoclonal p- and m-IgA induced migration of PMN in a similar dose-dependent manner(Fig.1). When IgAs were also added to the cell suspension in the upper chamber, the migration was inhibited, indicating that the activity of them was mainly of a chemotactic type. IC containing p-IgA prepared as above induced a similar response(39.5 ± 12.4) to p-IgA antibody(0.1mg/ml) alone(39.7 ± 13.7).

Effect of IgA on FMLP-induced chemotaxis. IgA was shown to modify the chemotactic movement of PMN induced by N-formylmethionyl-leucyl-phenylalanine(FMLP). When p-IgA(0.3mg/ml) was added to the cell suspension in the upper chamber, a leftward shift of the dose-response curve of FMLP-induced chemotaxis was observed(Fig.2a). When p-IgA was added to FMLP in the lower chamber, PMN chemotaxis towards every concentration of FMLP (except for 10^{-7}M) was enhanced(Fig.2b).

The present study revealed that rat IgA could induce the chemotactic movement of PMN in the absence of complement. Although the chemotactic potency of IgA seems weak, it can exhibit significant synergy with other chemoattractants, thus favoring mobilization of PMN from the circulation to the locus of bacterial infection on the mucosal surface, where IgA is abundant and complement is not.

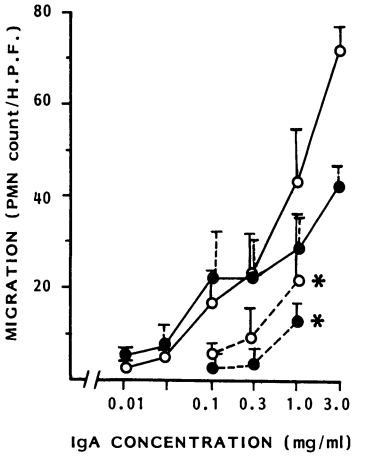

Fig.1. PMN migration induced by IgA. Degrees of migration (PMN count - the negative control count) are plotted against the concentration of p-IgA (O) and m-IgA (●). Each point represents the mean±SD (n=9).

* p- or m-IgA (0.3mg/ml) was also added to the cell suspension in the upper chamber, respectively.

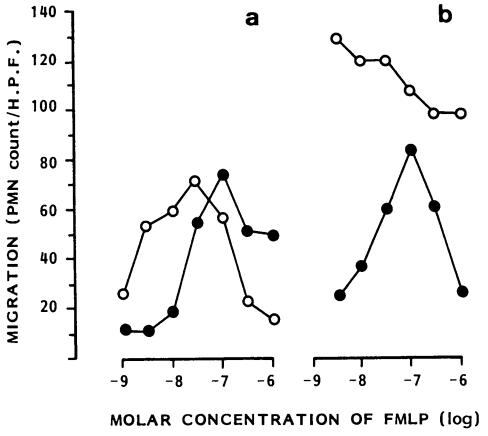

Fig.2. Effect of IgA on FMLP-induced chemotaxis of PMN. Migration towards FMLP (●) and its change after addition of p-IgA(0.3mg/ml)(O) to the upper(a) or lower(b) chamber, as described in the text(n=3).

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Comparison of IgA and IgG anti-mannan antibody mediated phagocytosis using double label fluorescent zymosan assay

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INTRODUCTION

IgA anti-yeast mannan antibodies are opsonic for the phagocytosis of zymosan by human peripheral blood polymorphonuclear leukocytes (PMN) (1), through an Fc receptor (2). IgG anti-mannan antibodies are ineffiecient opsonins in free suspension (1,3).

In order to investigate the relative efficiencies of the two antibody classes and their ability to interact with PMN, a double label zymosan assay has been employed.

MATERIALS AND METHODS

Anti-mannan antibodies were purified from the serum of a patient with Crohn's disease, previously identified, by ELISA, as having high levels of IgA and IgG antibodies. Purification involved ammonium sulphate precipitation followed by sequential affinity chromatography on protein A sepharose and jacalin agarose.

Double label fluorescent zymosan assay. The assay described below is an adaptation of Hed's assay (4). Zymosan was labelled with either fluorescein or rhodamine isothiocyanate. Fluorescent zymosan was then optimally opsonised with affinity purified immunoglobulins; rhodamine zymosan with IgA, fluorescein with IgG. After washing in RPMI 1640, equal numbers of particles were incubated with PMN at a target:effector ratio of 10:1. Phagocytosis was allowed to proceed for 30 mins at 37°C either after pelleting by centrifugation or with continuous agitation. Phagocytosis was stopped by the addition of PBS containing 2mg/ml trypan blue. 100 PMN were then counted for adherence and ingestion by phase-contrast and fluorescence microscopy. Attached and ingested particles were differentiated on the basis of trypan blue quenhing of external particles. Non-opsonic uptake was determined in parallel incubations and subtracted from opsonized values. Assays were carried out on single incubations as reading must be rapid to avoid quenching of internalized fluorescence. Asays were repeated three times.

RESULTS AND DISCUSSION

On the basis of protein A binding characteristics, the anti-mannan response in this serum lacked significant amounts of IgG3. Table 1 shows the results of a typical fluorescence experiment.

TABLE 1. Opsonized - unopsonized values/100 PMN

	suspension		pelleted	
	external	internal	external	internal
No. IgA zym	28	16	22	126
No. IgG zym	15	6	10	4
IgA + PMN	7	6	3	19
IgG + PMN	0	3	2	6
IgA + IgG PMN	8	2	0	15

IgA is clearly the more efficient opsonin. A greater number of PMN are capable of IgA mediated adherence and uptake than IgG. The presence of IgA and IgG zymosan in the same PMNs indicates that Fc receptors for both IgG and IgA are coexpressed on atleast some of the cells. As the results may be dependant on the affinities of the respective Fc receptors, no further conclusions as to receptor expression may be drawn. These results support previous findings (1,3) that in contrast to IgG IgA is an effective opsonin in free solution in the absence of complement. Furthermore, IgA is more active opsonin when the cells have been pelleted. Fanger et al (5) found IgA to be incapable of supporting the phagocytosis of ox erythrocytes in the absence of IgG. This difference may be explained by a synergy between IgA ang IgG receptors in the erythrocyte study, and a synergy between the Fc_{α} receptor binding and the yeast beta-glucan binding activity of CR3 in the present study.

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Release of PAF and oxygen radicals by human neutrophils and rat mesangial cells stimulated with IgA and IgG immune aggregates

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The mechanisms of tissue injury in immune complexes (IC) glomerulonephritis are incompletely understood. Most of studies have been dedicated to IgG IC. However, few data exist about the pathogenetic mechanisms of IgA mediated diseases. In this paper we have explored the possibility that neutrophils and mesangial cells exposed to IgA immune complexes, can mediate the production of platelet activating factor (PAF) and oxygen radicals (OR).

MATERIAL AND METHODS

Mesangial cells (MC) were cultured from isolated rat glomeruli.Confluent mesangial cells are present after 18 to 21 days in culture.Cells in primary culture were used in these studies. Human peripheral blood polymorphonuclear leukocytes (PMN) were isolated from blood of healty donors by using Ficoll/Hypaque gradient,gelatin sedimentation and hypotonic lysis of red cells.
Human IgA was isolated from serum of a patients with IgA myeloma.Aggregation was performed by heating 10 mg/ml of IgA or IgG for 150 or 30 min, respectively,at 60ºC in 0.15M Tris buffer saline (TBS).Approximately 500,000 MC(scrapped off their flask) or 5 x 10^6 PMN were incubated with varying concentrations of immune aggregates in a final volume of 1 ml for various periods of time up to 60 min.
Production of PAF by MC and PMN was measured by lipid extraction of cells and supernatant followed by bioassay. O_2^- production was assayed as reduction of ferricytochrome c by supernatants of MC or PMN incubated with different concentrations of immune aggregates (Aggr).

RESULTS

IgA-Aggr stimulated PAF and O_2^- production by PMN and MC in a concentration dependent fashion (not shown). The oxygen release was significantly ($p<0.05$) higher in MC than in PMN for the same dose of protein (Table I). MC stimulated with these Aggr produced as much PAF as PMN did. The most striking difference between IgA and IgG Aggr was noted in O_2^- production by MC (Table I). Aggr-induced PAF and O_2^- release was not affected by cytochalasin B, an inhibitor of phagocytosis.

TABLE I. Production of PAF and O_2^- by PMN and MC stimulated with Aggr.

	PMN	MC
PAF		
IgA	237 ± 120	243 ± 210
IgG	368 ± 134	222 ± 143
O_2^-		
IgA	5.8 ± 0.9	19 ± 2
IgG	4.1 ± 0.5	9 ± 1

PAF is expressed in pg/mg protein; O_2^- in nmol/10^6 cells/30 min. M ±SD

DISCUSSION

It is well established that IgGIC can cause tissue injury. In vitro, preformed IgG IC stimulate the release by phagocytic cells of various mediators such as O_2^- and PAF. Far less is known about the pathophysiology of IgA-mediated tissue injury despite its importance in human diseases such as IgA nephropathy, dermatitis herpetiformis and others. Recently, it has been shown that IgA IC selectively stimulates dose dependent O_2^- and H_2O_2 production by alveolar macrophages (1). However, to our knowledge, the possibility of PAF release by phagocytic cells induced by IgA IC has not been approached.

In this paper we have shown that IgA Aggr (akin to IC) induces the production and release of O_2^- and PAF by two different cells as human PMN and rat MC. The cytochrome c reduction provoked by the two Aggr, in both types of cells, was almost completely inhibited by 10 ug/ml of superoxide dismutase, suggesting, therefore, that reduction was O_2^- mediated. The characterization of PAF was done upon strict criteria. This lipid comigrated in thin-layer chromatography in the same Rf as synthetic PAF. The ^3H serotonine release was inhibited by a specific PAF antagonist and finally, the biological activity was lost after phospholipase A_2 treatment. The synthesis of O_2^- and PAF by IgA Aggr in similar amounts to IgG Aggr is surprising. Although IgA Fc receptors have been described in PMN, monocytes and other cells, there is no data that the same occurs in MC. It is important that the amount of PAF released by MC stimulated by IgA Aggr is in the same range as described by Sedor et al. (2) when incubated with IgG IC. Although cultured MC used in our study were well characterized there always exists a certain population of Ia^+ cells. Since the amount of O_2^- relased by MC in response to IgA Aggr was greater than that generated by PMN, we think that contamination by macrophages could not explain the magnitud of O_2^- production. Recently Neuwirth et al (3) have shown PAF formation by rat MC stimulated with IgG IC. In conclusion, our data suggest that IgA IC, when deposited in the mesangium, can stimulate intraglomerular release of O_2^- and PAF from MC.

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Fc-receptor mediated phagocytosis of IgA- and IgG-coated red cells by human alveolar macrophages

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1. Introduction

Receptors for the Fc portion of IgG subclasses and IgA are present on the cell surface of alveolar macrophages (AM) from mice, rat, and humans (1-5). Fc-receptors for IgG (Fcγ-R) are known to induce a series of phagocyte functions including the respiratory burst, degranulation and phagocytosis (6). By contrast, there is little information on the function of Fc-R for IgA (Fcα-R). In the present *in vitro* study, we evaluate the Fc-αR mediated phagocytosis in normal human AM and compare it to the Fcγ-R mediated phagocytosis. Furthermore, the effect of *in vivo* macrophage activation on the Fc-R is investigated by studying AM from patients with pulmonary sarcoidosis.

2. Materials and Methods

ALVEOLAR MACROPHAGES. AM were obtained by a standard bronchoalveolar lavage (BAL) in 5 normals and 9 sarcoidosis patients, all non smokers (5).

Ig-COATED CHROMIC CHLORIDE (CrCl3)-TREATED SHEEP RED BLOOD CELLS (SRBC). After 5 washes in PBS, 100 µl of packed SRBC were mixed either with 100 µg of polyclonal (PC) milk secretory IgA (sIgA), or with 100 µg of serum PC IgG, or with medium. Then, 10 ml CrCl3 (0.1%) were added dropwise to the SRBC. After 10 min of incubation and 3 washes, SRBC were resuspended at 0.5%.

PHAGOCYTOSIS OF SRBC BY AM. BAL cells (1.5×10^5) in 100 µl of PBS were incubated for 5 min at 22°C with either IgA- (IgA-SRBC) or IgG-coated SRBC (IgG-SRBC) or SRBC (control). After centrifugation, cells were further incubated for 15 min at 37°C and resuspended in NH_4Cl lysing buffer, then washed in PBS, cytocentrifuged and stained with peroxydase-Giemsa. Phagocytosis was estimated by the percentage of AM containing 3 or more SRBC; 400 AM were counted in each sample.

INHIBITION OF PHAGOCYTOSIS. For inhibition studies, AM were preincubated with 40 µg of sIgA or PC IgG and washed prior to incubation with, respectively, IgA- and IgG-coated SRBC.

3. Results

BAL cells from normals and sarcoid patients consist, respectively, of 94.4% AM with 5.4% lymphocytes, and of 82.5% AM with 16.5% lymphocytes. The results of phagocytosis tests are shown in Fig. 1. Respectively, 53% and 56% of normal AM ingested IgA- and IgG-coated SRBC, significantly (both $P < 0.05$) more than 32% of AM ingesting control SRBC. AM from sarcoid patients had similar phagocytosis profiles as normal AM. In AM from 5 normals, preincubation with IgA significantly reduced the percentage of AM ingesting IgA-SRBC (from 53% to 28%, $P < 0.05$). Preincubation of AM with IgG also significantly decreased the percentage of AM with internalized IgG-SRBC (from 56% to 27%, $P < 0.05$).

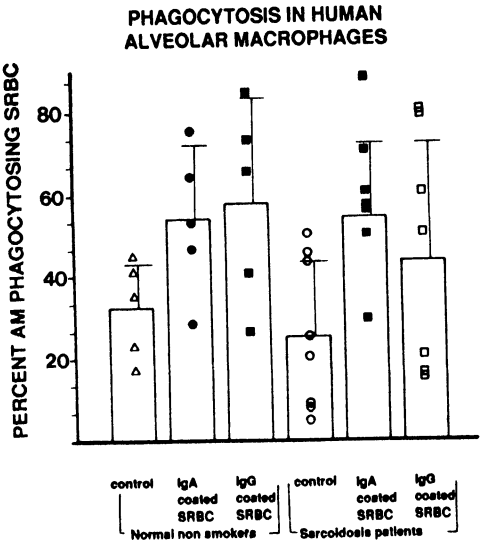

Figure 1.

4. Discussion

After the recent demonstration of Fcα-R on human AM, we now provide evidence for IgA-mediated phagocytosis by human AM *in vitro*. This IgA-mediated phagocytosis can be blocked by preincubation of AM with IgA, and is of the same order of magnitude as IgG-mediated phagocytosis. Several reports suggest that AM from sarcoid patients are "activated" in vivo and release *in vitro* increased amounts of mediators (7). Here, we did not observe an enhanced IgA- or IgG-mediated phagocytosis in AM from sarcoid patients compared to normals. This could be related to previous observations by our group and others (8) that AM from sarcoid patients express the same number of Fcγ-R and of unoccupied Fcα-R as AM from normals. In conclusion, the newly described Fcα-R on AM is functional, suggesting that interaction between IgA and AM in the lower respiratory tract could contribute to immune reactions in the lung.

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Porcine-murine *heterohybridomas* as stabb fusion partners for the production of porcine IgA

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Introduction

The immunoprophylaxis of neonates against intestinal pathogens remains a challenge. The approach is generally based on the entero-mammary linkage of the gestationing animal. However, a clear correlation between the virulence of the pathogen and IgA inducing capability exists. Therefore, our investigation aimed at the possibility of inducing mucosal immunity via the anti-idiotype pathway. Recent advances in human MAB production (for review Teng, N.N. 1985) and IgA producing hybridomas (Colwell, D., 1986, Dean, C., 1986) made research on this concept in a veterinary species feasible. PM-1 is a stable, non-Ig producing murine-porcine heteromyeloma capable of high fusion rates and stable porcine MAB production upon fusion with porcine lymphocytes of various sources (triple heterohybrids). PM-1 was obtained by intensive subcloning, in selective medium, of hybridomas resulting from the fusion of the murine myeloma P3x63-Ag8.653 and the porcine lymphoblastoid cell line P-16. Fusions of PM-1 with PBL, MNL, TDL and SL resulted fusion rates of 20-100% (hybridomas/wells inoculated). A total of 359 hybridomas were monoclonal Ig-producers. The majority of those analysed produced the IgM isotype. Only one heteorhybrid, YF 42-1, resulting from a PM-1 x PBL fusion was identified as producing dimeric IgA. Fusion with TDL, obtained from pigs after surgical removal of the mesenteric lymph nodes, resulted in 16 IgM and 2 IgG producing hybridomas. No IgA producing hybridomas were obtained from PM-1 x TDL fusions.

Materials & Methods: CONSTRUCTION OF HETEROMYELOMA LINE PM-1:

PM-1 was constructed from a fusion between the 2-Mercaptoethanol (2-ME) dependent-porcine lymphoblastoid cell line P-16. (Hammerberg et al., 1985) and the murine myeloma (HGPRT) P3x63Ag8.653 using a strategy described by Teng et al (1983). Selection was based on 1) absence of Ig production 2) aminopterin sensitivity (to select for desirable murine genetic characteristics) and 3) 2-ME dependability (to select for porcine genetic characteristics). Surface markers of the heteromyelomas were analyzed by flow microfluorometry (FMF) using MABs with specificity for porcine MHC Class I (Peskovitz et al, 1984), MHC Class II (Hammerberg et al, 1985), IgM (Paul et al, 1985), IgG and IgA (Hollingshead et al, 1985) and allo antisera with specificity for

murine MHC Class I and Class II (Tonkonogy et al, 1982) Karyotyping was done according to Johnson (1987).
FUSION OF THE HETEROMYELOMA PM-1 WITH PORCINE LYMPHOCYTES: PBL were obtained from heparinized blood. MNL from minced and sieved jejuno-ileo mesenteric lymph nodes, SL from minced and sieved spleen tissue and TBL from heparinized thoracic duct lymph. The mononuclear cell fraction of each cell preparation was obtained using Ficoll-Paque. fusion was performed with the non-adherent cell fraction using PEG-1000. Cloning and subcloning was performed using limiting dilutions (min. 0.25 cells/well). Swine used as sources for TDL were treated similarly to the protocol described for the production of IgA hybridomas in the rat. (Dreyer, et al, 1984). Briefly: week 1, oral infection with 10^5 $TCIC_{50}$ of porcine coronavirus (De Buysscher and Berman, 1980), week 2, surgical removal of jejuno-ilial mesenteric lymph nodes (barbital + fluothane anesthesia), week 5, oral infection with 10^5 $TCID_{50}$ porcine coronavirus followed 48 hrs. later by thoracic duct canulation. TDL's were analyzed by FMF using the MABs described above. In addition, TDLs were analyzed for cytoplasmic IgA using fluorescent Ab microscopy.

Results and Discussion

Karyogram analysis of the heteromyeloma, PM-1 indicated that this "stable" clone contains 83(±3) chromosomes. Most of the chromosomes are of P3X63Ag8.653 origin. Three to five centermeric chromosomes and one long unpaired acrocentric chromosome were derived from the porcine p-16 parent. PM-1 contains, in addition, 1-2 double minute chromosomes, which are not observed in the karyogram of either parent. PM-1 lost the expression of the porcine Class I (SLA-A or SLA-C) and murine Class I ($H\text{-}2K^d$) expressed by the parent P-16 and P-3. Interestingly, the Ig-producing hybridomas resulting from fusion of PM-1 x porcine lymphocytes did reexpress the class I surface glycoproteins of both species.

PM-1 is a very efficient fusion partner. From 8 fusions with PBL an average fusion rate (% of wells inoculated containing at least 1 hybridoma, varied from 16.3% (30 in 184) to 100% (336/336). When PBL were stimulated with LPS before fusion with PM-1 (four fusions) the fusion rate was 81-100%. Monoclonal Ig-synthesis and secretion was found in 236 out of 1,138 hybridomas (20.7%). The isotype secreted was mostly IgM, only one hybridoma, YF-42-1 (PM1 x PBL, no LPS) produced a dimeric IgA with undetermined specificity. From 3 fusions with TDL, 76 hybridomas were obtained (20.6%) and 18 synthesized monoclonal Ig. However, none produced the IgA isotype. These results are quite different from those obtained in the rat model (Styles et al). We analyzed Ficoll-Paque isolated mononuclear TDL and found 90-100% positive for MHC class I (SLA-A/C), 20% had surface IgM and 10% had surface IgG Staining for surface IgA (3 MABs) or cytoplasmic IgA (polyclonal rabbit anti-α chain) showed at most 1% of cells positive.

All MABs used did bind very well to their respective isotypes in Western blotting and ELISA. These findings were similar for animals (n= 2, 70lbs, fem.) with surgically removed jejuno-ileal lymph nodes or intact animals (n= 2, 70lbs, fem.). These data are at variance with data on swine intestinal lymph, but might be possibly explained by the particular lymphocyte recirculation pathway in the pig (Bennell and Husband 1981; De Buysscher and Berman 1980).

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SECTION L: ONTOGENY AND AGEING

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Ontogenesis of the human secretory immune system

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ABSTRACT. Immunglobulin (Ig)-producing cells and the epithelial expression of secretory component (SC) and HLA determinants, were studied by immunohistochemistry to obtain information on the development of the secretory immune system. Tissue specimens were obtained from 20 fetal and 42 postnatal salivary glands, including 17 specimens from sudden infant death syndrome (SIDS) victims. SC was present in small amounts in early fetal life and increased towards delivery. A few IgM-, occasional IgA-, but no IgD- or IgG-producing cells were seen (ratios, IgM:IgA:IgD:IgG~5:1:0:0). No epithelial HLA class II expression was observed during fetal life, whereas class I determinants were expressed in some acini and most ducts. During the first year postpartum, SC and epithelial HLA-DR expression increased markedly along with a growing number of IgA- and IgD-producing cells (IgM:IgA:IgD:IgG~2:4:1:1). These features probably reflected local activation of the immune system in response to environmental factors. SIDS victims had significantly ($p<0.01$) increased numbers of IgM-, IgA-, and IgG-producing cells compared with controls, suggesting overstimulation of the local immune system.

1. Introduction

Discrepant reports have been published about the ontogeny of the human secretory immune system [1]. This disparity may be explained by methodological problems, differences among various secretory sites, large individual variations, and difficulties in determining the exact age of the fetus. The present study was carried out to obtain information about the development of the secretory immune system in major salivary glands. Previous reports [2,3] concluded that active synthesis of IgA does not occur during fetal life. This is in contrast to a recent study [4] suggesting that at least some of the secretory antibodies often detected in neonatal saliva have a fetal origin. Also, in preliminary studies we observed scattered IgA-producing cells in some fetal specimens [5].

The reported expression of HLA class II determinants in normal major salivary gland epithelium raised the question whether this was a constitutive feature [6]. Discrepant findings have been reported with regard to fetal HLA class II expression in various secretory tissues [7,8], and fetal salivary glands have not been studied previously in this respect. A recent observation suggested

that respiratory hypersensitivity is involved in the pathogenesis of SIDS [9]. We therfore included salivary-gland specimens from SIDS victims and compared them with postnatal control specimens from babies who died of other causes.

2. Material and Methods

Tissue specimens from 20 fetal and 42 postnatal (0-15 months) major salivary glands were after excision from forensic postmortem cases, immediately fixed in cold 96% ethanol and processed for paraffin embedding [10]. Serial sections were cut at 6 μm and incubated with various pairs of primary antibody reagents [5,6] in a three-step immunofluorescence staining method [11].

3. Results

3.1. FETAL MATERIAL

SC was present in small amounts in early fetal life and increased towards delivery. A few IgM-, occasional IgA-, but no IgD- or IgG-producing cells were seen (ratios, IgM:IgA:IgD:IgG~5:1:0:0). IgA1 positive cells predominated (median, 80% and range, 0-100%) and they were mostly J-chain positive. No epithelial HLA class II expression was observed.

3.2. POSTNATAL MATERIAL

3.2.1. Controls. During the first year postpartum, SC and epithelial HLA-DR expression increased markedly along with a growing number of IgA-and IgD-producing cells (IgM:IgA:IgD:IgG~2:4:1:1). The median proportion of IgA1 positive cells was 65% (range, 55-100%) and they were mostly J-chain positive.
3.2.2. SIDS victims. These specimens contained significantly increased numbers of IgG-, IgM-, and IgA-producing cells ($p<0.01$) compared with controls, whereas no such difference was observed for the IgD class ($p>0.1$, Wilcoxon's two-tailed test for unpaired samples).

TABLE 1. Distribution of Immunoglobulin-producing cells (cells/mm^2)

	IgA median (range)	IgM median (range)	IgG median (range)	IgD median (range)
CONTROLS	6 (8-15)	5 (0.5-12)	1 (0-4)	2.8 (0-7)
SIDS	18 (8-30)	14 (5-30)	2.5 (0-9)	2.3 (0-9)

4. Discussion

Epithelial expression of HLA-DR along with remarkably small amounts of HLA-DP and -DQ (ratios, HLA-DR>DP>DQ 10:1:1), was first seen after delivery; this

suggests that antigenic and mitogenic stimuli are important for epithelial class II expression.

Mellander et al. [4] recently observed SIgA and SIgM antibodies to Escherichia coli and poliovirus type I in early saliva samples from an infant of a hypogammaglobulinaemic mother who had received regular intravenous immunoglobulin injections but lacked IgA and IgM antibodies; this finding supported a fetal origin of at least part of the secretory antibodies often detected in neonatal saliva. The prescence of J-chain positive IgA-producing cells in some fetal specimens is in keeping with this notion.

A larger number of IgM- than IgA-producing cells in fetal specimens was in accordance with an early salivary IgM response [12]. We did not observe any IgD- or IgG-producing cells until after delivery. Production of IgD in secretory tissues seems to be rather unique for the upper aeroalimentary region [13]. IgD-producing cells were in fact surprisingly numerous in the early postnatal period and might reflect a putative sequential switcing of B-cell phenotype postulated to be involved in the regulation of this part of the secretory immune system [14]. Increased levels of IgD in newborn saliva [15] support this suggestion.

The increased immunoglobulin production in salivary glands from SIDS victims probably reflected overstimulation of the local immune system in response to environmental factors. A recent study showing increased concentrations of IgG and IgM (and to a lesser extent IgA) in lung lavage fluid and lung tissue [9] is in agreement with our results. We have previously reported greatly raise hypoxanthine concentrations in vitreous humor in SIDS, indicating that prolonged cerebral hypoxia plays a major role in the death mechanism [16]. Babies dying of SIDS have often evidence of a viral upper respiratory tract infection; our results may reflect that SIDS victims have local hyperimmunity to such usually harmless infections.

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Distribution of IgG subclasses in human fetus

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INTRODUCTION

In the early fetal age, immunoglobulins in human composed largely of IgG isotype which transfered from mother through the placenta. A passive transfer of maternal IgG increases with gestational age and maternal IgG begin to disappear at birth. There are four IgG subclasses in human. It has been reported that all four IgG subclasses are also transported from mother across the placenta and may be present in fetal circulation at about 6 weeks of gestation. Initial synthesis of IgM has been detected in the lymphoid tissue of human fetuses at approximately 10 weeks of gestation. A small number of IgG-producing cells appear at 12 weeks and IgA-producing cells at 26 weeks of gestation. Although IgG-producing cells are present in human fetuses, a little is known as to the distribution of IgG subclass-producing cells in human.

We have examined the distribution of IgG subclass-producing cells in human fetus with respect to secretory immune system.

MATERIALS AND METHODS

Ten percent buffered formalin fixed paraffin embedded samples (salivary gland, small intestine, GALT, lung, BALT, liver, thymus and spleen) were obtained from 30 embryos with the fetal age from 16th to 36th gestational week on the legal abortions. The gestational age was determined by crown rump length, fetal foot length and fetal palm length. Thin sections were made and an avidin-biotin peroxidase complex (ABC) method was applied to sections. In brief, depaffinized section were treated with 0.1% pronase for 10 min and endogenous peroxidase activity was blocked by incubation with methanol containing 0.3% H_2O_2 for 20 min. Washed sections were incubated for 20 min with 10% normal horse serum to block non-specific reaction. Appropriately diluted mouse monoclonal antibodies to IgG subclass were applied to tissue sections for 45 min at room temperature. Mouse monoclonal antibodies to IgG were IgG1 (code : NL16), IgG2 (code : HP6014), IgG3 (code:HP6047) and IgG4 (code:6025). Washed sections were incubated for 45 min with biotinylated horse anti-mouse IgG dissolved in 1% bovine serum albumin-PBS and then with avidin peroxidase for 45 min.

Sections were immersed in 0.05%, 3',3' diaminobenzidine solution containing 0.01% H_2O_2 for 10 min and were counterstained with Mayer's hematoxylin.

RESULTS

Although all subclasses of IgG were detected at 16th gestational weeks, a small number of IgG subclass-producing cells begun to appear in fetal tissues at approximately 20th gestational week. The number of subclass-producing cells increased with gestational age in general. A few IgG1-producing cells were found in the medulla of thymus, perivascular area of large blood vessels in liver, submucosa of bronchus, around the large bronchioles of lung and the periphery of lymphoid tissues near large bronchus which considered to be bronchus-associated lymphoid tissue (BALT)at 26th gestational week. At the same weeks, positive cells were observed in small intestine and lymph node near small intestine which coresponded to gut-associated lymphoid tissues (GALT). In small intestine, more IgG1-producing cells were localized in the perivascular area of lamina muscularis mucosae compared to lamina propria of small intestine. In salivary glands, positive cells appeared at 28th week in the connective tissue near acinar cells and duct. The number of IgG1-producing cells gradually increased after 30th week in all fetal tissues examined.

IgG2-producing cells were observed in the perivascular area of large blood vessel in liver and spleen, the medulla of thymus and GALT at 20th weeks. At 26th week, positive cells appeared in the lamina propria of bronchus, salivary gland, lung, BALT and small intestine. Similar to IgG1-producing cells, more positive cells were localized in the lamina muscularis mucosae in small intestine. The increased number of IgG2-producing cells were found in BALT, small intestine and GALT at 28th week and thereafter. The number of IgG2-producing cells exceeded that of IgG1-producing cells through whole gestational ages.

Similar to IgG2-producing cells, IgG3-positive cells appeared in liver, spleen, thymus and small intestine at 20th gestational week with similar distribution patterns. Positive cells were detected in lung at 24th week and salivary glands at 28th week. The number of IgG3-producing cells was the same to that of IgG2-producing cells. The appearance of IgG2- and IgG3-producing cells in fetal tissues was earlier than that of IgG1-producing cells. Almost no IgG4-producing cells were found in fetal tissues except for liver and thymus. Only a few positive cells were present in liver and the medulla of thymus at 26th week.

CONCLUSION

1). At 20th gestational week, IgG2-and IgG3-producing cells were detected in liver, thymus and spleen, but not IgG1- and IgG4-producing cells.
2). IgG1-producing cells appeared at 26th week and almost no IgG4-producing cells in fetal tissues except for the presence of a few cells in thymus and liver.
3). The predominant IgG subclass in the number were IgG2- and IgG3-producing cells. The number of subclass-producing cells increased with gestational age.

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Development with age of the lymphatic tissue in the normal human appendix vermiformis

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Little is known about the development of the human gut-associated lymphoid tissue (GALT) after birth and its maintenance during life. We have studied the human appendix vermiformis (AV), a GALT organ, to obtain information on (1) the postnatal development of the lymphoid tissue and the time of appearance of plasma cells containing immunoglobulins (Ig); (2) the distribution of the Ig classes in mucosal plasma cells versus age; (3) the size of the organe and the amount of lymphoid tissue during adult life.

MATERIALS and METHODS

We have examined 190 human AV which had been removed "incidentally" from individuals 1 day to 89 years old. Specimens without histologic signs of active inflammation or luminal obliteration were fixed in 4% buffered formaldehyde solution. Specimens fixed in sublimate-formaldehyde were used for immunohistochemistry. The AV were processed by standard histologic techniques and embedded in paraffin wax.

We measured weight, length and diameter of the specimens and volumes. Three cross sections taken from the proximal, middle and distal parts were examined separately. We counted the lymphatic follicles and germinal centers, measured the mucosal areas, and the areas of the lymphatic follicles and germinal centers. Mean values were calculated for each AV. Serial sections were stained by the peroxidase-anti-peroxidase (PAP) method for IgA, G, M, and E, respectively. For each Ig class, all stained cells were counted in the whole mucosal area of 3 sections taken from the proximal, middle and distal part.

RESULTS

Median and mean values of the morphometric parameters were almost identical. In the first year after birth, the elongation rate exceeded the increase in volumen; length and volume reached maximum values in the first decade and remained constant during adult life; there was no decrease in volume in the old age.

The mucosal area increased rapidly, reached a maximum during the first year and remained constant thereafter, even in old persons.

There were only very few lymphatic follicles in the AV of the newborn. Germinal centers did not appear until the 4th week after birth. Thereafter, the number of both structures increased in parallel and reached a peak in the second decade. After a more rapid decrease in the third decade, these values decreased very slowly. As the number of lymphatic follicles in the newborn AV, the total follicular area was low. From the postnatal 1st month to the 10th year of life, the ratio of the numbers of germinal centers/lymphatic follicles was larger than in the 2nd decade, while the germinal center area decreased after the 2nd decade. Both parameters decreased slightly after the 5th decade.

There were few plasma cells present during the first year after birth. The highest plasma cell number were measured in the first decade. After a decrease in the second decade, their number remained almost constant until old age.

In the AV of the newborn, we did not observe cells containing immunoglobulins, but the secretory component of IgA was already detectable in the epithelium. The first mature plasma cells appeared 2 weeks after birth. After 4 weeks, there was marked increase in IgA and IgM cells; the IgM cells even exceeded that of IgA cells. Twenty weeks after birth, the number of IgA cells reached a maximum value that changed little until old age. Two months after birth, IgM and IgG cell numbers showed an inverse realationship until old age: The IgM cell population decreased while the number of IgG cells increased. The IgE cell number was low (1 to 2%) in all age groups with a small peak during the first 2 weeks after birth.

DISCUSSION

For the first 2 weeks after birth, the AV lacks mature plasma cells and secretory IgA. Germinal centers were not seen in the first 4 weeks, but there were already sparse mature plasma cells present in the mucosa. Later, the development of lymphatic follicles and germinal centers paralleled that of plasma cells. The development of germinal centers depends on antigenic stimulation and is always associated with humoral antibody production, but there is obviously no direct relationship to the early occurrence of plasma cells in the AV.

The number of IgM cells rises rapidly after birth while the rise of IgA cells is much slower. Later, IgM and IgG show an inverse relationship. The high proportion of IgG plasma cells (30%) of the AV differs markedly from the much lower values observed in the other gut mucosa.

There appears to be no decrease in size of the normal AV and only a slight involution of lymphoid tissue with age. Even in old persons, there are still well developed lymphoid tissues and many mature plasma cells present. Our findings contrast with the reported involution with age of the lymphatic tissue in other organs. Thus, structure and presumably function of the GALT differ from those of extraintestinal lymphatic tissue.

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A longitudinal study of immunoglobulin and antibody concentrations in lower respiratory tract secretions from very premature neonates

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The purpose of this longitudinal study was to determine the time course of the appearance of immunoglobulin and antibody in lower respiratory tract secretions in extreme prematurity.

Materials and methods

Thirty-three premature infants were enrolled. The median gestational age was 27 weeks (range 25-30 weeks). All required immediate postnatal intubation and ventilation for hyaline membrane disease and/or prematurity. Tracheobronchial aspirates were collected at weekly intervals from birth and throughout the duration of intubation (median 27 days; range 14-39 days). Ten neonates remained intubated beyond 35 days. Measurements of total IgG, IgA, IgM, secretory IgA and secretory IgM concentrations were performed as described previously[1]. Enzyme immunoassays were used to measure isotype-specific antibody concentrations to a pool of eight *E coli* serotypes[2].

Results

Samples obtained at birth contained IgG, IgA and IgM in 100%, 93% and 79% of samples respectively. The median concentrations (μg/mg total protein) of IgG, IgA and IgM in the tracheobronchial aspirates with increasing postnatal age are shown in table 1. Median anti-*E coli* antibody concentrations in the same samples are shown in table 2. Immunoglobulin and antibody concentrations were not correlated with gestational age. The proportions of neonates with detectable IgG, IgA and IgM anti-*E coli* antibody in aspirates with increasing postnatal age are shown in table 3. There were no significant correlations between cord serum and tracheobronchial aspirate IgG, IgA, or IgM concentrations at birth. Secretory-component associated IgA and IgM was detectable in paired tracheobronchial aspirates tested at birth and at four weeks of age from 12 neonates. Three of nine of these paired samples tested at four weeks postpartum contained secretory component-associated anti-*E coli* antibody but this antibody was not found at birth.

Discussion

Very premature neonates have been shown in this study to acquire increasing concentrations of IgA and IgM in lower respiratory tract secretions with increasing postnatal age. IgA and IgM were present in the majority of samples even at birth, and secretory component-associated IgA and IgM were detectable at birth, indicating respiratory tract mucosal production of at least some

of the immunoglobulin. IgA and IgM antibody concentrations to pooled *E coli* antigens also increased postnatally, with an increasing proportion of neonates having detectable antibody with increasing postnatal age. Extremely premature neonates, although able to acquire increasing concentrations of IgA and IgM immunoglobulin and antibody postnatally in secretions, appear to do so at a slower rate than in gestationally older infants[2], suggestive of slower postnatal maturation of mucosal antibody production and transport than at term.

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Table 1: Median concentrations of IgG, IgA and IgM in tracheobronchial fluid samples in ug/mg total protein.

Age n samples	Birth (n=28)	Week 1 (n=27)	Week 2 (n=29)	Week 3 (n=21)	Week 4 (n=18)	Week 5 (n=12)	Week 5.5 (n=10)
IgG	61.5	57.8	46.1	37.1	27.1	22.9	22.6
IgA	0.7	0.9	0.6	0.7	1.4	3.4	5.8
IgM	0.6	2.1	2.1	2.6	2.8	3.3	2.9

Table 2: Median concentrations (range) of IgG, IgA and IgM anti-*E coli* antibody in tracheobronchial fluid samples in ng/mg total protein.

Age n samples	Birth (n=28)	Week 1 (n=27)	Week 2 (n=29)	Week 3 (n=21)	Week 4 (n=18)	Week 5 (n=12)	Week 5.5 (n=10)
IgG	19.1 (0.0-189.5)	8.7 (0.0-218.7)	6.2 (0.0-72.9)	5.6 (0.0-34.2)	3.8 (0.0-22.5)	2.9 (0.0-16.8)	3.2 (0.0-16.4)
IgA	0.0 (0.0-1.6)	0.0 (0.0-1.2)	0.0 (0.0-10.5)	0.0 (0.0-0.6)	0.0 (0.0-0.5)	0.0 (0.0-19.8)	0.1 (0.0-54.6)
IgM	0.0 (0.0-7.7)	0.0 (0.0-5.7)	0.3 (0.0-8.9)	0.3 (0.0-1.6)	0.3 (0.0-1.0)	0.2 (0.0-2.1)	0.2 (0.0-2.6)

Table 3: Percentage of tracheobronchial aspirates with detectable IgG, IgA and IgM anti-*E coli* antibody with increasing postnatal age.

Age n samples	Birth (n=28)	Week 1 (n=27)	Week 2 (n=29)	Week 3 (n=21)	Week 4 (n=18)	Week 5 (n=12)	Week 5.5 (n=10)
IgG	90.2	81.4	83.0	76.4	89.1	75.2	70.3
IgA	11.1	18.5	21.4	23.8	33.3	41.7	50.0
IgM	30.4	40.7	62.1	66.7	61.1	58.3	60.0

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Development of local immunity in infants after oral colonisation with non-pathogenic *E.coli*

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In the present study we evaluated the onset of secretory immunity, the information about which is still highly discrepant, by measuring the development of the levels of sIgA and sIgM in the saliva of children from their birth up to six months of age. The second experimental approach was the study of the influence of artificial colonization of children with non-pathogenic strain of E.coli O-83 on the levels of secretory immunoglobulins in children's saliva.

Four groups of healthy children aged 5 days to 6 months were followed. Group 1: bottle-fed children perorally colonized with non-pathogenic strain E.coli O-83, group 2: breast-fed similarly colonized children, group 3: bottle-fed non-colonized children, group 4: breast-fed non-colonized children. The colonization and saliva collection were done as in /1/.

All immunoglobulins, ie. sIgA, sIgM, IgA1, IgA2 and IgG were quantitated by the modified enzyme-linked immunosorbent assay /2/, using polyclonal (Sevac, Prague) or monoclonal /3/ antibodies in the first layer and peroxidase conjugated antisera in the final antibody layer (Sevac). Anti E.coli O-83 antibodies were determined similarly by ELISA, but for coating of the plates, sonicated E.coli O-83 was used.

The amount of secretory immunoglobulins in children's saliva was variable in the age period followed. sIgA was found earlier and in higher levels in breast-fed children (0.041 mg/ml at day 6, 0.185 mg/ml at day 7) than in bottle-fed children, but the highest production of sIgA was found in 11 to 13 weeks in both tested groups. The levels of sIgM were much lower (0.006 mg/ml) in the first week. The highest secretion of sIgM was found earlier than that of sIgA (in 4 - 8 weeks of age) and again it was higher in breast-fed children. The levels of both secretory immunoglobulins decreased simultaneously after weaning.

Total levels of IgA1 and IgA2 were measured at the age 5,6 and 13 weeks only. The levels of IgA1 were several times higher in all the tested samples than the IgA2 levels, without any correlation to the type of feeding. The amount of IgG in saliva was very low, no differences between both groups of children were found.

Microbial colonization is known to play an important role in the development of the secretory immune system /4/. Colonization with E.coli O-83 has a positive effect in prevention and therapy of infantile diarrhea /5/, but the mechanism of its action is not fully understood. We proved that this treatment strongly increased the levels of sIgA in bottle-fed children, but this increase lasted only 5 weeks. At the same time, no effect of colonization on the production of total sIgM was found. Colonization caused significant elevation of specific anti E.coli O-83 antibodies of IgA class (mostly IgA2) and IgM class. This enhanced production of sIgA antibodies, most of all of the IgA2 subclass, could contribute to the protection of the mucosae by their non-specific agglutination of some strains of bacteria /6/. The preferential production of IgA2 antibodies could be the result of the response to the main immunogen of E.coli - lipopolysaccharide, which was shown to induce mainly the IgA2 response in adults/7/.

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Serum and salivary immunoglobulins and food antibodies in normal elderly subjects

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INTRODUCTION

Normal aging is accompanied by diverse alterations in cellular and humoral immunity. Most studies of humoral immunity in elderly humans have been confined to serum. There is little information on secretory immunity in this group. Studies of mixed saliva in elderly subjects have reported normal or increased levels of secretory IgA with an impaired antibody response to an oral antigenic challenge (1).

MATERIALS AND METHODS

We studied 43 normal elderly subjects, 29 women and 14 men, age range: 68-94 years (mean 79.3), and 37 normal younger controls, 23 women and 14 men, age range: 26-50 (mean 36.2), from which a detailed dietary and medical history was obtained.

Pure parotid saliva was collected after 5 min of stimulation by citric acid 5%, given in aliquots of 0.5 ml every 1 min, and using a Carlsson-Crittenden cup placed over the parotid duct. Serum and saliva specimens were frozen at -70°C, until further analysis.

Samples were analyzed for total immunoglobulins: IgA, IgM and IgG; and specific antibodies of IgA, IgM and IgG isotypes to three dietary proteins: gliadin, beta lactoglobulin (BLG) and ovalbumin (OVA), using an enzyme linked immunoassay (ELISA). The results were expressed in μg/ml for immunoglobulins in saliva, and IU/ml in serum; and as a percentage of the optical density from a positive standard serum, for specific antibodies.

The Mann-Whitney U test was used to compare immunoglobulins and antibody levels in both groups.

RESULTS

Salivary immunoglobulins: IgA, IgM and IgG were elevated in the

elderly group ($p<0.01$, $p<0.05$ and $p<0.01$ respectively), with a wide range of values, particularly for IgA.

Salivary antibodies of the IgA isotype to gliadin ($p<0.01$), BLG ($p<0.05$) and OVA ($p<0.05$), were higher in the elderly group. IgG antibodies to gliadin and OVA were also significantly elevated in this group, although such antibodies were undetectable in 24 elderly and 32 control subjects for gliadin, and 11 elderly and 30 controls for OVA. There were no differences in IgM antibody levels.

Serum IgA concentrations were also elevated in the aged group ($p<0.01$), conversely, serum IgM level was decreased ($p<0.01$), and no differences were found in serum IgG.

Finally, there were no significant differences in IgA and IgG food antibodies in serum, between the two groups; whereas serum IgM antibodies to the three dietary protein antigens were lower than the control group.

DISCUSSION

We tried to define the possible alterations in healthy elderly subjects in salivary and serum immunoglobulins and antibody levels to three common dietary proteins, and/or differences in antibody responses in the two compartments.

The results obtained do not exhibit the age-related decline in immunocompetence that has been described in the elderly population (particularly the IgA isotype). Nevertheless, normal or increased immunoglobulin levels do not mean that this group have a normal immune function, and other activities of secretory antibodies (eg. anti-microbial functions) could be impaired. Aberrations in immuno-globulin synthesis, with the appearance of ineffective broken IgA fragments, or unusual amounts of monoclonal immunoglobulins, have to be also considered.

Immunoglobulins and antibody titres in saliva and serum expressed different patterns in accordance with specific and separate regulatory mechanisms which apply to both compartments. Salivary immunoglobulin levels, IgA above all, showed a wide range of values in the elderly group, as compared with the results obtained in younger controls.

Cumulative responses to environmental/dietary antigens, with increased secretion of antibodies, and possibly a delay in the removal process, could explain the high level of IgA antibodies in saliva. Moreover, differences in the amount and nature of the antigenic challenge throughout life, could determine the wide range of values obtained among the elderly group.

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Ontogeny of salivary IgA and IgA subclass antibody to oral *streptococci*

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Introduction

Infants encounter a variety of antigenic challenges to their developing secretory immune systems from microorganisms which colonize mucosal surfaces. Evidence for the capacity for early mucosal immune response is seen in the presence of salivary IgA antibody to *Escherichia coli* and poliovirus in young infants who receive extensive environmental exposures to these microorganisms [1]. Vaccines administered in infancy often elicit a salivary IgA response [2,3]. In addition, infants are exposed to infectious challenges with oral streptococci soon after birth. Different oral streptococcal species colonize the oral cavity sequentially during early life [4]. In this report, we present evidence for early salivary immune responses to organisms occupying oral ecological niches.

Materials and Methods

Whole salivas from infants, three weeks to 54 months of age, were reacted in Western blot analyses with oral streptococcal culture supernatant antigens which had been separated by polyacrylamide gel electrophoresis. *Streptococcus mitis* and *S. salivarius* culture supernatants were selected as antigens since both species had been demonstrated in cultures obtained by oral swabbing these young infants, thus indicating early exposure to antigens of these species. Culture supernatants from *S. mutans* were also tested in Western blot. Mutans streptococci do not colonize the oral cavity until after tooth eruption and are not cultured from the teeth of a majority of children until after two years of age [4]. Secretory IgA fractions were also analyzed for antibody activity in Western blot after gel filtration of several whole salivas on Superose 6 (Pharmacia). Western blots were developed with affinity-purified goat anti-human alpha chain (Zymed) or mouse monoclonal anti-human alpha chain (Tago) reagents.

Results and Discussion

Salivas from infants as young as five weeks of age contained IgA antibody which reacted with components in both culture supernatants. Only the IgA1 subclass was detectable in some of the reactive salivas [5]. Whole salivas and the respective gel filtered fractions which eluted coincident with a SIgA standard and which contained both alpha chain and SC determinants, gave similar reaction patterns in Western blot. Among the infants who were between three and 21 weeks old, a significantly higher frequency ($p<0.02$) of salivas contained IgA antibody which reacted with components in the *S. mitis* (20/22), than with components in the *S. salivarius* (7/17) preparations. Most of these salivas contained IgA antibody which reacted with at least two *S. mitis* components, although the patterns of reactive components exhibited considerable variation among subjects. Nonetheless, a 70-75 kD *S. mitis* component was most often recognized. Patterns developing with culture supernatants from *S. salivarius* were less complex. The principal reactive component was approximately 170 kD. Antibodies to *S. mitis* and *S. salivarius* were never detected before the respective streptococci could be cultured from the infant. However, infection could be demonstrated prior to detectable salivary IgA antibody on some sampling occasions. Mutans streptococci could not be cultured from any of these predentate infants and no reaction was observed when salivas from this group of infants were

incubated with *S. mutans* blotted components.

Longitudinal and cross-sectional measurements of salivary IgA antibody activity to culture supernatant-components of *S. mitis, S. mutans* and *S. sanguis* were made by Western blot in young children who were seven to 54 months of age. All had teeth at the time of saliva collection. Thus each had the requisite sites for colonization of *S. sanguis* and mutans streptococci. The number of components reactive with salivary IgA antibody and the intensity of reaction with salivary IgA antibody increased as the children grew older. For example, some 2-3 year old children's salivas reacted with 10-15 *S. mitis* components. IgA antibody was not detected in the absence of demonstrable infection with the respective streptococcal species, except with two children where salivary IgA antibody weakly reacted with 1-2 mutans streptococcal components prior to the culture of mutans streptococci on selective media.

Antibodies in both IgA1 and IgA2 subclasses were detected in the seven to 54 month old population. Reaction was seen with the 70-75 kD and the 90 kD components of *S. mitis* in nearly all salivas tested. Somewhat less staining occurred with the IgA2 reagent, although most bands could be visualized with either IgA subclass reagent. In certain salivas, quantitative and qualitative differences were seen in the patterns of reaction of bacterial components with the IgA1 versus the IgA2 antibody. These differences in patterns of reactivity did not necessarily reflect the relative concentrations of IgA1 and IgA2 in the respective salivas.

These results indicate that secretory IgA antibody to some oral streptococcal components can be detected in the saliva by the second month of life. During this period, antibody is likely to be elicited by the species of streptococci to which the antibody was detected since 1) antibody was detected only after the respective bacterial species was recovered from the oral cavity, 2) significantly more salivas contained antibody which was reactive with *S. mitis* than with *S. salivarius*, reflecting the proportions of these streptococci in the oral cavity, and 3) no reactions were detected with mutans streptococcal antigens until after 18 months in these children. The heterogeneity observed among the IgA, and the IgA subclass antibody specificities in different salivas to the reference bacterial strains may reflect both the unique antigenic nature of the respective indigenous floras and the differing stages of maturation of the secretory immune response.

Acknowledgements

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Effect of cyclosporin A (Cycl.A) on systemic IgG response to soya protein in the pig at weaning

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Morphological changes and an increase in IELs in the gut associated with weaning in the pig may result from a transient hypersensitivity to dietary antigens (Miller et al., 1984). Arnaud-Bettandier et al. (1986) demonstrated that Cycl.A will prevent small bowel rejection following transplantation in the pig. McDonald and Spencer (1988) also demonstrated that Cycl.A will inhibit a T cell mediated crypt hyperplasia in a human fetal gut explant. The aim of this study was to investigate the effect of Cycl.A on the systemic IgG response to weaning onto a soya based diet and the associated morphological changes in the small intestine.

MATERIALS AND METHODS : Pigs from four sows from the Babraham herd were allocated at 14d of age to four experimental groups:

(1) Weaned (2) Weaned + treated with Cycl.A
(3) Unweaned (4) Unweaned + treated with Cycl.A.

Cycl.A was given orally (25mg/mg/d) in cooking oil at 9.30am each day from 14d age. Non-treated groups received cooking oil only. Groups 1 and 2 were weaned at 21d. Piglets were sacrificed at either 26 or 27d of age. Timed bloodsamples were collected from the anteria vena cava and tissue samples taken from both 25% and 75% along the small intestine. Serum samples were analysed for Cycl.A using a Sandimmun Kit, anti-soya IgG by ELISA and soya proteins by radioimmunoassay. Tissue samples were fixed in phosphate buffered glutaraldehyde and stained with Haematoxylin and Eosin. Morphological measurements were made using an image analysis system (VIDS III).

RESULTS AND DISCUSSION: Serum Cycl.A levels were found to be variable (<50-1800ng/m) rising up to at least 6h after dosing but to be undetectable by 24h, i.e. 1h after dosing on day 27. Groups 1 and 3 were all negative. Only piglets in Group 1 showed a serum anti-soya IgG response indicating that Cycl.A had suppressed the response to soya in group 2. In contrast, serum levels of soya as measured by radioimmunoassay were low or undetectable in Groups 1, 3 and 4. but present in Group 2 (range 19-105ng/ml). The lack of detectable soya in Group 1 is probably an artefact due to interference with the assay by the presence of anti-soya IgG. When samples from Group 1 with anti-soya IgG were mixed either with a 50ng/ml soya standard or with soya containing serum from Group 2, there was a significant depression (range 50-90%) in the soya levels measured by the radioimmunoassay.No difference in crypt depth was observed between any of the groups at either the 25 or 75% level. Unweaned pigs showed a crypt hyperplasia in comparison with previous work: (Miller et al., 1986). Villus height was significantly longer at the 25% level in unweaned pigs and unaffected by Cycl.A in both weaned and unweaned pigs. In contrast Cycl.A treatment was associated at the 75% level with longer villi in

unweaned pigs, yet shorter villi in weaned pigs, and there was no difference between non-treated weaned and unweaned animals. Such a confusing picture prevents any meaningful conclusion as to the effect of Cycl.A upon the morphological changes associated with weaning. Clearly, all the unweaned piglets used in this work did not show classical unweaned morphology suggestive of some prior infection. Interestingly, Bernard et al. (1985) demonstrated that Cycl.A treated pigs responded normally to both rotavirus and TGE infections.

CONCLUSIONS: (1) Cycl A treatment suppressed the systemic anti-soya IgG response to soya in the weaned diet.

(2) The presence of serum anti-soya IgG substantially interfered with the detection of serum soya protein by radioimmunoassay.

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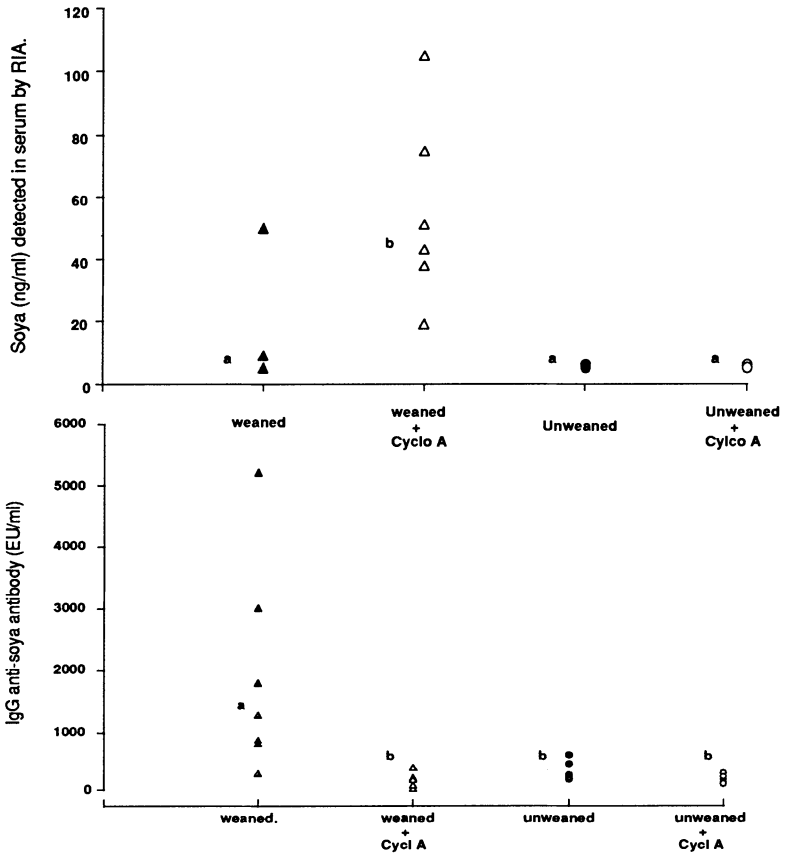

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Development of the intestinal B and T cell compartment in the pig

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INTRODUCTION

The number of intestinal immunoglobulin secreting cells (Ig-SC) of mice rises steeply during the first 3 weeks after weaning (1). In adult mice, the majority of all Ig-SC are located along the small intestine (SI) (2). About 99 percent of these intestinal Ig-SC secrete IgA, but also in the period before weaning IgA predominates among the Ig-SC (1).

Immunohistological studies of the porcine SI revealed that in pigs next to IgA- also high numbers IgM- and IgG-containing cells are found (3). In the first weeks after birth IgM-containing cells predominate in the SI of pigs, while it is even suggested that the SI remains one of the major sites of IgM and IgG production in adult pigs (3).

Therefore, in this study we investigated the kinetics and isotype distribution of the intestinal Ig-SC with 2, 4, 14, and 40 weeks old pigs and the results were compared with those of spleen, mesenteric lymph node (MLN) and Peyers patches (PP).

MATERIALS AND METHODS

Animals: Pigs were selected from the SPF breeding stock of our institute.

Enumeration of Ig-SC: Cell suspensions of the SI and other lymphoïd organs were prepared as described previously (4). Ig-SC in the cell suspensions were quantitated with a Reversed-Spot-Assay (5) by using monoclonal antibodies specific for the porcine Ig-isotypes (6).

RESULTS

Cell suspensions were prepared from dudodenum, jejunum, and ileum of 2, 4, 14, and 40 weeks old SPF pigs. These suspensions were tested for the number of IgM-, IgG-, and IgA-SC to study the development and the isotype distribution of the intesinal Ig-SC response (Figure 1). The number of Ig-SC in the small intestine increased exponentially after 4 weeks of age. Relatively high numbers of IgM-SC were already counted in intestinal cell suspensions of 2 weeks old pigs. After 4 weeks of age

IgA was the predominant isotype produced. The development of the IgA-SC response in ileum lagged behind the development in duodenum and jejunum.

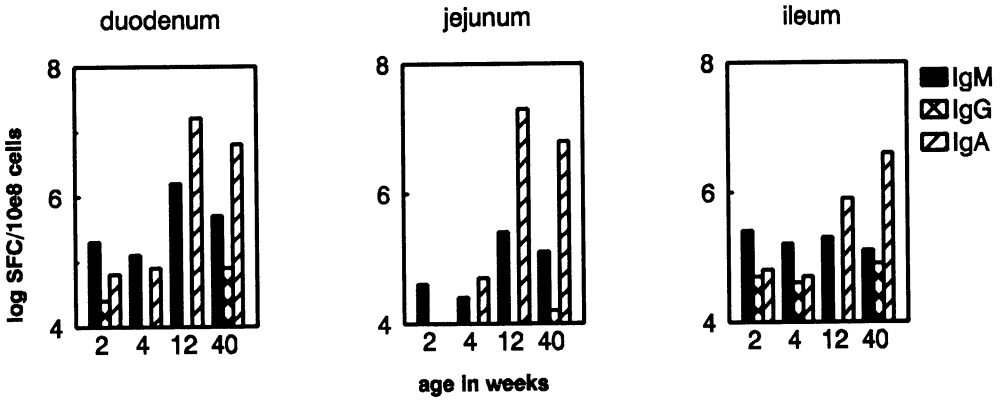

Fig. 1 Number of Ig-SC/10^8 duodenal-, jejunal-, and ileal cells in 2, 4, 14, and 40 weeks old pigs.

DISCUSSION

The results of this study clearly indicate that the mucosal tissues are the major site of "background" Ig-production in the adult pig. The IgM isotype predominates during the first weeks of life, while the "background" Ig-response switches from IgM to IgA between 4 and 12 weeks of age. This is in contrast with the results obtained in mice , where IgA predominates in the intestine from birth (1).

Although, we confirmed with our results the earlier observation of Browne and Bourne (3) that a high number of IgM producing cells are found in the porcine small intestine, we were not able to confirm their conclusion that the porcine small intestine is also the major site of IgG production.

The regulation of the spontaneous Ig production in the porcine small intestine is subject to further experiments in which the development of the number of Ig-SC in the small intestine is investigated in the period around weaning and as a function of immune status of the pig.

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Specificity of IgG pinocytosis in the neonatal piglet

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ABSTRACT. Widdowson reported in 1976 that the intestine of the newborn pig increases in mass and protein content if suckled by the mother. This experiment led us to search for intestinal trophic factors in colostrum. We have repeated Widdowson's observations. There was a visible alteration in the length and diameter of the intestine obtained from piglets suckled for 24 hours. During the first day of life, the weight of the small bowel doubled and the protein increased five-fold. These results confirm Widdowson's 1986 report.

1. Feeding Experiments

In 24-hour feeding experiments, we tested bottle-fed pig colostrum (C), colostral whey (CW), IgG-depleted pig colostral whey (ID), and a cows' milk formula for piglets (Soweena). The bottle-fed (CW) and dialyzed protein produced the same results observed in the suckled pig. ID and Soweena were without effect. SDS-gel electrophoresis of the CW, ID, and the IgG isolated from colostrum was performed. A thiophilic absorption gel was used to remove and isolate the IgG from pig colostrum. The quality of IgG separation and isolation was confirmed by SDS reducing gel electrophoretic patterns from the pig serum. The patterns demonstrated the selective absorptions of IgG in those pigs receiving either purified IgG or IgG-containing colostrum.

In order to increase the number of pigs studied, the feeding period was reduced to 6 hours. The total weight and protein content of small intestine increased in pigs that were fed isolated IgG (IG) and IgG-containing dialyzed (DW) or reconstituted (RC) colostral wheys. The results were similar to those of maternally-fed (MF) piglets. The ID, even when concentrated (CI) to match the protein content of the IgG-containing whey had negligible effects on the weight and protein content of the small intestine.

2. Mucosal Electrophoresis

Intestinal homogenates were collected from the ileums of 6-hour-old pigs fed the diets. The presence of heavy and light chain

immunoglobulin protein fractions contributed high peaks which overshadowed all other peaks in the homogenates.

3. Light Microscopy

Histologic studies confirmed the selective storage of IgG in enterocytes. In the trichrome-stained histologic specimen of the suckled pig intestine at 6 hours, red storage material was seen within individual enterocytes. In contrast, this material was absent in the intestine of the piglet fed CI. Feeding IG resulted in the presence of red inclusions similar to those seen in maternally suckled pigs.

4. Electron Microscopy

EM examination of jejunal mucosa from 24-hour suckled piglets revealed a mixed family of filled vesicles. A similar appearance was observed when DW was fed to newborn piglets for six hours. The electron-dense vesicles were observed when IG, and were absent when ID, wheys were fed for six hours. These vesicles were similar in appearance to the Russell bodies observed in plasma cells.

5. Immunofluorescent Microscopy

Immunofluorescent studies using a rabbit anti-pig polyclonal antibody revealed that the feeding of IG was associated with brush-border binding and enterocyte internalization of the purified IgG. This material was also seen in the vascular space of the lamina propria.

6. Summary

Widdowson's experiment has been confirmed. On the basis of the experimental effects of IgG feedings on intestinal weight, the evidence of large amounts of IgG present in intestinal homogenates, and the reduced trophic response to ID feedings, we conclude that Widdowson's observations in suckled pigs are largely explained by the retention of maternal milk IgG in the enterocytes. Intestinal protein content varied as a function of the nature of dietary protein intake. The increase in small intestinal protein content (relative to newborn intestine), divided by protein intake, is expressed as a percentage of intake. Approximately 20% of the dietary protein was retained in the intestine when DW, IG, and RC wheys were fed. Less than 10% of the ID or CI was fed. This finding suggests a specificity for protein retention related to the presence of immunoglobulin in the diet of the suckled newborn pig.

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A flow cytometric study of activation of lymph node cells at weaning in the rat

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1. Introduction

Weaning in the rat occurs spontaneously from day 15 and extends over 10-14 days with progressive reduction in suckling but increase in consumption of solid food [1]. We have previously shown that maturation of the small intestine at weaning has similar features to an immunological reaction with intestinal crypt hyperplasia, an increase in mesenteric lymph node (MLN) weight, and activation of mucosal mast cells (MMC) that peaks at 22 days of life [2-4]. As these may be mediated by T lymphocytes, we have studied interleukin-2 receptor (IL-2R: T cell activation marker) expression on pooled MLN mononuclear cells using flow cytometry, and compared these to systemic spleen cells.

2. Materials and Methods

DAxPVG^c rats litter pups remained with the dam rat until used at 19, 20, 21, 22, 23 and 25 days of life. At each age interval, 6-9 pups were killed and the MLN and spleens pooled. Mononuclear cells were purified using a Ficoll-Paque gradient which reduced negative labelling to <3% of total cells. Anti-IL-2R (OX-39) and -CD4 (W3/25) monoclonal antibodies were used to label cells in an indirect technique which included double mixing of the two antibodies to determine the proportion of IL-2R positive cells within the CD4 poulation. The results are expressed as the ratio of IL-2R to CD4 cells, as recovery of lymphocytes varied. We found that IL-2R positive cells were exclusively CD4 positive.

3. Results

TABLE. IL-2R expression of MLN and spleen mononuclear cells at progressive age intervals during weaning in the DAxPVG^{c} rat.

	IL-2R/CD4%	
Day	MLN	Spleen
19	7	
20	14	3
21	74	
22	97	8
23	10	40
25	18	

4. Discussion

These results show that MLN mononuclear cells (but not systemic spleen cells) are activated at day 22 and also suggest a reciprocal relationship between MLN and spleen cells. We conclude that activation of MLN cells is closely associated with the events of intestinal maturation and MMC activation.

5. Acknowledgements

We are very grateful to Mr A. Bishop (Department of Human Immunology, Institute of Medical and Veterinary Science, Adelaide) who performed the flow cytometry.

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Influence of the time of weaning on the spontaneous (background) immunoglobulin production in the murine small intestine

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INTRODUCTION

Shortly after birth, the mucosal B cell compartment is only partially developed. Maternal immunity, transferred via placenta or milk from mother to offspring, protects young animals from infectious diseases. The ontogeny of the mucosal B-cell compartment has been studied only partially. The small intestine (SI) of many newborn mammals contains Peyer's patches, which develop in the absence of antigenic stimulation. The newborn intestinal tract contains few plasma cells or intraepithelial lymphocytes and the occurrence of those cells does depend on antigenic stimulation. The effect of maternal antibodies on the development of the B-cell compartment in the SI has not been reported, although circulating maternal antibodies have been reported to suppress not only systemic immune responses, but antigen-specific responses in the intestine before weaning as well. In this paper we describe the influence of the time of weaning on the development of the murine B-cell compartment in the SI.

RESULTS

To study the influence of the time of weaning on the development of Ig-SC half of each litter of C3H/He mice was weaned when 19 days old, while the others were kept with their mothers until 29 days old. At 29 days Ig-SC in SI, spleen, bone marrow, MLN and PP of all mice were quantitated by the protein A plaque-forming cell assay (1,2; Fig. 1). The number of IgA-SC in the SI of early-weaned mice was 90% higher than that of their late-weaned littermates. In MLN and PP the number of IgG- and IgA-SC was about six times higher in early-weaned mice.
The number of Ig-SC in bone marrow differed little between early- and late-weaned mice, although slightly more IgG- and IgA-SC were detected in early-weaned mice. In contrast, the number of IgM- and IgG-SC in the spleen of late-weaned mice was 40% higher than that of their early-weaned littermates.

DISCUSSION

Before weaning hardly any Ig-SC were detected in the SI, whereas spleen and bone marrow already contained a relative high number of Ig-SC at

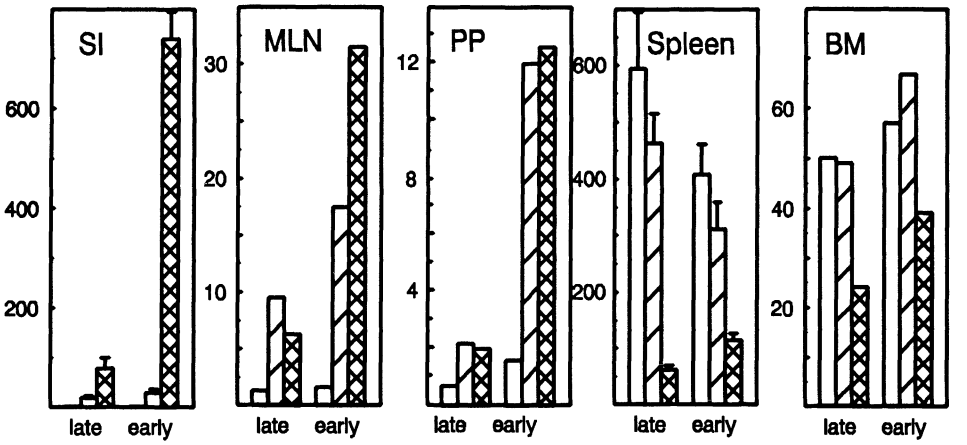

Fig. 1 Number of Ig-SC (x 10^{-3}) in the small intestine (SI), mesenteric lymph nodes (MLN), Peyer's patches (PP), spleen and bone marrow (BM) in 29-days-old late- and early-weaned C3H/He mice.

that moment (1). To study the influence of the age of weaning on the development of Ig-SC, we quantitated the number in the SI and other organs of 29-day-old mice that were either weaned early (at 19 days of age) or not weaned. The late-weaned mice presumably started eating their mothers' diet at day 18 to 20, while continuing to suckle. The results clearly showed that weaning itself and not the age determines the number of Ig-SC in SI, MLN and PP. In contrast, the number of Ig-SC in spleen and bone marrow does not depend on weaning. The number of IgM- and IgG-SC in the spleen of late-weaned mice was even greater than in early-weaned mice. Circulating maternal antibodies suppress the development of specific immune responses in offspring presumably by masking of the antigenic determinants, not only before weaning but after weaning as well (3). Circulating maternal antibodies do not notably affect the development of the "spontaneous" Ig-SC in the spleen or bone marrow. Moreover, the number of Ig-SC in the intestine increases immediately after weaning. Therefore, we conclude that circulating maternal antibodies do not significantly suppress the development of "spontaneous" intestinal Ig-SC as well.

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Maturation of the small intestine at weaning in the nude hypothymic rat

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1. Introduction

Maturation of the rat small intestine has similar features to an immunologically mediated reaction, with intestinal crypt hyperplasia, increased intraepithelial lymphocytes (IEL), eosinophils and mucosal mast cells (MMC), activation of MMC with systemic release of rat mucosal mast cell protease II (RMCPII), and increased interleukin-II receptor (IL-2R: T cell activation marker) expression on mesenteric lymph node (MLN) cells at day 22[1-3].These suggest that intestinal maturation should be delayed in the nude hypothymic rat.

2. Material and Methods

We have therefore investigated intestinal maturation in hypothymic nude (n=8) and heterogygous (n=8) CBH rats at 22 days of life. Immune activity was measured by relative MLN weight, jejunal count of IEL, eosinophils and MMC, serum RMCPII concentration, and immunoperoxidase staining of jejunum for IL2-R expression. The results are expressed as mean±SD.

3. Results

	$CBH^{rnu/-}$ (heterozygote)	$CBH^{rnu/rnu}$ (nude)	(mean±SD)	p
Body weight	39.9±4.9	24.8±3.9	g	<0.0001
Relative MLN weight	0.078±0.016	0.060±0.012	g/100 g	0.033
Villus area	0.276±0.041	0.280±0.069	mm^2	NS
Crypt length	105±10	90±12	µm	NS
Crypt cell production rate	5.9	6.7	cells/crypt/h	NS

	CBH$^{rnu/-}$ (heterozygote)	CBH$^{rnu/rnu}$ (nude)	(mean±SD)	p
IEL	58±29	33±8	/mm	0.06
Eosinophils	78±14	142±56	/mm	0.0006
MMC	14±3	28±7	/mm	0.0003
Serum RMCPII	384±153	447±211	ng/ml	NS
IL-2 receptor staining	normal	increased		

4. Discussion

Intestinal maturation and immune measures were equivalent (or increased) in nude hypothymic rats. A possible explanation is engraftment by maternal milk-derived lymphocytes and activation of these immunocompetent cells in the infant gut [4].

5. References

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Part II
Mucosal Tissues and Organs

SECTION M: SALIVA AND DISEASES OF THE ORAL CAVITY

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Phenotypic and functional analysis of oral mucosal macrophages and lymphocytes: the ins and outs of cell isolation

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1.Introduction

The murine oral mucosa is a complex tissue composed of a keratinized squamous stratified epithelium supported by a dense connective tissue where collagenous, elastic and reticulin fibers trap different structures (nerves, minor salivary glands, blood and lymphatic vessels). Macrophages, T and B lymphocytes are found at different levels in the tissue (1,2). Those cells should play a critical role in the event of any disruption of the integrity of the oral tissue. However, little is known about their functional activities in the mouse oral mucosa. The phenotypic and functional analyses of those cells called for their isolation in single cell suspension. Enzymes would have to be used to digest the intercellular bridges of epithelial cells and loosen the connective tissue. But what about the effect of the isolation procedures on the phenotype and functions of these different cells?

2.Materials and Methods

2.1.ANIMALS

BALB/c and C3H/HeJ male mice, 16-24 weeks old and 6-9 weeks old respectively, were obtained from Charles River Breeding Farms (St-Constant, Québec).

2.2.PREPARATION OF SINGLE CELL SUSPENSIONS AND THEIR PHENOTYPIC AND FUNCTIONAL ANALYSES

In each of these experiments the entire oral mucosa, which includes cheeks and hard and soft palates, was digested following various protocoles described below. Digested tissues were passed through a nylon mesh, and cells were washed (with dispase in some experiments). DNAse was added for a further wash. After oral mucosal excision, splenic and peritoneal cells were treated in the same way as the mucosa, as a control and as a reference for further comparison with the oral mucosa.

Cells were either characterized by immunofluorescence (T cells) or by a modified rosetting assay (macrophages) using fluorescent microspheres according to published methods (1,3).

Lactobacillus murinus was used as a target for phagocytosis by macrophages as described elsewhere (2). YAC-1 cells were used as targets in the NK cell assays while WEHI-164 cells were used as targets in the NC cell assays. Firstly, we measured their activity in the spleen before and after enzymatic treatment. Secondly, we verified the effect of the enzymes on some antigens expressed by splenic NK cells. Complement-dependent cell lysis was used for each of these antigens and the residual NK activity was then measured. NK and NC cell activities were both assayed in the oral mucosa.

3.Results and Conclusions

Collagenase (15U/mg) alone for a 2 to 4 hour treatment gives the best viable cell yield. Collagenase (0.5mg/ml) treatment does not affect the detection of Mac 1, Ia antigens and of Fc receptors on peritoneal and oral macrophages (assumed). Neither cell adherence on glass or plastic nor opsonophagocytosis of bacterial targets were impaired by this enzyme. Hyaluronidase (1mg/ml) and a lower concentration of collagenase (10U/mg) proved to be more efficient in digesting the mucosa than collagenase alone. The use of dispase (2mg/ml,0.75h) in a two-step digestion procedure further increased the viable cell yield by almost 10 %. This two-step digestion procedure (collagenase+ hyaluronidase/3h; dispase+DNAse /0.75h) did not affect either splenic NK or NC cell activity nor the expression of some NK cell markers. NC, but not NK, cell activity was demonstrated in the oral mucosa. However this two-step digestion procedure resulted in a complete destruction of Lyt-2 and L3T4 markers and a net decrease of Thy-1.2 on spleen cells as shown by cytofluorometric analysis. As expected, neither Lyt-2^+ nor L3T4^+ cells were found in similarly treated mucosal cell suspensions. When we used a lower concentration of both collagenase (2.5U/mg) and hyaluronidase (14U/mg) for a 3 hour digestion followed by a pulse of the cell suspension in dispase (0.1h, 50µg/ml) the three T cell surface markers were well preserved on spleen cells. When used on mucosal tissues, the cell yield decreased by about 28%, viability was kept over 90% and the incidence of T cell subsets is similar to that found on 6 µm sections of oral tissue (immunohistology not shown).

We report here the differential effect of enzymatic digestion on macrophages and lymphocytes. From our results we can conclude that:

1. Collagenase alone, at the concentration used, does not affect the phenotype (Ia, MAC1, Ig Fc receptors) nor the functional capacity (opsonophagocytosis, adherence) of peritoneal macrophages.
2. A two-step enzymatic procedure (collagenase+hyaluronidase/3h; dispase +DNAse /0.75h) did not affect NK and NC cell activity in the spleen, nor the expression of some markers on NK cells (NK-2.1, Thy-1.2, AsGM-1 and $H2^k$) as demonstrated by the NK functional inhibition assay.
3. Collagenase, hyaluronidase and dispase all constitute potential damaging agents for the three T cell markers studied (Thy-1.2, Lyt-2.2 and L3T4) as demonstrated by immunofluorescent analysis.
4. FACS profiles obtained after enzymatic digestion indicate a differential susceptibility of T cell markers to enzymatic treatment.
5. The isolation of mucosal cells for phenotypic and functional analysis should therefore be conducted under controlled digestion conditions taking into account the relative density of specific cell surface markers, their differential susceptibility to enzymatic treatment and the sensitivity of the immunodetection method used.

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Contact sensitivity in the murine oral mucosa: an experimental model of delayed type hypersensitivity at mucosal surfaces

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ABSTRACT. Aiming at developing an animal model of delayed type hypertsensitivitty (DTH) reactions at mucosal surfaces, we have examined the potential of the murine oral mucosa to serve as inductive and/or expression site of contact sensitivity reactions. Histological and immunohistochemical analyses of oral mucosa specimens from hapten sensitized and unsensitized animals, as well as adoptive transfer expreiments demonstrate the DTH nature of these reactions. This model should be valuable for studying T cell mediated inflammatory reactions at mucosal surfaces. (Supported by grants from the Swedish Medical Research Council).

1. Introduction

Immune reactions at mucosal surfaces in general, and in the oral mucosa in particular, have essentially been studied in terms of B cell mediated antibody responses, that are typified by secretory IgA. To date, very little is known concerning the potential of mucosal membranes to serve as inductive and/or expression site(s) of effector T cell responses. We have examined the potential of a most accessible mucosa, the buccal mucosa, to seve as inductive and /or expression sites of delayed type contact hypersensitivity (DTH) reactions.

2. Material and methods

2.1. IMMUNIZATON REGIMENS

Animals were sensitized with either oxazolone (OXA) or picryl chloride (TNCB), applied topically on the buccal mucosa (regimen 2) or onto the abdominal skin (regimen 1); one week later, animals were challenged by topical application of either hapten on the buccal mucosa.

2.2. HISTOLOGICAL AND IMMUNOHISTOCHEMICAL EVALUATIONS

Unless otherwise mentioned, the intensity of the reactions in the oral mucosa was evaluated 24 hours after elicitation, by routine histological examinations of fixed and paraffin embedded specimens, and the total number of nucleated cells was determined on an arbitrary unit of tissue. Where indicated, ear skin reactions were evaluated by standard mechanical technique.
The nature of infiltrating cells was determined by enzymeimmunohisto-chemical technique (peroxidase based ABC technique) performed on frozen sections. A paired immunohistoautoradiographic method was also employed to examine the proliferarive activity of infiltrating mononuclear cells.

2.3. ADOPTIVE TRANSFER EXPERIMENTS

Unfractionated, B cell, as well as T cell depleted lymph node cells, or serum, from skin sensitized and cyclophosphamide treated donors were injected into the buccal cheek of naive syngeneic mice; the later recipients were challenged 5 days after transfer with the pertinent hapten. DTH reactions were evaluated histologically as above.

3. Results

3.1. BUCCAL MUCOSA OR EPIDERMAL SENSITIZATION INDUCES HAPTEN SPECIFIC DTH IN THE MURINE ORAL MUCOSA

Regardless of the site of initial sensitization (skin or buccal mucosa) inflammatory cells appeared in the oral mucosa within 2 hrs after buccal elicitation with the pertinent sensitizing hapten (Fig. 1). Mononuclear cell infiltrates peaked by 24 hrs after challenge, comprising mainly

macrophages (Mac 1 + ve) and T cells (of both helper and supressor subsets). *In vivo* incorporation of radiolabelled nucleotide by substantial numbers of macrophages and T cells, although to a lesser extent than by basal keratinocytes, was documented by paired immunohistoautoradiography, demonstrating the proliferative nature of infiltrating cells.

3.2. VALIDATION

Such oral mucosa inflammatory reactions failed to develop in nude mice, in intact unsensitized mice, as well as in animals sensitized with irrelevant hapten. That such reactions could be adoptively transferred by lymph node T cells but not B cells from sensitized animals, but not from unsensitized syngeneic mice nor by immune serum, confirmed the DTH character of these inflammatory responses (Fig. 2).

4 Conclusions

A mouse model has been developed to study DTH reactions induced and/or expressed at oral mucosal sites . The observations that the oral mucosa may serve as inductive site of DTH reactions expressed not only at local mucosal sites but also at remote systemic (skin) sites indicate a marked heterogeneity between various mucosal surfaces, e.g. oral mucosa and gastrointestinal mucosa, in their potential to promote or to downregulate the development of T cell mediated reactions. This model should be valuable for studying the role of effector T cells in the development of inflammatory reactions involving oral mucosal surfaces.

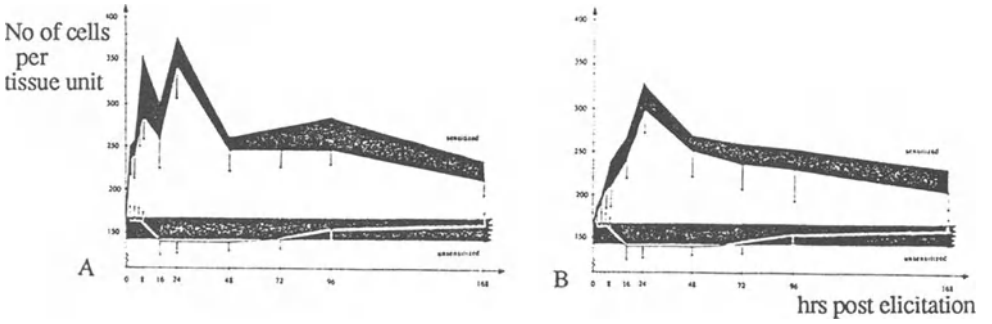

Figure 1. Kinetics of expression of DTH in the buccal mucosa of animals previously immunized at skin (A) and local oral mucosa (B) sites with oxazolone; data are expressed as mean cell counts per unit of tissue and upper shaded areas indicate the contribution of intraepithelial infiltrating cells.

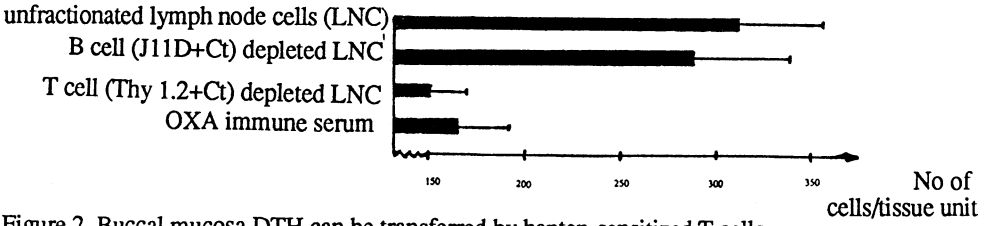

Figure 2. Buccal mucosa DTH can be transferred by hapten-sensitized T cells.

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Langerhans cells in chronic gingivitis and gingival hyperplasia

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INTRODUCTION

Langerhans cells (LC) are dendritic non-epithelial cells which form a network within stratified squamous epithelium. These cells are of stem cell origin and are important antigen gathering and presenting cells. In oral epithelium LCs increase in number as dental plaque accumulates (Walsh *et al.* 1985). The anti-convulsant drug Epanutin predisposes to gingival hyperplasia in patients with poor oral hygiene (Klar, 1973). The mechanism by which gingival overgrowth is initiated is not fully understood although there may be changes in the host response to plaque antigens and thus in the mucosal LC numbers. The present study compares the numbers of LCs in two forms of gingival inflammation, epanutin induced hyperplasia and chronic gingivitis, both before and after corrective therapy.

MATERIALS AND METHODS

In this study 4 patients with epanutin induced gingival hyperplasia were compared with 4 sex and age-matched patients with the common form of gingivitis, chronic gingivitis. At initial presentation and on completion of the hygiene phase of therapy clinical indices of plaque (PLI) (Silness and Löe, 1963) and gingivitis (GI) (Lobene *et al.* 1986) were recorded and a gingival biopsy, 2x2 mm in size, was obtained from the lower anterior labial gingival region of each individual. Frozen sections (6µm) were stained by an immunoperoxidase technique utilizing the monoclonal antibody OKT6 and counterstaining with haematoxylin. The number of LCs were enumerated at x400 magnification (approx. area 0.18mm^2) within 5 fields per specimen.

RESULTS

Table 1 shows the Plaque index (PLI), Gingival index (GI) and Langerhans cell (LC) numbers per unit area for each individual during both initial and post-treatment examinations together with the epanutin induced hyperplasia and gingivitis group mean scores. Differences in PLI, GI and LC numbers before and after treatment were statistically significant ($p<0.05$) in both epanutin induced hyperplasia and chronic gingivitis groups. The clinical indices and LC numbers were greater at the initial examination for the epanutin patients than for the chronic gingivitis patients ($p<0.05$). On completion of treatment the PLI and GI in both groups were not significantly different, however the mean LC numbers remained more than twice as high in

epanutin induced hyperplasia patients over chronic gingivitis patients ($p<0.05$). The epanutin group LC numbers both before and after treatment were approximately twice as high as the gingivitis group.

Table 1. The Plaque index (PLI), Gingival index (GI) and Langerhans cell (LC) numbers per unit area with standard errors for each individual for both initial and post-treatment examinations. The epanutin therapy and chronic gingivitis group means and standard deviations are included.

	INITIAL EXAMINATION			THREE MONTH REASSESSMENT		
SUBJECT	PLI	GI	LC NUMBERS	PLI	GI	LC NUMBERS
EPANUTIN GROUP						
E_1	2.2±0.1	2.8±0.1	14.4±0.6	0.5±0.1	0.3±0.2	3.5±0.3
E_2	2.3±0.4	2.5±0.8	15.2±1.3	0.2±0.1	0.2±0.1	4.2±0.5
E_3	1.9±0.4	2.4±0.2	14.3±0.9	0.3±0.2	0.4±0.1	3.4±1.0
E_4	1.8±0.5	2.6±0.4	13.2±0.6	0.2±0.1	0.2±0.2	2.2±0.3
MEANS (SD)	2.1±0.2	2.6±0.2	14.3±0.8	0.3±0.1	0.3±0.1	3.3±0.8
GINGIVITIS GROUP						
C_1	3.5±0.3	3.6±0.2	6.4±0.4	1.1±0.2	0.5±0.1	2.1±1.2
C_2	2.4±0.2	3.4±0.2	7.6±1.0	0.4±0.2	0.7±0.1	0.9±0.2
C_3	3.1±0.2	3.3±0.2	8.4±0.8	0.9±0.1	0.7±0.1	0.9±0.5
C_4	3.4±0.3	3.0±0.5	8.4±0.7	0.4±0.1	0.3±0.1	1.8±0.6
MEANS (SD)	3.1±0.5	3.3±0.3	7.7±1.0	0.7±0.4	0.6±0.2	1.4±0.6

DISCUSSION

This study suggests that LC numbers are elevated in epanutin induced hyperplasia. This proliferation of LCs may be due to epanutin or may be an indirect consequence of the hyperplasia induced by this drug. Histologically the tissue overgrowth comprises dense collagenous tissue with scattered chronic inflammatory cells and may be a result of the direct effects of epanutin or its metabolites on the gingival tissues (Modeer *et al.* 1982). Alternatively, epanutin may alter the host response to plaque antigens producing an exaggerated response and subsequent hyperplasia. This is supported by the fact that bacterial plaque is an essential aetiological factor (Nuki and Cooper, 1972). An increased response to plaque antigens may explain the large numbers of LCs present in the gingival mucosa and explain why during epanutin therapy, relatively minor amounts of plaque produce marked increases in gingival inflammation and hyperplasia.

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Antigens recognized by salivary antibody in leprosy patients and healthy contacts

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Abstract: The antigenic specificity of serum and salivary IgA in 9 healthy leprosy patients and 11 healthy contacts was investigated by ELISA using whole leprosy bacilli, the M.leprae-specific ML04 epitope of a 35 kDa protein, two serologically immunodominant antigens, a 65 kDa protein and lipoarabinomannan (LAM), and by immunoblotting to mycobacterial extracts. Salivary IgA antibodies were present only in healthy contacts and were specific for LAM and an 18 kDa protein of M.leprae. Serum antibody titres to LAM and the ML04 epitope were higher in patients than in contacts.

1. Introduction

Leprosy may be spread by respiratory droplet transmission and the nasal mucosa has been proposed as the site of initial infection [1]. Immunological tests have demonstrated infection or sensitization to M.leprae in healthy individuals within communities with endemic leprosy [1]. Serum and secretory IgA appear to differ with respect to antigenic specificity, molecular nature, function and relative proportion of the two IgA subclasses. If mucosal immunity plays a significant role in protection against leprosy, identification of the immunodominant antigens would be valuable both diagnostically and in vaccine development [2].

2. Materials and Methods

Antigens: whole gamma-irradiated leprosy bacilli, soluble extracts of Mycobacterium leprae (MLSE) and M.tuberculosis (MTSE) were prepared. LAM and the 65 kDa protein were gifts from Dr. P. Brennan and Dr. J. van Embden.
ELISA [3,4]: Microtitre plates were coated with whole leprosy bacilli (10^7/ml), 65 kDa protein (1 µg/ml), LAM (0.1 µg/ml) or MLSE (10 µg/ml). Non-specific binding was blocked and serial dilutions of saliva or sera added. Direct binding was measured with anti-human IgG or IgA peroxidase conjugates; epitope specific antibody levels were measured by competition for binding to MLSE with the monoclonal antibody MLO4 peroxidase conjugate.
Immunoblotting: MTSE and MLSE were separated into protein bands by SDS-PAGE, blotted on to nitrocellulose and developed with sera (1:100) or saliva (1:2,5,10,25) and anti-human IgG or IgA peroxidase conjugates.

3. Results

Salivary IgA antibody to whole leprosy bacilli was detected in four contacts and four patients. In one contact recently re-exposed to leprosy, salivary IgA antibody to LAM was demonstrated. Neither patients nor contacts had salivary IgA antibody to the 65 kDa protein. Two healthy contacts, both of whom had been working with leprosy patients for at least eight years, had salivary IgA antibody binding to an 18 kDa band of MLSE with no homologous band in the MTSE immunoblots. No IgA antibody binding to distinct bands of MLSE nor MTSE was present in saliva from patients with leprosy. Antibody to the ML04 epitope was not detectable in saliva from patients or controls.

In sera, both patients (5/9) and contacts (4/11) demonstrated an IgA response to the 65 kDa protein. Antibody titres (IgA) to LAM (30-40 kDa) were higher in patients (Log_5 mean$\pm$SD; 1.32 ± 0.94) than in controls (0.83 ± 1.14). Immunoblotting demonstrated an IgA response to the 33-36 kDa region of MLSE in two patients and no contacts. Although the antibody competition assay does not distinguish the class of immunoglobulin, antibody titres to the M.leprae-specific epitope ML04 of a 35 kDa protein were present in 4/9 patients and not in controls. An IgA antibody response to the 10-14 kDa region of MLSE and MTSE was observed in three healthy contacts (all laboratory technicians) and all patients.

4. Discussion

Levels of salivary IgA antibody have been shown to be higher in healthy contacts of leprosy patients [3]. This study has demonstrated salivary IgA antibody to LAM and an 18 kDa protein of M.leprae only in contacts of leprosy patients; IgA antibody to the serologically immundominant protein antigen (65 kDa) was not found in saliva. An 18 kDa protein with an epitope (L5) restricted to M.leprae has been described [5] and would merit study in the measurement of salivary IgA antibody. Mucosal IgA response to these specificities might be protective against leprosy and should be further explored.

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Regulation of antibodies to food proteins within the common mucosal immune system: lack of correlation between antibody titres in saliva and intestinal fluid

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Previous workers have studied saliva on the assumption that immunological changes reflect events in other gastrointestinal secretions. We tested this hypothesis by comparing total immunoglobulin levels and specific antibody titres in saliva and intestinal fluid obtained by gut lavage from 20 healthy subjects. An ELISA technique was used to measure total immunoglobulins and and specific antibody levels of the IgA, IgG, and IgM isotypes to three common food proteins, gliadin, ovalbumin and beta-lactoglobulin. No correlations between saliva and lavage fluid concentrations of IgA or IgM existed, although a positive correlation was found for IgG which was present in trace amounts in the secretions. Antibody titres in saliva and intestinal fluid did not correlate for any isotype. However in the case of IgM, comparison of saliva with serum, and lavage with serum showed significant positive correlations. This study suggests that saliva does not reflect immune events in intestinal secretions, that the gut must be studied directly, and that regulation of IgM antibody may not be segregated with respect to mucosal and systemic compartments, in contrast to IgA antibodies as is clearly demonstrated.

Introduction

Study of the gastrointestinal mucosal immune system in man has been hindered by practical and ethical problems of access. Investigators have tended to use in vitro studies of isolated tissue specimens obtained from patients at endoscopy or operation which may not be representative. The secretory aspects of gut mucosal immunity have been relatively neglected. The existence of a common mucosal immune system suggests that other mucosal secretions may reflect immunoglobulin and specific antibody status in gut secretions. To determine if parotid saliva is

representative of gastrointestinal secretions, we assayed immunoglobulins and antibodies in serum, saliva, and intestinal fluid obtained by whole gut lavage.

Materials and methods

Twenty subjects were recruited for the study, and comprised healthy volunteers and patients with an eventual diagnosis of functional abdominal problems. The subjects were given the PEG-electrolyte solution (osmolality 260 mosmol/L) and asked to drink at a rate of 1 litre per hour, to a total of 4 litres consumed. The stools were discarded until a clear fluid was passed. Approximately 100-200mls were then collected and immediately treated with soybean trypsin inhibitor, EDTA, phenylmethylsulphonylfluoride, sodium azide and fetal calf serum to inactivate proteases and to prepare a suitable fluid for analysis. This was aliquoted and stored at -70^{0c} until assay. Assay of total IgA, IgG, and IgM, and isotype-specific antibodies of these classes to three food protein antigens, gliadin, ovalbumin, and beta-lactoglobulin was carried out using an ELISA technique. The results for antibody levels were expressed as a percentage of a high positive control.

Results

No correlations existed for antibody titres to the food antigens between saliva and intestinal lavage in any isotype. Antibody titres of the IgM isotype in saliva and intestinal fluid were reflected by serum antibody levels (saliva vs serum antigliadin $r=0.31$, $p=0.011$, $n=20$; antiova $r=0.49$, $p=0.001$, $n=18$; lavage vs serum antiova $r=0.6$, $p=0.013$, $n=9$). Concentrations of total IgG were weakly correlated in saliva and intestinal fluid ($r=0.452$, $p=0.03$), but no such relationship existed for IgA or IgM. There was no correlation in either fluid between total immunoglobulin levels and specific antibody titres.

Discussion

Despite the existence of a common mucosal immune system, this study failed to find any correlation in healthy subjects between two mucosal secretions for either specific antibody titres, or total immunoglobulin concentrations with the exception of IgG. We feel that although antigen-specific effector and memory cells can be found throughout the mucosal immune system after antigen challenge, that final antibody levels may be largely determined by local antigen concentrations. This study suggests that saliva does not reflect immune events in intestinal secretions, that the gut must be studied directly, and that regulation of IgM antibody may not be segregated with respect to mucosal and systemic compartments, in contrast to IgA antibodies as is clearly demonstrated.

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Thyroid influence on the salivary secretory immune system

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INTRODUCTION

Thyroid hormones are known to play an integral role in the regulation of cellular dynamics and immune function of both lymphoid tissue and intestinal mucosa[1–3]. Because these hormones also stimulate the growth and differentiation of the submandibular gland[4], the present study sought to determine whether the thyroid influences the expression of the salivary secretory immune system. For comparison, studies were also conducted to assess the impact of hypothyroidism on IgA levels in tears, bile, serum and intestinal secretions.

MATERIALS AND METHODS

Adult female Sprague-Dawley rats were obtained from Forsyth Dental Center or Zivic-Miller Laboratories. Hypothyroidism was induced either by feeding animals diets supplemented with 0.02% propylthiouracil (PTU) or by performing thyroidectomy with parathyroid transplant (Zivic-Miller). Control rats were fed isocaloric diets without PTU enrichment or were subjected to sham thyroid/parathyroid surgery. The success of PTU treatment or thyroidectomy was verified by measurement of serum triiodothyronine and/or thyroxine levels.

Saliva was collected for a period of 15 minutes following pilocarpine (0.5 mg/kg) injection, as previously described[5]. Tears, upper intestinal secretions and serum were obtained according to published techniques[5,6]. Bile was collected through duct cannulation for a 5 minute interval. Submandibular glands were removed and homogenized for the preparation of cytosol. For immunizations, thyroidectomized or control rats were administered primary and secondary injections of glucosyltransferase (GTF; isolated from *S.sobrinus*; 7) in the vicinity of the salivary gland by reported procedures[8].

Concentrations of IgA, IgG and secretory component (SC) were analyzed by radial immunodiffusion or by radioimmunoassay[5,9,10]. Specific IgA antibodies to GTF were evaluated by enzyme-linked immunosorbent assay (ELISA; 8).

RESULTS

Effect of PTU-induced hypothyroidism on IgA, IgG and/or SC levels in saliva and the submandibular gland

To determine the influence of drug-induced hypothyroidism on the salivary secretory immune system, rats (n - 6–7/treatment group) were fed diets with or without (control) PTU supplementation; after 6 weeks of this dietary regimen, salivas and submandibular glands were collected and tested for IgA, IgG and/or SC content. As shown in Table 1, the concentration and secretion rate of IgA and IgG were significantly ($p<0.05$) reduced in saliva of PTU-treated rats, as compared to levels in control animals. These salivary effects were paralleled by a significant ($p<0.05$) decrease in IgA (C = 4,160 ± 1,800 μg IgA/g tissue; PTU = 1,013 ± 259 μg IgA/g tissue, but not IgG or SC, content in submandibular glands of hypothyroid rats.

Table 1: Influence of PTU-induced hypothyroidism on salivary IgA and IgG levels

Treatment	*IgA (μg/ml)*	*IgA (μg/min)*	*IgF (μg/ml)*	*IgF (μg/min)*
Control	137 ± 27	3.0 ± 0.3	127 ± 52	2.7 ± 1.0
PTU	67 ± 12*	1.9 ± 0.3	31 ± 10*	0.8 ± 0.3*

Saliva was obtained 6 weeks after feeding rats diets with or without (control) 0.02% PTU. Numbers equal the mean ± SE. * Significantly ($p < 0.05$) less than control

Impact of thyroidectomy on SC and/or IgA levels in saliva, tears, bile, serum and intestinal secretions

To establish whether PTU-induced alterations in the salivary secretory immune system were due to hypothyroidism, as compared to specific drug effects, female rats (n = 11–13/treatment group) underwent thyroidectomy (T) and parathyroid transplant or sham surgery (C). Saliva, as well as tears, bile, serum and intestinal secretions (by luminal flush with saline), were collected 2.5, 3.5 and/or 50 weeks after surgery. Thyroid ablation had no influence on IgA or SC content in the various mucosal fluids. In contrast, removal of the thyroid gland did result in a significant ($p<0.01$) increase in serum IgA concentrations (3.5 weks post surgery: C = 256 ± 15 μg IgA/ml; T = 1,674 ±462 μg IgA/ml).

Influence of thyroid absence on the salivary and serum IgA immune response to GTF

To examine the effects of hypothyroidism on the salivary immune response to a defined antigen, GTF was injected into the salivary gland vicinity of thyroidectomized or sham-operated rats (n = 8–10/group). Primary immunizations (day 0) were performed 5.3 months after surgery and secondary challenges were administered on day 109. Extirpation of the thyroid had no consistent influence on the salivary (Table 2) or serum IgA antibody response to GTF exposure, as compared to reactions in control animals.

Table 2: Effect of thyroidectomy on the salivary IgA antibody response to GTF

	IgA (ELISA units)/ml					
Treatment	*Day 12*	*Day 21*	*Day 109*	*Day 119*	*Day 130*	*Day 195*
Control	7 ± 1	5 ± 1	8 ± 1	50 ± 24	34 ± 17	6 ± 1
Thyroidectomy	13 ± 3	4 ± 1	9 ± 3	44 ± 23	55 ± 31	7 ± 2

Thyroidectomized and sham-operated rats (n = 8–10/group received primary and secondary GTF injections on days 0 and 109. Numbers represent the mean ± SE.

DISCUSSION

These findings indicate that PTU suppresses the salivary mucosal immune system. However, this effect appears to be drug-, not thyroid-, associated. Of interest, others have also demonstrated that PTU interferes with immune function[11]. Removal of the thyroid does not seem to modify salivary secretory immunity.

ACKNOWLEDGEMENTS

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The variability of immunoglobulins and albumin in saliva of normal and IgA-deficient adults

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1. Introduction

This study investigated the daily variability of salivary immunoglobulins and albumin within- and between-subjects and the influence of fasting, flow rate stimulation, age, sex and IgA-deficiency on the concentrations and detection rates of IgA, IgG, IgM and albumin.

2. Materials and Methods

Salivary IgA, IgG, IgM and albumin were measured by electroimmunodiffusion as previously described (Gleeson M. *et al.*, Aust NZ J Med, 1982, 12, p255) in whole mixed saliva collected from healthy subjects aged 5-75 years (n=99). Statistical analyses were performed on log transformed data.

3. Results

3.1 *Variability of Salivary Immunoglobulins and Albumin*

The concentrations within-subjects varied between-days by a factor of 2.53 for IgA, 3.14 for IgG and 3.12 for albumin. The variance between-subjects was significantly greater than the variance within-subjects ($p<0.001$). No significant differences were observed between levels in saliva of samples collected mid-morning and mid-afternoon. There were no significant age ($p>0.10$) or sex effects ($p>0.10$) and there was no significant interaction of age with sex ($p>0.10$) in any of the proteins studied.

3.2 *Effects of Fasting and Stimulation*

The levels of salivary IgA and albumin and the detection of IgM in fasting non-stimulated samples were significantly higher than the

levels in either fasting-stimulated or post-prandial samples ($p<0.001$). The levels of IgA were also significantly higher in fasting-stimulated samples compared to post-prandial samples ($0.02<p<0.05$).

3.3 *Effect of IgA-Deficiency*

The proportion of samples with detectable levels of IgG and IgM was significantly greater in the IgA-deficient subjects compared with normal subjects ($x^2=101.70$, df=1, $p<0.001$ for IgG; $x^2=44.08$, df=1, $p<0.001$ for IgM). The concentrations of IgG and IgM were significantly higher in the IgA-deficient subjects ($0.025<p<0.05$ and $p<0.0005$, respectively), however, albumin concentrations were not significantly different ($p>0.40$).

3.4 *Reference Ranges*

Reference ranges and detection rates (DR) are presented for immunoglobulins and albumin in unstimulated saliva collected from fasting (n=26), non-fasting (n=99) and IgA-deficient subjects (n=26).

Subjects	IgA		IgG		IgM		Albumin	
	(mg/l)	(DR)	(mg/l)	(DR)	(mg/l)	(DR)	(mg/l)	(DR)
Fasting	25-600	100%	<108	42%	<19	69%	19-370	100%
Non-Fasting	9-125	100%	<25	40%	<8	17%	9-112	100%
IgA-Deficient	-	0%	<56	77%	<40	88%	7-169	100%

4. Conclusions

The results of this investigation indicate that standardised collection conditions are required for the assessment of mucosal immunocompetence using salivary immunoglobulins. In order to maximise the power of detection of significant differences between study groups the use of non-fasting/non-stimulated collections of saliva is recommended.

The between-subject variances of each protein were significantly greater than the within-subject variances and validates the use of single sample collections from each subject for most simple comparisons. The levels on average varied within-subject by a factor of three for IgA, IgG and albumin and results from isolated collections should be considered in this context.

The results indicated that there were no significant influences of age or sex on the levels of salivary immunoglobulins in adults and children over the age of 5 years. Both IgM and IgG were detected in saliva more frequently and in significantly higher amounts in the IgA-deficient subjects, suggesting that in the salivary glands both classes of immunoglobulins compensate for the lack of SIgA.

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IgM and IgG levels in serum and saliva do not correlate to susceptibility of upper respiratory tract infections of HLA in individuals with selective IgA deficiency

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ABSTRACT. Serum and unstimulated whole saliva samples were collected from 63 IgA deficient (IgAd) individuals and the total levels of IgA, IgG and IgM were determined. The immunoglobulin levels were compared with human leucocyte antigens (HLA), episodes of upper respiratory tract infections and the intake of antibiotics prescribed for upper respiratory tract infections. Healthy IgAd individuals did not have increased concentrations of serum IgM or IgG compared to infectious-prone patients. Nor was there any correlation found between proneness to infections and HLA antigens or between serum IgM or IgG levels and HLA antigens in this patient material.

1. Introduction

Selective IgA deficiency is the most common form of immunodeficiency in Sweden [1] and the frequency of upper respiratory tract infections is increased in 30-36% of the IgAd individuals. Low or no detectable levels of serum IgA has been reported [1, 2], with normal or increased concentrations of IgM and IgG. Certain HLA antigens are found in an increased frequency in IgAd individuals and have been suggested to be a weak association with the infection rate [3].

The present study was undertaken to determine immunoglobulin in healthy and infectious prone IgAd individuals and the possible influence of HLA on antibody levels.

2. Materials and Methods

2.1 PATIENTS. Sera were collected from sixty-three individuals with selective IgAd with levels $\leq$0.02 g/l in serum. One of the IgAd investigated donors also had a concomitant IgG subclass deficiency. Fifty-eight IgAd individuals were tissue typed (HLA) for A/B and forty-six persons for HLA-DR. Saliva samples were collected from sixty-three individuals all lacking secretory IgA (sIgA) in saliva (<0.01 mg/l). In all IgAd individuals there was no apparent infection at the time of sampling.

A clinical questionnaire with special emphasis on incidence of upper respiratory tract infections was completed by forty-six IgAd individuals. The upper respiratory tract infection days were divided into five different groups (0 days, 1-7 days, 8-15 days, 16-30 days and $\geq$ 31 days). Forty-five IgAd individuals completed the part of the questionnaire related to antibiotic therapy ordinated for upper respiratory tract infections and they were divided into three groups (untreated, 1-3 and $\geq$ 4 antibiotic treatments).

2.2 SERUM AND SALIVA COLLECTION, TISSUE TYPING AND ANALYSIS. Determination in detail has been described elsewhere [4].

2.3 STATISTICAL COMPARISON. The Mann-Whitney test and Spearman correlation was used for the statistical analysis.

3. Results

The patients were subdivided according to number of infectious episodes during the year in which the serum and saliva samples were taken. As can be seen in Fig. 1 there was no significant correlation between salivary and serum IgM and IgG. No correlation between infection rate and levels of IgG or IgM in serum (Fig. 2) was found, nor was there any correlation when the data was pooled together into two groups, 0-7 days and $\geq$8 days. Statistical analysis of the number of courses of antibiotic treatment, necessitated by upper respiratory tract infection and serum levels of IgG and IgM showed no significant correlations (Fig. 3). Nor was there any correlation when the data were pooled into two groups, untreated and $\geq$1 antibiotic treat-

ments. Furthermore, there was no significant difference of IgG or IgM in serum between the individual HLA antigens A28/non-A28, B8/non-B8, B40/non-B40 or DR3/nonDR3 (data not shown). Individual HLA antigens were also compared to the number of antibiotic treatments and days with upper respiratory tract infection and no significant difference was found (data not shown).

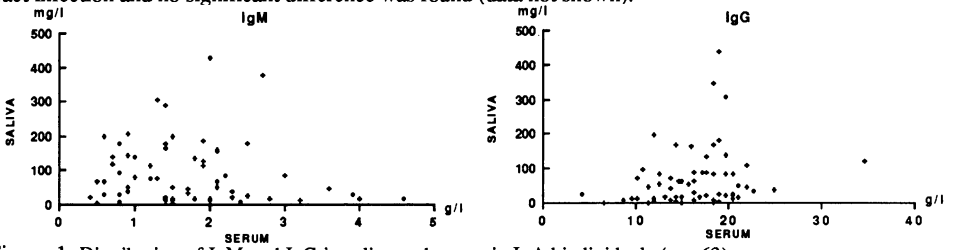

Figure 1. Distribution of IgM and IgG in saliva and serum in IgAd individuals (n = 63).

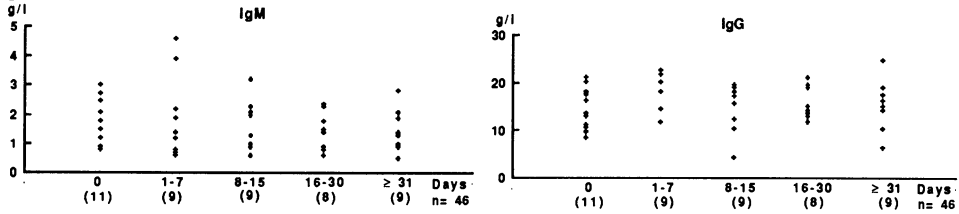

Figure 2. Serum levels of IgM and IgG in IgAd individuals according to the number of upper respiratory tract infection days.

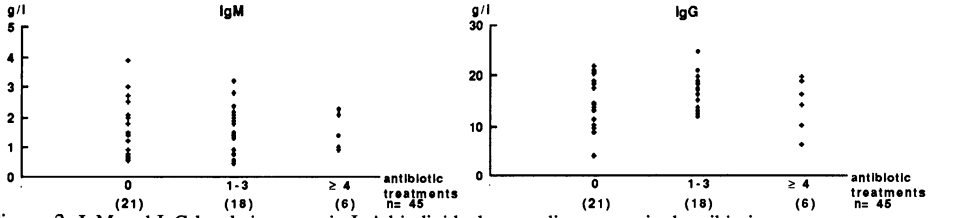

Figure 3. IgM and IgG levels in serum in IgAd individuals according to required antibiotic treatments.

4. Discussion

Individuals with IgAd normally lack IgA in both serum and secretions and around two-thirds of the IgAd individuals show no increased frequency of upper respiratory tract infections. Healthy IgAd individuals showed no compensatory rise in salivary [5] or serum IgM as compared to infectious-prone IgAd individuals. Increased levels of IgM and IgG has been reported in saliva [5], but was not found in serum in the same patient material. Nor was there any correlation between proneness to infections and HLA, or between IgM and IgG in serum and saliva, or between serum IgM and IgG and the different HLA antigens [3], in this patient-material. In agreement to previous study our data confirm the high frequency of HLA-B8/DR3 in IgAd individuals [3, 5] as compared to a normal population. Determination of antigen-specific antibodies may be a more informative then measuring the total levels of different immunoglobulin classes.

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Mucosal immunodeficiency in smokers is reversible and dose-dependant

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Cigarette smoking influences the risk of aero-alimentary disease in both protective (ulcerative colitis) and inductive (squamous tumours of pharynx, larynx, and oral cavity) roles. In order to study the effects of smoking on mucosal immunity, salivary immunoglobulins were measured in pure parotid saliva from groups of healthy non-smokers, ex-smokers, and current smokers. Smokers had significantly lower salivary IgA and higher IgM concentrations than did non-smokers. The effect on IgA was dose-related, and reversible after cessation of smoking.

INTRODUCTION

Despite strong evidence that smoking has a role in certain inflammatory and neoplastic aero-alimentary diseases, the influence of smoking on mucosal humoral immunity has been neglected. We measured salivary immunoglobulins in pure parotid saliva obtained from tee-total subjects in order to investigate a possible effect of smoking on mucosal immunity.

METHODS

Parotid saliva was collected from 30 healthy non-smokers, 47 current smokers, and 39 ex-smokers in Cairo using Carlsson-Crittenden cups with a simple modification. Salivary flow was stimulated by 2 mls of 5% citric acid and stored at -70^{o}C until analysis. ELISA was used to assay total IgA, IgG, and IgM.

RESULTS

In healthy individuals smoking strikingly decreased salivary IgA ($p=<0.00001$), and increased IgM ($p=<0.00001$, figure 1). There was a strong inverse correlation between salivary IgA levels and the number of cigarettes smoked per day ($r= -0.52$, $p<0.00001$, figure 2): no relationship was apparent for IgM or IgG. After 2 years of stopping smoking IgA levels had increased, and by 5 years were similar to

non-smokers. The effects on IgM were less consistent, with a return to normal levels after 2 years but higher concentrations at 5 years.

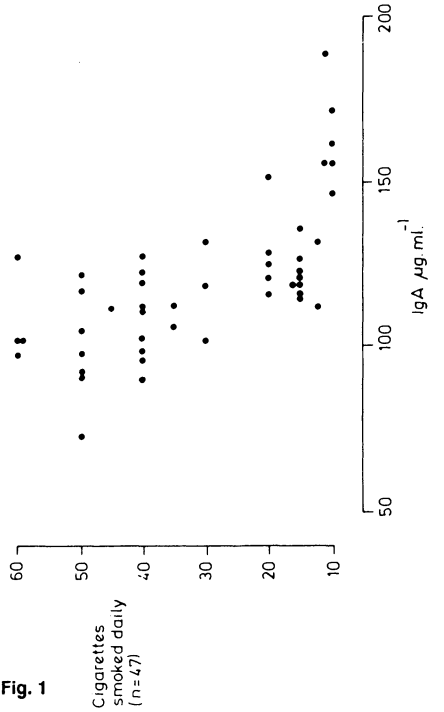

Fig. 1

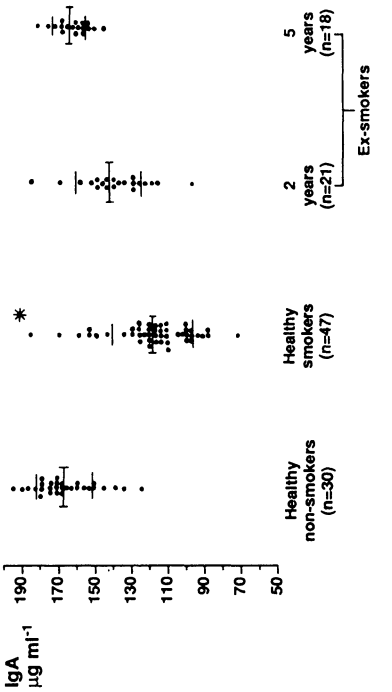

Fig. 2

DISCUSSION

Identical and striking alterations in IgA and IgM concentrations in two different groups of smokers provides strong evidence that cigarette smoking does exert an effect on mucosal immunity. The suppression of IgA in smokers seems to be strongly dose-dependant and reversible, and this adds credence to the evidence of a smoking-induced effect. That many smokers drink alcohol to excess often confounds clear interpretation of studies on cigarette smoking: fortunately the Egyptian subjects were largely non-drinkers, thereby removing that particular problem. We have not yet studied the mechanism by which smoking alters the immune profile of saliva; intuitively we favour the concept that smoking impairs T cell regulation of B cell differentiation and maturation, although other studies suggest that effects on antigen presenting cells may be important. If changes in secretory immunoglobulins reflect abnormalities of other components of mucosal immunity, new avenues of the pathogenesis of smoking-related diseases may emerge.

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Detection of rotavirus specific antibodies in saliva

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INTRODUCTION

We have previously demonstrated production of secretory antibodies against a systemic pathogen, measles virus [1]. With many oral vaccines now being tested, a simple and efficient method for determination of immunity to mucosal pathogens is needed, especially for very small children. Since the antibody response in saliva may reflect stimulation of the mucosal immune system by antigen presentation in the Peyer's patches, and since saliva is easily obtained even from infants, we wished to determine whether the presence of salivary antibodies may be indicative of protective immunity in rotavirus infections.

MATERIALS AND METHODS

The subjects in this study were residents of a kibbutz in southern Israel (children and adults) in which there occurred two sequential outbreaks of rotavirus gastroenteritis (GE), caused by serotypes 3 and 1, respectively. A description of the outbreaks has been published [2].

Acute and convalescent whole saliva, serum, and stool samples were collected from patients with GE and their close associates. Saliva samples collected by expectoration from subjects aged > 3 years (regular samples) were tested at a dilution of 1:10 in PBS. From children less than three years of age, saliva was collected by saturation of sterile cotton swabs and elution into 0.4 ml of PBS. The swab eluate was tested at a further dilution of 1:4.

Rotavirus-specific (rv) antibodies in saliva were detected by radioimmunoassay of saliva samples applied to commercial rotavirus antigen and control antigen. Saliva samples were tested in quadruplicate or in triplicate with bound antibodies detected by 125-I labeled rabbit anti-human IgA (Dakopatts). The net ratio of cpm bound to viral antigen and control antigen was determined (binding ratio, BR). A binding ratio of >1.36 was considered indicative of the presence of rv antibodies. Rotavirus antigen in stool was detected with the Rotavirus ELISA kit (Dakopatts). Rv IgM and IgA in serum and rv IgA in stools were determined by an ELISA developed in our laboratory [3]. Rv IgG in serum was determined by an immunoperoxidase assay previously described [4]. The presence of viral antigen in stool, rv IgA in stool, rv IgA or

IgM in serum, or a significant change in rv IgG titer in serum was considered confirmation of rotavirus infection.

RESULTS

Among subjects with confirmed rotavirus infection, symptomatic or asymptomatic, 77% (24/31) had BR≥1.36. Persons over age 3 with symptomatic rotavirus infection had positive saliva samples (10/10), as did most symptomatic children under age 3 (6/7). Older asymptomatically infected persons usually had positive saliva samples (8/11), but asymptomatics under age 3 were negative (3 children).

The breakdown of subjects with 'negative' BRs (<1.36) was: 11 (61%) were close associates who were not ill and had no evidence of infection; 5 (28%) were not ill, but did have evidence of infection; and 2 (11%) were ill, but had no evidence for rotavirus infection.

Table 1 shows, for persons present in both outbreaks from whom samples were available, the correlation of illness in the second with saliva BRs above or below 1.36 in the first.

Table 1. Correlation of illness in the second outbreak with BR above or below 1.36 in the first.

BR in Outbreak 1	Age	Ill in Outbreak 2	
		number	%
>1.36	>3	3/20	15
	<3	3/ 7	43
	all	6/27	22
<1.36	>3	3/ 9	33
	<3	0/ 5	0
	all	3/14	21

CONCLUSION

We conclude that rotavirus specific antibodies may be detectable in nearly all patients with rv GE, and in about half of asymptomatically infected persons. The appearance of rotavirus antibodies in saliva does not seem to be predictive of protection from symptomatic infection one year later, at least in the case of serotype 3 followed by serotype 1. The relationship between the appearance of mucosal antibody and protection from mucosal pathogens merits further investigation.

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Validity of salivary secretions for the assessment of mucosal immunocompetence

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1. Introduction

This study investigated the daily variation of immunoglobulin and albumin levels in saliva of children and the correlation between daily IgA and albumin levels. The effects of age and upper respiratory tract infection (URTI) were also investigated.

2. Materials and Methods

Unstimulated whole saliva was collected on consecutive days from 33 children, for periods ranging from 16 to 26 days. Age categories were defined as: 0.5-1 yr (n=12), 2-3 yr (n=9) and 4-5.5 yr (n=12). URTI periods were defined as the period of apparent symptoms (runny nose, fever and sore throat). Salivary IgA, IgG, IgM and albumin concentrations were measured by electroimmunodiffusion as previously described (Gleeson M. *et al.*, Aust NZ J Med, 1982, 12, p255).

3. Results

3.1 *Salivary IgA*

The between-subject variance for IgA (0.11) was 2.8 times the within-subject variance (0.04). The within-subject median IgA levels increased with age (Spearman rank correlation [SRC]=0.71, n=32, $p<0.001$) as did the interquartile ranges for each child (SRC=0.61, n=32, $p<0.001$). Elevations in IgA levels in URTI commenced 1-4 days ($\bar{x}=2.2$) after the appearance of symptoms and peaked at 2-6 days ($\bar{x}=4.6$). Geometric mean IgA levels within-subjects were consistently higher during URTI compared to non-infection periods. The effect was statistically significant only in the oldest age class

(z=2.5, p=0.013). There were significant correlations between IgA levels for samples collected one day ($\bar{r}[1]=0.09$, $p<0.05$) and three days ($\bar{r}[3]=0.05$, $p<0.05$) apart. Auto-correlations were significantly greater during URTI compared with periods of non-infection (U=69, $0.02<p<0.05$).

3.2 Salivary Albumin

The between-subject variance for albumin (0.046) was 1.3 times the within-subject variance (0.034). The within-subject median albumin levels increased with age (SRC=0.51, n=33, $0.01<p<0.01$) as did the interquartile ranges for each child (SRC=0.45, n=33, $0.01<p<0.02$). The geometric mean albumin levels within-child did not differ significantly between periods of infection and non-infection (z=1.3, p=0.196). There were significant correlations between albumin levels for samples collected one day ($\bar{r}[1]=0.14$, $p<0.01$) and two days ($\bar{r}[2]=0.12$, $p<0.05$) apart. This effect declined with increasing age (SRC=-0.434, n=33, $p<0.02$, for $\bar{r}[1]$).

3.3 Salivary IgG

IgG was detected in only 30% of samples and increased significantly with age ($x^2=6.33$, df=2, $p<0.025$). IgG was detected more frequently in samples during URTI than during non-infection periods, however, the association was not statistically significant ($x^2=3.33$, df=1, $p>0.05$).

3.4 Salivary IgM

IgM was detected in only 15% of samples and increased significantly with age ($x^2=4.03$, df=2, $p<0.05$). There was no consistent or statistically significant association with infection symptoms ($x^2=0.027$, df=1, $p<0.75$).

4. Conclusions

The larger between-subject than within-subject variances of IgA and albumin are important in statistical comparisons between study groups and validates the use of single sample collections from each subject for most simple comparisons. The geometric mean albumin levels within-subjects did not differ significantly between periods of infection and non-infection, indicating that the increase in salivary IgA levels and detection of IgG were due to locally produced antibodies and not serum-derived. The autocorrelation data indicates that in serial studies of mucosal immunity in children, samples collected within 3 days of each other cannot be viewed as independent samples. The significantly higher auto-correlation between IgA levels in samples during infections compared to non-infection periods may be a reflection of a more persistent and constant stimulus during infections.

SECTION N:
OCULAR SYSTEM

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Optimising the expression of antibody in tears: manipulation of the common mucosal immune response?

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ABSTRACT. The IgA in rat tears (200ug/ml) is supplied almost entirely by plasma cells in the lachrymal gland. We researched methods to elicit IgA responses in tears, with the aim of optimising vaccination protocols. Serum and tear responses after ocular topical (OT) or gastrointestinal (GI) immunisations with a particulate antigen (DNP coupled to Pneumococcus) were examined over several months. Though responses in serum (IgM/IgG) and bile (IgA) were similar, highest titres in tears were elicited by OT stimulation. Primary GI immunisation generated specific tear antibody, but also relatively downregulated IgA produced after subsequent OT doses. Addition of cholera toxin to the GI antigen doses did not overcome this effect, though it elevated anti-DNP antibody in both serum and bile. It was concluded that local stimulation of ocular tissue gives rise to cells in the gland which are more effective at promoting tear IgA than those arising after immunisation at a remote mucosal site.

1. INTRODUCTION

As with other secretions associated with the mucosae, the predominant immunoglobulin in tears is IgA. Secretory IgA occurs in normal rat tears in high concentration (ca. 200 ug/ml), and has been shown to be contributed almost entirely by plasma cells residing in the lachrymal gland [1,2]. Strategies to increase specific IgA in tears must therefore logically concentrate on means of raising the numbers of IgA plasma cells in the gland. We have compared: a) immunisation via ocular topical application of antigen, to directly stimulate eye-associated lymphoid tissue, and b) immunisation of the gut mucosae, in order to supply cells to the lachrymal gland via the 'common mucosal immune system' [3], so leading indirectly to IgA secretion in tears. Our

previous results indicated that OT stimulation was much more effective at eliciting IgA in tears than GI immunisation [4]. This result was discouraging in the context of vaccine development against ocular pathogens, since it is easier to envisage a safe vaccine given *via* the gut than one which has to be applied to the eye to be effective. However, we thought it possible that an initial GI dose may just as effectively prime the system for subsequent antigen encountered OT, and also that increasing the GI response might correspondingly enhance the tear IgA levels. This approach is examined in the experiments described here.

2. MATERIALS AND METHODS

Female Fischer 344 rats were used throughout. All methods have been fully described previously [4]. Briefly, rats were immunised with killed *Streptococcus pneumoniae* (0.5mg) coupled to DNP (DNP-Pn). Antigen was applied either ocular-topically (OT) by dropping 5ul of antigen suspension directly onto the surface of each eye, or by gastric intubation (GI) in 500ul. In both cases, antigen was given equally over three consecutive days. Samples were obtained before immunisation and twice weekly thereafter. Blood (1ml) was taken from the tail, and tears (10-12ul), collected by capillary pipettes from both eyes, were expelled into 50ul PBS/1% BSA. Pools of serum and tears were made per group and all samples stored at -20 C until assay at the end of the experiment. Bile samples were collected on selected days, by cannulation of the common bile duct, and assayed individually.

Anti-DNP IgA, IgG and IgM was measured in samples by radioimmunoassay [4], using DNP.BSA adsorbed to PVC plates, and iodinated rabbit anti-rat H chain reagents, against an anti-DNP serum standard for IgM and IgG, and bile for IgA.

In certain experiments, Cholera toxin (Sigma Chemicals) was added to the GI doses at 10ug or 50ug as indicated in the Results section; in this case the DNP-Pn was suspended in 0.2M sodium bicarbonate.

3. RESULTS

3.1. GI priming combined with secondary or tertiary OT stimulation

DNP-Pn was given to 2 groups of 7 rats. While both groups were given the first antigen dose GI, Gp A received secondary and tertiary doses *via* the OT route, while Gp B received a secondary GI and a tertiary OT dose. The secondary and tertiary anti-DNP responses were followed in serum and

tears. In general, the serum IgM and IgG responses were somewhat higher in Gp B than in Gp A (not shown). In tears, neither group responded to antigen as well as in previous experiments [4] where antigen was only given OT. However, the tear IgA responses were higher for Gp A than for Gp B (Fig. 1). Moreover, the sum tertiary IgA response in Gp B was virtually the same as the secondary IgA response for Gp A even though both groups had received their first OT doses at these respective times. In fact, since it was of longer duration, averaged on a daily basis, the tertiary Gp B response was actually under 70% of the secondary and totalled around 50% of the tertiary Gp A response. It seemed therefore that giving a prior dose of antigen GI tended to moderate the tear IgA response to OT stimulation.

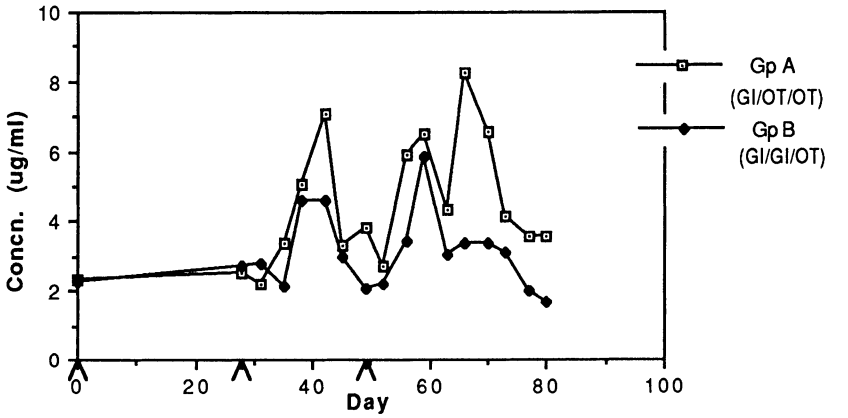

Fig. 1 IgA anti-DNP in tears of 2 groups of 7 rats immunised as shown in inset. First days of antigen administration are arrowed.

3.2.1 Tear IgA after cholera toxin addition to GI doses

Cholera toxin (CT) has been reported to be an excellent adjuvant for gut immune responses to other antigens [5]. We therefore tested whether adding CT to the GI doses of DNP-Pn would increase the appearance of IgA anti-DNP in tears. Two different doses (10ug and 50ug) of CT were added to DNP-Pn and administered as indicated in Fig. 2.

Serum IgM and IgG and tear IgA was followed in secondary and tertiary responses. It was found that, despite a rather late primary response in some Gp A rats, observed in both serum IgG (not shown) and tear IgA, the Gp A tear response was lower than Gps B or C. In the secondary response, Gps B and C appear similar. However, by the tertiary response, Gp C rats were responding substantially less than Gp B rats which received less CT in the primary antigen dose.

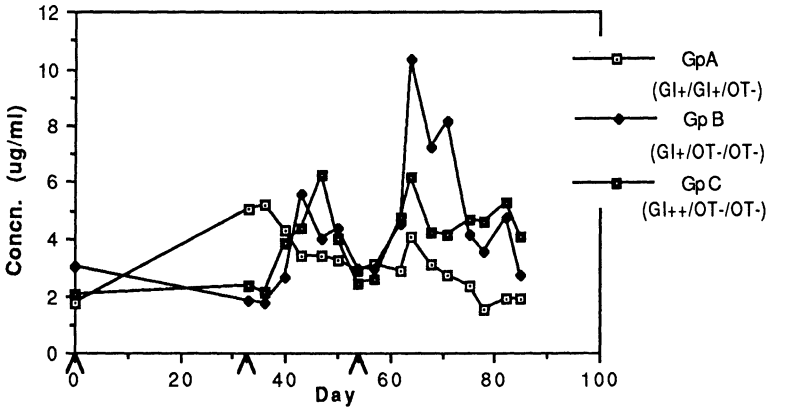

Fig. 2. IgA tear responses in 3 groups of at least 7 rats, immunised at shown in the inset, with 0.5mg DNP-Pn plus 10ug CT (+), 50ug CT (++) or no CT (-).

3.2.2 Anti-DNP response in the bile of CT treated rats

Again using CT added to DNP-Pn, IgA anti-DNP was measured in bile of 4 groups of 6 rats, on days observed previously to be at the peak of the secondary response, immunised as indicated in Table 1. The amount of IgA produced was greatest in the rats receiving the most CT.

TABLE 1. IgA anti-DNP responses in bile

Group	Immunisation protocol[a]	Days post 2° immunisation 14	15
A	GI+ / GI-	12.2 ± 5.8	23.8 ± 14.5
B	GI+ / GI+	32.2 ± 31.4	15.5 ± 10.6
C	GI+ / OT-	9.0 ± 5.0	13.3 ± 9.5
D	GI- / OT-	7.8 ± 3.1	11.0 ± 7.0

a 2 doses of DNP-Pn (0.5mg) with (+) or without (-) 10ug CT
b ug/ml IgA anti-DNP in bile; mean of 4 - 6 rats

These two experiments showed that CT increased the IgA titres local to the gut, but had no such effect on the remote site (tear) response. It thus seems that increasing stimulation of the gut leads to less IgA in tears compared with titres achieved after doses given only OT.

4. DISCUSSION

It should be held in mind that, whereas GI doses of antigen tend to reach only the gut, antigen administered to the eye in the manner described here is not similarly confined; using radiolabelled particulate antigen (unpublished data), we have estimated that as much as 50% of an OT dose may filter down the naso-lacrimal canal to the GI tract. Nevertheless, we have found that OT application elicits the better IgA response in tears compared to GI immunisation. Indeed, the more often the GI response was stimulated, the lower the IgA response in tears appeared to be. The addition of Cholera toxin to GI administered DNP-Pn, while increasing gut responses as evidenced by bile IgA titres, certainly did not improve this situation and may in fact have worsened a later response. Earlier work on the use of CT [5] suggested it acted as an adjuvant for gut immune responses, and recently published data suggests that it promotes downstream isotype commitment of surface-IgM positive B cells independently of T cell or macrophage activity [6]. Our findings concur, since they confirm the adjuvant effect of CT for anti-DNP IgA responses but suggest that this is a local effect only. It seems that although immunisation via the gut does lead the the expression of IgA in tears, a better response is promoted by local application of antigen, and thus the results suggest that whatever cells traffic to the lachrymal gland from the gut need to encounter further factors most efficiently stimulated by local immunisation and already resident in the lachrymal gland itself.

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Impact of antigenic exposure on the developmental appearance of IgA, IgG and IgM-containing cells in the lacrimal gland

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INTRODUCTION

Pronounced changes occur in the secretory immune system of the eye during postnatal development [1,2]. These alterations include a dramatic increase in the IgA-containing cell population of the lacrimal gland [2], which is the principal tissue involved in ocular mucosal immunity [3]. However, whether this immunological maturation is influenced by antigen presence on the surface of the eye remains to be established.

The objective of the present investigation was to determine whether local antigenic stimulation might play a role in the postnatal appearance of Ig-containing cells in the lacrimal gland. Towards that end, we: 1) analyzed the temporal accumulation of IgA-, IgG- and IgM-containing lymphocytes in lacrimal tissue during development; 2) examined whether prevention of antigenic exposure to the ocular surface by unilateral tarsorraphy might inhibit lymphocyte immigration into the ipsilateral lacrimal gland; and 3) assessed whether a non-invasive antigen, after placement on the ocular surface of pre-weanling rats, undergoes retrograde transfer to the lacrimal gland.

MATERIALS AND METHODS

Male Sprague-Dawley rats were obtained from Zivic-Miller Laboratories, Inc. and experimentally utilized at 6, 10, 12, 15, 18, 21, 24 and 27 days of age. Pups were separated from foster mothers at 22 days of age. Animals were housed in constant temperature rooms with light/dark intervals of 12 hours length. When indicated, tarsorrhaphies were performed on the left eye of 10 day old rats; right eyelids underwent sham surgery. These operations were conducted by the surgical staff at Zivic-Miller and their success was verified by trypan blue dye exclusion at the time of sacrifice.

To permit the immunofluorescent identification of IgA-, IgG- and IgM-containing cells, left and right lacrimal glands were removed, weighed and fixed in an ethanol-glacial acetic acid solution (19:1) at 4°C. After fixation, tissues were dehydrated, embedded in paraffin and cut into 5 μm sections. Tissue sections were transferred to gelatin coated slides, deparaffinized and stained with specific immunological reagents as previously described [2,4]. For quantitative analysis of Ig-containing cells, 2-4 sections/tissue and approximately 30 microscopic (312.5 x magnification) fields/tissue were examined with a Zeiss fluorescence microscope. To calculate the total number of IgA-, IgG- and IgM-containing cells in lacrimal glands, reported correction factors were employed [2].

To evaluate antigenic clearance from the ocular surface, tears were removed from both eyes of 18 day old rats and replaced with a solution (3 μl/eye) containing ^{125}I-monomeric albumin (97% precipitable by trichloroacetic acid), unlabeled bovine serum albumin (30 mg/ml) and 0.15M sodium phosphate buffer, pH 7.5. Inclusion of unlabeled albumin served to compensate for the loss of tear proteins (typical concentration > 30 mg/ml). Two hours following ocular radiolabel exposure, tears, lacrimal glands, lids, globes, stomach and blood were collected and processed for radioactivity measurements. Statistical analysis of the data was performed with Student's t test.

RESULTS

To determine the temporal appearance of IgA-, IgG- and IgM-containing cells in the lacrimal gland during postnatal development, right and left lacrimal tissues were obtained from 6, 10, 12, 15, 18, 21, 24 and 27 day old male rats (n = 5-6/age) and examined by immunofluorescent microscopy [5]. IgA-containing cells began to appear in lacrimal tissue between 10 to 12 days after birth, followed by a slight , but significant ($p < 0.01$), accumulation by 15 days. At this age eyelids opened, which event served as a prelude to respective 10- and 2-fold increases in the total number of lacrimal IgA-containing cells by days 18 and 21 (Table 1). The number of IgA-positive cells plateaued in lacrimal tissue from days 21 to 28. The pattern of IgM-containing cell accumulation was analagous to that of IgA. IgG-containing cells were not evident until 18 days of age and cell densities/microscopic field did not vary over the next 10 days. The accumulation of Ig-containing cells during development was identical in both left and right lacrimal glands. Moreover, IgA-

positive cells were the predominant cell type, equaling 76 to 100% of the total Ig-containing cell population in lacrimal tissue from 12 to 28 days.

Table 1. Effect of age on the total number of Ig--containing cells in the lacrimal gland

	Total Ig-Containing cells (x 10^4)/Lacrimal Gland		
Age (days)	IgA	IgM	IgG
15	0.333 ± 0.096	0.057 ± 0.017	0
18	3.595 ± 0.615 **	0.385 ± 0.083 **	0.044 ± 0.042
21	8.045 ± 1.322 **	0.871 ± 0.176 *	0.136 ± 0.035

Lacrimal glands were obtained from age-matched rats (n = 5-6/group, 2 tissues/rat) and results from both tissues were combined. Numbers equal the mean ± SE. Significantly ($p < 0.05$) * or ($p < 0.005$) ** greater than value of previous age group. Data from [5].

To evaluate whether prevention of antigenic exposure to the ocular surface might reduce the accumulation of Ig-containing cells in the lacrimal gland, the closed left lids of 10 day old rats (n = 6) were kept shut by unilateral tarsorrhaphy; right lids underwent sham-operations and opened during the experimental time course. Eight days after surgery, lacrimal glands were processed for immunofluorescence [5]. The tarsorrhaphy-induced inhibition of antigen access to the left eye had no effect on the Ig-conaining cell populations in the ipsilateral gland: the total number of IgA-, IgM- and IgG-containing cells in both left and right lacrimal glands were the same.

To examine whether non-invasive antigens might undergo retrograde transfer from the ocular surface to the lacrimal gland, ^{125}I-monomeric albumin (560,000 cpm/eye) was placed on each eye of 18 day old rats (n = 9) [5]. Within two hours after radiolabel exposure, antigen was almost completely cleared from the ocular surface: only 0.2% of the iodinated albumin was present in individual tear samples. Analysis of exorbital lacrimal glands demonstrated no significant accumulation of radioactivity. Less than 0.05% of the administered dose was associated with lacrimal tissue, which counts could possibly be accounted for by uptake of free ^{125}I from the blood. Lid tissues accumulated 33,261 ± 5908 cpm, which amount was 36-fold higher than that radioactivity associated with globes. However, other experiments with adult rats [5] indicated that lid and globe counts were due primarily to superficial adsorption. Of interest, over 12% of the radiolabel was found in the stomach. It should be noted that clearance of ^{125}I-labeled albumin from the ocular surface could not be explained by deiodination, because tear samples did not exhibit deiodinase activity [5].

DISCUSSION

These experiments demonstrate that a rapid development of the rat lacrimal secretory immune system occurs between 15 and 21 days of age. This process does not appear to be dependent upon local antigenic exposure. Furthermore, our results indicate that a non-invasive antigen, when applied to the ocular surface, does not undergo retrograde transfer to the lacrimal gland. Instead, antigen appears to be cleared chiefly through the gastrointestinal tract.

ACKNOWLEDGEMENTS

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Hormonal influence on autoimmune expression in lacrimal tissue of MRL/Mp-lpr/lpr and NZB/NZW F1 mice

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INTRODUCTION

Autoimmune disorders often exhibit gender-related differences, with estrogens increasing disease severity in females and androgens decreasing immunopathology in males [1]. In fact, androgen administration has been utilized to significantly diminish autoimmune symptoms in animal models of systemic lupus erythematosus, thyroiditis, polyarthritis and myasthenia gravis [1]. Quite possibly, androgen therapy may also provide an effective treatment for Sjogren's syndrome. This syndrome is an autoimmune disorder observed primarily in females and is characterized lymphocytic infiltration in the lacrimal gland, disruption of acinar and ductal tissues and keratoconjunctivitis sicca [2]. Because androgens modulate lacrimal gland immunity and regulate lacrimal structure and function [3], these hormones may have therapeutic value. Therefore, the objective of the current experiments was to determine, by using mouse models of Sjogren's syndrome, whether androgen administration might ameliorate autoimmune sequelae in the lacrimal gland.

MATERIALS AND METHODS

Age-matched female MRL/Mp-lpr/lpr mice (MRL/lpr; 4-5 months old; n = 6-11 mice/treatment group) and NZB/NZW F1 (F1; 6 months old; n = 8-10 mice/treatment group) were administered subcutaneous implants of placebo- or testosterone (10 mg)-containing pellets after the onset of disease. These pellets were designed for slow release of physiological amounts of hormone. Lacrimal glands, and for comparison, submandibular glands, were collected from sacrificed mice immediately prior to androgen administration (pre-treatment controls) and following 34 (MRL/lpr) or 51 (F1) days of maintained hormone exposure. Tissues were fixed in 10% buffered formalin, embedded in historesin, cut into 3 μm sections (4 sections/gland) and stained with H&E. Sections were examined with a Zeiss image analysis system to quantitatively determine the percentage of lymphocyte infiltration (% = [area of lymphocyte infiltration/area of entire section] x 100), as well as to measure acinar area and density. Data was statistically analyzed by using Student's t test.

RESULTS [4,5]

Prior to the initiation of androgen therapy (pretreatment controls), lacrimal glands of F1 and MRL/lpr mice showed distinct differences in disease severity. Tissues from MRL/lpr animals contained extensive areas of lymphoid infiltrates, whereas F1 mice harboured significantly fewer lymphocyte foci and smaller infiltrates (Table 1). This differential autoimmune expression, which has been previously described [6], reflects the earlier onset and accelerated disease of MRL/lpr mice [7]. However, as shown in Table 1, the magnitude of this lymphocyte infiltration increased significantly in placebo-treated controls of both strains during the time course of this study. In contrast, testosterone administration dramatically reduced lymphocyte infiltration in lacrimal tissue: following 34 days of treatment, the percentage infiltrate had undergone a 14-fold decrease in glands of MRL/lpr mice. Similarly, androgen exposure in F1 mice caused a 25-fold reduction in the extent of lymphocyte infiltration in lacrimal tissue. These hormone actions involved significant abrogations in both infiltrate size and number. Moreover, no evidence of fibrosis or destruction of acinar and ductal tissues was found in glands of testosterone-treated mice. Of interest, androgen exposure also induced a significant 2-4 fold rise in lacrimal gland weight and/or acinar area and a 2-fold reduction in acinar density/field, compared to values in placebo-treated controls. Hormone effects on tissue weight could not account for the associated reduction in percentage lymphocyte infiltration.

With regard to the submandibular gland, androgen administration significantly decreased the extent of lymphocyte infiltration in both MRL/lpr and F1 mice, as compared to that found in placebo-treated controls. This action also involved a diminution in the density and area of individual infiltrates.

DISCUSSION

Our results demonstrated that androgen therapy has a significant influence on autoimmune expression in MRL/lpr and F1 mice. Testosterone exposure caused a precipitous decrease in the number and area of lymphocyte infiltrates, as well as the total extent of lymphocyte infiltration, in both lacrimal and submandibular glands. Moreover, no evidence of fibrosis or glandular destruction was found in lacrimal or submandibular tissue following androgen administration.

The similarity of hormone action in both strains of mice was intriguing, given the known differences between MRL/lpr and F1 mice in disease immunopathology and glandular lymphocyte populations [7,8,9]. One possible mechanism to account for androgen effects may involve hormonal regulation of Ia expression, which is increased in exocrine glands during autoimmune disease and may be susceptible to androgen control [1].

In summary, our findings indicate that androgens ameliorate autoimmune symptoms in lacrimal and submandibular glands in mouse models of Sjogren's syndrome.

Table 1. Influence of testosterone administration on autoimmune expression in lacrimal glands of MRL/lpr and F1 mice

Treatment	Number of Lymphocyte Foci/Tissue Section	Focal Infiltrate Area (x 10^4 μm^2)	Lymphocyte Infiltration (%)
MRL/lpr			
Pretreatment	6.8 ± 0.6	22.1 ± 1.6	14.7 ± 2.2
Placebo	8.3 ± 0.4 †	20.3 ± 1.2	21.9 ± 1.3 †
Testosterone	3.6 ± 0.5 **	6.1 ± 0.5 **	1.6 ± 0.3 **
F1			
Pretreatment	1.4 ± 0.3	5.5 ± 0.6	0.9 ± 0.2
Placebo	3.1 ± 0.7 †	13.7 ± 1.9 †	4.9 ± 1.3 †
Testosterone	1.0 ± 0.3 *	2.7 ± 0.3 **	0.2 ± 0.1 **

Mice (n = 6-11/group) were administered testosterone (10 mg)- or placebo-containing pellets and lacrimal glands were collected 34 (MRL/lpr) or 51 (F1) days after hormone treatment. Numbers equal the mean ± SE. Significantly ($p < 0.05$) † lower or ($p < 0.05$) * higher than pretreatment control; Significantly ($p < 0.005$) ** less than pretreatment and placebo controls. Data from [4,5].

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Endocrine and neuropeptide control of secretory component synthesis by acinar cells from the lacrimal gland

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INTRODUCTION

Recently, it has been demonstrated that androgens regulate the secretory immune system of the eye [1]. These steroid hormones modulate the secretion of IgA and secretory component (SC) by the lacrimal gland and stimulate the accumulation of IgA antibodies and SC in tears. This hormone effect, however, is significantly reduced in diabetic animals [2] or by interruption of the hypothalamic-pituitary axis [3]; these latter findings suggest that androgen action may be modified by insulin or substances of neuroendocrine origin. To better understand the control of the ocular mucosal immunity, the current studies were designed to investigate the potential role of neural and endocrine factors in the regulation of androgen-induced SC synthesis by acinar cells from the lacrimal gland.

MATERIALS AND METHODS

Animals: Six week old male Sprague-Dawley rats were purchased from Zivic-Miller Laboratories.

Cell culture and SC analysis: Lacrimal glands were removed from CO_2-euthanized male rats and acinar cells were isolated as previously described [4]. Acinar cells were cultured for 4 days on reconstituted basement membranes (Matrigel) in media containing defined supplements and specified hormones or peptides [5,6]. Media SC levels were measured by radioimmunoassay [7].

Hormones and peptides: Vasoactive intestinal peptide (VIP), substance P, somatostatin, alpha-endorphin, beta-endorphin, methionine-enkephalin, leucine-enkephalin, oxytocin and arginine-vasopressin were purchased from Boehringer-Mannheim. Insulin, 8-bromoadenosine-3'5'-cyclic monophosphate (cAMP), 8-bromoguanosine-3'5'-cyclic monophosphate (cGMP), 5 alpha-dihydro-testosterone (DHT), alpha-melanocyte stimulating hormone (MSH) and melatonin were obtained from Sigma. Adrenocorticotrophic hormone (ACTH) and cholera toxin (CT) were purchased from Calbiochem-Behring.

Insulin binding assay: Binding of insulin to the surface of isolated acinar cells was measured by a method modified from that described by Kelleher *et al* [8]. Suspended acinar cells (2×10^6 cells per sample in a total volume of 0.25 ml) were incubated for 90 min at 20°C with radiolabeled insulin (^{125}I-insulin) in the presence or absence of excess unlabeled insulin. Quadruplicate samples were filtered to separate free from bound insulin. Specific binding was calculated as the difference between total (without) and non-specific (with excess unlabeled insulin) binding.

RESULTS

Influence of endocrine and neural factors on the androgen regulation of SC synthesis by acinar cells [6]

In experiments designed to examine the influence of various hormones and neuropeptides on the androgen control of SC synthesis, acinar cells (2×10^6) were cultured on Matrigel for 4 days in defined, supplemented media. Exposure to DHT significantly increased cellular SC output, as compared to that of control cultures (Table 1). The magnitude of this effect was markedly enhanced by the addition of VIP to culture media; in fact, VIP caused heightened SC production by both control and androgen-treated cells (Table 1). In contrast, incubation of cells with substance P, somatostatin, met-enkephalin, leu-enkephalin, alpha-endorphin, beta-endorphin, ACTH, melatonin, MSH, oxytocin or arginine-vasopressin (1 μM) had no influence on media SC levels in vehicle- or DHT-containing cultures.

To determine whether VIP action might be mediated through effects on cyclic AMP, acinar cells were exposed to cAMP or cholera toxin, which increases intracellular cAMP levels, in the presence or absence of DHT. As shown in Table 1, cholera toxin and cAMP stimulated enhanced SC synthesis by both control and DHT-treated cells. This effect was not duplicated by inclusion of cGMP (2×10^{-4}M) in the culture media. These results indicate that intracellular concentrations of the second messenger cAMP may be important in regulation of SC output by acinar cells.

Effect of insulin on the androgen control of SC production by acinar cells [5]

To explore the possible role of insulin in androgen-induced SC synthesis, initial studies involved culture of lacrimal gland acinar cells in media containing reduced (1/4 concentration) or recommended levels of "ITS" (Sigma; 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenous acid). Results demonstrated that cultures containing decreased 'ITS" levels produced at least 2-fold less SC/cell in response to DHT (1 μM), than did those with recommended "ITS" concentrations. Additional experiments, which selectively deleted insulin, transferrin or selenous acid from culture media, identified insulin as the important component of "ITS": insulin absence in culture media significantly ($p < 0.05$) diminished the DHT stimulation of acinar cell SC output, as compared to cultures with 5 μg/ml insulin.

To evaluate whether insulin receptors might be present on the surface of acinar cells, cells were incubated with ^{125}I-insulin with or without excess unlabeled insulin. Results showed specific cellular binding of insulin, which was dependent upon cell density, time of incubation and media pH. This insulin-acinar cell association was similar to that found in other cell types [5,8].

Table 1. Effect of VIP, CT and cAMP on androgen-mediated SC synthesis

	Media SC (ng)			
	Media	+ VIP	+ cAMP	+ CT
Control	123 ± 7	227 ± 11^	320 ± 18^	293 ± 35^
DHT	378 ± 32 *	570 ± 19 *~	515 ± 16 *~	692 ± 60 *~

Acinar cells (n = 5 cultures/group) were cultured with or without DHT (10^{-6}M), VIP (10^{-6}M), cAMP (2 x 10^{-4}M) or CT (10 μg/ml).^ Significantly ($p < 0.0005$) higher than 'media' control; * Significantly ($p < 0.01$) greater than corresponding control group; ~ Significantly ($p < 0.01$) higher than 'DHT' control.

DISCUSSION

The present results demonstrate that VIP enhances both constitutive and androgen-stimulated SC synthesis by lacrimal gland acinar cells. The mechanism underlying this effect may relate to VIP's known action on cAMP, because cAMP also increased acinar cell SC output. These findings indicate that VIP may play an important role in SC production and IgA transport in the lacrimal gland. This contention is supported by the presence of VIP-containing nerve terminals surrounding acinar cells in this tissue [9]. Our results also showed that insulin binds to acinar cells and modulates androgen control of SC synthesis. Future studies will further analyze these and possible VIP binding sites to better explain the relationship among the nervous, endocrine and ocular mucosal immune systems.

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Neural–immune interactions: role of ocular innervation in the secretory immune system of the lacrimal gland

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INTRODUCTION

An elaborate interrelationship is known to exist between the nervous and immune systems [1,2]. In fact, research has shown that direct neural innervation of lymphoid tissue and small intestinal mucosa, coupled with neurotransmitter release, has a profound impact on the immigration, distribution and function of lymphocytes, including IgA-positive cells [3,4]. Given this neural-immune interaction, the current study was designed to evaluate whether ocular nerves might influence the distribution, density and output of IgA-containing cells in the lacrimal gland. Experiments focused upon the role of the: 1) tempero-facial nerve division; 2) superior cervical ganglion, which serves as the primary source of sympathetic nerves to lacrimal tissue and reportedly modulates the IgA cell number in this gland [5]; and 3) optic nerves, because light is known to regulate T cell function within the eye [6].

MATERIALS AND METHODS

Adult male Sprague-Dawley rats (6 weeks old) were purchased from Zivic-Miller Laboratories, Inc., and housed in controlled temperature rooms with light/dark intervals of 12 hours length. Animals underwent one of the following surgical protocols: ablation of the superior cervical ganglion or denervation of the tempero-facial nerve division or optic nerve. Ganglionectomies and denervations were performed on one side and sham-operations on the contralateral side. Additional groups of rats were exposed to bilateral sham or complete optic nerve disruption. All surgical procedures were performed by surgeons at Zivic-Miller Laboratories, Inc. Success of the superior cervical ganglion removal was verified by the presence of ptosis in the ipsilateral lid.

Tears were collected from the eyes of anesthetized rats as previously described [7]. Lacrimal glands were fixed in ethanol/glacial acetic acid (19:1) at 4°C. Following fixation, tissues were dehydrated, embedded in paraffin and cut into 5 μm sections. When indicated, lacrimal glands were serially sectioned. Tissue sections were transferred to gelatin-coated slides and deparaffinized before staining.

Tear IgA levels were measured with a specific, double antibody radioimmunoassay [8]. The procedure for the immunofluorescent analysis of IgA-containing cells in lacrimal gland sections followed reported techniques [9]. For quantitative determination of the density of IgA-positive cells, at least 2 sections/tissue and approximately 15 microscopic fields (312.5 x magnification)/ section were evaluated with a Zeiss Photoscope II fluorescence microscope, equipped with epiillumination and fitted with a xenon lamp, a 460-490 nm excitation filter and a 528 nm barrier filter. To calculate the total number of IgA-containing cells in lacrimal glands, the cell density/field was multiplied by gland weight (mg) and by an appropriate correction factor [10]. Data was analyzed statistically with Student's t test, or, for tests of randomness, chi square goodness of fit.

RESULTS

Distribution of IgA-containing cells in the lacrimal gland [11]

Analysis of serial sections of lacrimal tissue from non-operated rats demonstrated that a marked heterogeneity was present in the frequency distribution of IgA-containing cells. Statistical analysis of this distribution showed that the topographical location of IgA-positive cells per section or through the gland was not random ($p < 0.0001$). This heterogeneity did not appear to be altered by interrupting various nerves (i.e. tempero-facial, sympathetic, optic) to the eye.

Neural impact on tear IgA levels and the number of IgA-containing cells in the lacrimal gland [11]

To assess the influence of the temporo-facial nerve division on immune expression in the lacrimal gland, rats ($n = 13$) were subjected to tempero-facial denervation (D) on the left side and sham-surgery (S) on the right. Tears and lacrimal glands were collected 4 weeks after surgery. Analysis of tears demonstrated that nerve severance had no effect on IgA content ($S = 771 \pm 160$ ng; D =

761 ± 175 ng). Similarly, neural disruption did not modify either the density (S = 7.40 ± 0.71 cells/field; D = 7.46 ± 1.70 cells/field) or total number of IgA-containing cells in the lacrimal gland.

To examine the role of sympathetic innervation in lacrimal gland immunity, rats (n = 26) underwent unilateral superior cervical ganglionectomy and sham-surgery on the opposite side. As demonstrated in Table 1, inhibition of the sympathetic nerve supply to lacrimal tissue for 2 weeks did not alter the level of tear IgA or the density or total population of IgA-containing cells in the lacrimal gland.

Table 1. Influence of the superior cervical ganglion on lacrimal gland immunity

Treatment	Tear IgA (ng)	IgA-Containing Cells (x 10^5)/Lacrimal Gland
Sham-Surgery	886 ± 177	3.13 ± 0.27
Ganglionectomy	887 ± 151	3.07 ± 0.20

Tears and lacrimal glands were obtained from rats (n = 26) 2 weeks after undergoing superior cervical ganglionectomy or sham-surgery on opposite sides. Numbers represent the mean ± SE. Data from [11].

To determine the possible impact of the optic nerve on IgA content in tears and the lacrimal gland, rats (n = 9/treatment group) received one of the following surgical procedures: 1) optic nerve denervation on one eye, and sham-surgery on the contralateral eye; 2) bilateral optic nerve denervation; and 3) bilateral sham-surgery. Results showed that unilateral or bilateral optic nerve denervation or sham surgery had no significant influence on either tear IgA amounts or the IgA-containing cell population in lacrimal tissue.

DISCUSSION

These results indicate that the topographical distribution of IgA-containing cells in the lacrimal gland has an apparent organization, because lymphocyte location is not random. However, it appears that the sympathetic, tempero-facial or optic nerves are not involved in this cellular distribution. Moreover, these nerve supplies do not regulate IgA expression in the lacrimal gland or tears. Whether ocular innervation plays a role in the mucosal immune response to defined antigens remains to be determined.

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in vitro functional studies of conjunctival lymphocytes after ocular *Chlamydia trachomatis* infection

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ABSTRACT. During ocular chlamydial infection, the conjunctiva becomes inflamed and follicles develop. Using a cynomolgus monkey model of trachoma, we have performed in vitro functional studies demonstrating the chlamydia-specificity of the conjunctival infiltrate. By limiting dilution analysis, we have shown that the frequency of chlamydia-specific cells in conjunctiva is 30-100 times higher than observed in peripheral blood.

1. Introduction

Although Chlamydia trachomatis is a leading cause of preventable blindness in the world, the immunopathogenesis of this disease is poorly understood. Our laboratories have used a cynomolgus (Macaca fasicularis) monkey model of trachoma to study the role of local and systemic immunity in the pathogenesis of ocular chlamydia infection [1-3]. Previous studies showed the presence of a marked T cell infiltrate surrounding B cells within follicles during the inflammatory response to infection [3]. It was of great importance to determine how much of this inflammatory infiltrate was associated with a local chlamydia-specific immune response, since this would further our understanding of the possible immune basis of the blinding sequelae of this disease and influence the approaches to vaccine development. This report summarizes our recent functional studies.

2. Materials and Methods

2.1. THE MONKEY MODEL

Monkeys were infected with Chlamydia trachomatis (TW-3, C serovar; or HAR 13, A serovar). Chlamydial elementary bodies (EB) were grown and Percoll-purified using standard methods. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by Ficoll-Hypaque separation [4], and conjunctival lymphocytes (CL) were obtained by collagenase digestion [5].

2.2. LYMPHOPROLIFERATIVE ASSAY

Suspensions of PBMC (10^6 cells/ml) and CL (2 x 10^5 cells/ml) were cultured in the presence of varying concentrations of chlamydial EB or concanavalin A (Con A) for 4 days [5]. During the final 16-20 hr of culture, cells were pulsed with 1 μCi of ^{3}H-thymidine. Proliferative responses were expressed as the stimulation index. Negative control samples of PBMC were obtained prior to infection; normal conjunctiva contains insufficient cells to perform in vitro assays.

2.3. LIMITING DILUTION ANALYSIS OF CHLAMYDIA-SPECIFIC CELLS

Limiting dilution analysis of chlamydia-specific precursor frequencies was performed using standard methods. Briefly, serial dilutions of PBMC or CL were co-cultured with irradiated (3300R) autologous PMBC as feeder cells and proliferative responses compared for wells containing chlamydial EB approximately 10^6 IFU/ml or medium [6]. Chlamydia homologous to the in vivo ocular infection were used in each experiment. Results were analyzed using a program provided by Dr. A. Donnenberg of this institution. Frequencies are expressed as the number of antigen-specific precursors/10^6 cells, $p < 0.001$.

3. Results

Lymphoproliferative responses by PBMC and CL to chlamydial EB were assayed at varying times after ocular infection. Typical responses for 3 different monkeys are shown in Table 1. Dose-dependent responses to chlamydia by PBMC and CL were seen in each case, and all monkeys showed similar responses to Con A and PWM (not shown). PBMC from normal monkeys generally exhibit stimulation indices of ≤ 4.0.

Since the conjunctiva is the site of chlamydial ocular infection, and also the site of immunopathogenic events which are associated with chlamydial antigens [2,7], it was important to determine if the frequencies of antigen-specific cells in conjunctiva surpassed those observed in

peripheral blood. We performed limiting dilution assays on parallel samples of PBMC and CL obtained during ocular infection. Typical results for CL are shown in Table 2. Cells were collected 6-7 wks after ocular infection, at the peak of conjunctival inflammation. While PBMC showed antigen-specific precursor frequencies ranging from 10-100, the frequencies observed in conjunctiva were 30-60 times higher. PBMC from uninfected monkeys exhibited frequencies of $\leq 10/10^6$ cells.

4. Discussion

Our recent studies demonstrated for the first time the presence of chlamydia-specific lymphocytes at the site of infection. Follicles are known to be a hallmark of ocular chlamydial infection, and it was not surprising to find that at least some of the conjunctival B lymphocytes are chlamydia-specific [5]. The combined lymphoproliferative and limiting dilution assays provide further information regarding the antigen-specific responses to ocular infection. While PMBC and CL both respond specifically to chlamydia in vitro [5], the frequencies of antigen-specific cells are much higher in conjunctiva then in the peripheral blood (not shown). We have recently reported studies where a noninfectious chlamydial antigen induces an ocular DTH response in immune monkeys [2,5,6], and found similar high frequencies of chlamydia-specific conjunctival cells [6]. Information provided by these and future functional studies of lymphocytes isolated from the conjunctiva will be helpful in developing appropriate anti-chlamydial therapy, particularly in devising effective vaccine candidates.

5. Acknowledgments

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TABLE 1. Anti-chlamydial proliferative responses by peripheral blood and conjunctival lymphocytes

Monkey No.	Chlamydial Serovar	Stimulation Index[a] PBMC	CL
B107	C	22	15
C7	A	12	4
A7	B	17	6

[a]Cells removed between 3 and 6 weeks post-infection with Chlamydia trachomatis; responses to EB (10^6 IFU/well) are shown. Responses to Con A and PWM were similar for all monkeys.

Table 2. Chlamydia-specific precursor frequencies in conjunctiva after ocular infection

Monkey No.	Expt. Time	Precursor Frequency (No./10^6 cells)
B107	7 wk	119
C6	7 wk	630
B100	12 wk	361

Limiting dilution analysis of conjunctival lymphocytes after ocular infection; PBMC contained 30-100x lower precursor frequencies.

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The expression of MHC Class II antigens by conjunctival epithelial cells in trachoma: implications concerning the pathogenesis of blinding disease

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Introduction

Trachoma is a major cause of blindness, second only to cataract in many parts of the world. It is believed to affect some 500 million people, of whom 7 million are blind (1). In its early stages, trachoma is a chronic follicular conjunctivitis affecting principally the subtarsal conjunctiva, often with corneal involvement in the form of punctate keratitis or vascular infiltration (pannus). Chlamydia trachomatis can often be isolated from conjunctival swabs or scrapings at this stage.

As the inflammatory changes subside, they are frequently replaced by conjunctival scarring and fibrosis, which if severe may lead to distortion of the lid margin, entropion and hence blindness secondary to prolonged corneal trauma. C. trachomatis can rarely be isolated from patients with scarring disease.

The pathogenesis of pannus and conjunctival scarring in trachoma remain unclear; in particular it is not clear why scarring should progress in subjects from whom the organism cannot be isolated. It has been proposed that such patients may have latent chlamydial infections, and chlamydial antigen has been detected in a minority of isolation negative patients with conjunctival scarring (2). An alternative hypothesis is that an initial chlamydial infection may trigger certain immunological changes in the conjunctival epithelium, leading to chronic immunopathological damage. We have explored the role of MHC class II expression by conjunctival epithelial cells in the pathogenesis of trachomatous pannus and scarring.

Patients and Methods

The study was carried out in the Gambian village of Jali. All patients were examined by the same observer (DCWM) with a x4 illuminated monocular loupe. The diagnosis of active trachoma was made on the basis of follicular and papillary changes in the subtarsal conjunctiva, according to the criteria of Dawson et al (1).

A total of 78 subjects were studied. All were children aged 17 years or less. 40 had active disease and 38 were trachoma- free controls. Among the 40 children with active disease, 21 had pannus, and 17 had conjunctival scarring. Among the children without active disease, pannus was present in 3 and conjunctival scarring in 7.

Swabs for detection of MHC class II expression were taken from the upper subtarsal conjunctive by firmly rubbing it 3 to 4 times with a cotton wool tipped swab. The swab was immediately expressed into 1ml of transport medium (Phosphate buffered saline + 5% bovine serum albumen), and stored at 4 C for up to 4 hours, after which it was cytocentrifuged. Cytocentrifuge preparations were air dried, fixed in acetone and transported with dessicant at -20 C to Southampton.

Detection of MHC class II antigen

The following monoclonals were used: TAL 1B5 (anti-DR) and B7/21 (anti-DP) courtesy of Dr. J. Bodmer; TU 22 (anti-DQ), courtesy of Prof. A. Ziegler. The second layer was rabbit anti-mouse polyclonal serum, and the third layer alkaline phosphatase complexed with monoclonal mouse anti-alkaline phosphatase.

Results

MHC Class II Expression and Conjunctival Inflammatory changes

MHC class II expression was observed in conjunctival epithelial cells from 28 of 40 subjects with active trachoma (70%) (fig 4), but in only 10 of 38 controls (26%). This difference is highly significant ($x = 13.2$; $p < 0.001$). There was no difference between resukts obtained with antibodies to DR, DP and DQ.

MHC Class II Expression and Corneal Pannus

HLA class II expression was significantly more prevalent in those with pannus (19/24, 79%) than in those without (19/54,35%) ($x = 11.2$; $p< 0.001$). However, pannus was very much more prevalent in those with active disease (defined according to conjunctival inflammatory changes) than in those without (21/40 vs 3/38; $x = 16.2$, $p < 0.001$), making it difficult to assess the relationship between HLA expression and either pannus or conjunctival changes separately. We attempted to look for a specific relationship between MHC class II expression and pannus by considering those with and without active trachoma separately. MHC class II expression was observed in 17 of 21 subjects with active disease and pannus (81%), and in 11 of 19 with active disease without pannus (58%); this difference is not significant ($x = 1.55$; $p> 0.1$).

MHC Class II Expression and Conjunctival Scarring

MHC class II expression was observed in 16/24 subjects with conjunctival scarring (67%) and in 22/54 without scarring (41%). This difference is not significant ($x = 3.49$; $p>0.05$). Among active cases, MHC class II expression was observed in 13 of 17 subjects with conjunctival scarring (76%) and among 15 of 23 without scarring (65%). This difference is not significant ($x = 0.18$, $p>0.1$).

MHC Class II Expression and Evolution of Disease

Of the 38 controls without evidence of active trachoma, 12 had active trachoma at the previous or subsequent survey, carried out one year before or one year after that in which specimens were taken for MHC detection. 7 of these 12 subjects expressed MHC class II (58%), compared with 3 of 26 who were inactive at previous and subsequent surveys (12%). This difference is significant ($x =7.0$, $p<0.01$), implying that MHC class II expression may precede overt disease and persist after it has resolved.

Conclusions

MHC class II antigens are commonly expressed by conjunctival epithelial cells in patients with active trachoma. Class II expression is correlated with the severity of conjunctival follicular changes and papillary hypertrophy, and with the presence of corneal pannus. Since these three variables are all interrelated however, it is difficult to determine whether class II expression is specifically associated with the presence of pannus. It will be necessary to study larger numbers of patients to elucidate this relationship more clearly.

Among those without active disease, MHC class II expression was significantly more common in patients with active disease at a previous or subsequent survey. This implies that the expression of class II antigen precedes the development of active disease, and persists after it has resolved. It is possible therefore that MHC class II expression plays a part in the progression of conjunctival scarring which appears, on epidemiological grounds, to continue after the inflammatory process has subsided and *C. trachomatis* can no longer be isolated. We have failed to show a correlation between class II expression and conjunctival scarring among those with active trachoma. It would be instructive to study the relatonship between scarring and class II expression among older subjects without inflammatory disease.

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The roles of cell mediated immune responses in chlamydial ocular infection: the effects of cyclosporin A on an animal model of trachoma

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1. INTRODUCTION

The aim of this study was to test the hypothesis that T-lymphocytes are important in trachoma, using an animal model. The model is the guinea-pig infected with guinea-pig conjunctivitis (GPIC) agent, an oculo-genital strain of Chlamydia psittaci. Ocular reinfection produces a phase of acute severe inflammation lasting about seven days, during which GPIC agent is present, followed by a phase of moderate chronic ocular inflammation in which GPIC agent can not be detected. Repeated ocular infection produces chronic conjunctivitis, conjunctival scarring, lid deformities, which are characteristic features of trachoma [1]. There appear to be two components of the partial microbiological immunity which follows ocular infection [2]. The first is short-lived (<3 months). It increases the amount of GPIC agent required to initiate infection, and it is probably mediated by anti-chlamydial secretory IgA. The second is long-lived (>1 year). It restricts the ability of GPIC agent to grow in previously infected animals, and is probably mediated by T-lymphocytes. In order to examine the role of T-lymphocytes in responses to GPIC agent, we studied the effects of cyclosporin A (CsA), which inhibits T-lymphocyte activation, on skin-test responses and ocular reinfection.

2. MATERIALS AND METHODS

Guinea-pigs were infected with GPIC agent before the start of the study. Animals which had been infected at least one month before were used to study skin responses, and animals which had been infected at least three months before were used for ocular reinfection. Animals were treated with CsA (10mg/kg/day i.p.) or an equivalent volume of vehicle, before, during and after challenge. Dermal challenge, evaluation of skin responses, ocular reinfection, clinical assessment of eyes and collection of ocular specimens were carried out as previously described [3,4]. Tears were collected by placing cellulose sponges inside the lower eye-lid and leaving them there for five minutes. CsA levels were

estimated by radio-immunoassay (CYCLO-Trac, IDS, Tyne and Wear).

3. RESULTS AND CONCLUSIONS

CsA treated altered skin responses to GPIC agent. At 24 hours, induration and increase in skin-fold thickness were unaffected, while the erythematous response was significantly inhibited. These observations suggest that recruitment of inflammatory cells to the site of the antigen is unaffected by CsA, but their function is inhibited.

Treatment with CsA also altered responses to ocular reinfection. The phase of severe ocular inflammation was prolonged, and GPIC agent inclusions were present in larger numbers and for longer than in animals treated with vehicle. The numbers of inflammatory cells in conjunctival scrapings were similar in both groups of animals, but the duration of the cellular response was prolonged in the group treated with CsA. Levels of chlamydial antibodies in blood were the same in both groups. The prolonged phase of severe ocular inflammation, GPIC agent infection and inflammatory cell infiltration continued until treatment had ceased and the level of CsA in tears had fallen below 200ng/g tears. This suggests that the inflammatory cells in the eyes of animals treated with CsA were unable to function normally. CsA inhibits the activation of T-lymphocytes, but does not affect the activities of polymorphonuclear cells, monocytes or macrophages [5]. It is therefore likely that the enhanced ability of GPIC agent to grow in the eyes of previously infected animals treated with CsA is a consequence of the inhibition of T-lymphocyte activation by CsA.

These results support the hypothesis that T-lymphocyte responses limit the growth of GPIC agent in previously infected animals. They also indicate that an effective vaccine against trachoma must evoke T-lymphocyte responses as well as antibody production, and that immunosuppressed or immunodeficient individuals may be more likely to develop disseminated disease following infection with C. trachomatis.

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SECTION O:
MAMMARY GLANDS
AND
THEIR SECRETIONS

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Identification of tumor necrosis factor alpha (TNF-α) and transforming growth factor beta (TGF-β) in murine milk

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Introduction
Breast milk is well known for its protective role in the immature intestine of the neonate. This protection may relate to immunocompetent cells, immunoglobulin, and antimicrobial substances [1]. Breast milk also has a role in promoting intestinal maturation; cytokines, growth factors and hormones have been identified in the milk [2,3,4]. We sought to detect tumor necrosis factor alpha and transforming growth factor beta in murine milk by the Western blot technique.

Methods
Following barbital anesthesia and oxytocin stimulation, milk was obtained from lactating mice by manual expression. After centrifuging at 800 x g to remove milk cells, the supernatant was defatted by ultracentrifugation at 16,000 x g for 1 hour and was dialyzed against distilled water. The dialysate was lyophilized and the resulting milk powder was resuspended in 1/10th of the original volume of PBS. The concentrated milk, as well as recombinant murine TNF-alpha and purified porcine TGF-beta were reduced and denatured by treatment with sodium dodecyl sulphate and 2-mercaptoethanol. The samples were subjected to polyacrylamide gel electrophoresis using a discontinuous buffer system. The separated proteins were transferred to nitrocellulose paper. The nitrocellulose paper was incubated with affinity purified, polyclonal rabbit anti-TNF-alpha or rabbit anti-TGF-beta antibodies. The antigen-antibody complexes were identified by addition of biotin-conjugated, goat anti-rabbit IgG antibody followed by an avidin-alkaline phosphatase complex. In the final step, chromogenic substrate was added to produce a color reaction.

Results
After electrophoresis of rTNF-alpha and development with antibody to TNF-alpha, a single protein band with an apparent M.W. of 17 kD was seen. Following electrophoresis of milk and development with anti-TNF-alpha antibody, a similar band was seen in the same M.W. range. Bands of higher M.W. were also seen. Absorption of the anti-TNF-

alpha antibody with 500 ng of rTNF-alpha abolished its reaction with the 17 kD protein band. Purified porcine TGF-beta was subjected to electrophoresis and developed with the anti-TGF-beta antibody; a band of protein with an approximate M.W. of 12 kD was seen. Following electrophoresis of milk and development with anti-TGF-beta antibody, a protein band of approximately 12kD was detected. Bands of higher M.W. were also observed.

Discussion

In this study we identified TNF-alpha and TGF-beta in murine milk. The principal protein band of TNF-alpha was identified in the 17 kD region. This finding is in agreement with the reported molecular structure of TNF-alpha [4]. TNF-alpha is a 35 kD disulfide-linked dimer that is denatured into two 17.5 kD monomers. TGF-beta in milk was identified as a single band of protein of approximately 12.5 kD. This molecular size corresponds to the reduced form of the activated 25 kD disulfide-linked dimer [5]. The bands of higher molecular weight probably represent polymeric forms of TGF-beta. Other investigators have previously reported the presence in milk of TNF-alpha [6] and TGF-beta [7], as well as interleukin-1 and epidermal growth factor on the basis of the biological activity of milk samples. In the present study, TNF-alpha and TGF-beta were identified on the basis of their molecular size and antigenic properties.

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IgA1 and IgA2 subclass antibodies to food proteins and salmonella antigens in secretions

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1.Introduction

IgA is the predominant immunoglobulin in secretions, although with the exception of colostrum, exocrine fluids contain less IgA than serum. In other hand, the percentage of IgA1 and IgA2 subclasses are quite different in secretion compared to serum levels (1,2). The IgA2 amounts about 40% of total IgA in secretions and only 10% in serum.
With regard to IgG subclass, a restriction of subclasses response is known. Proteins mainly induce IgG1 and IgG3 antibodies whereas polysaccharide induce IgG2. It is not so clear if a similar IgA subclass restriction occurs. We had previously reported a predominant IgA1 antigliadin response in coeliac patients.
The principal aim of this study was to investigate the behaviour of IgA1 and IgA2 subclass antibodies against several common bacterial (4 types of Salmonella) and food antigens (lactoglobulin, ovalbumin and gliadin) in secretions. In other hand, we tried to know if there is a good coincidence between antibody levels in saliva and colostrum. This finding would permit to predict, before the delivery, the presence of antibodies in colostrum from salivary data.

2.Subjects and methods

Colostrum and saliva were simultaneously taken from 50 normal mothers, 36-75 hours after delivery. All infants were normal and term-born. Colostrum was taken by manual extraction and saliva was also spontaneously got, without chemical stimulation.
Antibodies were determined by ELISA. Briefly, the wells of microtiter plates (Dynatech Lab.) were coated with 100 µl of carbonate buffer (pH 9.6) containing gliadin (20 µg/ml), ovalbumin (50 µg/ml), lactoglobulin (50 µg/ml) or Salmonella ($2x10^5$ bact/ml). The plates were saturated with 0.1% BSA. The colostrum samples were diluted 1/320 and the saliva 1/20, in both subclass studies. Monoclonal anti-IgA1 (1/5000) and anti-IgA2 (1/2000) antibodies (OXOID) were used. A final incubation was made with peroxidase-conjugated antibody (1/2000) against mouse IgG. The OD was read by a spectrophotometer (Dynatech) and 3 samples were utilized as correction factor among the different plates. The statistical significance was calculated by an Apple Macintosh computer (Stat-View 512+) using the Spearman's non-parametric correlation test.

3.Results

3.1.IgA1 and IgA2 food antibodies were detected in almost all samples, but the percentage of positive antibodies to Salmonella was smaller. Especially, IgA2 antibodies were never found in saliva. IgA1 antibodies were more frequently detected than IgA2 antibodies in saliva ($p<0.001$), but this did not occurred in colostrum.

ANTIBODIES	COLOSTRUM		SALIVA	
	IgA1	IgA2	IgA1	IgA2
LACTOGLOBULIN	98%	96%	96%	87%
OVALBUMIN	96%	94%	96%	83%
GLIADIN	86%	74%	98%	56%
S.PARATYPHI A	48%	36%	29%	0%
S.PARATYPHI B	44%	46%	13%	0%
S.PARATYPHI C	24%	32%	23%	0%
S. TYPHI	40%	30%	11%	0%

3.2.The correlation between IgA1 and IgA2 food antibodies was always high in colostrum ($p<0.001$) and generally lower ($p<0.05$) for Salmonella antibodies. This correlation was also low in saliva, even for foods.

3.3.Correlations between colostrum and saliva data were not found, except for some IgA1 antibodies to Salmonella.

3.4.When we compared antibodies each other the correlation among food antibodies was always very high ($p<0.001$) in colostrum and saliva, but this did not occur for saliva salmonella antibodies. Finally, the correlation food-salmonella antibodies was very infrequent and poor, and this finding seems to rule out the existence of a polyclonal IgA response.

4.Conclusions

4.1.It is possible to detect salmonella and especially food antibodies in most normal colostrum and saliva. The IgA1 response is generally stronger than the IgA2 response.
4.2.The correlation between IgA1 and IgA2 antibodies is higher in colostrum than in saliva.
4.3.The correlation between colostrum and saliva antibody levels is quite poor, so the prediction of breast milk levels from saliva data seems to be inaccurate.
4.4.IgA1 and IgA2 antibodies had a different behaviour against food or salmonella antigens, and it is also quite independent in saliva and colostrum.

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Anti-pili and anti-LPS antibody producing cells in mammary glands of immunized rats

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ABSTRACT. The number of antibody secreting cells, specific in production of antibodies directed against bacterial LPS- and pili-antigens, in the mammary gland of immunized rats, were estimated. Six rats were immunized in the Peyer's patches with *Escherichia coli* 06K13, carrying type 1 pili, one day after delivery. Eight days later the mammary glands were excised and a lymphocyte suspension was prepared. The ELISPOT assay was used to estimate the number of plasmacells. IgG anti-LPS antibody producing cells dominated in all 6 rats compared to IgG anti-pili producing cells. The same pattern was seen in the distribution of IgM producing cells. On the other hand 4 of 6 rats had higher number of IgA anti-pili producing cells than IgA anti-LPS producing cells. Possible explanation for this difference could be a selective homing of the IgA anti-pili producing cells to the mammary gland or a clonal expansion of this cells in the gland.

1. INTRODUCTION

After antigen stimulation B-cells leave the gut associated lymphoid tissue and start to migrate to distant mucosal and glandular sites where they settle and mature to plasmacells. It is of great importance to gain more knowledge about factors influencing this migration. In order to do so we have studied the significance of the antigen as one such factor. The number of antibody producing cells, with certain antigen specificities, were estimated in the mammary glands of immunized lactating rats.

2. MATERIALS AND METHODS

Six female Spraque- Dawley rats (250-300 g) were immunized in the Peyer's patches with 0.1 ml of formalin killed *E.coli* 06K13, carrying type 1 pili, at a concentration of 10^{10} bacteria/ml, one day after delivery. Eight days later the mammary glands were excised ,homogenized and treated with trypsin and a lymphocyte suspension was prepared. According to FACS analysis 20% of the cells were positve for B-cell staining (Ox 33).

The ELISPOT assay was performed as described by Czerkinsky et al.(1), and the number of cells producing antibodies against either the 06-LPS - or the type 1-pili -antigen, was determined.

3. RESULTS

Plasmacells produing IgA, IgG and IgM antibodies against both the 06-LPS- and the type 1-

pili antigens were detected. Cells producing IgG and IgM antibodies against the LPS antigen dominated. Conversely 4/6 rats had a higher number of cells producing IgA anti-pili than anti-LPS antibodies.

3.1 Table

TABLE 1. Number of antibody secreting cells found in mammary gland of rats immunized in the Peyer's patches with *E.coli* 06K13, carrying type 1 pili. Each spot represent one antibody producing cell.

	No .of spots/10^6cells					
	IgA		IgG		IgM	
Rat no.	α–pili	α–LPS	α–pili	α–LPS	α–pili	α–LPS
1	7	37	20	63	13	33
2	21	3	13	77	19	16
3	57	13	15	68	21	39
4	28	12	32	80	80	40
5	1	8	0	14	1	3
6	13	10	0	3	40	0

4. DISCUSSION

We have earlier reported that IgA anti-pili antibodies dominates in milk while anti-LPS IgA antibodies prevail in bile of rats immunized in the Peyer's patches (2). In this report we showed that there was a higher number of IgA anti-pili ,than IgA anti-LPS producing cells in the mammary gland. An explanation could be a selective T-cell dependent homing of the IgA anti-pili producing cells to the mammry gland. The pili-antigen is a T-cell dependent antigen and 70% of the T-cellpopulation in the mammary gland of rats are T_H-cells (3) and a extensive T-cell dependent clonal expansion of the cells predestined to produce anti-pili antibodies could take place.

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Reduced frequency of acute otitis media in breast-fed infants

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ABSTRACT

The finding that breast-milk inhibits bacterial attachment in vitro(1) provided the basis for a clinical study of the protection by breast-feeding in children below one year of age. 493 infants were enrolled in the study. Breast-fed infants between 4 and 12 months had a significantly lower frequency of upper respiratory tract infections compared to the other feeding groups. In the age group 0 to 3 and 8 to 12 months of age the exclusively breast-fed children had a significantly lower frequency of acute otitis media.

INTRODUCTION

Breast-feeding has been suggested to protect the baby against infection (2). Human milk contains immunoglobulin and other anti-bacterial substances like lysozyme, lactoferrin and peroxidase (3). Further more human milk contains components which interfer with the attachment of S.pneumoniae and H.influenzae to nasopharyngeal epithelial cells (1). These inhibitors are present both as free oligosaccharides corresponding to the cellbound receptors for attaching pneumococci, and as glycoproteins e.g. in the casein fraction (4). Bacterial adherence probably facilitates the colonization of the nasopharynx, permitting prolonged persistence of bacteria and thus indirectly enhancing their opportunity to cause infections. The intermittent administration of milk with anti adhesive substances into the nasopharynx of the nursing child may, thus, reduce the extent of bacterial colonization and protect against infection.

MATERIAL AND METHODS

Study population: 493 infants were cultured during regular visits every third month. During each visit, a detailed history of the feeding patterns was obtained.
Bacteria: Nasopharyngeal cultures were obtained with a cotton tipped metal swab.

RESULTS

There were 233 girls and 238 boys. Colonization with *S. pneumoniae* increased with age and was 30 percent at 12 months of age. *H. influenzae* also increased with age but only to 13 percent.
Feeding pattern: At 3 month of age 78 percent of the children studied where exclusively breast-fed and 14 percent received breast milk with other foods. At 8 to 12 month of age only 10 percent were exclusively breast-fed.

Upper respiratory tract infection in relation to breast feeding. Breast-fed infants between 4 and 12 months had a significantly lower frequency of upper respiratory tract infections compared to the other feeding groups.
Acute otitis media in relation to breast feeding (TABLE 1). In the age group 0 to 3 and 8 to 12 months of age the exclusively breast fed children had a significantly lower frequency of acute otitis media compared to those receiving other foods only.
Adhesion inhibition by milk. The milk from each mother was tested for its capacity to inhibit the attachment of a highly adhering *H.influenzae* strain. Breast-milk samples received from mothers with children between 0 and 3 months of age inhibited H.influenzae to 54 percent compared to a buffer control. The inhibitory activity was higher (65 %) for breast-milk from mothers with children between 4 and 7 months and highest (70 %) for 8 to 12 months babies.

DISCUSSION

Breast feeding protects the infant against infection. Protection may be due to lower exposure to environmental microorganisms, to a reduced microbial colonization of mucosal surface with potential pathogenes or to a direct interference with mechanisms of pathogenesis. The frequency of nasopharyngeal carriage of *S.pneumoniae* and *H. influenzae* was low during the first 3 months of age. This is in contrast to the colonization frequencies previously reported, e.g., from Papua, New Guinea (5).

The precent study shows that breast-fed babies have a reduced frequency of acute otitis media compared to age matched children who receive mixed feeding or other foods.

TABLE 1 **Acute otitis media in relation to feeding pattern.**

Age (months)	*feeding*	*Acute otitis media, % positive*	
0-3	breast milk	1	
	mixed feeding	5	p<0,05*
	other	6	p<0,05*
4-7	breast milk	5	
	mixed feeding	5	
	other	13	
8-12	breast milk	0	
	mixed feeding	8	
	other	19	p<0,01*

** Chi square compaired to breast milk.*

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Isolation of secretory immunoglobulin A and free secretory component from porcine whey

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Introduction

The importance of obtaining more information about the mucosal immune system of swine is shown by two facts. Firstly, the pig is regarded as a model for studying the primary immune response. In this species the placental barrier does not permit the transfer of immunoglobulins and high molecular weight antigens to pig embryos. They receive passive protection by colostrum and milk.

Keeping colostrum deprived piglets germfree one can study influence of route and form of antigen application on the ontogenic development of local and systemic humoral immune response without interference by maternal antibodies.

In addition, a better understanding of sIgA system (including the transport mechanisms of polymeric Ig) as the protection system for newborn piglets in their first weeks of life is crucial, because these piglets are very susceptile to infections.

Isolation of sIgA has been reported by several authors. However, no information is available concerning the isolation of porcine free secretory component. Presently we developed a procedure to purify sIgA and FSC from the same material: Sow milk from late period of lactation.

Materials and Methods

Milk was collected from sows during their late period of lactation. Fat was removed by precipitating caseine and centrifugation of the material at 2000 g for 30 min. at 20° C.

Dialysing the whey for 48 h against 0.01 M potassium-dihydrogenphosphate, 0.001 M di-potassium-hydrogenphosphate buffer precipitated the euglobulines, which could be separated by centrifugation at 2000 g for 30 min. at 4° C.

The supernatant was treated with ammoniumsulfate to be saturated to 33 %. The resulting precipitate was collected after centrifugation (2000 g for 20 min. at 20° C) for further sIgA purification steps, the supernatant was stored at -20° C, until used for isolation of FSC.

For sIgA isolation a second precipitation step with 33 % ammoniumsulfate saturation was performed. The supernatant was removed and the precipitate was fractionated by reverse-flow exclusion chromatography on Sepha-

rose CL-4B after extensive dialysis against water and then against 0.01 M NaCl buffer, pH (7.3) which was used for elution. The fractions were pooled, concentrated by ultrafiltration and applied to a column of DEAE-Sephacel, which were then eluted by stepwise changes of molarity at the same pH: 0.05; 0.075; 0.10; 0.125; 0.15; 0.2; and 1 M.
Some fractions were further purified by affinity chromatography using a column of protein-A bound to Sepharose CL-4B and 0.02 M Tris buffer, pH 7.3 as elution buffer. Purity has been checked by immunodiffusion, immunelectrophoresis and SDS-PAGE.
FSC was isolated using the supernatant of the first precipitatation step with 33 % ammoniumsulfate-saturation. This material was further treated by adding ammoniumsulfate to a saturation of 55 %. The precipitate was collected after centrifugation for 20 min. at 20° C. This precipitation step has been repeated once.
Then the precipitate was applied to a DEAE-Sephacel column, after exhaustively dialyses against water and then against 0.01 M Tris 1N NaCl buffer, pH 7.3. Samples were pooled, dialysed against elution buffer and concentated before applicated to the column. Purity was tested as described for sIgA preparation.

Results

For sIgA preparation gelfiltration on Sepharose Cl-4B led to elution of 5 peaks. The third one contained mainly sIgA, contaminated with IgG, as was shown by immunodiffusion and immunelectrophoresis. After anion-exchange-chromatography most fractions from elution with 0.125 and 0.15 M NaCl were tested to contain only sIgA. Others gave weak reactions in immunelectrophoresis with antiserum against porcine IgG. No contamination were seen after carrying out affinity chromatography with Sepharose bound protein-A.
FSC were eluted from ionexchange-chromatography with NaCl molarity of 0.16 and 0.18 M. Further purification on Sephadex G-100 led to fractions containing pure FSC.
When tested in SDS-PAGE porcine FSC was shown to be a glycoprotein with an apparent molecular weight of 70 kD. No further reduction were noticed after treatment with 2-mercaptoethanol.

Discussion

With the method described we were able to isolate pure porcine sIgA and FSC as has been tested by various methods, including ELISA (data not shown). Sow milk from a late period of lactation seems to be a suitable material for isolating both proteins. The results obtained with FSC are in aggrement with findings reported for FSC from other species.
Having both proteins the next step will be the attempt to develop different monoclonal antibodies against sIgA and FSC.

Acknowledgement This work was supported by Fraunhofer-Gesellschaft, München, Germany

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Influence of colostrum and colostral immunoglobulins on immune development in piglets

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Colostrum is essential for the survival of piglets in a normal environment since there is no placental transfer of maternal immunoglobulins to the foetus. However the protective effects of colostrum persist beyond the survival time of maternal antibodies in the piglets' circulation. In this study we tried to evaluate the role of colostrum, (C) and colostral immunoglobulins (clg) in particular, on active development of general immune competence and specific immune responsiveness in piglets in a controlled environment.

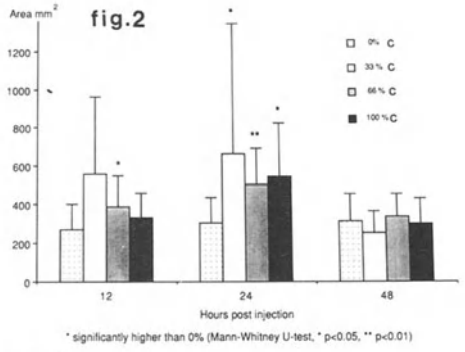
ANIMALS

Piglets (Group 1: n = 48, Group 2: n = 18) were catch farrowed under semi-sterile conditions and reared in a purpose built SPF isolator unit, fed on a cows milk based milk replacer until 4 wk when they were weaned onto conventional soya based diets. During the first 20h, the piglets were stomach tubed with 4 x 50 ml of sow colostrum/milk replacer mixture to form 2 x 4 experimental groups; Group 1 - 0%C (no colostrum), 33%C, 66%C or 100%C (= 200 ml colostrum) or Group 2 - 0% clg, 33% clg, 66% clg, 100% clg fed the immunoglobulin (Ig) fraction of the equivalent amount of colostrum. Blood samples were taken at 24h, from 1-10 wk then 14, 17 and 20 wk. All piglets were immunised intramuscularly at 3 wk of age with 1 mg ovalbumin (OVA) in Freund's complete adjuvant.

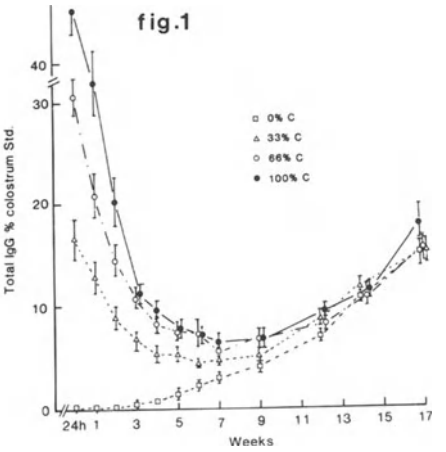
METHODS

General immune competence was evaluated by total Ig levels (IgG, IgM, IgA), measured by the Mancini R.I.D. technique, and the lymphocyte transformation (LT) response to the mitogens PHA, ConA and PWM.

Specific immune responsiveness to OVA immunisation at 3 wk was monitored by OVA specific antibodies (IgG, IgM, IgA) and LT responses with DTH skin challenge at 5 wk only. At the end of the experiment histological samples were taken from the GI tract and the incidence and distribution of IgA, IgG and IgM producing plasma cells were investigated.



RESULTS



All statistical evaluations were by Mann-Whitney U-test (* $p<0.05$, ** $p<0.01$).

Humoral As expected, throughout the study the colostrum/colostral Ig deprived (0% C and 0% clg) pigs displayed significantly lower total serum IgG than C/clg fed littermates (Fig. 1). This delay in serum IgG expression was very marked up to 7 wk age ($p<0.01$). The serum antibody (IgG and IgM) response to OVA was delayed in both C and clg deprived piglets though anti OVA activity was more markedly depressed in the latter group. No IgA anti OVA response was observed in any of the piglets.

Cellular Neither colostrum nor colostral Ig deprived pigs appear to have any impairment of their general (mitogen-induced) cellular responsiveness, although ConA response appeared to be lower across all C treatment groups between 10 and 12 wk. This may be due to addition to the feed at this time of the antibiotic, chlorotetracycline, or to increased antigen load. Similarly there was no statistically significant difference in LT response to OVA between treatment groups (0%-100% or 0% clg-100% clg) although generally cells from 100% groups showed highest stimulation with OVA. *In vivo* the DTH skin response to both OVA and PHA was significantly lower in 0% C and 0% clg pigs at both 12h and 24h post intradermal challenge (e.g. Fig.2).
Histologically there was dichotomy between the incidence of IgA and IgG plasma cells in small intestine and colon. In the small intestine, the incidence of IgA plasma cells was significantly greater in the colostrum deprived (0%C) pigs compared to colostrum fed littermates. However in the colon, the incidence of IgG plasma cells was significantly lower in the 0% pigs compared to the 100%C group.

DISCUSSION

This study has demonstrated a selective stimulatory effect of colostrum or colostral Ig on immune ontogeny in pigs. This is evident from the increase in both the kinetics of active humoral immune development (total serum IgG, 1° and 2° anti OVA responses) and in the magnitude of *in vivo* cell mediated responses (DTH to OVA and PHA). The increased incidence of IgA plasma cells in the small intestine of colostrum deprived pigs may be reflective of the higher bacterial antigenic load in these pigs.

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Influence of antigenically primed colostrum on the specific immune response of piglets

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At birth the immune system of the piglet is functionally immature and the newborn is totally dependent on its mother's immune system in the immediate postnatal period via ingestion of colostrum. Colostral antibodies are present in the piglets serum up to 6-7 w of age. However the intake of colostrum seems to have a profound influence on the development of a functional immune system since colostrum deprived piglets continue to be more disease susceptible even after maternally derived antibodies are no longer present.In this study the possible influence of specific maternal antigenic experience on the subsequent immune responsiveness of piglets recieving the "primed" colostrum has been evaluated.

MATERIALS & METHODS

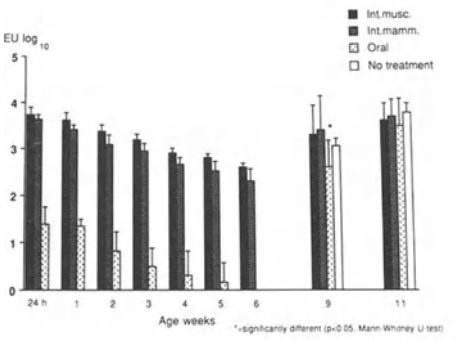
Sows were immunised with ovalbumin (OvA) as follows:

1. *Intramuscular* (Int. musc.n=2) with 5 mg OvA in Freund's complete adjuvant.and boosted at 5 and 3 w before farrowing with OvA in incomplete Freund's adjuvant.
2. *Intramammary* (Int. mamm. n=2) with 1 mg OvA+2 mg Quill A at 6 and 4 w before farrowing.
3. *Oral* (n=2) with 20 whole fresh eggs/day/sow for 7w until 1w before farrowing.

Piglets (n=28) were catch farrowed from 3 concurrently farrowing, non-immunised sows and randomised across the treatment groups; T1 (n=6) colostrum from int.musc. immunised sows, T2 (n=6) colostrum from int.mamm. immunised sows, T3 colostrum from orally immunised sows and T4 (n=5) non-immunised colostrum. A total of 200 ml of colostrum was given at 3h interval within 20h of birth. The piglets were artificially reared in a SPF isolator fed on cow's milk based milk replacer. All piglets were immunised i.p. with 0.5 mg OvA in saline at 2 w of age and boosted i.m. 4w later with 1mg OvA in Freund's complete adjuvant.

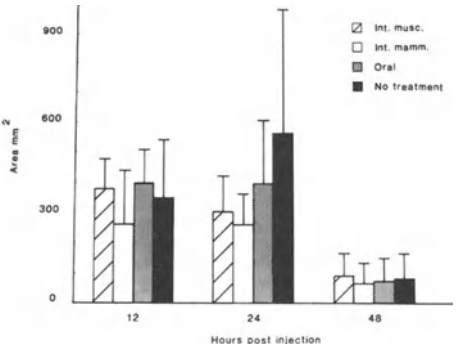
Immune response Specific antibody response to OvA (IgG, IgM, IgA) in colostrum and piglet serum was measured by ELISA. *In vitro* and *in vivo* cellular responses to OvA and the mitogen PHA were evaluated by lymphocyte transformation (LT) and DTH skin test.

Fig1



IgG anti OvA levels in piglets

Fig2.



DTH skintest response 9w of age

RESULTS

All immunisation routes of the sows induced IgG, IgM and IgA anti OvA in colostrum. The oral route, however, was significantly lower for all classes than the other two routes. From the piglets anti OvA profiles it was apparent that these antibodies were absorbed from colostrum in all three groups (fig.1)and decayed following their natural halflife. In terms of active antibody formation the group recieving orally primed colostrum T3 showed a significantly reduced response ($p<0.05$, Mann-Whitney U-test) than either group T1 and T2 2w after challenge with OvA in adjuvant (fig.1). However this supression was not significantly mirrored by the IgM response or maintained until 4w after challenge. No IgA anti OvA response was seen in any of the piglets. All piglets showed a very low LT response to OvA with no apparent differences between the groups. However, at 9w of age the 24h DTH skin response to OvA in groups T1 and T2 were significantly lower ($p<0.05$) than that of the unprimed group T4 (fig 2)

DISCUSSION

From this preliminary study of the influence of maternal antigenic experience and colostrum mediated regulation of piglet immune responses, it would appear that colostral factors from orally primed sows can supress the secondary IgG antibody formation. High levels of specific anibodies from colostrum may also supress the subsequent specific cellular response.

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IgA secretion by human breast milk cells (HBMC) in culture

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Human breast milk (HBM) provides effective protection for the neonate against diarrheal diseases and has been associated with a marked reduction in infant morbidity and mortality from infectious diseases regardless of sociologic standing. Improved understanding of the beneficial effects of HBM might be applied to the treatment and prevention of adult diarrheal disease.

It is well recognized that HBM from the first 48-72 hours post partum (colostrum) provides particularly high concentrations of passive immunoglobulin (Ig) as well as immunocompetent cells. We attempted to establish the amount of IgA, IgM and IgG released by washed HBMC in unstimulated cultures over 7 days. This slow release of antibody might reflect cellular delivery of specific antibody to the intestinal microenvironment.

METHODOLOGY

Healthy volunteers 2 to 3 days post-partum are enrolled in the research study with informed consent approved by our institutional human use committee. Following a sterile skin preparation of the nipple area, 20 to 30 ml of HBM are collected using an electric pump. The HBM is diluted with Dulbecco's balanced salt solution (without calcium and magnesium) and centrifuged at 1200 rpm for 10 minutes. The cellular pellet is resuspended and washed in one of two media: MEDIA #1: RPMI 1640 (Whittaker MA Bioproducts, MD) with 20% heat inactivated fetal calf serum, Penicillin (100 units/ml), Streptomycin (100 mcg/ml), Gentamycin (40 mcg/ml) and additional L-glutamine (4 mM) at pH 7.4; MEDIA #2: OPTI-MEM I serum free media (Gibco Laboratories, Inc., OH) but with antibiotics as above.

Cultures are set up at 1 X 10E6 cells per ml in 1 ml volumes using Falcon 2058 tubes (Falcon, Oxnard, CA) at 37 degrees C. and 5% CO2 in air. A sample cell media is saved for future assay as a baseline time 0 value. After 7 days in culture, supernatants are collected and frozen at minus 0 degrees C. A time course study for testing days 0, 1, 3, 5, and 7 is set up for those donors where adequate cell numbers are available.

An isotype specific enzyme-linked immunosorbent assay (ELISA) is standardized for the measurement of human IgA, IgM and IgG using well described procedures and commercially available affinity purified, polyclonal antisera and reference standards (TAGO, Inc., CA). All test samples are assayed in multiple dilutions for each isotype with 8 dilutions of the same standard on each microtiter plate. Results are expressed in micrograms per ml of antibody.

RESULTS

Colostrum was collected from 8 women within 72 hours after an uncomplicated delivery. All HBMC supernatants are assayed in duplicate and in at least 4 dilutions in the ELISA assays. Mean IgA, IgM and IgG concentrations are listed in Table 1 reflecting 7 days of culture and the two media conditions. IgA secretion is comparable in both medias with wide variations in concentrations between donors. Table 2 summarizes the time course study for IgA concentrations in HBMC supernatants of 2 of the 8 donors on days 0, 1, 3, 5, and 7.

TABLE 1. IgA, IgM & IgG concentrations in HBMC supernatants (in mcg/ml) in media # 1 versus media # 2 (N = 8).

HBMC CODE	IgA		IgM		IgG	
	#1	#2	#1	#2	#1	#2
MEAN	40	37	2.5	1.7	0.5	0.4
S.D.	19	14	0.9	0.6	0.2	0.2

NOTE: By non-parametric statistics, no significant differences.

TABLE 2: Time course of IgA secretion (mean results in mcg/ml with standard deviation) by 2 donors in Media # 1 and # 2.

TIME (DAY #):	0	1	3	5	7
A - MEDIA 1	16(3)	36(8)	66(8)	60(9)	58(9)
MEDIA 2	12(4)	19(5)	35(9)	43(8)	57(7)
B - MEDIA 1	20(4)	40(8)	32(6)	48(7)	55(6)
MEDIA 2	18(4)	21(5)	20(4)	30(5)	48(5)

CONCLUSION

Cells derived from HBM continue to release IgA over 7 days of culture in media with or without fetal calf serum. The largest proportion of IgA release occurs in the first 24 to 72 hours suggesting preformed release rather than synthesis. IgM and IgG secretion occurs in lower concentrations, but with a similar time course.

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Immunological aspects of colostrum and milk of mothers delivering low birth weight neonates

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1. Introduction

Several studies have been performed in order to evaluate immunological and nutritional factors of the milk from mothers whose infants are preterm. The obtained data are controvert. Regarding the mother's milk of small-for-gestational age infants, its composition was not evaluated enough, with findings equivalent to the milk of preterm infants' mothers. (Barros & Carneiro-Sampaio, 1984: Garza et al, 1981: Pamblanco et al, 1986.)

The purpose of this study was to analyze some immunological and nutritional characteristics of colostrum and milk of mothers of low-birth weight neonates.

2. Methodology

Samples of colostrum and milk on the 7th, 15th, 30th and 60th days were collected from 67 well-nourished mothers divided into three groups according to their infants' birth weight and gestational age: G1- full-term small-for-gestational age (T-SGA) (n=16); G2-preterm appropriate-for-gestational age (PT-AGA) (n=21) and G3-full-term AGA (T-AGA) (n=30). The following factors were studied: osmolality, total protein, total IgA (simple radial immunodiffusion); lysozyme (activity evaluated by litic effect on M.Gaglysodeikiticus suspension under agar gel), antibody titres anti-poliovirus types 1,2,3 (neutralization assay), anti-herpes virus, anti-smallpoxvirus and anti-cytomegalovirus (ELISA). For statistical analysis, we used Mann-Whitney and Friedman methods.

3. Results

No differences in human milk composition related to gestational age (G2 X G3) and to birth weight (G1 X G3) were observed in osmolality, total protein, lysozyme activity and antibody titres against virus. Higher levels of IgA were shown on PT-AGA and T-SGA groups (see figure 1). IgA antibodies anti-herpesvirus, anti-smallpoxvirus and anti-citomegalovirus were undetectable in all the samples.

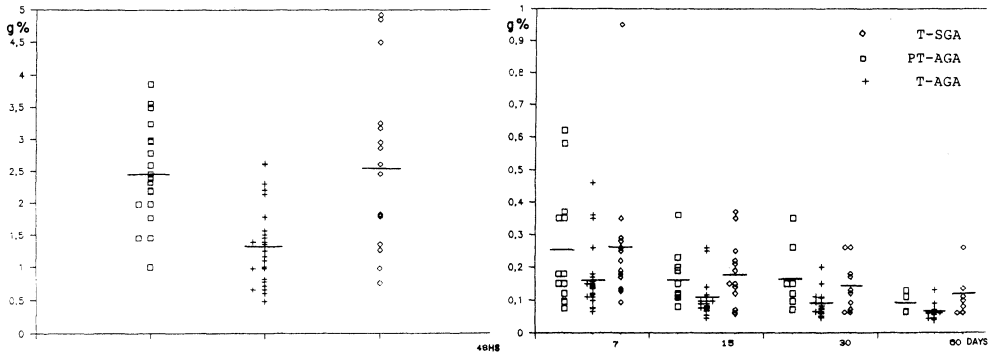
4. Discussion

Pregnancy duration has been advocated as having a critical role in maturation of the mammary gland. Our results do not support the idea that this factor is the only one involved, since the milk of full-term SGA infants' mothers have shown similar patterns as preterm milk. We have also measured total lipids, sodium, calcium, magnesium, protein fractions and the findings were equivalent in the both low-birth-weight groups. It is interesting that IgA levels were similar in both low-birth weight groups and the other factors were not different for all the three groups.

It is possible that some immunological activation occurred and could be associated to the low-birth weight.

It has been shown that hormonal profiles of pregnant women can be used to identify intrauterine growth-retardation and that their hormonal levels are comparable to those of women in earlier stages of pregnancy. Placenta is the main source of hormones in the last trimester of pregnancy. Preterm placentas are immature and in some cases of growth-retardation, placental insufficiency occurs. In both cases, the physiological action of placental factors may have comparable influence on histologic, metabolic and immunological maturation of mammary gland and consequently on the quality of milk to be produced. Further investigations are necessary to confirm this hypothesis and the physiological basis to explain this adaptive phenomenon still remains unknown.

Figure 1. IgA in colostrum and milk samples.



5. Acknowledgements

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SECTION P: IgA AND THE HEPATOBILIARY SYSTEM

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Relationships between IgA and the liver

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ABSTRACT. During the past 10-15 years a great deal of evidence has shown that the liver is very much involved in the secretory IgA system. Much of our understanding of the details of the synthesis, cell-surface expression, and intra-cellular migration of secretory component (the polymeric immunoglobulin receptor) has come from studies in the rodent liver. Also, very remarkable species differences in the clearance of IgA from plasma have been observed: whereas certain animals (rats, mice, rabbits) efficiently clear circulating IgA by a secretory-component mediated, vesicular transport across hepatocytes, several other species (including man) lack this transport pathway. On the other hand, the IgA1 subclass in man might bind to and be internalized or transported by the asialoglycoprotein receptor on hepatocytes. The major biological functions ascribed to the secretion of IgA into bile are enhancement of immunological defense of the biliary and upper intestinal tracts and clearance of harmful antigens from the circulation as IgA-antigen complexes. The true importance of these proposed activities, however, deserve careful investigation. Abnormalities in the metabolism of IgA have been described in many liver diseases, most notably alcoholic liver disease, in which characteristic deposits of IgA1 are common along hepatic sinusoids and, to a certain extent, in the renal mesangium.

1. TRANSPORT OF IgA BY THE LIVER

In the mid-1970's it was discovered that secretory IgA (sIgA) and secretory component (SC), the cellular receptor for polymeric immunoglobulins (pIg), are major proteins in rat bile (1). This discovery prompted intensified interest in the sIgA system. The results of several congruent studies established that, in the rat, biliary IgA is derived nearly entirely by the active hepatic transport of IgA polymers from the circulation and that the transport is an SC-mediated process. It was found, for example, that a) pIgA was effectively transported, whereas there was minimal or no detectable transport of sIgA, preformed complexes of pIgA with SC, or monomeric IgA (2); b)

polymeric Fc fragments of IgA, which could bind to SC in vitro, were actively transferred in vivo into bile, whereas the corresponding Fab fragments, which could not bind to SC, were not transported (3); c) IgA polymers recovered from rat bile were entirely or mostly complexed with SC (2); and d) heterologous IgG antibodies to rat SC injected into the circulation of rats were transported into bile, whereas control, nonimmune IgG was not (2,3).

Those reports on the hepatic transport of IgA in the rat were followed by similar studies conducted on several other species, with remarkable differences being recorded. It soon became clear that although the rabbit, like the rat, could transport much pIgA from plasma into bile (4), the guinea pig (5), dog (6) and sheep (5) could not transfer the immunoglobulin nearly as efficiently. Man fell into the category of the inefficient hepatic transporters of IgA (7,8). The quantitative differences in hepatic transport of IgA among species are quite striking: whereas the per cent of an injected dose of pIgA transported from plasma into bile is in the range of 25-95% during a 2 to 5-hour period in the rat and rabbit, the corresponding value is no more than 3% in humans, dogs and guinea pigs (summarized in reference 9). These species differences in hepatic transport of IgA are also reflected in differences in the amount of IgA that is delivered into the intestine by way of bile and in the proportions of biliary IgA that are derived from the clearance of plasma pIgA vs. synthesis of pIgA in the hepatobiliary tissues: whereas the biliary excretion of pIgA is about 35 mg/kg body weight/day in rats and rabbits, it is only about 1 m/kg/day in humans, guinea pigs and dogs (6,8); and whereas about 90% of biliary pIgA in the rat is derived from the plasma, the corresponding figure in man is only about 50% (8,10). The source of the remaining pIgA present in human hepatic bile has not been directly identified, but we (11) found large numbers of plasma cells containing IgA and J chain surrounding the accessory glands in the extrahepatic bile ducts, so we suspect that the biliary duct mucosa is the source. IgA-secreting cells within the liver also may contribute to the content of IgA in bile (12).

The species differences in hepatic transport of pIgA are clearly related to the cellular locations of SC in the hepatobiliary tissues. In those species in which transport is moderate or high, SC has been found on hepatocytes. Moreover, by immunoelectron microscopy the SC is seen on the external surface of the sinusoidal plasma membrane of the cells, ideally situated to serve as a receptor for IgA polymers circulating in sinusoidal blood. In contrast to the situation in those species, the species that transport IgA poorly from blood to bile (man, dog, guinea pig) do not express SC on hepatocytes; rather they express it on intrahepatic and extrahepatic biliary epithelium (16; summarized in reference 9). Although this point was initially contested (14,15), it has been verified by reports from about 7 different laboratories (9). The relationships between the capacity for hepatobiliary transfer of pIgA and the cellular location of SC in hepatobiliary tissues are summarized in Table I.

TABLE I. Relationships between the capacity for hepatobiliary transfer of pIgA and the cellular location of SC

Species	Capacity for transfer	Location of SC
Rat	Large	Hepatocytes
Rabbit	Large	Hepatocytes
Chicken	Large	Unknown
Mouse	Moderate	Hepatocytes
Human	Small	Biliary epithelium
Monkey	Small	Unknown (not hepatocytes)
Dog	Small	Biliary epithelium
Guinea pig	Small	Biliary epithelium
Sheep	Small-moderate	Unknown

2. CELL BIOLOGICAL ASPECTS OF IgA TRANSPORT BY THE LIVER

The discovery of the SC-mediated pathway for IgA polymers through the rat liver (a much convenient organ to study than epithelia) greatly facilitated detailed studies on the biosynthesis and trafficking of SC and the transcellular routing of IgA during secretion. Some of the most significant findings are mentioned in the following paragraphs. SC is synthesized as a transmembrane glycoprotein (mSC). Like most integral plasma membrane proteins, mSC is synthesized in the rough endoplasmic reticulum, processed in the Golgi and transported to the plasma membrane. Since SC is also a soluble protein in secretions, though, it follows an especially complicated intracellular biosynthetic pathway. Several studies have established that the SC is synthesized as a molecule larger than the form that is present in secretions. Notably, Mostov and Blobel (17) demonstrated, by means of biosynthetic pulse labeling/chase experiments in HT29 human colonic adenocarcinoma cells, that the secreted form of SC (77 kD) is derived from the NH_2-terminus of a larger, transmembrane form of SC (95 kD). We (18) and others (19) have found that forms of SC in rat liver membranes also are larger than the immunologically related form present in bile. In experiments with biosynthetically labeled rats, SC appeared sequentially in the Golgi, the plasma membranes and the bile (20). From the sinusoidal membrane, mSC is routed across the hepatocyte to the canalicular plasma membrane, where it is proteolytically converted to the smaller, soluble form that is secreted into the bile (17-19). The location of the proteolytic step is not clarified yet, but the results of experiments with cultured primary hepatocytes suggest that it may occur at the canalicular surface (21). mSC reportedly is phosphorylated at serine residues located within the cytosolic tail of the molecule; consequently, the biliary form of SC is not phosphorylated (22,23).

A most remarkable feature of the synthesis and transcellular migration of SC is that those steps do not require the presence of the ligand, pIgA. Both in liver perfusion studies (24,25) and experiments with cultured cells

(23,26), SC followed its usual intracellular route even in the absence of IgA. This observation suggests that determinants of the complex intracellular sorting mechanisms for SC reside within the mSC molecule itself. Indeed, studies by Mostov and colleagues (27), using expression of a normal or mutated mSC gene in cultured, polarized epithelial (MDCK) cells, have suggested that the cytoplasmic portion of mSC is responsible for the initial targeting of mSC to the basolateral (sinusoidal) domain of the cell, whereas the extracytosolic portion of mSC (or SC) contains the appropriate signals for routing mSC to the apical domain of the cell.

The presence of mSC on the sinusoidal surface of the rat hepatocyte, though, is responsible for the efficient binding, endocytosis and transcellular migration of pIgA. Circulating pIgA binds to mSC, which is located diffusely along and in invaginations of the sinusoidal plasma membrane (13). In isolated hepatocytes, the binding of IgA induces clustering of mSC-IgA complexes; the clustering is sensitive to cytochalasin B, which can disrupt microfilaments (28). After pIgA binds mSC on the sinusoidal surface of the hepatocyte, the IgA-mSC complexes are internalized into coated endocytic vesicles (13,29). Soon after endocytosis, the IgA-mSC complexes are localized in a vesicular-tubular network (29). In this network, the sorting of IgA from other endocytosed ligands presumably occurs. The sorted, IgA-mSC- containing vesicles then migrate across the cell, avoiding interaction with lysosomes, and fuse with the bile canalicular membrane (13,30). The culmination of the IgA delivery pathway involves the previously mentioned proteolytic solubilization of the IgA-mSC complex at the bile canalicular pole of the hepatocyte.

Although the above studies in the rat have been important sources of fundamental information about the physiology of SC and IgA in the hepatocyte, they do not have much applicability to the issue of IgA secretion into human hepatic bile, because of the absence of SC from human hepatocytes. Nevertheless, human hepatic bile does contain both monomeric and polymeric forms of IgA, although at much lower concentrations than in the rat. As mentioned, we feel that some of the pIgA secreted into human hepatic bile is derived from plasma cells located in hepatobiliary tissues. pIgA synthesized by those cells likely is transferred into bile directly across SC-containing biliary epithelial cells (16). However, about 50% of pIgA and all of the monomeric IgA present in human hepatic bile reportedly come from plasma (8). How IgA is transferred into bile from plasma is unclear. Our electron micrographic studies suggest that there might be some transfer across periductular capillaries, thence to SC-containing biliary epithelial cells (16). Another route that has been proposed is that involving the asialoglycoprotein receptor (ASGPR), which is expressed on the sinusoidal surface of human hepatocytes. Support for this proposed route are the observations that 1) human IgA1 subclass proteins can bind to and be internalized by the ASGPR on Hep G2 cells (31), and 2) human IgA1 was cleared from plasma in the mouse by the ASGPR (32). The affinity of IgA1 for the ASGPR evidently is due to the presence of a galactose-terminated, O-linked oligosaccharide in the hinge region of IgA1. Usually, circulating asialoglycoproteins that bind to the ASGPR, are internalized into clathrin-coated vesicles, and the asialoglycoprotein is dispatched to

lysosomes for degradation. However, a small portion of the internalized ligand evidently can be spared this fate; in rat hepatocytes, for example, human pIgA1 can be internalized via the ASGPR, and once inside the cell, it dissociates from the ASGPR and binds to mSC, with consequent transport to bile (33). Whether such a pathway for pIgA exists in human hepatocytes remains to be resolved.

3. BIOLOGICAL ACTIVITIES OF IgA ANTIBODIES IN BILE

Two major biological benefits have been proposed for the secretion of IgA antibodies into bile: enhanced immunological protection of the biliary and upper gastrointestinal tracts and the clearance of harmful antigens from the circulation in the form of IgA-antigen complexes. Critical analysis of both of these propositions is indicated, however.

Several kinds of specific IgA antibodies have been demonstrated in bile, either after experimental immunization or in the natural state (summarized in reference 9). Not surprisingly, many of the "natural" antibodies are directed towards antigens on bacteria that are common in the gastrointestinal tract. It has also been shown that IgA antibodies in bile can be protective against cholera toxin-induced intestinal secretion in the experimental animal (34). However, it is quite another matter to show that the IgA biliary antibodies are effective in protecting either the biliary tract or the upper gastrointestinal tract in the physiological state; it is clinically obvious, for example, that patients whose bile is diverted from the upper gut do not suffer especially from enteric infections. Probably IgA antibodies in bile, like in other secretions, are but one of the numerous components of the mucosal defense system.

The hepatic transfer of circulating IgA-antigen complexes into bile via the SC-mediated pathway has been frequently demonstrated in the rat and mouse. This observation has prompted speculation that the disposal of such complexes represents a major role of the liver in protecting the host from harmful antigens, in particular those antigens that might have inadvertently violated mucosal barriers. However, since the efficiency of hepatic transport of IgA-antigen complexes is directly related to the transport of IgA itself, the human liver likely is not very effective in clearing IgA-antigen complexes into bile via the SC-mediated pathway. This then raises the question of whether that "defect" in IgA clearance renders the human especially prone to the development of diseases that involve the deposition of IgA or IgA immune complexes in tissues.

4. IgA IN HEPATOBILIARY DISEASES

Alterations in the concentration, subclass distribution and molecular configuration of serum IgA, and the presence of deposits of IgA in various tissues, have been observed in many kinds of liver diseases, most notably alcoholic liver disease (ALD). The finding that has attracted the most attention is the deposition of IgA along the margins of hepatic sinusoids in ALD (35-38). Indeed, the IgA deposits are regarded by some as diagnostic of ALD (38), although others (37) have challenged the specificity of

the deposits. I have concluded that the literature clearly indicates that IgA deposits in the liver are at least very common in ALD, if not entirely specific for that disease. A particularly interesting feature of the IgA deposits is that the IgA quite convincingly is IgA1 subclass (36). Why IgA1? Perhaps the answer lies in the affinity of IgA1 for the ASGPR. Is it possible that alcohol somehow alters the ability of the ASGPR to internalize and dispose of IgA1 or IgA1-antigen complexes and, consequently, the IgA becomes deposited in the liver. Equally provocative is the observation that the IgA that is deposited commonly in the kidneys of ALD patients is of the IgA1 subclass. Is this deposition also linked somehow to a disturbance in the handling of IgA1 by the ASGPR in the liver? These and many other questions concerning relationships between IgA and the liver in human diseases deserve much investigation.

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Secretory component (SC): comparative ontogeny in rat gut and liver

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ABSTRACT. Using a sensitive sandwich radioimmunometric assay, secretory component (SC) levels were measured in sonicated homogenates of isolated small intestinal crypt and villous cells, and of livers, in rats killed at various times after birth. There was very little SC expressed in both tissues before day 20, whereafter SC levels increased in parallel for both tissues up to 40 days of age, and then remained relatively stable up to 60 days. The simultanous appearance of SC expression in both tissues confirms the importance of the liver in the rat intestinal humoral defenses.

1. Introduction

Secretory component (SC) is the extracellular portion of the transmembrane receptor protein of epithelial cells (1) responsible for the selective transport of polymeric (p-) immunoglobulins (p-IgA and IgM) in various exocrine secretions (2) such as milk, saliva, tears, intestinal, genito-urinary and bronchial secretions, and bile. In some species like rabbits, rats and mice (3-5), the very high level of IgA in bile is due mainly to hepatocytes which strongly express SC at their sinusoidal pole and selectively transport pIgA from plasma or the space of Disse into hepatic bile against a concentration gradient. In other species, such as humans, monkeys, dogs, sheep and guinea pigs (4-6), hepatocytes do not express SC and the much smaller selective IgA enrichmnet of their bile results mainly from SC-mediated transport of epithelial cells in small and large bile ducts and in gall bladder (7,8). Besides its main transport function, SC is also known to stabilize p-IgA against proteases (9) and denaturing agents (10). In rats, the importance of biliary IgA for intestinal humoral immunity has been clearly outlined. For instance, about 90% of IgA found in rat upper intestinal washings is derived from bile (11), and high IgA antibody titers occur in bile of rats after repeated intestinal immunizations (12,13). It would seem logical that the epithelial expression of SC during development should precede or be concomittant with the appearance of plasmacytes secreting p-IgA in the mucosae.

In humans, SC has been occasionally detected by immunohistochemistry as early as at 8 weeks of gestation. The presence of SC has been regularly demonstrated at 13-17 weeks in bronchial, intestinal and kidney epithelia, but not in liver (14,15). This appearance of SC occurred long before any immunoglobulin-secreting cell could be found.

In rats, SC was detected in urine at 5 weeks of age, 2-3 weeks before secretory IgA (sIgA) was evidenced in the same secretion (16). Large numbers of IgA-plasmacytes only appeared in rat intestinal mucosa around 4 weeks of age (17,18). The ontogeny of SC in rat intestinal epithelium has been investigated in detail. Only minute amounts of SC were detected in rat enterocytes before 20 days post-partum, whereafter SC rapidly increased to reach adult levels at about day 40 (19). In the villus-crypt unit, the typically decreasing crypt-to-villus SC-concentration gradient became evident during the fourth week of life. Interestingly, corticosterone and thyroxine, two well known activators of "intestinal closure", a

process believed to play a major role in rat intestinal maturation, did not induce a precocious increase, but rather a decrease, in the expression of SC by rat enterocytes at 10-13 days of age; however, insulin was recently shown to significantly increase the minute SC content of enterocytes and hepatocytes in rats aged 13-20 days (20).

Because rat bile represents an important contribution in the establishment of rat intestinal immunity, it could be expected that the ontogenic expression of SC in rat hepatocytes would closely follow that in rat enterocytes. The present study deals with this question, and compares the expression of SC measured in liver homogenates and in homogenates of enterocytes of rats killed at various ages. Significant expression of SC by rat hepatocytes was initiated at 20 days of age, in good agreement with the SC ontogeny described in the rat small intestine.

2. Materials and Methods

2.1. ANIMALS

Wistar rats were used throughout. The day of birth was called day 0. Sucklings remained with their mother from day 0 (six rats per litter) and had free access to the nipples. Pups were weaned on day 18 onto a chow pellet diet. Six rats from the same litter were killed every other 5th day from day 0 to day 40 for liver cells or at days 10, 14, 20, 28, 40 and 60 for intestinal cells. Adult rats (60 days old) were issued from different litters.

2.2. RAT LIVER HOMOGENATES

After killing the rats by decapitation, the liver was dissected and a specimen of 30 mg of wet tissue was weighed from each animal for all age groups. The specimen was homogenized with a Potter (tight pestle) homogenizer for 30 sec at 2,600 rpm at 4°C in 3.0 ml of 0.1 M Tris-HCl buffer, pH 7.4 (representing a 100-fold dilution), and then sonicated for 30 sec at 21 watts at 4°C i a B12-Sonifier (Branson Sonic Power, Danbury, CT). Occasionally, 1% Triton X-100 (v/v) was added (30 min, 37°C) to the sonicated specimen to solubilize the remaining vesicles and membranes. A 1.0 ml aliquot of homogenate served for total protein quantitation (21) with bovine serum albumin as standard. Two other aliquots were used for SC titration.

2.3. RAT INTESTINAL EPITHELIAL CELLS

Sequential release of epithelial cells from villous tips to the bases of crypts was performed according to Weiser's (22) procedure modified by Raul et al. (23). Jejunal segments were removed, everted, and rotated (170 rpm) in PBS containing 1.5 mM EDTA and 0.5 mM dithiothreitol at 37°C for successive 10 min periods. The successive cell fractions obtained were collected, washed and homogenized as for the liver specimens. The percentage of cells isolated in each fraction was determined by the proportion of cell protein isolated in a given fraction, assuming that the total sum of protein corresponds to 100% of cells.

2.4. IMMUNORADIOMETRIC TITRATION OF SC

The procedure has been described earlier (19). In brief, the IgG fraction (20 mg/ml) of rabbit or goat antiserum against rat free SC (24) was used to coat polystyrene beads at pH 9.5 in 0.05 M glycine-NaOH buffer. After three washes with phosphate-buffered saline, pH 7.2 (PBS), beads were incubated for 4 h at 37°C with 0.2 ml of liver homogenate, undiluted or diluted 1/100 in 10% goat serum in PBS. After three more washes, incubation was pursued with 0.25 ml of iodine-labelled (25) affinity-purified goat or rabbit anti-SC antibodies (± 1,000 cpm/µl) for 18 h at room temperature. After three further washes, the radioactivity bound to the beads was counted. All individual liver homogenates were titrated in duplicate. The SC standard was pure rat bile free SC (24) or serum from bile duct ligated rats which was standardized with pure SC. The standard dilutions were in 10% (v/v) fetal calf serum in PBS. Standards were run with each new as-

say. The data were expressed as ng of SC per mg of liver or intestinal epithelial protein.

3. Results

3.1. INTESTINAL SC EXPRESSION.

The increasing postnatal expression of SC by intestinal villous and crypt cells of rats killed at various ages is illustrated in Fig. 1A. Up to 20 days of age, litlle SC was found in both villous and crypt cells. At 14 days, crypt cells expressed slightly more SC than villous cells (mean ± sem : 18.8 ± 5.9 versus 11.8 ± 0.5 ng of SC/mg cell protein, respectively). During the third and fourth postnatal weeks, SC expression abruptly increased in crypt cells and less markedly in villous cells. The crypt-to-villous decreasing concentration gradient typical of SC was reached at about 30 days of age and maintaind up to adulthood (60 days).

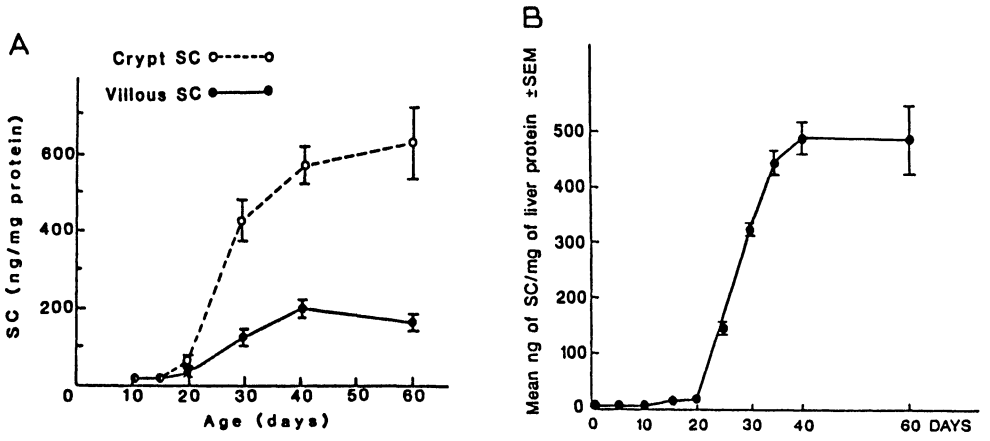

Figure 1. Appearance of SC expression in isolated intestinal villous and crypt cells (A) and in liver (B) of rats killed at various times after birth. Each point represents the mean ± sem of 6 rats.

3.2. HEPATIC SC EXPRESSION.

Only traces of SC were expressed by liver cells up to 20 days, as illustrated in Fig. 1B. Thereafter, liver SC expression increased rapidly to reach a plateau at 40-60 days of age. SC was also measured in liver homogenates from rats killed at various times after birth, in the presence or absence of 1% Triton X-100. Mean levels of SC ± sem (in ng per mg of total protein) in the presence of Triton were 10.95 ± 4.3, 184.1 ± 21.0 and 571.5 ± 100.3 at 5, 25 and 40 days of age, respectively, and 12.08 ± 1.3, 162.7 ± 10.8 and 552.8 ± 49.1 in the absence of Triton (n = 6).

4. Discussion

The parallelism of the development of SC expression between rat intestinal and hepatic cells is strongly outlined by these data. For both cell types, little SC was expressed before 20 days of age, whereafter SC production strongly increased. Apparently, adult SC-expressions in crypt enterocytes and in liver cells were roughly equal (500-600 ng SC per mg protein). At 20 days, however, the SC content of crypt enterocytes (60 ng/mg) was larger than that of liver cells (20 ng/mg). This suggests a stronger early IgA-transport in gut than in liver epithelium, and could relate to the first occurrence of large numbers of IgA-secreting cells in the gut lamina propria, together with very low levels of circulating polymeric IgA.

Our assays probably measured total SC content, including both membranous and soluble forms of SC. The anti-SC antisera used in these studies only reacted with the extracytoplasmic part of the polymeric Ig-receptor, and not with its transmembrane and intracytoplasmic domains. As the cells were both strongly "potterized" and sonicated, we believe that intact vesicles which could have hidden some SC inside closed membranous structures were not left. Also, cleavage of hepatocyte membranous SC has been shown after cell disruption (26). Moreover, our hepatic SC levels in the presence of Triton X-100 were not significantly higher than those without Triton.

The parallelism in the ontogeny of intestinal and hepatic SC-expression in the rat further confirms the major role which the rat liver plays in the rat intestinal secretory immune system.

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The concentration of IgA in hepatic lymph

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ABSTRACT. In order to find out about the extent of IgA synthesis in the liver, samples of hepatic lymph were collected from 7 rats and 6 sheep, together with corresponding samples of venous blood. The concentrations of total protein and of IgA were measured in each sample of lymph plasma or blood serum. All the IgA in the peripheral lymph from the livers of sheep could be accounted for in terms of ultrafiltration from the blood. In rats, the mean concentration of IgA in hepatic lymph was 93.5µg/ml., some 5 times higher than the concentration in the blood. A consideration of the flow rates of hepatic lymph etc. showed that even if all the lymph-borne IgA were synthesised in the liver it would probably account for no more than 6% of the output of IgA in the bile. It was concluded that the livers of healthy, adult rats are quantitively unimportant in the synthesis of IgA.

INTRODUCTION

The classical experiments of Miller and Bale [1] showed that the liver can synthesise all plasma proteins except immunoglobulins. This fact, together with the observations that the liver can transport actively large amounts of endogenous (from the gut) or exogenous IgA dimer from blood to bile [2,3] encourages the belief that most biliary antibody is of extra-hepatic origin. Recently, it has been proposed that local antibody synthesis by cells in the laminae of the bile ducts and the portal tracts may make a contribution to biliary antibodies [4,5]. In order to get some measure of such intrahepatic Ig synthesis we have investigated the composition of hepatic lymph as described below.

MATERIALS AND METHODS

Animals and surgical procedures

Cross bred whethers weighing approx. 30kg were used. Under general anaesthesia, an afferent duct leading to the hepatic node was cannulated directly with polythene tubing with an O.D. of 1.0mm [6]. No attempt was made to collect all the hepatic lymph from sheep; the efferent ducts from the node or other afferent ducts were not ligated,

and in all cases the cannulated duct drained from the region of the gall bladder. The lymph was collected into heparinised, polythene bottles and flowed for up to 40 days. After operation samples of blood were taken by direct, percutaneous jugular venepuncture.

Adult Wistar rats ($RT1^u$) weighing 300g were anaesthetised with ether and the main hepatic lymph duct was cannulated by the standard procedure [7] using polythene tubing of 0.6mm O.D. After recovery from anaesthesia the rats were kept in a Bollman cage and the lymph collected quantally for 2-3 days, after which the rats were exsanguinated.

Preparations which did not yield a continual and spontaneous flow of lymph were excluded from the series.

Assay of proteins

Total protein was measured by the Bicinchonic acid assay, using the manufacturers instructions and standards (Pierce Chemicals, Cheshire, U.K.). Total IgA was measured by solid phase RIA [8], using an affinity purified or monoclonal antiα reagents and standards of dimeric IgA of the appropriate species.

RESULTS

The salient results are shown in Tables 1 and 2. Table 3 presents the data in a derived form embodying known physiological constants.

TABLE 1. Principal characteristics of blood serum and peripheral hepatic lymph plasma collected from six unanaesthetized sheep. Mean and range.

	Total Protein (mg/ml)	Total IgA (µg/ml)
Blood	81.5 (69.5 - 91.5)	178.1 (58.7 - 313.9)
Lymph	56.1 (54.3 - 74.8)	83.7 (23.1 - 122.3)
	Flow rate of lymph = 1.23 (0.3 - 8.0) ml/hr	

TABLE 2. Principal characteristics of blood serum and hepatic efferent lymph plasma collected from seven unanaesthetized rats. Mean and range.

	Total Protein (Mg/ml)	Total IgA (µg/ml)
Blood	62.1 (47.4 - 71.8)	16.9 (6.3 - 28.0)
Lymph	43.2 (32.9 - 51.5)	93.5 (10.9-208.9)
	Flow rate of lymph = 0.08 (0.03 - 0.13) ml/hr.	

TABLE 3. Dynamics of IgA Production in Rats

Total hepatic lymph flow at 4ml/day, containing 93.5µg IgA/ml	= 374µg IgA/day
Total bile flow at 24ml/day, containing 250µg IgA/ml	= 6000µg IgA/day
Daily output of IgA in hepatic lymph thus	= 6.2% of Biliary output
Daily output of IgA in hepatic lymph	= 28µg IgA/g wet wt hepatic tissue/day
Total intestinal lymph flow at 100ml/day, containing 100µg IgA/ml	= 10,000µg IgA/day
Daily output of IgA in intestinal lymph	= 2857µg IgA/g wet wt of small gut/day
	i.e. 100 times that of the liver

DISCUSSION

The concentration of IgA in peripheral hepatic lymph from sheep was consistent with the view that the bulk of this Ig was derived from the blood. This does not exclude the possibility that tiny amounts of IgA might have been added by plasma cells in the biliary tract, but substantial local synthesis, as occurs in the gut, always endows the local lymph with IgA concentrations above those of blood, even in sheep where IgA is not the only secretory Ig [9]. Nonetheless, sheep do actively transport IgA from blood to bile [10].

The concentration of IgA in rat hepatic lymph was five times higher than that of blood. Some of this would have come from the blood, some (about 7%, data not shown) was combined already with S.C., and some may have come from plasma cells in the hepatic node. However, even without taking these erosions of the total figure into account, the daily output of IgA in hepatic lymph was small. Of course, we did not collect all the hepatic lymph, some would have drained into lymphatics accompanying the hepatic veins but we took this into account by doubling the observed flow rate for the purposes of our calculations. In any case the hepatic lymph, as collected, would have included most lymph coming from the biliary tree. Even so, it accounted for only about 6% of the biliary output of IgA. The question arises as to the ratio of the lymphatic output of IgA to the output of directly secreted IgA. This ratio cannot be measured directly; a reasonable assumption is that the ratio is 1:1 but it is an assumption. However, a direct comparison with the gut is possible; there the daily output of IgA in the lymph is some 10mg a day, i.e. some 25 times more than that of the liver, in absolute terms, and 100 times more when expressed in terms of the relative weights of the two organs. This puts the contribution of intrahepatic IgA synthesis into perspective; it does occur, it can be measured, and it is small. Of course, were the liver to become the site of a substantial lymphoid infiltrate, as

occurs e.g. in hepatitis the amount of local Ig synthesis might well be increased but that would be pathology, not physiology.

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Immune activity in the rat liver

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Abstract: Intravenous (IV) or intra Peyer's Patches (IPP) immunization of rats with horse red blood cells (HRBC) results in an increased number of mononuclear cells being associated with the liver. Those involved in antibody secretion are in the minority but are held responsible for much of the specific antibody activity in bile. By far the majority express T-cell markers ($W3/13^+$) with $OX8^+$ cells predominating over $W3/25^+$ cells. The estimated residence time of IgM secreting cells in the liver was assessed by splenectomizing rats which had been immunized 3 days previously. A 75% loss of specific IgM antibody in bile within 10 hours was followed by a steady output for a further 12 hrs. This suggests both transient and longer term residency of secreting cells. IPP immunization and the subsequent labelling of PP cells with FITC revealed the migration of labelled cells to the liver with $OX8^+$ cells showing a preference to localize. These results demonstrate that the liver is a major site of lymphocyte activity during an immune reponse.

Introduction

The sources of specific antibody against foreign erythocytes in rat bile resulting from intestinal (1,2) or systemic (3) immunization are considered to be from plasma via an active transport system and from synthesis within the liver. Evidence for the latter is based on the detection of antibody forming cells (AFC) in the liver following immunization (4,5). The working hypothesis of our group is that a portion of the AFC which arise in lymphoid tissue after antigenic challenge migrate to and localise in the liver where they secrete antibody into bile. The processes involved in the localization and secretion are not clearly understood. Here we address the issue of the functional time that AFC spend in the liver. Also it was noted that liver AFC are only a minor component of the lymphoid cells which migrate to the liver (4). We report here on the nature and likely source of the cellular infiltrate in the livers of rats immunized IPP.

Materials and Methods

Animals and immunizing regimens. Wistar rats (10-12 weeks old) were obtained from the Animal Unit of the School of Microbiology. Groups of rats were injected IV with 1.0 ml of a 7.5% or 1.0% suspension of washed horse erythrocytes (HRBC; CSL, Melbourne, Australia) in 0.15M phosphate buffered saline (PBS), pH 7.2, or IPP with 0.05 ml of a 15% suspension of HRBC. The spleens of some rats were removed through a small incision in the abdominal wall after ligation of the appropriate blood vessels.

Sample collection and serology. Serum samples were prepared from blood taken from a tail vein and bile was collected according to the method of Lambert (6). Haemagglutinin activity (HA) in serum and bile was measured in a standard haemagglutination assay.

Lymphoid cell preparation and staining. The preparation of mononuclear cell suspensions from liver and

lymphoid organs has been described (7). The immunoperoxidase method of Barclay (8) was used to assay the proportions of T and B-cell subsets. Briefly, cells were smeared onto glass slides, fixed in acetone and treated with the mouse monoclonal antibodies MRCOX8, W3/25, W3/13 or MRCOX12 (Seralab, Sussex, England; see (9) for reactivity of these antibodies) for 1 h. After the slides were washed, horseradish peroxidase-labelled anti mouse immunoglobulin (Dako, Glostrup, Denmark) was applied and incubated for a further 1 h. The slides were then washed and treated with Hanker-Yates reagent (Polysciences, Detroit, Michigan) for 1 h. and then washed again. A Leitz microscope was used to count a minimum of 200 cells in order to determine proportions staining with each antibody.

In one experiment, 1 day after IPP immunization with HRBC, the Peyer's patches (PP) of a group of 3 rats were injected with fluorescein isothiocyanate (FITC, Isomer 1; Sigma, St Louis, Missouri) solution prepared by the method of Butcher and Ford (10). Five days later cell preparations from PP and liver were treated with MRCOX8 or W3/25 monoclonal antibodies and then with rhodamine-conjugated sheep anti-mouse IgG (Silenus Laboratories, Melbourne, Australia). The cells were fixed in 1% formaldehyde in PBS and examined using a Leitz microscope equipped with a 100W mercury/75W Xenon vapour lamp epi-illumination and exiter/barrier filter combinations for fluorescein and rhodamine (TRITC). A minimum of 100 $FITC^+$ cells were counted per sample and their phenotype determined by switching to filters to detect rhodamine. The number of $TRITC^+$ cells was expressed as a percentage of the total number of $FITC^+$ cells counted. For comparison, cells were prepared from PP and livers of rats injected only with FITC (5 days previously) and then phenotyped as above.

Results

When groups of 4-6 rats were immunized IV or IPP with HRBC, approximately 10-20 fold increases occurred in the numbers of mononuclear cells which could be isolated from the livers (results not shown). Also, the increases were found to be primarily due to infiltrating small lymphocytes which expressed T-cell markers (Table 1). It is especially noted that while the $OX8^+$:$W3/25^+$ ratio remained close to 2:1 after IV challenge, the ratio dramatically shifted to 6:1 following IPP immunization.

While increased numbers of $OX12^+$ cells were not as obvious, we have previously reported on the appearance of large numbers of anti-HRBC haemolytic plaque forming cells in the liver following immunization (4). To gain some insight into the time a liver AFC actually delivers antibody to bile, a group of 10 rats was immunized IV with HRBC (1%) and their bile ducts cannulated 3 days later. At that time 5 rats were splenectomized. The results shown in Fig. 1 indicate that removal of the spleen causes a decline in biliary activity to approximately 25% of the initial level within 10 hours whereas the levels in the bile and serum of the unsplenectomized animals continued to rise. After 10 hours the splenectomized rats continued to secrete antibody at a steady rate. Splenectomy at the time of immunization abrogated both serum and biliary antibody responses.

The final matter relates to the nature and possible source of the infiltrating T cells following IPP immunization. The experiment is described above and is part of a larger study to be reported elsewhere. The results given in Table 2 show an increased proportion of $FITC^+/W3/25^+$ cells in PP following immunization. Further, $FITC^+$ cells were detected in the livers of both groups of rats (at levels much in excess of those present in blood-results not shown). Interestingly the proportion of liver $FITC^+/OX8^+$ cells increased significantly ($P<0.05$) while the proportion of $FITC^+/W3/25^+$ cells decreased significantly ($p<0.05$) as a result of immunization.

Discussion

The results presented in this paper strongly support the idea that the liver is a significant site for lymphocyte accumulation following immunization. Early studies (11) demonstrated that hepatic lymph is a particularly rich source of lymphocytes, suggesting that the liver should be considered in discussions of lymphocyte traffic. It must be stated that no evidence has been presented here which indicates whether cells detected in the liver are destined for hepatic lymph or are merely retarded in their passage in blood.

The clearest indication of significant interaction between lymphoid cells and the liver is the indentification of in situ antibody synthesis for secretion into bile. Results from the present and previous studies (3,4) indicate the spleen as the source of liver IgM AFC following IV immunization and that a continuous input is required to at least maintain the level of biliary antibody. It is apparent then that IgM-

Table 1. Lymphocyte subsets in the livers of rats immunized with horse erythrocytes

	Numbers of cells per liver staining positively with monoclonal antibodies		
Monoclonal antibody	Unimmunized	IV (day 6) immunized	IPP (day 5) immunized
OX 12	50,000 ± 7,300	51,250 ± 16,500	132,000 ±22,400
W3/13	162,500 ± 74,105	1,275,000 ± 203,150	4,900,000± 42,000
W3/25	92,300 ± 13,694	321,000 ± 91,000	660,000 ± 14,000
OX8	195,000 ± 7,071	600,000 ± 105,000	3,900,000 ± 84,550

Details of experiment are given in text. Cells were enumerated in mononuclear cell preparations following immunoperoxidase staining. Values are the mean numbers (± SD) from 4-6 rats.

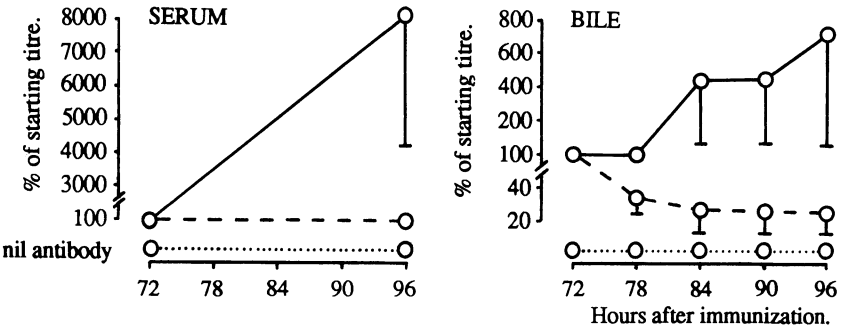

Figure 1. The effect of splenectomy on haemagglutination responses in serum and bile of rats after HRBC injection (IV). Individual titres are expressed as percentage of the initial titre. Intact O——O. Splenectomy at immunization (day 0): O·····O. Splenectomy 72 hours after injection: O - - - O.

Table 2. The migration of Peyer's patch T-cells to the rat liver.

	% FITC $^+$ cells reactive with monoclonal antibodies			
Tissue	W3/25	OX8	W3/25	OX8
	Unimmunized		Immunized	
Peyer's patches	14.8 ± 0.3	3.9 ± 0.7	36.3 ± 5.9	2.7 ± 1.4
Liver	23.9 ± 2.3	47.8 ± 2.9	10.9 ± 2.4	61.9 ± 1.0

The experiment is described in the text. Values are the mean percentages (±SD) of at least 100 FITC$^+$ cells from each of 3 rats which were reactive with the respective monoclonal antibodies as detected with TRITC labelled anti-mouse IgG serum.

AFC at least are but transient residents although it is tempting to suggest that a minor portion do reside for longer periods as seems to be the case for IgA secreting cells (5). This is consistent with the finding that little or no IgM is transported from plasma to bile (3). The detection of IgA AFC in sheep hepatic lymph after immunization (12) suggests a pathway from blood to hepatic lymph. A further possibility is that the liver is a site for further maturation of antibody secreting cells, especially as cells with definite plasma cell morphology are rarely seen. Finer studies including histological analysis should help resolve these issues.

As far as T-cell localization in the liver following immunization with the 'bland' antigen HRBC is concerned, there is as yet no functional activity which has been highlighed which would allow a quantitative assessment of the nature of the interaction and its role in the immune response. It is quite apparent that the lymphocyte content in the liver represents a discrete population (13,14) and on teleological grounds it is reasonable to expect them to express an individual function. It is noted in passing that the predominance of $OX8^+$ cells and the considerable numbers of large granular lymphocytes (15) is similar to the make up of intra-epithelial lymphocytes of the gastro-intestinal tract.

The detection of $FITC^+$ cells in the liver in numbers in excess of those in blood indicates a pathway of migration of the major T cell subsets from Peyer's patches to this organ. T cells exit from mucosal sites after antigenic challenge and their subsequent pathways of migration have not been extensively studied although the emigration of T helper (16) and T suppressor/cytotoxic cells (17) from PP has been documented. Pertinent to the current study, Jeurissen et al. (18) demonstrated that suppressor T cells are the major emigrants from PP. The current results and those to be published elsewhere support this finding and futher suggest that the liver is a significant site for the transient localization of T cells with the suppressor/cytotoxic phenotype having an enhanced capacity to interact.

Acknowledgement

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Altered mucosal immune response in CCl_4-induced liver cirrhotic rats

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ABSTRACT. Changes of immunological response in GALT were examined in CCl_4-induced liver cirrhotic rats. In these rats, the number of transported lymphocytes through intestinal lymph especially T cell population was significantly suppressed with marked lymphangiectasia of small intestine. The population of pan-T cells especially suppressor/cytotoxic-T cells were significantly decreased in the intestinal mucosa and mesenteric lymph nodes. On the other hand, mucosal IgA containing cells and macrophages were remarkably increased. In spite of increased IgA containing cells, specific antibody production against cholera toxin appeared to be decreased judging by anti-cholera toxin containing cell count in the mucosa and by ELISPOT assay of intestinal lymph cells in liver cirrhosis. In conclusion, other than altered systemic immunity, there are significant alterations in mucosal immune response which may closely associate with lymphatic disturbances in liver cirrhosis.

INTRODUCTION

In liver cirrhosis, disturbance in lymphatic transport of macromolecules through intestinal lymph has been reported (1). Liver cirrhosis may affect the immunological response of gut-associated lymphoid tissues (GALT), which are closely associated with intestinal lymphatic system, however, there are paucity of data about the changes of mucosal immune system of gut. In this study, therefore, the effect of liver cirrhosis on mucosal immune response was examined in CCl_4 -induced cirrhotic rats.

MATERIALS AND METHODS

Male Wistar rats 200g were used for the experiments. Liver cirrhosis was induced by the subcutaneous injection of carbon tetrachloride to rats at the doses of 0.3ml/100g B.W. as a 50% solution in olive oil twice a week for 12 weeks. Intestinal lymph flow and lymphocyte number were determined after the cannulation into intestinal lymphatics according to Bollman et al(2). Lymphocyte subsets in peripheral blood and intestinal lymph were determined by fluorescence activated cell sorter using monoclonal antibodies against rat lymphocytes. Lymphocytes which are positive against monoclonal antibodies W3/13HLK, W3/25, OX8 and OX6 are regarded as pan-T cells, helper-T cells, non-helper-T cells and Ia positive cells respectively.

Mucosal weight and the area of Peyer's patches of the small intestine were compared in control and liver cirrhotic rats. Small intestinal mucosa and mesenteric lymph nodes were fixed with PLP and cryostat sectioned. Lymphocyte subsets and macrophages in these tissues were determined by monoclonal antibodies against rat lymphocytes and anti-rat macrophage antibody Mar-1 (gifted from Dr. A. Yamashita, Hamamatsu Medical College, Japan) using enzyme immunohistochemistry. Immunoglobulin containing cells in the tissues were also determined. Number of positive cells per 1000 nuclear cells in the tissues were compared in control and liver cirrhotic rats.

Specific immunological response against cholera toxin (CT) was compared in control and cirrhotic rats. Anti-CT antibody was made by the immunization of rabbit with purified CT(Sigma) and IgG fraction of sera was purified and peroxidase labeled. Purified CT was administered into the duodenum of rats at the dose of 10 μg/100gB.W. and the same dose of CT was administered intraduodenally two weeks later as booster. Five days later, small intestine and mesenteric lymph nodes were removed, fixed with PLP and cryostat sectioned. After blocking of intrinsic peroxidase, tissues were incubated with CT (l μg/ml) for 1 hour and then reacted with peroxidase-labeled anti-CT antibody.

Specific antibody production against CT was also determined in intestinal lymph lymphocytes using solid phase enzyme-linked immunospot (ELISPOT) assay according to Czerkinsky et al (3). After coating of petri dishes with purified CT (10 μg/ml), lymphocytes collected from intestinal lymph (10 ml with 5%FCS) were incubated for 6hrs at 37°C in 10% CO_2 After washing, peroxidase-labeled anti-rat IgA was added, then reacted with paraphenylenediamine and H_2O_2 in agar. Dark spots were recognized as spot-forming cells and these numbers were compared in control and cirrhotic rats.

RESULTS

After a full course of CCl_4 injections, the livers of these rats had developed cirrhosis. In small intestine, marked lymphangiectasia was observed in intestinal mucosa of CCl_4-treated rats and the central lacteals of villi of these rats appeared to be dilated even at fasting state. There was about twice increase in portal vein pressure in liver cirrhotic rats. There was significant decrease in the area of Peyer's patch in the ileum of cirrhotic rats. It should be noted that intestinal lymph flow was significantly enhanced in rats with liver cirrhosis. Despite of the marked increase in lymph flow, total lymphocyte number transported through intestinal lymphatics was significantly decreased suggesting the disturbed lymphocyte traffic (Table 1). Percentage of T cells especially non-helper-T cell subset tended to be decreased in intestinal lymph of liver cirrhotic rats.

From immunohistochemical study, pan-T cells detected as W3/13HLK positive cells were mainly observed in the lamina propria of intestinal villi and in Peyer's patch especially in thymus dependent area in control rats. There was a significant decrease in T cell number in cirrhotic rats, although there was no significant changes of their distribution pattern. The non-helper T cells detected by OX8 were observed lining just below the epithelial cells in control rats, however, there was a dramatic decrease in this population in cirrhotic rats (Table 1). In contrast to the changes in T-cells, there was a significant increase in IgA containing cells in liver cirrhotic rat intestinal mucosa, suggesting the increased synthesis of IgA in the intestinal mucosa. There was also a significant increase in the number of Mar-1 positive cells in the lamina propria of intestinal mucosa of cirrhotic rats. The changes of lymphocyte subsets and immunoglobulin containing cells in mesenteric lymph nodes showed similar changes compared to those seen in the intestinal mucosa. These changes were more dramatically observed in the small intestine of rats with ascites compared to those in rats without ascites.

To further determine the specific immunological reaction, response against CT was observed in two groups. The number of anti-CT containing cells was about 40 in 10000 nuclear cells in control small intestine and about 500 in mesenteric lymph nodes. There was remarkable suppression of the appearance of anti-CT containing cells to 25 in intestinal

mucosa and to 380 in mesenteric lymph nodes cirrhotic rats. Similarly, the decreased response against specific antigen was observed in intestinal lymph lymphocytes. Spot-forming cells in intestinal lymph determined by ELISPOT assay were 100 cells per 10^6 lymphocytes in control groups, while this number was significantly decreased to 75 in liver cirrhotic rats.

TABLE 1. Lymphocyte number of intestinal lymph, lymphocyte subsets and immunoglobulin containing cells in lamina propria of the small intestine of control and liver cirrhotic rats

	Control	Liver cirrhosis	P
Lymphocyte number of intestinal lymph ($x10^6$/h)	10.8 ± 1.3	8.6 ± 1.6	0.05
Positive cells in intestinal mucosa (per 1000 nuclear cells)			
W3/13HLK (pan T)	337 ± 35	255 ± 34	0.01
W3/25 (helper T)	320 ± 30	210 ± 44	0.01
OX8 (non helper T)	280 ± 25	167 ± 19	0.001
Mar-1	319 ± 90	230 ± 23	N.S.
IgA	208 ± 31	315 ± 86	0.05
IgG	90 ± 19	97 ± 22	N.S.

Values are Mean ± S. D.

DISCUSSION

Recently gut-liver interactions in the immune system have been paid attention from the standpoint of IgA system. It is known that liver can function as an IgA pump transporting polymeric IgA from serum to bile and in alcoholic liver diseases significant rise in serum IgA has also been reported (4, 5).

The present study revealed that cellular immunity of intestinal mucosa was markedly altered under the cirrhotic condition. The most striking features are the decreased T cell number especially the decrease in suppressor/cytotoxic-T cell subpopulation and the increased IgA containing cells in the intestinal mucosa of cirrhotic rats. The exact mechanism for these changes are not known, however, one possibility is a condition of chronic stimulation of GALT because of increased amount of bacterial antigens in liver cirrhosis(6). It is also considered that blocking of lymphocyte migration through intestinal lymph may play major role in the changes of GALT components. The observation that ascites formation produced the more drastic change in immunological response of GALT suggests that these immunological changes closely correlate to the lymphatic disturbance in liver cirrhosis. Portal hypertensive states may associate with the disturbance of lymphatic drainage which should be important for clearance of luminal antigens. The immunological changes will further decrease the mucosal defense and allow the easy access of luminal antigens to the intestinal mucosa. In spite of increased IgA containing cells, specific immunological response against CT appeared to be decreased in liver cirrhotic rats. In this respect, further investigations concerning antigen-specific immunoregulation are necessary to elucidate the exact changes of mucosal immunity in liver cirrhosis.

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IgA subclass antibodies to *Streptococcus mutans, Escherichia coli, Veillonella parvula* and *Clostridia perfringens* in human peripheral blood, portal venous blood and bile. Evidence for local antibody production in bile

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1. Introduction

Human IgA occurs in two subclasses, IgA1 and IgA2, distinguished by antigenic, biochemical and biological properties (Mestecky & Russell 1986). The two subclasses are distributed in serum and external secretions, approximately 75% of serum IgA belongs to the IgA1 subclass, occurs predominantly in monomeric form, and originates from bone marrow. In contrast up to 60% of the IgA in external secretions belongs to the IgA2 subclass, is produced locally in secretory glands and tissues and is predominantly in polymeric form. The proportion of the polymeric and monomeric form of IgA is significantly increased in patients with cirrhosis (Kutteh et al., 1982; Newkirk et al., 1983; Kalsi, Delacroix & Hodgson, 1983). These observations suggest that the liver plays a role in the maintenance of normal levels of secretory and serum IgA. Human bile contains high levels of polymeric IgA,but the origin of this IgA is not clear. In this study we have compared the concentration of IgA antibody and its subclasses in 22 matched samples of human portal and peripheral venous blood and bile. Antibodies of each isotype to two oral bacteria were compared with those to two non-oral bacteria. In addition, the specific activity of antibodies in each fluid was calculated to determine if evidence for local production in bile could be found.

2. Materials & Methods

IgA was assayed in 22 matched samples of peripheral venous blood, portal venous blood and bile obtained from patients undergoing abdominal operations. Total IgA, IgA1 and IgA2 concentrations were assayed by modified antibody capture method using ELISA and specific monoclonal or polyclonal antisera (Challacombe et al., 1987). Microtitre plates were coated with anti- human IgA (Dakopatts) and incubated overnight at 4° C. After washing and blocking, serum samples were added at appropriate dilutions. Specific anti- human IgA (monoclonal, Oxoid), IgA1 (Nordic) or IgA2 (Becton & Dickinson), antibodies were added, as required in the experiment. The assay was completed by adding enzyme linked conjugate and then substrate. The absorbance was measured at 405nm. Representative bacterial strains of Streptococcus mutans, Escherichia coli, Veillonella parvula and Clostridia perfringens were grown in Todd Hewitt broth, washed, harvested and resuspended to a concentration of $2x10^9$ (Czerkinsky et al 1983). IgA, IgA1 and IgA2 antibodies to each of the bacteria were assayed by a solid phase ELISA using methyl-glyoxal attachment of whole bacterial cells as previously described (Czerkinsky et al (1983). Specific activity of IgA, IgA1 or IgA2 antibodies were expressed as antibody units per ug of the IgA, IgA1 or IgA2 concentration.

3.Results

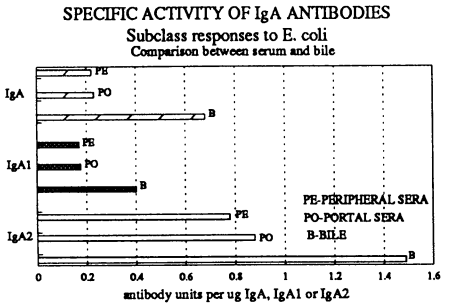
Serum IgA subclass antibodies to S. mutans and to C. perfringens were predominantly of the IgA1 subclass whereas those to E. coli and V. parvula were equally distributed between IgA1 and IgA2. There was considerable variation between individual subjects and some subjects for example had no detectable IgA2 antibodies to C. perfringens. The mean IgA antibody concentrations were significantly lower in bile than in serum. In addition a greater proportion of the antibody was of the IgA2 subclass to each of the bacteria examined. Measurement of total IgA, IgA1 and IgA2 levels allowed specific activity to be calculated. Striking difference between

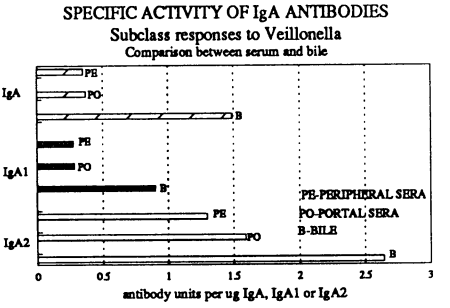
serum and bile were evident (figure 1.) The specific activity of antibodies to E. coli and to V. parvula were much greater in bile than in serum, and this was evident with antibodies of both IgA1 and IgA2 subclasses (figure 1).

4.Discussion

In this study we did not find any significant differences between portal and peripheral serum IgA or IgA subclass responses to the bacteria examined in individual patients. This was not unexpected, though portal blood may contain greater concentrations of secretory IgA and polymeric IgA (Challacombe et al.,1987). The proportion of specific IgA antibody in serum which was polymeric was not examined in this study. The IgA1/IgA2 antibody ratios to S. mutans and to C. perfringens were higher than those to V. parvula and E.coli in both peripheral and portal sera. There was proportionally a much greater IgA2 antibody response to the two Gram-negative bacteria. This proportionally greater response in the IgA2 subclass to E.coli and V. parvula may indicate that subclass responses are directed to specific antigens such as lipopolysaccharide.

In bile the total IgA2 concentration was greater than IgA1. This was reflected in antibody values and bile contained a greater proportion of IgA2 antibodies than IgA1 to both E. coli and V. parvula than was found in portal or peripheral blood. The specific activity of bile antibodies for IgA and for IgA1 and IgA2 was significantly higher than in serum (see figure). These findings suggest that in man IgA antibodies in bile do not directly reflect those in serum and strongly suggest a contribution by local synthesis.





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Local synthesis as the likely source of specific and natural IgA antibody in human bile

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Introduction

In humans, diffusion and low level transport from blood as well as local production (Dive and Heremans 1974, Delacroix et al. 1982) all contribute IgA to bile. We have been studying natural biliary IgA antibody against Escherichia coli O serotypes and the specific bile IgA that can follow tetanus vaccination as to their occurence and whether they arrive secondhand from blood or are made in the hepatobiliary tract.

Materials and Methods

Patients from whom bile could be collected participated in the study. Serum samples were taken at bile collection. Some patients received Tetanus Vaccine, Adsorbed (5Lf, CSL, Parkville, Victoria). IgA antibody was detected by indirect ELISA using plates coated with either soluble tetanus toxoid (TT, 10 Lf/ml, CSL) or E. coli O type 01 antigen (Ahlstedt et al. 1978) at 10 µg/ml. Bound antibody was detected using an alkaline phosphatase anti-human α chain conjugate (Kirkegaard Perry Lab., Gaithersburg, Md.). Bound Secretory Component (SC) was detected with a rabbit a/human SC and anti-rabbit Ig HRP conjugate (Dako, Denmark.). Titres of IgA a/TT were ascribed relative to a reference positive serum and titres to E. coli 01 were taken at a cutoff point 0.1 O.D. units above background (Dahlgren et al. 1986). Selected samples were retested by ELISA after absorption with specific and non-specific antigens. Total IgA concentrations in serum and bile were measured by rate nephelometry using Beckman Protein Array equipment (Beckman, Brea, Calif.). Immunoglobulin fractions in selected samples were separated on Ultrogel AcA22 (LKB, Sweden).

Results and Discussion

Tetanus immunization of patients led to increased serum IgG antibody and produced a short lived IgG response in bile (14 patients, result not shown). Of these, 9 also made an IgA response in both serum and bile (Figure 1a.). IgA antibody could be found in both hepatic and gall bladder bile, and was selectively removed by absorption with the original vaccine, but not adsorbed ovalbumin. A comparison of antibody per total IgA in serum and bile showed that this antibody was proportionately more abundant in bile (not shown). Preliminary work suggests that biliary IgA antibody is not SC bound. It may however by dimeric as it elutes in the major IgA region when filtered on Ultrogel AcA22.

Natural IgA antibody to E. coli was detected either in both serum and bile, or serum alone, or bile alone (Figure 1b.). Both hepatic and gall bladder could contain natural IgA antibody, confirming the observations of Dahlgren and colleagues (1986). Values for antibody per mg. of total IgA in serum and bile were seldom comparable; bile was either enriched or depleted for IgA antibody relative to serum (not shown). Absorption of antibody-positive samples with E. coli 01 specifically removed antibody to this serotype whereas absorption with E. coli 08 did not.

These results suggest little link between the antibody simultaneously present in paired serum and bile, and appear to preclude the serum as source of these biliary antibodies. Rather, the biliary antibody may be made locally as occurs in rats after both systemic (Jackson et al. 1985) and intestinal (Altorfer et al. 1987) immunization. The usefulness of intrahepatic synthesis of IgA for bile remains unclear; in rats it appears redundant given their constitutive SC transport of IgA from blood to bile. It does however operate early in

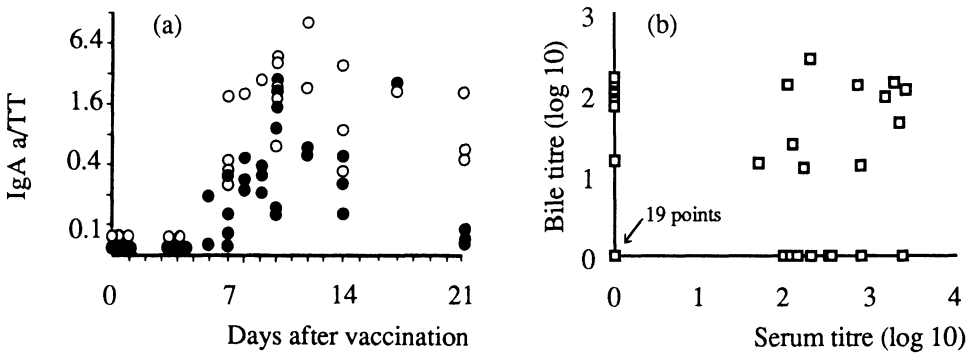

Figure 1 (a). IgA antibody response in the serum and bile of 9 subjects after tetanus toxoid immunization. Values for available samples are shown, samples for every patient on each day were not obtained. Serum ○ bile●. 1 (b). Natural IgA antibody to Escherichia coli 01 in paired sera and biles.

their intestinal immune response (Altorfer et al. 1987), raising the possibility that its purpose in this species is to protect hepatic precincts, while transported IgA is intended more for the proximal intestine.

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Dissociation of IR22 IgA myeloma protein during hepatobiliary transfer after thermal injury in the rat

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Introduction: The 90% decrease in biliary secretory IgA content observed after thermal injury in the rat [1] may relate in part to a decrease in supply of polymeric IgA (p-IgA) to the hepatocyte or to a decrease in hepatocyte transfer of p-IgA into bile. In this study, we examined the transfer of ^{125}I-p-IgA into bile after thermal injury.

Methods: Purified IR22 rat IgA myeloma protein consisting of both monomeric (m-IgA) and p-IgA was labelled with Na^{125}I by the chloramine T method. Sprague-Dawley rats (160-180 gm) received a 20%-30% body surface area scald burn or sham treatment, under ether anesthesia. 20 hr later, the bile duct was cannulated and ^{125}I-IgA was injected into the tail vein. Bile was collected under light ether anesthesia for 3 hr and blood was obtained by cardiac puncture at 3 hr. Total, TCA precipitable, and immunoprecipitable radioactivity was assessed. The molecular character of the radioactivity was examined by gel permeation on a BioGel A1.5 column.

Results: After i.v. injection of unfractionated ^{125}I-IR22 IgA myeloma protein, or the ^{125}I-p-IgA or ^{125}I-m-IgA fractions, no significant differences were seen in total, TCA-precipitable or immunoprecipitable radioactivity in bile from burn-injured or sham-treated animals. Bile obtained from rats injected with unfractionated ^{125}I-IR22 IgA myeloma protein was subjected to gel permeation; the radioactivity in bile from sham-treated animals (28.3% of the injected dose) eluted in the region of p-IgA as expected. The radioactivity in the bile from burn-injured animals (23.6% of the injected dose) eluted equally in the p-IgA and m-IgA regions. In serum from both control and burn-injured rats, the percentage of radioactivity appearing in the same fraction as p-IgA was markedly reduced in comparison to the radioactivity eluting in the m-IgA peak. After injection of the ^{125}I-p-IgA fraction 33% of the radioactivity appeared in the bile of sham-treated animals compared to 29% in the bile of thermally injured rats. Bile of sham-treated rats was subjected to gel permeation; the resulting fractions contained primarily p-IgA. Bile from burn-injured rats contained a mixture of p-IgA and m-IgA. The radioactivity in the serum from both groups eluted predominantly in fractions corresponding to p-IgA. After injection of isolated ^{125}I-m-IgA, only small amounts of radioactivity

were transferred into bile (<1%). This finding suggests that the monomeric IgA in bile after injection of unfractionated ^{125}I-IgA was not due to transfer of m-IgA from the circulation into bile. Additional controls were considered. After mixing of ^{125}I-p-IgA with blood in vitro, gel permeation did not reveal evidence of fragmentation of p-IgA in the serum of burn-injured animals. Incubation of ^{125}I-p-IgA with bile from control and burn-injured rats for 3 hr in vitro did not produce evidence for breakdown of the ^{125}I-p-IgA to ^{125}I-m-IgA.

Discussion: No quantitative difference was seen in the transfer of radioactivity into bile suggesting that hepatobiliary vesicular transport of p-IgA remains intact after burn-injury. There was however, a qualitative change in the IgA reaching bile; a marked increase in m-IgA was noted after burn-injury. Several mechanisms might account for the greater amount of m-IgA in bile of burn-injured rats. 1) p-IgA breakdown in the circulation and presentation of increased amounts of m-IgA to the liver: but the profile of circulating radioactivity was the same in burn-injured or sham-treated rats. Following i.v. injection of p-IgA into burn-injured rats, we failed to detect large amounts of m-IgA in the circulation at 3 hr. 2) Ability of the liver to transfer m-IgA into bile: but less than 1% of the dose injected entered the bile of either burn-injured or sham-treated rats. 3) Transfer of intact p-IgA into bile with breakdown in bile: but no breakdown occurred after 3 hr incubation of ^{125}I-p-IgA with bile from burned injured and sham-treated rats. 4) Transfer of p-IgA into the liver, dissociation of p-IgA into m-IgA in the hepatocyte and subsequent release into bile: we favor this last explanation. In burn-injured animals, endocytic vacuoles containing p-IgA might encounter lysosomal granules and be subjected to their degradative actions. There is however no evidence that the contents of such vacuoles would be released into bile nor indication that lysosomal degradation would have such limited effect on p-IgA so as to produce m-IgA rather than more extensively degraded products [2]. Human p-IgA is known to be susceptible to dissociation under mild reduction and alkylation [3,4]. Possibly, enhanced levels of glutathione [5] that might accompany burn-injury are involved in reduction of p-IgA in-vivo in burned animals leading to the appearance of m-IgA in their bile.

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Ammonium and α-mannosidase are key factors determining natural killer (NK) activity in patients with liver cirrhosis

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ABSTRACT. Natural killer (NK) cells are a group of lymphocytes which destroy in vitro a variety of tumor cells in a nonselective way. The serum α-mannosidase activity was significantly higher in patients with liver cirrhosis (LC) than healthy subjects. α-mannosidase inhibited NK activity *in vitro* at low concentrations comparable to the serum level. NK activity was demonstrated to be directly proportional to serum ammonia level in patients with LC. These results suggest that α-mannosidase and ammonium are key factors determining NK activity in patients with LC.

1. Introduction

It has been thought that natural killer (NK) cells comprise an important component involved in immune surveillance system against cancers[1]. Previously we reported that NK activity of peripheral blood is significantly decreased in patients with liver cirrhosis (LC) compared with healthy subjects[2]. In the present study we investigated the relationship between NK activity and an exoglycosidase, i.e., α-mannosidase, or ammonium.

2. Materials & Methods

The cytotoxicity of peripheral blood MNC isolated on Ficoll-Hypaque density gradient from heparinized venous blood against chromium-labelled K-562 cells was examined at an effecter : target ratio of 50 : 1. The result was expressed by %cytotoxicity. Serum α-mannosidase activity was determined using 4-methylumbelliferyl-α-D-mannoside as a substrate. The result was expressed in terms of nM/ml/h[3]. Serum ammonia level was determined by a conventional method using Ammonia-Test (Wako Pure Chemicals, Osaka, Japan). Group data were compared by Mann-Whitney U-test and correlation was analyzed by the least square regression method.

3. Results

3.1. IN VITRO EFFECT OF α-MANNOSIDASE ON NK ACTIVITY

When MNC from 5 healthy subjects were incubated at 37 °C for 20 h in the serum-free RPMI-1640 medium containing α-mannosidase at concentrations corresponding to the enzyme activity of 20 and 40 nM/ml/h, NK activity was inhibited by 3.83 (± 3.89) % (mean ± SD) and 12.53 (±

4.72) %. But MNC from LC patients with hyperammonemia (> 86 μg/dl) were not affected.

3.2. SERUM α-MANNOSIDASE ACTIVITY

The mean (±SD) of serum α-mannosidase activity was 32.84 (±8.55) nM/ml/h in 23 patients with LC, which was significantly higher than the value (18.36±4.40) in 95 healthy subjects ($p<0.0001$).

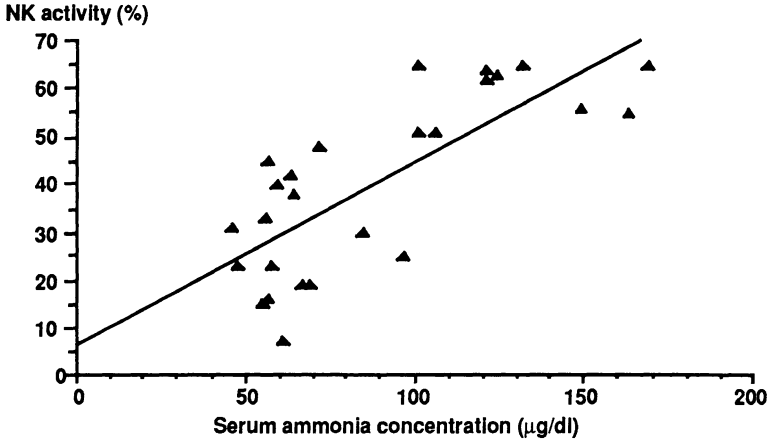

Figure 1. Correlation between serum ammonia level and NK activity in patients with LC. In 26 patients with LC there was a significant correlation ($Y=6.88+0.378\ X$, $R=0.761$, $p<0.001$).

3.3. NK ACTIVITY AND SERUM AMMONIA LEVEL IN PATIENTS WITH LC

In 26 patients with LC there was a significant correlation between NK activity and serum level of ammonia (Figure 1). There was no significant difference of NK activity between 11 LC patients with hyperammonemia and 95 healthy subjects. NK activity in 15 LC patients with normal level of serum ammonia was significantly lower than healthy subjects ($p<0.0001$) or LC patients with hyperammonemia ($p<0.001$).

4. Discussion

It is well known that hepatocellular carcinoma (HCC) develops in longstanding LC at high frequency. It is reported that the frequency of HCC development in patients with LC whose liver dysfunction is severe is lower than that in patients whose liver dysfunction is slight[4]. This phenomenon may be explained by a hypothesis that high level of serum ammonia at advanced stage of LC enhanced NK activity by making NK cells resistant to α-mannosidase and that high NK activity suppresses the development of HCC as the result.

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Cellular cytotoxicity induced by liver specific idiotype-bearing antibody against Chang liver cells

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ABSTRACT. Liver-specific idiotype-bearing antibody (LSIA) was capable of being purified from the sera of patients with chronic hepatitis by affinity chromatography. It was demonstrated that LSIA bound to Chang liver cells and induced ADCC. It is suggested that ADCC induced by LSIA is related to liver cell damage in patients with chronic hepatitis (CH).

1. INTRODUCTION

Although the pathogenesis of CH is not completely understood, autoimmune responses against liver cell components have been considered to be involved. We produced a murine monoclonal antibody with human liver-specific reactivity, designated H2, and demonstrated that the serum level of the antibody sharing the idiotype with H2, which we named LSIA, was elevated in patients with CH. In the present study we tried to purify LSIA from the sera of patients with CH and examined whether antibody-dependent cell-mediated cytotoxicity (ADCC) against Chang liver cells was induced by LSIA.

2. MATERIALS AND METHODS

2.1. Preparation of anti-H2 idiotype antibody (anti-H2 Id)

A rabbit was subcutaneously immunized with H2 and its serum was collected. The serum was absorbed twice with mouse whole serum and IgG fraction was purified by affinity chromatography with protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden). Anti-H2 Id was purified by affinity chromatography with H2-conjugated Sepharose 4B.

2.2. Purification of LSIA from sera of patients with CH.

Sera were served from five patients, histologically diagnosed to be chronic active hepatitis. Two of these were positive for HBs and HBe antigens and others were negative for HB virus-associated antigens. Sera were heat-inactivated and IgG fraction was purified with protein-A Sepharose CL-4B. After passed through rabbit IgG-conjugated Sepharose 4B, LSIA was purified by affinity chromatography with anti-H2 Id-conjugated CNBr-activated Sepharose 4B.

2.3. Procedure of ADCC

Chang cells derived from a normal liver tissue, HCC-M cells established by us from hepatocellular carcinoma[1] were used as target cells and HeLa cells derived from uterus carcinoma were used as control. Mononuclear cells from healthy subjects were used as effector cells (E:T=50:1). Cytotoxicity was estimated by ^{51}Cr release method after incubation for 4 h. LSIA was used at a concentration of 5μg/ml because preliminary studies showed that this concentration was most effective. Percent ADCC was calculated according to the following formula: %ADCC=% cytotoxicity with LSIA - % cytotoxicity without LSIA, % cytotoxicity = (experimental release - spontaneous release / maximum release - spontaneous release) x 100

3. RESULTS

3.1. Binding of LSIA to culture cell lines

It was estimated that LSIA purified from patients with CH was about 1/700 of the original IgG. It was demonstrated that LSIA bound to Chang and HCC-M cells but not to HeLa cells by immunoperoxidase method and cellular ELISA.

3.2. ADCC

Percent ADCC against Chang, HCC-M and HeLa cells were 31.0±12.1, 4.2±2.8 and 1.8±1.8 (mean±SE), respectively . The ADCC activity against Chang cells was significantly higher than that against HeLa cells ($p<0.05$).

4. DISCUSSION

We have already reported that LSIA exists in the sera of patients with CH at a high rate[2]. In the present study LSIA was capable of being purified from the sera of patients with CH. It was demonstrated that LSIA bound to Chang and HCC-M cells derived from liver tissues. Furthermore LSIA induced ADCC against Chang cells but not to HCC-M and HeLa cells. LSIA bound to HCC-M cells but could not induce ADCC. These observation might be related to the fact that HCC-M cells were derived from a cancer tissue. LSIA bound to Chang cells derived from normal liver tissues and induced ADCC. From these results it is suggested that ADCC induced by LSIA plays a role in the pathogenesis of CH.

5. ACKNOWLEDGEMENT

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Effect of interferon-β on *in vitro* production of the liver specific idiotype-bearing antibody in patients with chronic hepatitis B

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1.Introduction

Recently interferon (IFN) has been used for the treatment of patients with chronic hepatitis (CH) B. It is known that IFN-ß has an anti-viral effect, but clinical effect of IFN-ß is limited. In Japanese cases of type B CH, complete eradication of hepatitis B virus (HBV) can not be achieved, however, partial suppresion of viral replication is possible. On the other hand, IFN-ß has also an immunopotentiating effect. To analyze the effect of IFN-ß on immune reactions to HBV and autoimmune reactions in patients with type B CH is thought to be important.

We produced a murine monoclonal antibody (McAb), designated H2, of which the reactivity was restricted to human hepatocytes and it is thought that H2 McAb possesses a liver-specific structure on its molecule, i.e., idiotype. In the present study, we investigated effect of IFN-ß on *in vitro* production of liver cell-associated antibodies and HBV-associated antibodies.

2.Materials and Methods

2.1.PATIENTS

Nine patients with type B CH who were serologically positive for HBsAg, HBeAg and HBcAb received IFN-ß (Feron, TORAY, Tokyo, Japan) 3 million IU daily by drip infusion for 28 days. All these patients had been revealed as chronic active hepatitis by liver biopsy. Other 31 patients with CH, 5 patients with acute hepatitis and 20 healthy subjects were also studied.

2.2. PRODUCTION AND PREPARATION OF ANTI-H2 IDIOTYPE-BEARING ANTIBODY

A rabbit was subcutaneously immunized with 0.5mg of H2 McAb with Freund's complete adjuvant. Serum was collected after booster injections. Immunoglobulin (Ig) was precipitated with ammonium sulfate and Ig was purified by affinity chromatography with Protein A-Sepharose CL-4B (Pharmacia Fine Chemicals, Uppsala, Sweden). This was absorbed with mouse whole serum followed by the absorption by passing through mouse IgG-conjugated Sepharose 4B (Pharmacia). The eluate was passed through H2 -conjugated Sepharose 4B and bound Ig was eluted by 0.1M acetate buffer (pH2.2). The solution was dialyzed against PBS.

2.3. MONONUCLEAR CELL CULTURE

Peripheral blood mononuclear cells (MNC) were isolated from heparinized venous blood and were suspended in RPMI1640 supplemented with 10% heat-inactivated fetal bovine serum and antibiotics at a concentration of 1×10^6/ml. MNC were incubated with 10μg/ml of pokeweed mitogen (PWM) (GIBCO, Great Island, NY, USA) and with or without 100IU/ml of IFN-ß at 37°C for 7 days and supernatants were collected.

2.4. ASSAY OF HBV-ASSOCIATED ANTIBODIES

HBcAb and HBeAb were assayed by radioimmunoassay (RIA) according to the methods described elsewhere*.

2.5. ASSAY OF LIVER-SPECIFIC IDIOTYPE-BEARING ANTIBODY (SANDWICH ENZYME-LINKED IMMUNOSORBEANT ASSAY [ELISA])

The procedure of sandwich ELISA was already described elsewhere*.

3. Results

3.1. EFFECT OF IFN-ß ON *INVITRO* PRODUCTION OF HBV-ASSAOCIATED ANTIBODIES

HBcAb was detected in the supernatant of culture with PWM in all the cases of type B CH. Further addition of IFN-ß had various effects on its production. There was no regular tendency in HBcAb production level. In patients treated with IFN-ß, HBcAb production varied case by case during the therapy and there were no regular shift in HBcAb level before and after the therapy.

In patients who were serologically positive for HBeAg, *in vitro* production of HBeAb was sporadically detected. In 2 cases of 9 patients HBeAb productions were enhanced by further addition of IFN-ß. In 5 cases, the production of HBeAb was detected at least once during the IFN-ß therapy. But *in vitro* effect of IFN-ß on HBeAb production varied. There was no regular shift in HBeAb production level before and after the therapy.

3.2. DETECTION OF LIVER-SPECIFIC IDIOTYPE-BEARING ANTIBODY

In vitro production level of liver-specific idiotype-bearing antibody was significantly higher in patients with chronic hepatitis than that of healthy subjects ($p<0.001$). But there was no difference of the antibody production level between HBsAg-positive and -negative patients. The level of such antibody was not increased in patients with acute hepatitis.

3.3. IFN-ß AND PRODUCTION OF LIVER-SPECIFIC IDIOTYPE-BEARING ANTIBODY

In 5 cases who were serologically positive for HBeAg and who received IFN-ß therapy, the production level of liver-specific idiotype-bearing antibody was sequentially assayed. In these cases, the production of such an antibody increased shortly after the injection during the drip infusion of IFN-ß and after that, it decreased quickly. The production of such an antibody was decreased 4 weeks after the start of IFN-ß therapy.

4. Discussion

The present study showed that *in vitro* production level of liver-specific idiotype-bearing antibody was significantly higher in patients with CH than that of healthy subjects and IFN-ß therapy decreased that level. Recent clinical trials using IFN-ß showed that IFN-ß decreased serum transaminase level, in other words, stabilized liver damage. In this point of view, present data suggest that such an antibody plays a part in the pathogenesis of CH.

There were some patients in whom *in vitro* production of HBeAb was detectable even if their serological examination showed that serum HBeAg was positive. This result suggests that in such patients the HBeAb producing clone already exist but the antibody production was suppressed by a certain mechanism.

5. Acknowledgement

This study was supported in part by the Grant-in-Aid for Science Research of the Ministry of Education, Science and Culture (#1770475).

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The polymeric immunoglobulin receptor is present on hepatocytes in human liver

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INTRODUCTION. It is widely believed (Brandtzaeg, 1985; Delacroix & Vaerman, 1983; Tomana, Kulhavy & Mestecky, 1988; Brown & Kloppel, 1989) that the polymeric IgA receptor (pIgAR) cannot be detected in or on human hepatocytes. In rats and several other species it is accepted that hepatocytes synthesize the pIgAR and use it to transfer polymeric IgA (pIgA) across the cell from blood to bile. At the biliary face of the hepatocyte, the pIgAR is proteolytically cleaved, releasing either secretory IgA (sIgA) when pIgA has been bound or free secretory component (FSC).

Human bile, like that of other species, contains sIgA and in some cases, FSC, although the amounts of the latter are extremely variable. Human bile/blood ratios for pIgA or sIgA also indicate that pIgA is actively transported from blood to bile. The transport in the absence of pIgAR has been explained by postulating that in Man pIgA is transferred solely via the bile duct lining cells, in which the receptor has been detected.

MATERIALS AND METHODS. a. Monoclonal antibodies were prepared after immunization of Balb/C mice with HPLC-purified FSC from human colostrum (gift from Linda Ambler, ADDR, Ciba-Geigy Pharmaceuticals, Horsham, UK). Antibody producing clones were selected by measuring binding of 125-I labeled FSC.

b. Liver samples for immunohistochemistry were obtained from the Addenbrooke's Hospital transplant programme. One was from unused donor liver, one from donor at operation, five from donor soon after operation, one from rejected liver, one from a removed liver from a region showing no morphological abnormality and two from cirrhotic livers removed at operation.

c. Frozen sections from liver samples were fixed in acetone for 5 min, then washed in PBS and stained for 60 min with monoclonal M9 (50 mg/ml). After a further PBS wash, the second antibody was a rabbit anti-(mouse IgG) conjugated with peroxidase (1:100) applied for 30 min in the presence of 10% normal human serum. After washing with PBS and incubating with 1.4 mM diaminobenzidine in Tris-buffered saline pH 7.6 plus 0.003% H2O2, sections were counterstained with haematoxylin.

d. Human bile samples were collected by endoscopy and immediately placed on ice, in the presence or absence of protease inhibitors (0.2 mM p-methylsuphonyl fluoride, 2.5 mM benzamidine and 2 mg/ml leupeptin). 125-I-FSC was incubated with bile samples without protease inhibitors for 18 hours at 25°C or added directly to bile samples with protease inhibitors at 0°C prior to gel electrophoresis.

e. Immunoradiometric assays. Monoclonal antibody coded M9 was selected for immunohistochemistry and as capture antibody for FSC and for sIgA in two-site

immunoradiometric assays by its ability to bind 125-I-SC in the presence of up to 0.075% w/v SDS and to immunoblot native, but not reduced, SC (FSC or sIgA) in human bile and colostrum (data not shown). For the assay of FSC, monoclonal antibody M7 was used as signal antibody; for the assay of sIgA, a monoclonal antibody of commercial origin anti-IgA was used as signal antibody.

RESULTS. a. Monoclonal antibodies,Five monoclonal antibodies were produced and their binding on SC epitopes examined. These monoclonals were found to bind three different epitopes on SC and were used in immunoassays of FSC and sIgA in serum and bile.

b. Immunohistochemistry with monoclonal antibody M9 produced even staining of the hepatoycte plasma membranes as well as bile duct lining cells in all sections examined from eight normal and three abnormal human livers.

c. Bile proteolytic activity. In preliminary studies, using M9 to immunoassay FSC in individual human bile samples collected with no special precautions, low and very variable levels were found. Breakdown of FSC was therefore examined by incubating 125-I-FSC in three different bile samples overnight at 25°C. Trichloroacetic acid precipitation showed that whereas 94 % of the radioactivity was acid-precipitable when bile samples containing protease inhibitors were mixed with 125-I-FSC immediately before precipitation, the same samples without inhibitors incubated at 25°C overnight showed increases of 2-22 % in acid-soluble radioactivity.

DISCUSSION. The human pIgAR which is known to be very similar in primary sequence to the pIgAR of rat and rabbit, has now been shown to be very similar in its distribution within the liver tissue of Man compared to the other two species.
The most important difference in the present study is the use of a monoclonal antibody. However the availability of very good samples of normal liver collected under ideal conditions has almost certainly contributed. SC is a difficult antigen to detect reliably since it is easily damaged by fixatives and very susceptible to proteolytic degradation.
If human hepatocytes possess the pIgAR, it seems likely that human liver is more important in the removal of pIgA from the blood to the bile than is currently believed.
Separate measurements of sIgA and FSC in bile samples have hitherto been difficult; the two-site assay presented makes it possible to measure FSC and sIgA levels in the same sample and should produce better data. The measurement of FSC and sIgA using samples collected under conditions of inhibition of proteolysis should prove a good starting point.

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SECTION Q: MUCOSAL IMMUNITY IN THE GENITOURINARY TRACT

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Secretory component production by immature rat uteri and uterine epithelial cells in culture

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ABSTRACT. The present studies demonstrate that incubation of uteri from immature rats treated with estradiol for 1, 2 or 3 days *in vivo*, results in the accumulation of increasing amounts of secretory component in culture media relative to saline controls. In addition, our findings indicate that isolated uterine luminal epithelial cells cultured on a matrix-coated permeable surface with separate apical and basolateral secretory compartments release secretory component into culture media (apical chamber) both prior to and following their growth to confluence.

Estradiol increases the levels of polymeric IgA in uterine secretions of rats in part by stimulating the synthesis of polymeric IgA (pIg) receptor (1). Also known as secretory component (SC), pIg receptor binds and transports IgA from blood and tissues to bile and mucosal surfaces where it exerts its protective effects (2-4). Previous studies have shown that free SC as well as SC bound to IgA increase in uterine secretions of adult ovariectomized rats treated with estradiol and that this effect is mediated through RNA synthesis (5). The purpose of the present study was to examine whether uteri from immature rats synthesize SC in response to estradiol and to determine whether uterine luminal epithelial cells produce SC *in vitro*.

Immature female Sprague-Dawley rats (26 days, 40-50 gms) were treated with estradiol (0.1 μg/0.1 ml/day) for 1, 2 or 3 days. Twenty-four hrs after the last injection, uteri were removed, slit lengthwise, pooled (3 uterine horns/group) and incubated in RPMI 1640 with glutamine at 37°C with 95% O_2-5% CO_2 for 24 hrs (100 rpm) as described previously (5). Secretory component levels in culture media were measured by radioimmunoasssay (6).

The effect of estradiol on the accumulation of uterine SC in incubation media is shown in Figure 1. Daily administration of estradiol for 1, 2 or 3 days significantly increased uterine SC levels in media when compared to that released by uteri from immature rats that received saline for 3 days. In other studies (not shown), we have found that the estradiol effect on uterine SC is reduced by protein and RNA synthesis inhibitors. When estradiol-stimulated uteri were placed in media either with cycloheximide (100 μg/ml) or actinomycin D (20 μg/ml), SC levels were significantly reduced.

To determine whether luminal epithelial cells from uteri of immature rats produce SC *in vitro*, individual slit uterine horns were incubated with 0.5% trypsin/2.5% pancreatin for 60 min at 4°C and 60 min at 20°C, to release luminal epithelial cells as described previously (7). Following removal of enzymes and vortexing of uterine horns in buffer, pooled supernatants were centrifuged for 5 min at 500 x g, after which plaques were removed by sedimentation. Collected cells were resuspended in DMEM high glucose and Ham's F-12 Nutrient mixed 1:1, containing BSA, HEPES and Pen/Strep, supplemented with 2.5% FBS and 2.5% Nu-Serum, and seeded onto Millicell HA membranes coated with Matrigel. As seen in Figure 2, apical incubation media contained increasing amounts of SC (ng/20µl/48 hrs) when uterine epithelial cells were incubated for 6-12 days. In other studies (not shown), SC release continued after epithelial cells reached confluence as measured by electrical resistance (days 10-15).

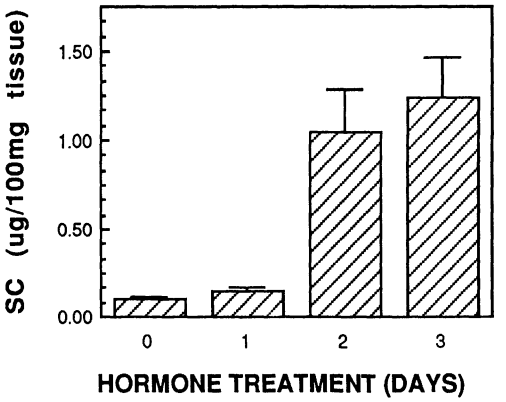

Figure 1. Effect of increasing lengths of estradiol treatment *in vivo* on uterine SC output *in vitro*. Immature rats were administered estradiol (0.1 µg/0.1 ml/day) for 1, 2 or 3 days and then sacrificed 24 hrs later. Control animals (0 days) received 3 daily injections of saline. Uterine horns were slit and placed in incubation flasks. Values equal the mean ± range; N = 2 flasks each containing 3 uterine horns.

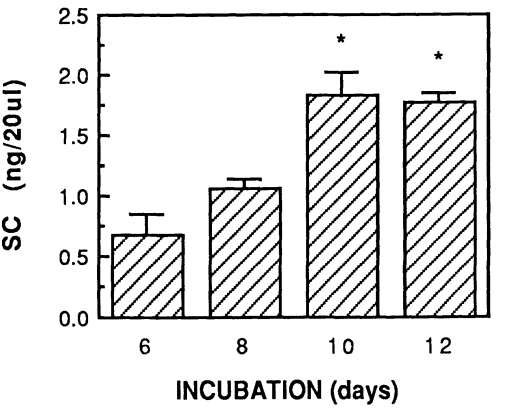

Figure 2. Time course of accumulation of secretory component in apical media by uterine luminal epithelial cells in culture. Media was exchanged every 2 days and collected from the upper chamber of Millicell HA coated wells containing uterine epithelial cells. Secretory component values represent the mean ± SE of media collected from 5-7 wells at the times indicated. (*), Significantly ($p<0.05$) greater than the level of SC measured at day 6 of incubation.

These results indicate that immature rat uteri, when stimulated by estradiol *in vivo*, respond by synthesizing SC as do adult uterine tissues. Furthermore, it demonstrates that, independent of mesenchymal elements, isolated uterine luminal epithelial cells in culture release SC both prior to and following growth to confluence. This study also indicates that hormone responsiveness is maintained in these cells since SC production is known to be estrogen dependent in immature and adult intact uteri. When considered in light of our previous results, these findings help to clarify the central role of estrogens in the uterine immune response.

ACKNOWLEDGEMENTS

We wish to thank Dr. B. Underdown (McMaster University, Hamilton, Ontario) for his gifts of SC and rabbit anti-rat SC and Ms. Carola P. Sandoe for excellent technical assistance. Supported by Research Grant AI-13541 from NIH.

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Characterization of secretory component from rat uterine fluid

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ABSTRACT. Secretory component (SC) in uterine fluid from intact rats at the proestrous stage of the reproductive cycle and from estradiol-treated ovariectomized rats were examined using high performance liquid chromatography (HPLC) and Western immunoblot analysis. HPLC analysis revealed a peak, which is associated with free SC from uterine fluid, with a molecular size of 105 kDa (range 100-115 kDa). Western blot analysis demonstrated that SC in denatured and rapidly reduced uterine fluid from both proestrous and estradiol-treated rats has a molecular mass of 87 kDa (range 83-93 kDa) with a minor band of 80 kDa. When SC in uterine fluid was compared to bile SC, both were found to have the same molecular masses. These results indicate that uterine fluid, whether from intact animals or ovariectomized rats treated with estradiol, contains SC that is identical in size to that found in bile.

INTRODUCTION. Knowledge of the function and regulation of the secretory immune system is important to an understanding of the antigenic response to the presence of pathogens in the reproductive tract and to how these protective mechanisms may best be enhanced for the prevention and treatment of sexually-transmitted diseases. Previous studies in this laboratory, using a female Sprague-Dawley rat model, have focussed on the role of the sex steroid hormones in the regulation of the secretory immune system of the uterus.

Immunoglobulin A (IgA) has been demonstrated to be the major antibody present in mammalian external secretions including the uterine fluid of the rat [1]. Secretory component (SC), the IgA transport protein synthesized by mucosal epithelia, is also present in large quantities in rat uterine and vaginal secretions during the reproductive cycle [2]. Levels of IgA and SC (both free and bound) have been shown to be significantly increased in ovariectomized rats following estradiol treatment [3]. Furthermore, these estradiol-induced alterations in IgA and SC levels appear to be specific for uterine and vaginal secretions and are not evident in saliva and intestinal fluid.

While biochemical characterization of SC from other mucosal sites (bile, milk) in the rat have been previously described [4], SC from rat uterine fluid has not been investigated to the best of our knowledge. Due to the pivotal role that SC plays in the regulation of the movement of IgA into uterine secretions and to the apparent differential regulation of IgA and SC levels by sex steroids in reproductive tissues, we examined some biochemical characteristics of uterine SC from the rat under native and denaturing conditions.

MATERIALS AND METHODS. Adult female Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were maintained in constant temperature rooms with 12 hr cycles of light and dark. Some animals were ovariectomized 7-10 days prior to a 3-day treatment with estradiol (1 μg/0.1 ml/day). The stage of the estrous cycle of intact rats was determined by daily vaginal lavage for at least 2 cycles. Rats were decapitated and uterine fluid collected from estradiol-treated ovariectomized rats and from intact rats at proestrus. Bile was obtained by cannulation of the bile duct and fluid obtained the second hour of cannulation was utilized. Following centrifugation (12000 x g, 4 min), samples were stored at 4°C until analyzed.

Immunoblot Analysis. SDS-PAGE was performed using a modified Laemmli method [5]. Fresh uterine fluid and bile samples were immediately reduced and denatured by boiling for 5 minutes with 2X sample buffer (0.125M tris-base, 4% SDS, 20% glycerol, 0.01% Brom phenol blue) containing 2-mercaptoethanol (9% V/V, Aldrich Chemical Co., Inc.) and electrophoresed on 10% polyacrylamide gels at 20 ma for 6-7 hrs (≈15°C). Proteins were transferred to nitrocellulose (Schleicher & Schuell, Keene, NH; 0.2 μm) in a Bio-Rad Transblot Apparatus in tris-glycine electrode buffer without SDS (100 ma for 14-16 hrs at room temperature). Nitrocellulose strips were then incubated (2X for 1 hr each) in 5.5% milk with 1% Tween in phosphate-buffered saline (PBS). After washing with PBS (6X), some strips were incubated overnight in rabbit anti-rat SC (1:10,000 dilution in 2.75% milk solution) at room temperature on a rotator (100 rpm). Control strips were incubated in normal rabbit serum (Sigma Chemical Co., St. Louis, MO; 1:10,000 dilution). Nitrocellulose strips were washed with PBS and incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Sigma Chemical Co., 1:1000 dilution) for 3 hrs at 100 rpm. Immunoreactive bands were visualized by using p-Nitro blue tetrazolium chloride (0.15 mg/ml, Research Organics, Inc., Cleveland, OH) and 5-Bromo-4-chloro-3-indoyl phosphate (0.05 mg/ml, Research Organics, Inc.) in substrate buffer (0.05 M Na_2CO_3, 0.01 M $MgCl_2$, pH 9.8).

High performance liquid chromatography. HPLC analysis was performed by injecting uterine fluid samples into a TSK-G 3000 SW column (Phenomenex, Rancho Palos Verdes, CA) equilibrated in PBS, (Dulbecco's, pH 6.8 with 0.02% sodium azide). The HPLC system included a Waters 840 work station equipped with two Model 510 pumps, a U6K injector, and a Beckman Model 165 multiwave length detector. Fractions (0.25 ml) were collected for 30 min at a flow rate of 0.5 ml/min. Each fraction was assayed for SC by RIA as described previously [3].

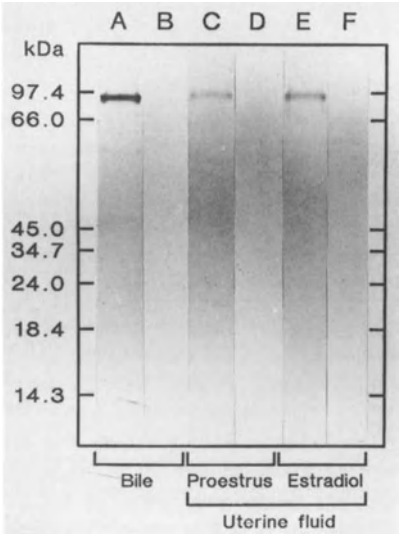

Figure 1. A representative immunoblot of SC from bile and uterine fluid from intact rats in proestrus and from estradiol-treated rats. Lanes A, C and E represent strips incubated with rabbit anti-rat SC, while lanes B, D and F represent controls incubated with normal rabbit serum. Molecular standards are indicated on left in kDa.

RESULTS. The results of the Western blot analysis of reduced uterine secretions and bile are shown in Figure 1. In proestrous and estradiol-treated rat uterine fluid (lanes C and E), SC exists as a protein band having a molecular mass of 87 kDa (range 83-93 kDa; N=10). A minor band of 80 kDa is found occasionally (range 76-84 kDa; N=6). No size differences were noted between uterine fluid from intact rats in proestrus and from estradiol-treated rats. Bile was also analyzed by the Western blot technique (lane A) and found to contain an SC protein band of the same molecular mass as that seen in uterine fluid.

A representative SC elution profile of native uterine fluid following HPLC analysis and RIA of fractions, is shown in Figure 2. Two peaks were observed, one with a large molecular mass eluting near the void volume of the column and a second peak of 105 kDa (range 100-115 kDa). Uterine fluid from both proestrous rats and estradiol-treated ovariectomized rats gave identical results both in peak positions and relative distribution of the two peaks. A peak of 40 kDa (range 35-50 kDa) was seen in 2 out of 5 experiments using uterine fluid from estradiol-treated rats; this may be a result of proteolytic degradation.

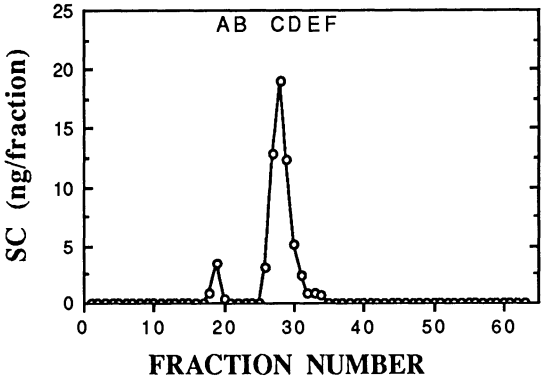

Figure 2. A representative profile of the SC content of fractions from HPLC analysis of uterine fluid. Molecular standards are indicated at top. Letters A-F indicate the elution points of standards with known molecular masses: A (thyroglobulin, 669,000), B (ferritin, 450,000), C (aldolase, 158,000), D (albumin, 67,000), E (ovalbumin, 43,000) and F (soybean trypsin inhibitor, 20,400).

DISCUSSION. Western immunoblot analysis revealed similar immunoreactive protein bands for SC from denatured uterine fluid obtained from intact rats in proestrus and ovariectomized estradiol-treated rats. These results indicate that although SC production is much greater in the estradiol-treated rats [2], the molecular mass of the induced SC is similar to that from intact rats. Furthermore, protein bands associated with SC from uterine fluid did not differ in mass from those of bile, indicating that although there is differential regulation of SC by sex steroids, the proteins are of similar size. In contrast, Altamirano *et al.* [4] found that purified bile SC had a molecular weight of 68,000. Differences might be explained by the rapid denaturation and reduction of uterine fluid and bile samples used in our study to minimize proteolytic degradation.

SC in native uterine fluid from estradiol-treated and intact rats revealed two major peaks, one with a large molecular mass and a second peak with a relative mass of 105 kDA. The large molecular mass peak likely represents either aggregates of SC or SC bound to polymeric IgA. Other experiments have shown that this peak also contains

IgA. (C. Wira, unpublished data). The second major peak of 105 kDa likely represents free SC. This value tends to be slightly higher than the molecular weights reported for SC from other sources [see 6, for review].

In conclusion, the present study demonstrates that SC in uterine fluid from intact or hormonally treated animals, when analyzed under denaturing and native conditions, results in similar molecular mass estimates of 87 kDa and 105 kDa, respectively. Further, it indicates that uterine SC has the same molecular mass as that seen in bile.

ACKNOWLEDGEMENTS. We thank Dr. Brian Underdown (McMaster University, Hamilton, Ontario) for his gifts of SC and rabbit anti-rat SC and Mr. Richard M. Rossoll and Ms. Carola P. Sandoe for excellent technical assistance. Supported by Research Grant AI 13541 from NIH.

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Effects of intravaginal and pelvic immunization on specific antibody in mouse vaginal fluid

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INTRODUCTION. Intravaginal (iv) immunization caused specific IgA secretion into mouse vaginal fluid, but lymphoid nodules were not detected in mouse vaginal mucosa (Parr et al., 1988). It is generally accepted that IgA responses are generated in mucosal lymphoid tissue, but regional lymph nodes may also be involved (Spencer et al., 1983). Also, immunization in the vicinity of mammary glands, salivary glands, or the respiratory tract can elicit specific IgA secretion into colostrum, saliva, and bile (Genco and Taubman, 1969; Taubman and Smith, 1974; Hall and Spencer, 1984). We therefore sought to identify non-mucosal sites in the mouse pelvis from which lymph drains to the lymph nodes that drain the vagina (iliac nodes), and to determine whether immunization at those sites would cause higher IgA titers in vaginal fluid than immunization at other sites. In addition, we studied the effects of adjuvants on responses to intravaginal (iv) immunization.

METHODS. Three non-mucosal pelvic sites were studied: the subserous space (ss) between peritoneum and pelvic diaphragm, the presacral space (ps) between sacrum and rectum, and the superficial perineal space (sps) between pelvic diaphragm and skin. Colloidal carbon drained to the illiac nodes after injection into the ss and ps but not the sps. Mice (5-6 per group) were primed and boosted with 50 ug horse ferritin adsorbed to 100 ug alum (AH) at the 3 pelvic sites, subcutaneously (sc) over the scapulae, intraperitoneally (ip), and iv. We also immunized mice iv daily for 5 days with tampons containing 500 ug of ferritin combined with AH, muramyl dipeptide (MDP), monophosphoryl lipid A (MPL), dimethyl dioctadecyl ammonium bromide (DDA) or cholera toxin (CT). Titers of anti-ferritin IgA and IgG were measured in vaginal fluid by ELISA.

RESULTS. In the pelvic immunization experiment, anti-ferritin IgG titers in vaginal fluid were essentially the same in all but the iv group, where only a small response was observed (Fig. 1). In contrast, IgA titers differed significantly between groups. Animals immunized sc developed a transient response that declined to zero after boosting, whereas immunization ip and in the ss and ps elicited IgA responses

that were significantly higher and better sustained. Immunization in the sps caused an intermediate response, and iv immunization caused only a small response. Additional mice were immunized sc or ps, using DDA, AH plus MDP, or RIBI as adjuvants. Anti-ferritin IgG titers in vaginal fluid reached similar levels in all groups (6.5-8.0 at 15 wks). Specific IgA titers in the ps groups at 15 wks were 3.0 (DDA), 2.2 (RIBI), and 1.0 (AH plus MDP), and had declined to zero in all sc groups. The most effective adjuvant for iv primary immunization was AH, while MPL was most effective for iv boosting. Primary iv immunization for 5 days with ferritin and AH followed by iv boosting for 5 days with ferritin and MPL elicited higher IgA titers in vaginal fluid (5.5 one week after boosting) than systemic priming and boosting with ferritin and AH (3.0) or systemic priming and iv boosting with ferritin and MPL (2.0). Systemically immunized animals exhibited the highest IgG titers in vaginal fluid (7.0).

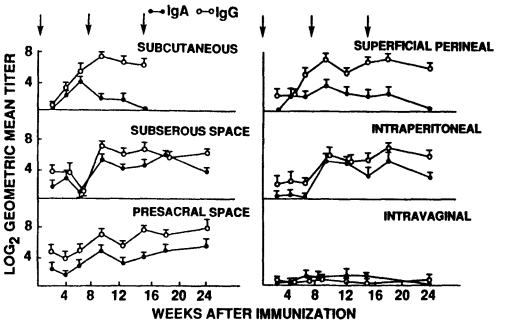

Fig. 1. Anti-ferritin IgA and IgG responses in mouse vaginal fluid after immunization with horse ferritin at pelvic and other sites.

DISCUSSION. Immunization at two non-mucosal sites in the mouse pelvis caused higher and better sustained IgA titers in vaginal fluid than either sc or local mucosal (iv) immunization. The non-mucosal pelvic sites permitted effective use of depot adjuvants to sustain IgA responses in the reproductive tract. Alum and MPL can enhance responses to iv immunization, but it should be noted that multiple applications of large doses of antigen were required.

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Antigen recognition in the female reproductive tract: I uptake of intraluminal protein traces in the mouse vagina

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INTRODUCTION. Local immunization in the vagina of several species elicits immune responses, but little is known about the uptake, processing and recognition of antigens at this site. Here, we investigated the uptake of intravaginally (iv) administered protein tracers into the vagina and draining lymph nodes (iliacs) during the estrous cycle in the mouse.

MATERIALS AND METHODS. Mice of the ICR strain 60-100 days old were used. Tracers included: fluorescein (control; 0.1 mg/ml), FITC-HRP (0.6 mg/ml), FITC-BSA (10 mg/ml) and FITC-horse ferritin (10 mg/ml). Expt. 1. Tracers were introduced into the vaginal lumen in tampons that contained 50 ul of tracer solution or by placing 20 ul of tracer solution at the external opening of the vagina with a micro-pipet without touching the vaginal mucosa in anesthetized mice. The mice were killed 1, 4, or 17 h later and the vagina and iliac lymph nodes were removed, fixed, and embedded in polyethylene glycol (PEG). Expt. 2. FITC-HRP or FITC-BSA was introduced into the lumen of the left uterine horn. 3 h later the left horn, cervix and vagina were removed, fixed and embedded in PEG.

RESULTS. Expt. 1. Fluorescent proteins administered into the vaginal lumen were taken up into the vaginal mucosa primarily at diestrus, to a lesser extent at proestrus and metestrus, and not at all at estrus (Table 1). The pattern of uptake into the vagina was similar with all protein tracers. They appeared in the vaginal stroma in small, spherical granules of various sizes in cells that were irregular in shape or very long and narrow. Tracers were also frequently observed in endothelial cells lining small and medium lymphatic vessels. The vaginal epithelium was largely devoid of tracers, but fluorescence was seen in dendritic cells within this layer (Fig. 1). Protein tracers were also detected in the iliac lymph nodes of a few mice that showed tracers in the vaginal mucosa after intraluminal administration. One to 4 h after administration, tracers were either absent in the nodes or were detected in phagocytic cells lining the marginal and/or medullary sinuses. Seventeen h after intraluminal administration, FITC-HRP was found in small cells, sometimes dendritic in appearance, in the paracortex of the iliac nodes. These cells resembled interdigitating cells (IDCs). There was an absence of fluorescence in all tissues when free fluorescein (control) was used as the tracer. Expt. 2. After administration of tracers into the lumen of a uterine horn, tracers were detected in the stroma of the cervix and/or vagina in some mice. The uterine horns of 5 mice showed tracer in small vesicles in the apical region of the luminal epithelia, but no tracer was observed in the uterine stroma of any mice.

Table 1. Uptake of tracers into the vaginal mucosa of mice after intraluminal administration

Tracer	Time (h) after administration	Stage of estrous cycle: Estrus	Metestrus	Diestrus	Proestrus
Fluorescein	4	0/2[a]	0/3	0/2	0/5
FITC-HRP	1	0/2	1/6	3/5	0/2
	4	0/16	2/2	8/10	4/5
	4[b]	0/5	2/3	5/5	N.A.
	17	0/4	2/7	6/7	0/1
FITC-BSA	4	0/9	1/10	6/6	3/4
	4[b]	0/5	1/4	9/9	4/4
	17	0/7	1/1	1/3	0/4
FITC-HSF	4	0/3	0/2	7/12	3/3
	4[b]	0/4	0/3	4/4	0/2
	17	0/1	0/1	2/4	0/9
HRP[c]	4	0/1	0/3	3/3	4/4
	4[b]	0/3	N.A.	7/7	N.A.

[a]Number of mice showing uptake into the vagina/ total number of mice examined.
[b]Tracer was administered by tampon.
[c]Uptake of tracer was based on its presence in dendritic cells in the vaginal epithelium.

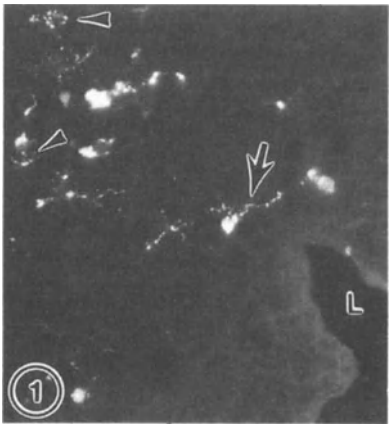

Fig. 1. FITC-HRP is present in a dendritic cell (arrow) in the vaginal epithelium and in the stroma (arrowheads). L, vaginal lumen.

DISCUSSION. These experiments show that the vagina although lined by stratified epithelium, is permeable to intraluminally administered proteins of different molecular weights (40-450 kDa). Passage of tracers into the vagina was not due to physical damage to the epithelium because uptake was observed in cases where the vaginal wall was not touched when tracers were administered. Passage across the epithelium was also not due to chemical damage because intrauterine administration of tracers resulted in uptake of tracers into the vagina and cervix but not the uterine stroma. Thus, at certain stages of the estrous cycle, proteins can pass from the lumen into the stroma of the vagina and cervix. Of particular interest in relation to the role of the vagina as a site of antigen recognition was the observation that dendritic cells in the epithelium endocytosed tracers. These cells in shape, size and location resemble those identified as Langerhans' cells (LCs) at this site (Young et al., 1985). Little is known about the function of LCs in the vagina but extensive studies of LCs in the epidermis have suggested that they probably represent the most peripheral outpost of the immune system and are responsible for antigen uptake, processing and presentation to T lymphocytes in vivo (Romani et al., 1989). It is possible that LCs in the vagina serve a similar role; i.e., they may act as antigen-presenting cells in situ or may become transformed into IDCs, migrate to the iliac lymph nodes and there present antigen to immune cells. The results of these experiments suggest that the vagina and cervix may be major sites of antigen recognition in the genital tract of the female mouse.

This work was supported by research grant 17337 from the NICHHD.

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Interaction of uterine mucosal large granular lymphocytes with human trophoblast *in vitro*

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The presence of abundant leucocytes in both pregnant and non-pregnant endometrium and decidua has long been recognised. It is now generally agreed that the predominant leucocyte population belongs to the family of Large Granular Lymphocytes accounting for about 70% of the total, with the remainder being HLA-DR+ macrophages (~20%) and mature CD3+ T cells (~10%). Very few B cells are found (1). The uterine LGLs stain strongly for the leucocyte differentiation marker Leu19 (CD56), but do not express other NK markers such as CD16 or Leu7. They appear to correspond to a small subset (~2%) of peripheral blood LGLs which also have the same phenotype being CD3-, CD16-, CD5-, CD2+, CD7+ and are designated Leu19+bright (2).

The LGL subset in peripheral blood appears to be responsible for Natural Killer (NK) activity. We have demonstrated NK activity using freshly violated decidual cell preparations against the NK sensitive cell line K562. However, we have shown that cultured first trimester trophoblast cells are resistant to lysis by decidual NK cells (3). These trophoblast cells have many phenotypic similarities to the invading extravillous trophoblast populations which lie in close opposition to decidual LGLs in the placental bed. Using cultured trophoblast cells as cold targets in a 51Cr release assay with decidual NK effectors against K562 we have shown to inhibition of K562 lysis (Fig.1). In addition, a single cell conjugate assay demonstrated binding of decidual CD56+ effectors to K562 cells but not to trophoblast cells (Fig.2).

These results indicate that the resistance to lysis of early human trophoblast to decidual NK cells is due to the lack of surface membrane structures required for NK recognition and binding. There appears to be no evidence for cellular intensities between uterine LGLs and trophoblast, so this functional relationship during pregnancy remains unclear.

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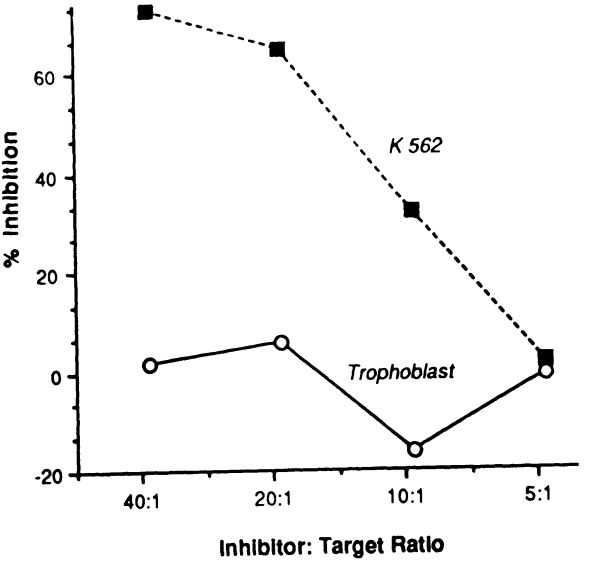

Fig.1 - A representative cold target inhibition assay using graded numbers of cultured trophoblast cells and K562 cells. 51Cr-labelled K562 cells (100uCi of Sodium Chromate for 90 mins) were added to each well ($1x10^4$ well). Decidual effector cells prepared by mechanical disaggregation as previously described (3) were added to achieve an effector target ratio of 100:1 in a total volume of 200ul. After 5hr at $37^{o}C$ 5%CO_2 100ul of supernatant was was removed and counted using a gamma counter. The results are expressed as a percentage of inhibition of specific 51Cr release.

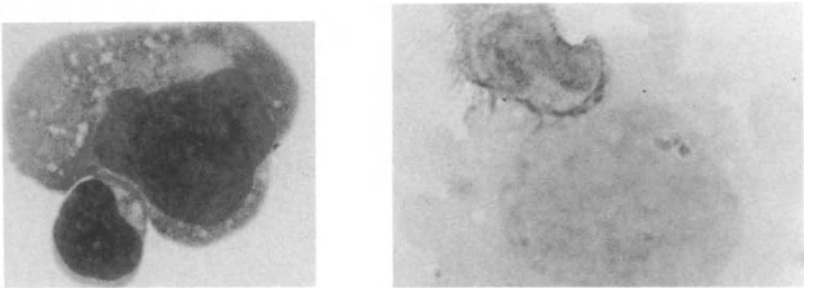

Fig.2a,b - Conjugates formed between decidual effectors and K562 cells in a single cell conjugate assay. Equal numbers of effector and target cells were mixed together, spun at 250g for 5 minutes and then incubated at $37^{o}C$ for 10 mins. 100ul of the supernatant was removed and the pellet resuspended. The number of conjugates counted using a phase contrast microscope. Cytospin preparations were stained with Giemsa or stained with Leu19 using an avidin-biotin peroxidase method (1). Fig.2a - The effector cell has indented the larger K562 cell and has a reniform nucleus typical of LGLs. Fig.2b - Immunostaining for CD56 (Leu19) shows the effector cells are positive.

SECTION R: IMMUNOHISTOLOGY OF MUCOSA IN MAN

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Expression of an adhesion related activation antigen on endothelial cells in human oral mucosal diseases

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1. Introduction

At sites of inflammation vascular endothelial cells (EC) become activated by inflammatory cytokines and exhibit altered functions including increased adhesiveness for circulating leukocytes.

In order to study EC activation further a monoclonal antibody 1.2B6 was developed by immunising mice with human umbilical vein EC activated for 6 hours with tumor necrosis factor-α (TNF) 320 U/ml. Antibody 1.2B6 recognises[1] the neutrophil adhesion related antigen ELAM-1 (Endothelial cell-leukocyte adhesion molecule-1) [2]. In culture ELAM-1 is not expressed on unstimulated EC but is rapidly induced on activation of EC with TNF, interleukin-1 (IL1) or bacterial lipopolysaccharide (LPS). Expression is maximal after 4 to 6 hours stimulation and returns to near baseline by 24 hours. The EC are then refractory to re-stimulation with the same agent but can be restimulated with another[3]. Since most studies of EC activation have been carried out using cultured large vein EC, there exists a need to determine whether EC in tissues behave in a similar fashion. The aim of this study was therefore to investigate EC activation in skin organ cultures and acute and chronic inflammatory conditions of the oral mucosa using mAb 1.2B6.

2. Materials & Methods

2.1. Organ Culture. Samples of normal human skin and oral mucosa were divided into 5mm squares and cultured for 0, 6 or 24 hours at 37°C in RPMI 1640 containing 10% foetal calf serum, L.glutamine and antibiotics +/- $_r$IL1 (10 U/ml) or $_r$TNF (320 U/ml) then snap frozen. Frozen sections of each specimen were stained using a streptavidin-biotin immunoperoxidase technique and primary antibodies 1.2B6 (activated EC), EN4 (all EC) and a negative control antibody.

2.2. EC Activation in Oral Mucosal Diseases. Specimens of a range of oral mucosal conditions (Table 1) were snap frozen and cryostat sections stained, as in section 2.1. The number of blood vessels and the number of activated blood vessels in each specimen were determined by counting respectively the number of 1.2B6 and the number of EN4 positive vessels per mm^2 for both superficial (within 2mm of epithelium) and deep corium and the proportion of activated vessels calculated in each case. Intensity of 1.2B6 staining was also scored semi-quantitatively (+/- to +++).

3. Results

Skin and mucosa organ cultures exhibited similar kinetics of 1.2B6 antigen expression on blood vessels as found on EC cultures ie:- little or no staining at 0 & 24 hrs, but strong staining at 6 hrs.

The number and proportion (Fig 1) of 1.2B6 positive activated blood vessels was significantly raised ($p<0.001$) in all mucosal diseases examined, both acute, such as non-specific ulcer, and chronic, such as lichen planus. The increase occured at both superficial and deep levels but the

increase in the proportion of vessels that were activated was greatest for deep vessels. 1.2B6 never stained more than 60% of vessels, and staining was mainly restricted to post capillary venules.

The intensity of 1.2B6 staining was also increased (Table 1) in oral mucosal diseases compared to normal tissue (with the exception of lichenoid change). Intensity was increased in acute and chronic conditions, but it was notable that staining was most intense in conditions associated with a neutrophil infiltrate (pyogenic granuloma, non specific ulcer and erosive lichenplanus).

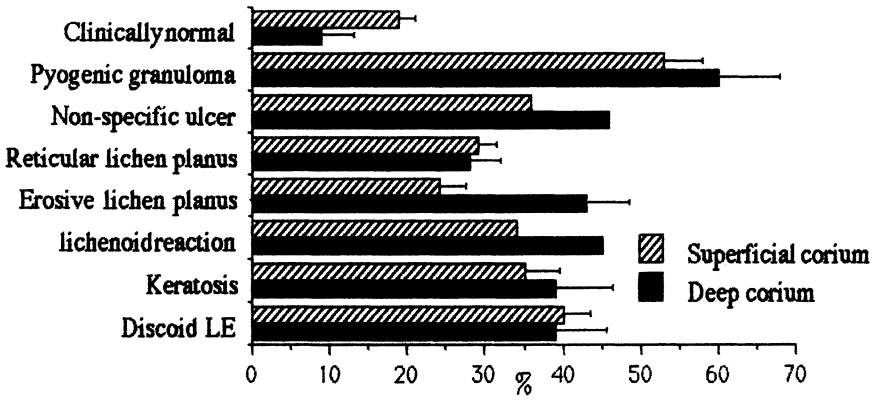
Table 1. Intensity of 1.2B6 staining

Clinical condition (n)	superficial	deep
Cinically normal mucosa (17)	+	+/-
Keratosis (7)	++	++
Pyogenic granuloma (4)	++/+++	++
Non-specific ulcer (2)	++/+++	++
Reticular Lichen Planus (14)	++	+
Erosive Lichen Planus (9)	++/+++	+
Lichenoid Change (2)	+	+
Discoid lupus erythematosus (5)	++	+

4. Discussion

EC activation, as detected by 1.2B6 staining, was a feature of all oral mucosal diseases examined. Although ELAM-1 is involved in mediating netrophil-endothelial cell adhesion[2], its expression was seen in conditions characterised by a lymphocytic infiltrate (eg: reticular lichen planus) and in conditions characterised by a neutrophil infiltrate (eg: pyogenic granuloma). However neutrophil accumulation was only seen in those conditions in which ELAM-1 expression was most marked. Activation of EC in cell or organ culture resulted in only transient expression of ELAM-1 but in oral mucosal diseases ELAM-1 expression was prolonged being a feature of acute and chronic conditions. These findings sugest that ELAM-1 expression is modulated differently in the oral conditions examined and *in vitro*. In addition ELAM-1 expression may have to exceed a certain threshold before neutrophil adhesion and migration into the tissues can occur.

Fig 1. PERCENTAGE OF VESSELS STAINED BY 1.2B6 (mean +/- SD)



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Human Peyer's patches and intestinal lamina propria contain remarkably different numbers of dendritic cells positive for S-100 protein and macrophages positive for L1 antigen

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1. Introduction

The monocyte-derived cell lineages include classical phagocytic macrophages and various antigen-presenting dendritic cell types. The latter are poorly phagocytic but play an important role as accessory antigen-presenting cells in the induction of immune responses. S-100 protein (a formalin-resistant cytoplasmic antigen) is a useful marker for the specialized dendritic cells in the T-cell areas of lymphoid tissues, the so-called interdigitating cells or IDC [1]. We have recently observed that reactive macrophages can be selectively labelled by staining for the formalin-resistant myelomonocytic L1 antigen [2]. As a monoclonal antibody (Mac 387) to the latter marker is now available [3], we were able to perform paired staining for macrophages and putative IDC on formalin-fixed biopsy material from normal human distal ileum, including Peyer's patches (PP).

2. Materials and Methods

Biopsy specimens (n=8) from normal human ileum, including PP, were fixed in formalin and processed for paraffin embedding. In addition, tissue samples (n=8) of PP with surrounding mucosa, obtained from eight kidney donors with artificially maintained respiration, were fixed in alcohol and processed for paraffin embedding [4].

Immunohistochemistry was performed by two-colour immunofluorescence staining (rhodamine and fluorescein) as described previously [5]. Briefly, polyclonal antibody to S-100 protein and monoclonal antibody (Mac 387) to L1 were applied on trypsinized formalin-fixed sections; polyclonal antibody to L1 and monoclonal antibody to HLA-DR (Becton-Dickinson) were applied on alcohol-fixed sections.

3. Results

L1-positive macrophages were diffusely distributed in the lamina propria of the ileum and in the dome region of PP. However, they were relatively scarce beneath the typical flattened follicle-associated epithelium (FAE) lacking

goblet cells. Conversely, S-100 protein-positive putative IDC were abundant beneath this part of the FAE, in addition to the interfollicular T-cell zones. The proportion of macrophages to IDC was thus significantly ($p<0.05$) lower in the dome region than in the lamina propria (median ratio $L1^+$/S-100 protein$^+$ = 20/80 vs. 72/28).

Most L1-positive macrophages were negative for HLA-DR, both in the dome region and in the lamina propria of the ileum (median, 6.2% and 6.8%, respectively). Beneath the typical FAE completely lacking goblet cells, 9 of 14 macrophages were HLA-DR-positive in two subjects, but in the remaining six in whom altogether 67 macrophages were evaluated, all were HLA-DR-negative.

4. Discussion and Conclusion

Macrophages are supposed to have a modulating function in primary antibody responses. Accessory activity of dendritic cells in cultures has been shown to be suppressed by macrophages [6], and the close spatial relationship in lamina propria supports interaction between L1-positive macrophages and dendritic cells.

The scarcity of reactive macrophages and abundance of dendritic cells positive for S-100 protein and HLA-DR (IDC phenotype) immediately beneath the FAE may favour primary immune responses in PP and be crucial for induction of mucosal immunity.

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Epithelial and plasma cell immunoglobulin distribution in the normal, inflamed and neoplastic salivary gland

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Abstract

IgA has long been reported in salivary glands [1] but many aspects of its topography remain to be described. We examined by immunofluorescence (IF) and immunoperoxidase (IP) normal, inflamed and neoplastic salivary glands. In normals, only serous acini were positive for IgA. We found intraepithelial IgA positive round cells (ERCs), presumed to be lymphocytes. IgA and IgG stromal plasma cells (SPCs) of salivary glands were enumerated and showed IgA was the most frequent isotype ($P \leq 0.05$). In sialadenitis, fivefold greater numbers of IgG SPCs were seen ($P \leq 0.001$). In atrophy (AT), intraepithelial IgA and IgA SPCs were decreased to 10% of normal ($P \leq 0.001$). In Sjogren's disease (SD) a 20:1 increase in IgG SPCs ($P \leq 0.001$) was seen. Adenoid cystic carcinoma (AC-CA) showed lesser intraepithelial IgA, but IgA SPCs were increased 2:1 ($P \leq 0.001$). Warthin's tumors (WT) had an infiltrate of IgG SPCs. We conclude that only serous cells secrete IgA, and that the most frequent SPC isotype in salivary gland is IgA. In sialadenitis, increased levels of IgG PCs were seen, explaining elevated IgG levels in saliva. AT results in disappearance of intraepithelial IgA and decreased IgA SPCs. AC-CA had increased numbers of IgA SPCs. WT had mostly IgG SPCs. A peripheral node origin for lymphocytes in WTs is suggested.

Introduction

The salivary immune system has been studied extensively [1-5], and its main immunoglobulin (Ig) is IgA [1-5]. We have shown that IgA cells in salivary glands originate in gut associated lymphoid tissue[6]. Little information exists on topography of SPCs in inflammation and neoplasia of the salivary gland.

Materials and Methods

Salivary gland sections were studied by IF and IP for IgA, IgM, and IgG as described [6-8], and assessed for Igs in the epithelium and SPCs per 10 high power fields. Slides were co-stained with Alcian Blue (AB) allowing discrimination of cells containing mucin and/or Igs in the same tissue.

Results(See Table)

Normal Salivary-IgA was present in serous cells only, by combined AB-IP. Only serous cells contained IgA in small vacuoles. Collecting ducts were uniformly positive for IgA. IgA SPCs predominated 17:1 over IgG cells ($P \leq 0.05$). IgA

ERCs thought to be lymphocytes were seen. IgA SPCs predominated 4:1 over IgA ERCs, ($P \leq 0.05$).

Inflammatory Conditions-Sialadenitis had a 5:1 IgG/IgA prevalence ($P \leq 0.001$) and decreased intraepithelial IgA. IgA ERCs were increased 50% over normals ($P \leq 0.001$). IgG SPCs were increased fivefold compared to normals ($P \leq 0.001$). In Sjogren's decreased intraepithelial IgA was seen, and intraepithelial IgG was absent. Interstitial IgG was increased. IgA SPCs were decreased 50% ($P \leq 0.001$), and a twenty-fold increment in IgG SPCs was noted ($P \leq 0.001$).

Neoplasms-In AC-CA intraepithelial IgA was decreased, but a twofold increase in IgA SPCs was observed ($P \leq 0.001$). WTs showed absence of intraepithelial IgA, and occasional IgA SPCs. IgG SPCs were abundant (20:1; $P \leq 0.001$).

Discussion

Salivary IgA is an important defense system in the oral cavity. Its protection includes a capacity to inhibit attachment of bacteria to epithelium [9] and to neutralize viruses and endotoxins [10]. Topography of IgA in salivary glands has been studied [1-4].

We saw a granular distribution of IgA in serous cells, a diffuse distribution of IgA in collecting ducts, and IgA ERCs that are probably lymphoid cells. All glands positive for IgA were positive for SC. In mixed acini, only serous cells had IgA.

In sialadenitis, most SPCs contained IgG but not the epithelium. Intraepithelial IgA was increased, but not IgA SPCs. AT showed decreased intraepithelial IgA and SPCs suggesting abrogation of attracting mechanisms. In Sjogren's, increased IgG SPCs, and increased intraepithelial IgA alone were noticed. We suggest that in Sjogren's, IgA transport by epithelia is enhanced. AC-CA revealed intraepithellial IgA suggesting a capacity to internalize IgA. Increments of IgA SPCs suggest increased attraction of cells. WTs contained no intraepithelial Igs but abundant IgG SPCs, suggesting IgG lymphoid cells result from entrapment of peripheral node structures.

TABLE: Mean # of IgA/IgG Positive Cells per 10 Fields(S.E.M.)

		IgA		IgG	
	n	Epithelial	Stromal	Epithelial	Stromal
Normal	6	102.7 (7.4)	447.5(23.7)	2.3(0.3)	26.0 (2.0)
Sialadenitis	3	171.0(10.7)	342.3(49.9)	16.0(2.3)	147.3(11.9)
Atrophy	3	5.7 (0.3)	48.7 (2.4)	2.3(0.3)	6.0 (1.0)
Sjogren's	3		225.3 (1.2)		519.7(25.1)
Warthin's	3		57.0 (8.5)		522.0(15.0)
Adencyst Ca	3		769.3(34.0)		60.3 (1.2)

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Two new mabs: one stains marginal zone B cells in gut associated lymphoid tissue (Galt)

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ABSTRACT. Two murine monoclonal antibodies (mAbs), 4D12 and 3D3 have been produced using a centrocyte like (CCL) cell rich primary B cell gastric lymphoma (4D12) or a follicular lymphoma (3D3) as immunogens. On spleen sections, they stained marginal and mantle zone B cells respectively. Immunochemistry showed that centrocytic lymphomas are $3D3^+$ $4D12^-$ while all mucosal lymphomas tested were $3D3^-$ but about 50% were $4D12^+$. None of the cell lines tested expressed either of these two antigens. The majority of $3D3^+$ cells are $sIgM^+$ and $sIgD^+$ and most $4D12^+$ cells are $sIgM^+$ and $sIgD^-$. Functional studies showed that both antigens play a role in B cell activation as the mAbs increase the mitogenic effect of *Staphylococcus aureus* (SAC) Cowan I and reduce TPA (12-O-tetradecanoyl phorbol 13 acetate) activation on tonsil B cells. These two mAbs recognize new B cell surface antigens and their reactivity reinforces the suggestion of homology between mucosal and splenic marginal zone B cells and they may be useful in determining the function of these populations of cells.

1. Introduction

Recent studies have suggested that there is a relationship between mucosal peri-follicular centrocyte like (CCL) cells which infiltrate follicle centers in gastric lymphoma and splenic marginal zone B cells (1). Both populations consist predominantly of IgM^+, IgD^- cells (1, 2), in contrast to mantle zone and peripheral blood (PB) B cells which are largely IgM^+, IgD^+.We have therefore produced mAbs to these sub-populations of B cells, studied their cellular distributions and their functions.

2. Materials and Methods

Balb/c mice were immunised with cells teased from either a follicular lymphoma (3D3) or CCL cell rich primary gastric lymphoma (4D12). The supernatants were screened on frozen sections of low grade lymphoma of Mucosal Associated Lymphoid Tissue (MALT) and spleen using indirect immunoperoxidase assay. A panel of lymphomas of MALT have been tested with 4D12 and 3D3 mAbs. A list is given in Table 1. Single staining was done on B cells from PB, tonsil and spleen. Double staining were done on tonsil and spleen B cells using FITC conjugated $F'(ab')_2$ fragments of rabbit anti human IgD and IgM (Dako) and biotinylated 3D3 and 4D12. Fresh tonsil B cells were cultured with different concentrations of SAC, TPA, and purified 3D3 or 4D12

mAbs at 37 °C under 5% CO_2. The cells were labelled on the 3rd day with 1 µCi ^3H thymidine for 8 hrs. They were harvested and the level of incorporation was measured in a scintillation counter

3. Results

4D12 stains splenic marginal zone B cells and a subset of follicle centre cells in spleen and tonsil. 3D3 stains mantle zone B cells and follicular denditric cells on spleen and tonsil sections (fig. 1). The resuls of staining on lymphoma are summarized in table 1

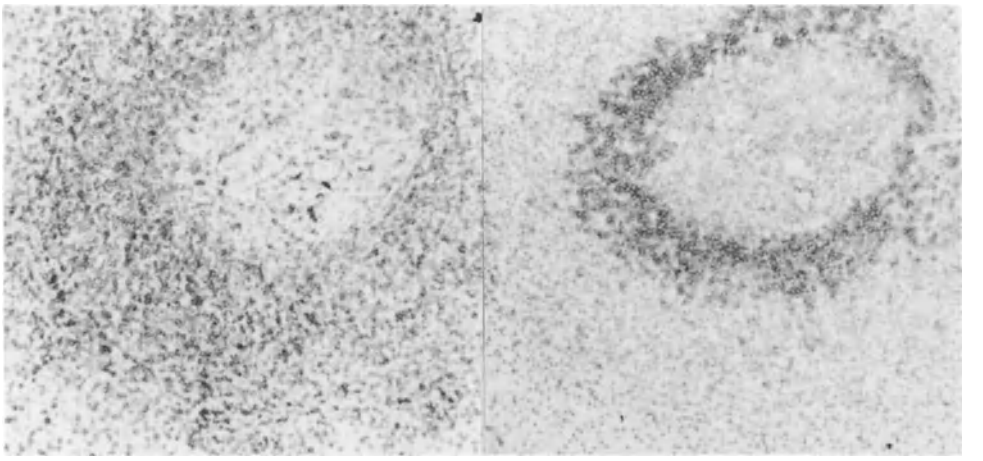

Figure.1. Staining patterns of 4D12 (left) and 3D3 (right) on spleen sections.

TABLE 1 Reactivity of 4D12 and 3D3 on a panel of lymphomas

lymphomas	4D12	3D3	CD22
Gastric	6/13	0/13	13/13
CB/CC	13/16	0/16	16/16
CLL	1/5	1/5	5/5
Centrocytic	0/6	6/6	6/6
Immunoblastic	0/5	0/5	5/5
pleomorphic B	0/1	0/1	1/1
plasmatic	0/1	0/1	1/1

Isolated splenic cells are 16% $4D12^+$, 40-50% $3D3^+$; tonsil B cells 5% $4D12^+$, 20-40% $3D3^+$ peripheral blood B cells <2% $4D12^+$, 20% $3D3^+$. No cell lines so far tested (23) express either 4D12 and 3D3. Double staining shows that $4D12^+$ cells expressed sIgM but not sIgD while $3D3^+$ cells are $sIgM^+$ and $sIgD^+$. Functional

studies demonstrate that these two mAbs enhance the stimulatory activity of SAC by 30-50% and inhibit activation of TPA by 30%.

4. Discussion

4D12 mAb has been raised by using mucosal CCL cells as immunogen and it recognises both mucosal and splenic marginal zone B cells but not mantle zone. These results suggest that there is a close relation between splenic marginal zone B cells and mucosal CCL cells as reported earlier (1) and that they are analogous populations in either their stage of maturation or their lineage. None of the B cells lines tested showed any reactivity with these two mAbs which could mean that they are expressed at early stage during B cell maturation. The majority of recirculating B cells are $4D12^-$ and a large proportion of them are $3D3^+$. Double staining confirms that $4D12^+$ cells and $3D3^+$ cells have the marginal and the mantle zone phenotype respectively. Functional studies reveal that both antigens are lost rapidly in culture and that they play a role in B cell activation.

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Nerve growth factor (NGF) receptor expression in human gut associated lymphoid tissues

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Introduction

NGF is a protein required for the survival and differentiation of sympathetic and sensory neurones[1]. Recently, it has become clear that NGF may have broader biological activity, including effects on immune cells[2]. NGF has been shown to enhance mitogen stimulated lymphocyte proliferation, when added to rat spleen mononuclear cell cultures[3]. The cell type mediating this response, presumably through binding to an NGF receptor (NGFR), is not known. In the human, data concerning the effects on NGF on lymphoid cells is not available. However, a recent study by Garin Chesa et al[4] demonstrated strong NGFR-immunoreactivity in follicles in lymph nodes and spleen. Because of our interest in mucosal immunity, we decided to see if any cells in the germinal centres of mucosa associated lymphoid tissues (MALT) also expressed the NGFR.

Materials and Methods

Fresh tonsils (20), appendices (5) and 'normal' areas of resected intestines (10) were fixed in acetic acid/ethanol (1:9, AA) before processing to paraffin. Some tonsils were frozen for cryostat sectioning or used for cell isolation. Mononuclear cells were separated from heparinized peripheral blood using Ficoll-Hypaque, cytospin smears prepared and fixed in formalin. MoAb 20.4 (NGFR, IgG1)[5] and 1B7.11 (TNP, IgG1) were purified from culture supernatants of hybridomas (ATCC, Rockville, MD). MoAb R4/23 (DAKO-DRC1, IgM) and Leu M1 (IgM, Becton Dickinson) were also used. Cell suspensions from dissected tonsillar follicles were fractionated by two methods: 1) preincubation with MoAb 20.4 or MoAb 1B7.11 (control) followed by magnetic beads coated with anti-mouse IgG (Dynabeads™ M-450, Dynac Inc. NY); and 2) separation into lower and upper fractions by sedimentation over 30% serum at unit gravity. Whole and fractionated cell populations were prepared as cytospins for immunoenzyme staining and fixed in 2% glutaraldehyde for electron-microscopy (EM). NGFR-immunoreactivity was demonstrated with MoAb 20.4 in an indirect peroxidase method using rabbit anti-mouse Igs, peroxidase conjugated swine anti-rabbit Igs, and aminoethylcarbazole (red). For double-staining this was followed by the localization of FDC using R4/23 in a biotin-streptavidin-alkaline phosphatase technique (SA) with fast blue BB. To prevent cross-reaction with the first monoclonal (20.4,IgG1),

a biotinylated anti-mouse IgM (Zymed, CA) was employed.

Results

Immunostaining of whole tonsillar cell suspensions revealed a 20.4^+ population of predominantly dendritic and occasional smaller mononuclear cells. NGFR-immunoreactivity was not seen in the blood smears. Sedimentation resulted in an upper fraction of single cells which were 20.4^- and $R4/23^-$; and a lower fraction containing large cells, single or in groups. The clumps contained dendritic 20.4^+ and $R4/23^+$ cells with attached small lymphoid cells. A proportion of the single cells were stained with both antibodies. Using the immunobeads with 20.4, we recovered a small percentage of isolated or clumped dendritic cells. Beaded cells were not seen with the irrelevant MoAb (1B7.11). EM confirmed that cells with the morphological characteristics of FDC were concentrated in the lower fraction. Immunohistochemistry on tonsil and gut showed that 20.4 stained the lymphoid follicles. This was limited to the germinal centres and suggestive of FDC. Serial sections stained for 20.4 and R4/23 (FDC), showed a similar distribution of positivity. In the tonsil, the basal epithelium was also 20.4^+, as were the neural elements in the gut. In double stained slides, these results were confirmed in all tissues. The majority of FDC were 20.4^+ $R4/23^+$, although there were small subpopulations of 20.4^+ $R4/23^-$ and 20.4^- $R4/23^+$ dendritic cells. Omitting either MoAb resulted in the development of a single colour only. Substitution of R4/23 by LeuM1 resulted in red staining of a dendritic pattern, with scattered blue granulocytes.

Discussion

The predominant dendritic NGFR-immunoreactive FDC population in germinal centres of MALT follicles suggests the possibility that NGF might act on this cell type. That peripheral blood cells and the majority of tonsillar mononuclear cells are <u>not</u> NGFR-immunoreactive would argue against a direct effect of NGF on most lymphocytes. The small population of lymphoid 20.4^+ and dendritic 20.4^+ $R4/23^-$ cells may be significant in terms of FDC heterogeneity. We speculate that the mononuclear cell proliferative response to NGF in rodents might be due to an effect on accesory cells.

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Distribution of Ia-positive cells in human gut-associated lymphoid tissue

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Introduction

Ia antigens are polymorphic membrane glycoproteins coded by genes within the major histocompatibility complex(MHC) class II determinants, which act as restriction elements in immune responses. The antigen-presenting function of cells is associated with the expression of Ia antigens on the cell surface. Distribution studies in several species, including man, have shown that different cells express Ia antigens[1)2)] and thus play an important role in the induction of the immune responses.[3)] In the gut, antigens applied to the intestinal mucosa can stimulate immune responses. Because of the importance of Ia molecules in antigen presentation, it was of interest to examine their tissue distribution in the intestinal mucosa.

Materials and Methods

Ia antigens were localized in cryostat sections of human intestine fixed with PLP by an indirect immunoperoxidase technique using monoclonal antibodies that recognized major histocompatibility complex(MHC) class II-restricted antigens, HLA-DR and HLA-DQ, and interleukin 1(IL-1) at light and electron microscopic levels.

Results and Discussion

The distribution studies showed that Ia antigens were present in three groups of cells; first, in some T and B lymphocytes, macrophges and dendritic or interdigitating cells, second, in endothelial cells, and third, in epithelial cells. Non-lymphoid cells with dendritic morphology within the follicle-associated epithelium over Peyer patches and subepithelial dome(Fig. 1) and in the reticular area inside of the T-dependent interfollicular area expressed MHC class II(HLA-DR and HLA-DQ) determinants. Intraepithelial T lymphocytes(IEL) between the epithelium over Peyer patches expressed HLA-DR antigen, whereas, those between the absorptive epithelium of intestinal villi lack HLA-DR antigen.

The follicular associated epithelium including M cells and absorptive epithelium in the distal 2/3 of the intestinal villi expresed only HLA-DR determinant. Particularly, M cells showed apical and basolateral expression of Ia antigen(Fig. 2). These HLA-DR antigen-positive cells virtually positive for interleukin 1.

It has been well recognized that isolated endothelial cells and enterocytes can replace macrophges as antigen-presenting cells in vitro. Although HLA-DR antigen are normally found on villous epithelial cells,[2] the presence of the antigen on M cells has been controversial.[4] The present study, however, clearly demonstrated HLA-DR expression on both apical and basolateral plasma membranes of M cells.

These characteristic distributions of Ia-positive cells in the intestinal mucosa support the view that these cells form an antigen-presenting system in gut-associated lymphoid tissue, and participate in regulation of lymphocyte differentiation and activation in the mucosal immune system.

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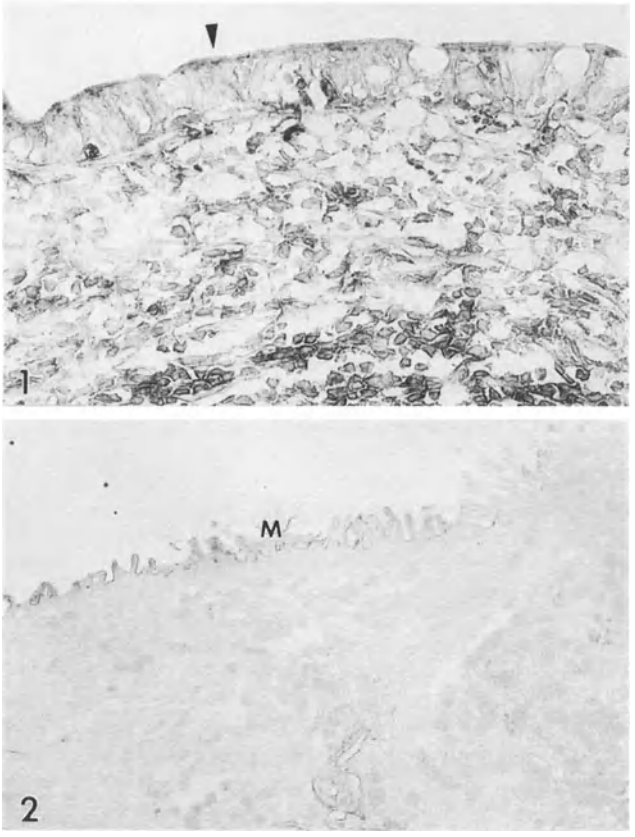

Figures 1 and 2: Immunohistochemical localization of HLA-DR in Peyer's patches. HLA-DR antigen is present on non-lymphoid cells and M cells (**M**) within the follicule associated epithelium(◄).

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Common antigenic determinant on ductular cells of normal pancreas, on mucosal cells of the gastrointestinal tract and on CEA

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INTRODUCTION:

CEA is widely used as a human tumor marker and was first defined by Gold and Freedman in 1965 as an antigenic component in cancers derived from gastrointestinal tract epithelium. It is a member of a large family of immunologically related glycoproteins that vary in size and tissue distribution. Studies with c-DNA clones for CEA and NCA reveal that this family consists only of a limited number of different proteins with variable glycosylation, due to posttranscriptional modifications. The complete gene family includes about 10 closely related genes. Recently it was published that in contrast to mRNA coding for CEA, mRNA coding for NCA was expressed predominantly in cancerous tissues. A monoclonal antibody recognizing an epitope expressed on both CEA and NCA could therefore be a useful diagnostic reagent.

MATERIALS AND METHODS:

Monoclonal antibody 82-F19 was established after immunizing mice with human breast tumor cell line MCF-7 and fusion of spleen cells with mouse myeloma cell line P3-NS1/1-Ag 4-1. Antibody binding to different cultured human tumor cell lines and immunohistochemical studies on fresh frozen tissue sections were performed by the indirect immunoperoxidase method. For biochemical analysis proteins of 82-F19 positive tumor cells were solubilized with 1% CHAPS, fractionated by SDS-gel electrophoresis or HPLC-separation according to size (column Du Pont GF-450, Zorbax). Antigen detection was performed by Western blot analysis or by coating fractionated proteins after molecular weight exclusion chromatography to microfluor W plates (Dynatech, Alexandria, VA) and screening fractions by ELISA techniques as described earlier. For detection of 82-F10 antigen in the sera of patients with gastrointestinal tumors, 82-F19 antigen was purified from tumor cell line HT-29 (electroelution of the positive 100 kD band after SDS-gel-electrophoresis) and mAb 82-F19 reactivity was

tested after preincubation with sera of tumor patients and healthy donors, by an inhibition ELISA. Positive sera blocked mAb 82-F19 binding to the purified antigen.

RESULTS

Immunohistological analysis performed on fresh frozen tissue sections show that mAb 82-F19 identifies a tumor-associated protein of the gastrointestinal tract. This antigen is expressed on numerous gastrointestinal tumors especially on human pancreatic adenocarcinoma. Most of the tumor cells in 5/5 pancreatic adenocarcinoma reacted with this antibody. In the normal pancreas only the ducts are detected by this mAb. The antigen was also expressed on the cell surface of in vitro cultured gastrointestinal cell lines (pancreatic, stomach and colon cancer cell lines). Detergent solubilization of proteins of these tumor cell lines, molecular weight exclusion chromatography, SDS-gel electrophoresis and Western blot analysis reveal that the molecules recognized by this mAb are proteins of about 200 and 100 kD. Using HT-29 colon cancer cells as antigen source the 100 kD band was detectable only. As shown by Western blot analysis and ELISA tests the mAb reacted with purified CEA. Therefore we tried to establish a sensitive inhibition ELISA test system to detect CEA in the sera of tumor patients. Our results were in good agreement with the results obtained by a commercial CEA-test-kit. In the group of 15 patients with pancreas carcinoma we were able to detect one additional and in the group of 15 patients with stomach carcinoma we detected two additional positive sera, which could not be assessed positively with the commercial CEA-test-kit.

DISCUSSION

Based on the results obtained by Western blot analysis and positive staining of granulocytes, mAb 82-F19 presumably recognized an epitope which is expressed on both CEA and NCA. Therefore the antibody may be a useful reagent for clinical diagnostics. The epitope recognized by mAb 82-F19 may also be relevant in view of the close association between ductular epithelia in the normal pancreas and the tumor cells in pancreatic adenocarcinoma. These results emphasize the antigenic heterogeneity of CEA, which belongs to the immunoglobulin supergene family.

This CEA-epitope may therefore represent a common determinant of at least some members of the CEA-family. It also appears to be relevant in view of the close association between ductular epithelia in the normal pancreas and the tumor cells in pancreatic carcinoma.

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Topography of immunoglobulins in normal and neoplastic endometrium

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Abstract

Reports of endometrial IgA secretory immunity have been controversial and non-quantitative [1,2]. We assessed the topography of different Ig isotypes in normal and neoplastic endometrium by immunofluorescence (IF) and immunoperoxidase (IP) techniques. We show, as others [1,2], that IgA is the predominant Ig in endometrium. IgG is present in greater numbers of stromal plasma cells (SPCs) than other tissues [25% of SPCs in the proliferative (PE) and secretory phases (SE)]. Atrophic endometrium (AE) shows negligible numbers of SPCs and intraepithelial Igs. Atypical hyperplasia (AH) shows a 50% increase in intraepithelial IgA and IgA SPCs ($P \leq 0.01$). Well-differentiated carcinoma (WD-CA) has a 100% increase of IgA PCs ($P \leq 0.001$), but intraepithelial IgA is decreased, whereas poorly differentiated carcinomas (PD-CA) show decreased IgA PCs ($P \leq 0.001$) and negligible intraepithelial IgA. Occasionally epithelial IgA positive round cells (ERCs), probably lymphocytes, are seen. We conclude, as others, that IgA is the prevalent epithelial and SPC isotype, especially in SE, suggesting hormonal control. In AH and WD-CA, increased IgA SPCs suggest the mechanism for attracting cells is increased. Carcinoma shows decreased intraepithelial IgA, especially in PD-CA. In conclusion, the degree of differentiation may determine intraepithelial IgA and SPCs in endometrium.

Introduction

Endometrium is more exposed to the environment due to denudation. IgA is one of its significant defense mechanisms [1], as attested by IgA PC increments in endocervical infection [2]. Controversy on endometrial IgA topography exists; some observe epithelial and SPC IgA [1], whereas others report their absence [3].

Materials and Methods

Using IF and IP for IgA, IgM, IgG and SC as described [4,5], we analyzed sections of 6 PE, 5 SE, 4 AH, 5 WD-CA, 3 PD-CA and 3 AE. Both SC and Igs in epithelium and SPCs were assessed in 10 high power fields.

Results(See Table)

Normal Endometrium-In PE, intraepithelial IgA was present apically. Some glands didn't contain IgA, all glands positive for IgA contained SC. IgA SPCs predominated 3:1 over IgG SPCs ($P \leq 0.0277$), IgM SPCs were negligible. SE showed

prominent intraepithelial IgA in infra- and supra-nuclear vacuoles, as well as apically. IgA SPCs predominated over IgG SPCs 3:1 ($P \leq .0001$), ERCs were observed. AE glands were negative for intraepithelial IgA and IgA SPCs. *Neoplastic Endometrium*-IgA was prominent apically and predominated over IgG 10 fold in SPCs ($P \leq 0.0003$). IgA SPCs were 50% greater in AH than SE ($P \leq 0.01$). WD-CA had smaller amounts of IgA concentrated in the brush border. IgA-SPCs were prominent (17-fold over IgG, $P \leq 0.0001$), but PD-CA showed negligible intraepithelial IgA and few IgA or IgG SPCs.

Discussion

Endometrium undergoes cyclic hormonal changes exposing it to the environment. IgA is a crucial defense mechanism in the endocervix and endometrium [1,3]. Several authors have not found Ig containing SPC's in endometrium [6], others report both SC and IgA in the epithelium but no SPCs [7], and yet others report few IgG and IgA SPCs in SE [8].

We agree with Lee Y-S. et al [9] that IgA cells prevail over IgG, but the study was not quantitative. IgA SPCs in PE range from 157 to 234, and are greater in SE (range = 349 to 501). IgA epithelial internalization and attraction may be more developed in SE, suggesting hormonal dependence; it is abrogated in AE.

AH contained more epithelial IgA and SPCs, indicating SPC attraction may be increased. WD-CA had more IgA SPCs than other normal or pathologic conditions ($p \leq 0.05$), but smaller amounts of intraepithelial IgA, suggesting IgA internalization may be defective in CA. In PD-CA negligible intraepithelial IgA and IgA SPCs were seen, implying a lack of ability to internalize IgA and/or recruit IgA lymphocytes. Interstitial IgG was strongly positive in all cases except atrophy, but no increase in IgG SPCs was seen. In normal endometrium, numbers of IgG cells were higher than in other mucosae. We conclude that the presence of IgA in endometrium may be hormone dependent and correlated with the degree of epithelial differentiation.

TABLE: Mean # of IgA/IgG Positive Cells per 10 Fields

	n	IgA	IgG	P<
Proliferative	6	214.5 (12.0)	79.3 (30.7)	0.0277
Secretory	5	425.8 (29.3)	122.6 (13.3)	0.0001
Atrophy	3	21.0 (2.5)	11.7 (9.2)	n.s.
Atyp Hyperplasia	4	619.3 (33.5)	57.8 (10.9)	0.0003
Well-Diff CA	5	973.4 (32.7)	59.0 (9.0)	0.0000
Poor-Diff CA	3	49.7 (13.8)	14.3 (5.4)	n.s.

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Immunoglobulin distribution in the epithelium and stromal plasma cells of normal and neoplastic prostates

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Abstract
IgA, the major secreted immunoglobulin (Ig) in the prostate [1], has been described both in normal and pathologic glands [2]. Reports on its histotopography are limited. By immunofluorescence (IF) and immunoperoxidase (IP) techniques, IgA was present in a variegated distribution suggesting only certain cells internalize it. Intraepithelial IgA round cells (ERCs), probably lymphocytes, were seen. In benign prostatic hyperplasia (BPH) IgA was seen luminally, with a threefold increase in stromal plasma cells (SPCs). Well-differentiated cancers (WD-CA) had decreased intraepithelial IgA, and a 6-fold increase of SPCs, whereas poorly differentiated cancers (PD-CA) had negligible intraepithelial IgA and half as many IgA SPCs. Our findings suggest that internalization of IgA as well as attraction of IgA cells is dependent on differentiation. We conclude that IgA is the predominant Ig in normal and WD-CAs. In BPH an impaired transport mechanism relegating IgA to the luminal border may exist, a phenomenon also seen in WD-CA. Most PD-CAs expressed negligible amounts of intraepithelial IgA, with decreased SPCs. It is possible that the mechanism for attracting cells is altered in PD-CAs.

Introduction
IgA is the main Ig in the prostate [1,2], and is elevated in prostatitis [3]. We observed in normal and neoplastic colon that intraepithelial Ig and SPCs were related to the degree of differentiation [4]. Others have explored the presence of IgA and secretory component (SC) in normal and neoplastic prostate [5]. None of these studies reported a detailed topography of Igs.

Materials and Methods
Sections were studied by double IF[4] and direct IP[6] for IgA, IgG, IgM, and SC in glandular epithelium and SPCs. IP was performed on 8 cancers and 3 BPHs for IgA, IgG and IgM, and cells counted in 10 fields.

Results(See Table)
Normal Prostate-IgA was identified in epithelial Golgi zones and apices. Glands positive for IgA were positive for SC. IgG was present in smaller amounts, and IgM was negligible. A variegated distribution of IgA was observed. ERCs, thought to be lymphocytes, and SPCs enumerated showed a prevalence of IgA SPCs over IgA ERCs($p \leq 0.0001$). IgA ERCs predominated over IgG ERCs

($p \leq 0.0001$) and IgA SPCs predominated over IgG SPCs in all groups ($p \leq 0.0001$). *Benign Prostatic Hyperplasia (BPH)*-IgA was relegated apically. IgA SPCs prevailed over IgG cells ($p \leq 0.0001$). IgA SPCs prevailed over IgA ERCs 2 to 1 ($p \leq 0.0001$). Three times as many IgA ERCs were seen ($p \leq 0.001$.)
Well Differentiated (WD-CA) and Poorly Differentiated (PD-CA) Carcinomas-WD-CA showed a sixfold increase in IgA PCs ($p \leq 0.001$) over normals. A decrease in intraepithelial IgA was seen in WD-CA. Intraepithelial IgA was rarely found in PD-CAs.

Conclusions

IgA in the prostate exists in a variegated pattern (i.e. with or without IgA), suggesting two types of epithelial cells. Internal and external glands differ endocrinologically, [7] and may affect IgA expression. The main Ig isotype in SPCs was IgA. IgA ERCs thought to be lymphocytes were also visualized. Increased numbers of IgA PCs in BPH and WD-CA appear to be related to the neoplastic process rather than an increment of epithelium alone. In PD-CA, epithelium appears to lose its capacity to internalize IgA, as suggested by occasional PD-CA areas expressing IgA. Thus a paucity of IgA SPCs cannot be the only cause for a lack of intraepithelial IgA. We suggest that differentiation is necessary for intraepithelial IgA expression and its transport receptor, SC. No IgA positive epithelium lacked SC. It is possible that homing in PD-CA of prostate is altered in proportion to differentiation.

TABLE: Mean # of IgA/IgG Positive Cells Per 10 Fields(S.E.M.)

		IgA		IgG	
	n	Epithelial	Stromal	Epithelial	Stromal
Normal	7	83.7 (2.7)	184.3 (7.3)	1.6(0.2)	11.9 (1.8)
Hyperplasia	7	258.9 (7.3)	580.9(13.1)	2.0(0.4)	18.7 (1.7)
Well Diff Ca	5	381.6(36.9)	1242.6(42.6)	1.3(0.7)	59.4(10.3)
Poorly Diff Ca	3	6.3 (0.9)	55.0 (0.0)	1.3(0.9)	5.7 (1.8)

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Isotype distribution of mucosal IgG-producing cells in patients with various IgG-subclass deficiencies

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1. Introduction

The aim of this study was to examine by immunohistochemistry the IgG-immunocyte subclass distribution in nasal and rectal mucosa of serologically IgG-subclass deficient patients. The availability of a clinically and serologically well-characterized patient material at the University of Göteborg [1], encouraged us to study possible associations between mucosal expression patterns of IgG-deficiency and different clinical manifestations in groups of patients with various serological IgG-subclass deficiencies. A preliminary study had suggested that the nasal expression of IgG-subclass deficiency does not necessarily conform with that in serum [2].

2. Material and Methods

Biopsy specimens of nasal and rectal mucosa were obtained in 18 adult patients, whereas only a nasal or a rectal sample was available from 9 similar subjects. Chronic lung disease was common in the patient groups with selective serum IgG1 deficiency (both patients) or combined IgG1 and IgG3 deficiency (four of six patients), whereas only two of four in the group with selective IgG2 deficiency suffered from this serious disease. The other categories of patients had mostly upper airway and other mild infections like the group with selective serum IgG3 deficiency where only one of ten suffered from chronic lung disease.

Paired immunofluorescence staining was performed on serial tissue sections by applying subclass-specific murine monoclonal antibodies followed by fluorescein-labelled conjugate to murine IgG and rhodamine-labelled conjugate to human IgG; the proportion of immunocytes belonging to each subclass could thereby be determined in relation to the total number of mucosal IgG-producing cells [2]. This method showed an excellent intra- and inter-observer reproducibility.

3. Results

Serum IgG2 or IgG3 deficiency was usually expressed also at the cellular level in rectal mucosa, and the proportion of rectal IgG1 cells was significantly

correlated (r=0.90, p<0.001) with the IgG1-subclass level. There likewise tended to be a decreased isotype expression at the cellular level in the nasal mucosa of patients with serum IgG1- or IgG2-subclass deficiency. Conversely, the median nasal proportion of IgG3 cells was remarkably unaffected by a deficiency of this subclass in serum and rectal mucosa. Interestingly, these patients rather seemed to have raised IgG3- and reduced IgG2-cell proportions in nasal mucosa, although this apparent local IgG3 compensation was nevertheless strongly correlated with the serum IgG3-subclass level (r=0.87, p<0.002).

4. Discussion

The observed discrepancies between the expression of IgG3-subclass deficiency at the two mucosal sites might reflect different antigenic and mitogenic loads, for example a persistent protein bombardment (virus) of the nasal mucosa that could locally override a B-cell maturation defect. Such stimulatory differences could explain the disparity between the rectal and nasal mucosa.

A fairly intact humoral defence afforded by IgG3 compensation in the upper respiratory tract of patients with selective serum IgG3-subclass deficiency might explain the relatively low incidence (10%) of chronic lung disease in this group. Clinicopathological evaluation of the patient material pointed out the group with single or combined serum IgG1 deficiency as the one mostly affected with chronic lung disease. Absence of IgG1 is usually associated with hypogammaglobulinaemia since IgG1 makes up the largest proportion (60-70%) of total IgG. Consequently, these patients often have a history of lung disease and increased susceptibility to pyogenic infections [3].

Genetic factors may additionally influence the IgG-subclass response. Immunoglobulin deficiencies may occur as a result of defects in heavy chain genes or in the regulation of heavy-chain gene switching on chromosome 14 where the genes for $\gamma 3$ and $\gamma 1$ and those for $\gamma 2$ and $\gamma 4$ are closely linked [4]. In several clinical situations the IgG isotypes appear to be regulated or expressed in patterns reflecting this gene arrangement.

Further studies of the mucosal IgG-subclass distribution in relation to clinicopathological features may contribute to a better understanding of the distinctive pathogenetic mechanisms interacting in these immunodeficient patients.

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Phenotype of bronchial T-lymphocyte population in the chronic inflammation of bronchiectasis

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1. INTRODUCTION

Bronchiectasis is a chronic disease characterised by irreversible dilation of one or more bronchi. The affected bronchi are seat of persistent inflammation which may lead to scarring and shrinkage of the lung, and in some cases to cor pulmonale and death. The understanding of the pathogenesis bronchiectasis is still poor. Recently a "vicious circle" hypothesis for the pathogenesis of the disease has been proposed by Cole. According to this, damaging insults to the bronchial tree or underlying disease may compromise the first line bronchial defence mechanisms of mucociliary clearance and predispose the individual to microbial colonization of the bronchial tree. The host's inflammatory response fails to eliminate these microbial colonists and becomes chronic, damaging adjacent lung disease in the process. There is immunohistological evidence that a significant cell-mediated immune response develops in inflamed areas of bronchiectatic lungs. This inflammation could be responsible, at least in part, for the progressive lung damage seen in the disease, either by the emergence of a cytotoxic T-lymphocyte population or by the activation of macrophages. Such mechanisms have been identified in several chronic inflammatory diseases such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease. We present here the phenotype of the T-lymphocyte population present in the bronchial wall of resection specimens of 23 bronchiectatic patients in comparison to non-bronchiectatic controls.

2. MATERIALS AND METHODS

Patients: Tissues from 23 patients with radiographically proved bronchiectasis were obtained at lobar resection or by bronchial biopsy during clinically indicated fibreoptic bronchoscopy. Controls: Tissues from 7 patients without bronchiectasis (four carcinomas, three pneumonias) were used as controls, being obtained by bronchial biopsy during clinically indicated fibreoptic bronchoscopy (three cases), or at resection for bronchial carcinoma, from non-compromised areas (four

cases). All samples were snap frozen in liquid nitrogen and cryostat sections of 6 microns preopared and some were stained for conventional histology. Indirect immunoperoxidase using a pan T-cell marker was employed to determine the distribution of T-lymphocytes in the bronchial mucosa. Double immunofluorescence was used to investigate the CD4/CD8 ratios in the bronchial wall and the co-expression of other molecules by the T cells using of a combination of specific anti-mouse antisera used in this study: CD7 (blast T cells); RFDR (Class II MHC); CD25 (Interleukin-2 receptor); CD38 (activated T-cells); UCHL1 (LCA-memory cells); CD45R (LCA-virgin cells);

3. RESULTS

TABLE1- Distribution of T-lymphocytes in the bronchial mucosa

	Epithelium	Lamina Propria	Submucosa
BX	26.9+10.4 (20)	29.7+21 (22)	28.7+20.8 (21)
C	7.1+1.6 (6)	5.4+4.1(7)	6.5+3.4 (5)
P	0.0001	0.0001	0.0001

BX= bronchiectatic samples C= controls p= p value (x) = number of samples. Both CD4+ and CD8+ subsets were observed but there was a consistent overall predominance of CD8+ cells. Further analysis of the membrane antigens by both subsets was carried out and is expressed here as the percent of doubly-labelled cells (SD ommited for clarity):

TABLE2 - Phenotype of CD4 and CD8 positive T-lymphocytes

	CD7		CD25		CD38		HLA-DR		UCHL 1		SN130	
	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8	CD4	CD8
BX	27.6	83.0	2.0	4.3	6.1	13.6	27.3	19.3	61.8	75.5	43.1	18.6
C	15.0	30.0	0	0	1.2	0	3.7	3.5	35.0	35.0	25.0	17.5
P	NS	.05	NS	.05	.05	.01	.01	0001	.0001	.0001	NS	NS

4. DISCUSSION

This paper showed that T-lymphocytes constitutes a major component of the inflammatory infiltrates present in the bronchial wall of bronchiectasis. The differences between bronchiectatic and control suggested that these cells may play a role in the pathogenesis of this condition. Also important was the predominance of the CD8+ cells when compared with the CD4+ subset. The CD8+ population expressed significant amounts of activation markers, as IL-2 receptor, CD7, CD38, and HLA-DR. The only activation marker expressed in significant amounts by the CD4+ cells was the HLA-DR. Differences between BX and C were noted in the proportions of CD8+UCHL1+ and CD4+UCHL1+, whereas no differences could be found in the proportions of CD8+CD45R+ or CD4+CD45R+. It has been demonstrated that after T cell activation, a switch of leucoyte common antigen expression occurs which is detected by the disappearance of CD45R and the appearance of UCHL1 positivity. The damage created by this inflammation in the bronchi and surrounding tissues in bronchiectasis indicates a cytotoxic role for the CD8 positive T cells, the overall characteristic of which reflect those seen in cell-mediated immune reactions.

SECTION S: IMMUNOHISTOLOGY OF MUCOSA IN ANIMALS

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Macrophage subpopulations, dendritic cells and high endothelial venules in the gastrointestinal tract of the mouse

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ABSTRACT. Macrophage subpopulations, dendritic cells and high endothelial venules (HEV) of the GI tract were studied using immuno- and enzyme-histochemistry. Most macrophages were detected in the lamina propria of the lower intestine. These cells displayed Ia-expression and were strongly AcPh+. Dendritic cells with strong Ia-positivity were recognized in PP and PP-associated villi. HEV were not only present in PP, but also at the base of villi.

1. INTRODUCTION

Macrophages (Mϕ) and dendritic cells (DC) play a key role in the immune response, as antigen presenting cells, regulator and effector cells (LeFevre et al., 1979; Steinman & Nussenzweig, 1980). To understand the role of the different parts of the GI tract in antigen uptake and in the induction and regulation of immune responses, it is of importance to study the distribution of Mϕ subpopulations and DC in these areas. In the present study we investigate the presence of such cells along the GI tract of the mouse using various monoclonal antibodies with special attention to the presence of HEV-like vessels through which lymphocyte traffic occurs (Jeurissen et al., 1987).

2. MATERIALS AND METHODS

BALB/c mice (CPB, Zeist, The Netherlands) were used. Tissue was sampled from the oesophagus, stomach, duodenum, jejunum, ileum, caeccum, colon, rectum and frozen in liquid nitrogen. Cryostat sections, fixed in acetone, pretreated with periodate 0,2% 20 sec, and were then incubated with one of the following monoclonals: MOMA-1, MOMA-2, Mac-1, ERTR9, F4/80 (Mϕ); NLDC-145, MIDC-8 (DC), MECA-325 (HEV), M5/114 (Ia antigen). Acid phosphatase activity was demonstrated with naphthol AS-BI phosphate (Sigma, USA) as substrate, and hexazotized pararosaniline as diazonium salt.

3. RESULTS

A heterogeneity of Mø subpopulations was found along the GI tract. Such cells displayed AcPh+. Most Mø were detected in the lamina propria of the lower intestine by MOMA-1, MOMA-2, Mac-1, ERTR9 and F4/80 antibodies. MOMA-1+ Mø have similar distribution pattern with ERTR9+ Mø, though they are separate subpopulations. Mac-1+ Mø were found in high numbers per villus. Few F4/80+ Mø were detected in the villi. Besides in the lamina propria of villous cores, MOMA-2+ Mø were also found at the base of villi and in the epithelium. Compared to other Mø subpopulations, MOMA-2+ Mø were more widely distributed both in the intestinal villi and PP. NLDC-145+ Mø were also recognized in the lamina propria of lower intestine. A typical NLDC-145+ DC with slender processes were detected in PP-associated villi as well as in PP. These cells showed MHC class II expression. HEV-like vessels were recognized by MECA-325 monoclonal antibody at the base of villi of the lower intestine. Unlike PP HEV, the endothelium of the venule in villi was flat. Few lymphocytes were seen in the lumen of HEV-like vessels.

In conclusion, the lower part of the intestine play important role in immune responses, since most Mø and DC were found in these areas. This correlates with the higher antigen load there. The presence of HEV-like vessels indicates that lymphocyte traffic mainly occurs in the lower part of intestine.

4. ACKNOWLEDGEMENTS

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Morphological aspects of nasal-associated lymphoid tissue (NALT) in rats

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ABSTRACT. Lymphoid and non-lymphoid cells of nasal-associated lymphoid tissue (NALT) were investigated morphologically. NALT is a well-organized lymphoid structure comprising B- and T-cell areas covered by epithelium which contained M-cells. This epithelium is infiltrated by several lymphoid subpopulations and Ia- and ED1-positive cells.

1. INTRODUCTION

The local immunological defense system of mucous surfaces in rats comprises characteristic lymphoid structures, known as mucosa-associated lymphoid tissue (MALT). This tissue is represented in the respiratory tract by bronchus-associated lymphoid tissue (BALT) and nasal-associated tissue (NALT). NALT is situated at both sides of the nasal entrance of the pharyngeal duct (Spit et al. 1989) and is already present at birth (Hameleers et al. 1989). In the present paper NALT was investigated by electronmicroscopy and immuno- and enzymehistochemistry.

2. MATERIALS AND METHODS

Young adult male albino Wistar rats (Cpb; WU Wistar random) were used. NALT was collected and frozen at -159°C. Cryostat sections, fixed in pure acetone, were incubated with one of the following monoclonals; W3/13 (T cell), W3/25 (T helper cell), Ox8 (T non-helper or T suppressor/cytotoxic cell), IgG clone Ox12, IgM clone MARM-4, IgA clone MARA-1, IgE clone MARE-1 and Mas 029c (Ia), all from Seralab (UK) and ED1, ED2 and ED3 (macrophages/monocytes) and ED5 (follicular dendritic cells). In addition, sections were incubated with the polyclonal rabbit anti-rat antiserum against laminin (E.Y. Labs, Inc., Sanbio, The Netherlands).

Acid phosphatase activity was demonstrated with the substrate naphthyl-AS-BI phosphate and a non-specific esterase with α-naphthyl-acetate (Sigma, USA); hexazotized pararosaniline was used as the diazonium salt.

For electronmicroscopy, glutaraldehyde-fixed sections were con-

trasted with uranyl-magnesium acetate and lead citrate.

3. RESULTS

NALT consisted of B follicles and interfollicular T cell areas, and was covered by a specialized epithelium. Many lymph and blood vessels were found in the T cell areas and a single myelinated nerve was observed. The B cell areas were traversed by capillaries. Most B cells were positive for surface IgM or IgG. Germinal centers were found infrequently and follicular dendritic cells were present in some B cell follicles. The follicles also housed T cells, mainly of the T helper type. In the T cell areas, T helper cells outnumbered T suppressor/cytotoxic cells, although the ratio varied markedly within NALT. Around high endothelial venules, T as well as B cells were observed. Most macrophages were ED1-positive. A few were ED2- or ED3-positive. Lymphocytes and macrophages infiltrated the epithelium. The majority were B lymphocytes with surface IgM or IgG. A few cells were T cells (W3/25+ or Ox8+) and macrophages (ED1). The epithelium contained ciliated and non-ciliated or M-cells, and only a few goblet cells. The M-cells had microvilli or merely an irregular outline of the apical cell membrane; they were Ia-positive and showed endocytic activity. Nasally-applicated horseradish peroxidase coupled with gold (HRP-gold complex) was found in vacuoles in the M-cells and not in the ciliated cells.

In conclusion, the lymphoid aggregates that are situated at the nasal entrance of the pharyngeal duct (denoted NALT) are organized lymphoid structures that share several morphological characteristics with other mucosal lymphoid tissues. The high number of M-cells and the presence of numerous, mostly IgM-positive B cells, and some T cells and macrophages in NALT epithelium of untreated rats (low antigenic load) suggests that NALT is easily activated. It is in addition situated strategically. NALT may therefore be an important part of the mucosal immune system in the respiratory tract.

4. ACKNOWLEDGEMENTS

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Ultrastructural characterization of microenvironmental elements associated with proximal colonic lymphoid tissue (PCLT) in the mouse

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Introduction

The mucosal immune system is comprised of both diffuse and aggregated lymphoid components, and acts as the primary immune barrier for ingested antigens. The elaborate studies of Owen (1) and others have clearly demonstrated that a specialized population of antigen sampling Membranous cells (M cells) is present in the follicle-associated epithelium (FAE) overlying Peyer's patches of the small intestine. However, in contrast to the antigen responsive role of secondary lymphoid tissue generally bestowed upon gastrointestinal associated lymphoid tissue (GALT), the terminal ileal Peyer's patch has been shown to be a primary source of B lymphocytes in sheep (2,3) and pigs (4). We have recently described proximal colonic lymphoid tissue (PCLT), which is a lymphoid nodule in the proximal colon of mice and rats displaying many characteristics of a primary lymphoid tissue (5,6). The majority population of B cells found within PCLT display a relatively immature B cell phenotype (10-20% of which are $CD5^+$), and appear unable to respond to mitogenic activation. In addition PCLT displays a unique steroid sensitivity among gut associated lymphoid tissues, losing up to 70% of its tissue weight after steroid treatment. Until now little attention has been focused on the stromal components associated with this unique tissue. Therefore it was the intent of these studies to define the non-lymphoid elements associated with PCLT in the mouse.

Results and Discussion

For these studies we have employed routine light microscopy (LM) of thick sections from plastic embedded materials, as well as both transmission and scanning electron microscopy (TEM and SEM, respectively). As this methodology is routine it will not be detailed here.

As we have previously described, PCLT was found consistently located 25% of the distance from the cecum to the rectum (5,6). Scanning electron microscopy clearly demonstrated the epithelial dome overlying this tissue. The PCLT itself demonstrated a tightly packed lymphoid architecture, with various non-lymphoid components obvious at the LM level. In TEM preparations the lymphocytes were medium to large, uniformly rounded with an undifferentiated cytoplasm free of organelles. No plasma cells were seen within PCLT, although such cells are often observed in Peyer's patches. These characteristics would morphologically be consistent with the classification of these cells as immature lymphocytes. However, in contrast to other primary lymphoid tissues, PCLT

lymphocytes showed a distinct lack of mitotic figures at both LM and TEM levels.

The population of "clear cells" which we have previously described in both mouse and rat (6) appears to consist of two morphologically distinct cell types distributed throughout PCLT. The first of these are clearly macrophages, as they can be seen at both the LM and TEM levels to contain phagosomes, large primary and secondary lysosomes and residual bodies. The second population of "clear cells" found in PCLT are similar in size to the macrophages, but differ morphologically and in their apparent state of activity. These "clear cells" are always large round cells, with a round euchromatic nucleus, extremely pale and undifferentiated cytoplasm. It is currently unclear whether these cells represent a distinct population of epithelial or hematopoietic cells, or whether they might be macrophages in an early state of maturation and cytoplasmic differentiation.

The area of FAE on the dome overlying PCLT contained striking examples of lymphoepithelial interactions. Lymphocytes and associated FAE cells often exhibited very active cell membranes, with numerous infolding and points of apparent contact. Coated vesicles were observed in the epithelial cells of the FAE. The lymphocytes were often associated with cells which morphologically resembled M cells, as judged in TEM sections by their less electron dense cytoplasm, poorly defined terminal web and short irregular microvilli. The presence of M cells in the dome FAE was confirmed by SEM comparison of the dome regions of PCLT and the terminal ileal Peyer's patch, and clearly showed that M cells are not confined to lymphoid aggregates of the small intestine. This represents the first morphological demonstration of M cells in the FAE overlying colonic lymphoid tissue in mice, although the presence of such cells has been shown in human colonic FAE (7,8), and suggested but unconfirmed in the rat colon (9).

Finally, we observed the presence of multivesicular bodies, described by Landsverk in the terminal ileal Peyer's patch of ruminants (10). Landsverk originally suggested an important role for these vesicles in B cell differentiation. In the current studies these structures were consistently found in association with the golgi apparatus of the cell. These findings would not be inconsistent with a secretory function for these organelles as suggested by Landsverk, however caution should be exercised in the interpretation of static observations until the actual in vivo function of these structures is resolved.

In summary, although a number of common non-lymphoid microenvironmental elements can be demonstrated in proximal colonic lymphoid tissue, we have confirmed the presence of M cells in the FAE associated with lymphoid tissue of the mouse colon. In addition, we have been able to demonstrate the presence of two morphologically distinct populations of clear cells in PCLT (macrophages and "clear cells"). However, the functional characteristics and interrelationships of these components and their potential roles in B cell differentiation and/or activation in colonic lymphoid tissue remain to be elucidated. (Supported by NIH grants AI 25222 and AI 25826)

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Possible role of the antrum in mediating immunological reactions in the stomach

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1. Introduction

In dogs and rats antigens given orally after systemic immunization cause a specific release of gastrin, a hormone that is produced by G-cells in the antrum and known to be a potent stimulator of acid secretion. Furthermore the mucosal blood flow is increased only in the antrum (Krämling et al 1987, Adv Exp Med Biol 216 A: 427-429, Teichmann et al 1983,Langenbecks Arch Chir Suppl 5-8). Thus the antrum mucosa seems to be involved in immunological reactions, which are antigenspecific and therefore immunocompetent cells are likely to mediate these reactions. The aim of the study was to investigate the number and distribution of immunocompetent cells in the antrum and to compare those with the corpus.

2. Methods

Stomachs of male Wistar rats were used. To identify immunocompetent cells in situ cryostat sections of the corpus and antrum were stained, after fixation in acetone, with Oxford monoclonal antibodies (Serotec) Ox 1 (1:500), Ox 6 (1:500), Ox 19 (1:200) and Ox 8 (1:200) for 30 min, followed by incubation with rabbit anti-mouse (Dako, 1:25, 30 min) and finally with mouse PAP complex (Dako, 1:100, 30 min). Positive cells were detected by developing in AEC. Mast cells were identified by Wright's stain. For detection of eosinophiles May-Grunwald and Giemsa stain was used. In order to calculate and compare the number of immunocompetent cells, a single cell suspension was prepared. Everted sacs of the antrum or the corpus were filled with pronase (5 mg/ml). After incubation in medium (37^{o} C, 90 min.), cells were carefully dispersed by using a magnetic stirrer for 1 h. This method prevents mucosal cells coming into direct contact with high concentrations of pronase (Viability always greater than 85%). Cytospins

were stained for mast cells, leucocytes and lymphocytes as described above (dilution of Ox 1 1:400, Ox 6 1:500, Ox 19 1:100, Ox 8 1:200). Mucosal mast cells were detected by staining with Alcian blue (1% in 0,7 N HCl) and Safranin (0,5% in 0,125 N HCl) after fixation in Carnoy solution. The proportion of positive, stained cells was calculated by counting a total of 1000 cells.

3.Results

In cryostat sections, as well as in cytospins of single cell suspensions, leucocytes (Ox 1^+), T-cells (Ox 19^+), T-suppressor cells (Ox 8^+), Ia-positive cells (Ox 6^+), mast cells and eosinophiles can be demonstrated. In sections, these cells are located in the lamina propria of both the antrum and the corpus mucosa, intraepithelial lymphocytes are rare. Epithelial cells do not stain for Ia. Quantification of immunocompetent cells in the suspension shows a significant difference between antrum and corpus. The antrum mucosa contains more leucocytes and lymphocytes as well as typical macrophages, mast cells and eosinophiles (Tab.1). All mast cells are mucosal mast cells as shown by the Alcian blue-Safranin staining of cell suspensions.

Table 1. Percentage of immunocompetent cells in gastric mucosal single cell suspensions (mean ± SEM):

	leucocytes	T-cells	T-supp.cells	Ia^+ cells	mast cells	eosinophiles
antrum(n=6)	1,85±0,29	0,65±0,12	0,40±0,03	1,10±0,07	1,50±0,16	1,10±0,20
corpus(n=5)	0,74±0,12	0,09±0,04	0,07±0,06	0,41±0,06	0,21±0,03	0,09±0,04

4.Discussion

Immunocompetent cells for a cell mediated immunological response (antigen presenting cells, T-cells) and for hypersensitivity reaction (mast cells, eosinophiles) can be demonstrated in the lamina propria of the gastric mucosa. In sections most of these cells seem to be located in the antrum. Morphometric quantification of different cells in sections is difficult. In cell suspensions calculation of stained cells is precise and most of the immunocompetent cells were in fact demonstrated in the antrum mucosa. In this part of the stomach gastrin release and increased blood flow after oral antigen challenge has been demonstrated. Mast cell mediators or lymphokines may be responsible for these reactions. Furthermore these results suggest that the antrum might be the more important region for antigen identification, immunological reactions and hormonal response.

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The evolution of mucosal lymphoid tissues in mammals

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INTRODUCTION

We have examined and compared the mucosal and peripheral lymphoid tissues of a representative monotreme, the Australian echidna (Tachyglossus aculeatus) and a representative marsupial, Antechinus swainsonii (marsupial mouse).

RESULTS AND DISCUSSION

The mucosal and peripheral lymphoid tissues of the echidna are simpler and less well organised than those of their metatherian and eutherian counterparts. Monotremes possess simple Peyer's patches but the constituent lymphoid follicles are separated by non-lymphoid tissue and are surrounded by a peri-follicular lymphatic space. The lymphatics drain into collecting vessels, in the lumen of which single lymphoid follicles are present (Figure 1). An earlier report [1] has shown Peyer's patches similar in structure to those of eutherian mammals; possibly both types of Peyer's patch exist in the echidna.

The mesenteric and peripheral lymphoid tissues of the echidna are unusual in that both primitive and more advanced lymphoid structures are present. Simple isolated lymphoid follicles represent the majority of the organised lymphoid tissue although two or three lymphoid follicles may be aggregated into non-capsulated or capsulated lymph nodules. There is no evidence of trabeculae and medullary tissue and the putative cortex is relatively unstructured.

The results of this study are in accord with earlier reports [2,3] and also demonstrate the presence of non-capsulated and capsulated lymph nodules in monotremes.

Marsupial mice possess Peyer's patches and peripheral and mesenteric lymph nodes which are histologically indistinguishable from those of eutherian mammals. However, the Peyer's patches are few in number their position in the intestine relatively constant and they are grouped in an anti-mesenteric or a lateral position. Although histologically indistinguishable it is not known if the two types of Peyer's patch serve the same or different functions.

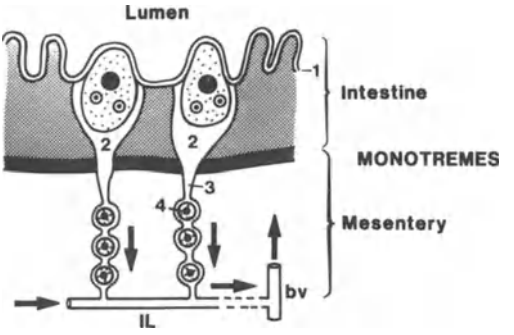

Figure 1 Monotreme Peyer's patch

Key:
1. Epithelial cell layer with intraepithelial lymphocytes
2. Perifollicular lymphatic space
3. Afferent lymphatic vessel
4. Lymphoid follicle with or without germinal centre

IL intestinal lymphatic
bv blood vessel

Thus the primitive lymphoid tissues observed in the peripheral tissues of the echidna are not retained in higher mammals. In contrast, the primitive mucosal lymphoid tissues observed in the echidna (and lower animals) have been retained along with the highly structured multifolliculate lymphoid tissues (Peyer's patches) in the mucosal associated lymphoid tissue of metatherian and eutherian mammals.

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Part III
Diseases of Mucosal Tissues in Man and Animals

SECTION T: HIV AND THE GASTROINTESTINAL TRACT

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The role of the gut-associated lymphoid tissue in the pathogenesis of the acquired immunodeficiency syndrome (HIV-infection)

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Abstract. Intestinal lamina propria T cells (LP-T) may be an important site of primary infection and replication of HIV. We studied the presence of HIV-infected cells and changes in lymphocyte subpopulations in the mucosa of HIV-infected patients by immunohistology. HIV-infected mononuclear cells were present in the intestinal lamina propria in 40% of HIV-infected patients. There was an increase in CD3+ and in CD8+ cells with a significant decrease of the CD4/CD8 ratio in the mucosa. The number of activated LP-T expressing IL-2 receptors (IL-2R) in the mucosa was decreased, whereas the percentage of HML-1+, Leu8+, Ki67+, and EBM11+ cells was not different in HIV-infected patients compared to controls. In addition, we investigated small intestinal structure and function in HIV-infected patients by three-dimensional morphometry and enzyme histochemistry. HIV-infected patients without additional intestinal infection had a lower villus surface area and a lower number of mitotic figures per crypt, the findings being most pronounced in patients with mucosal HIV-infected mononuclear cells. Brush border lactase/ß-glucosidase was significantly decreased or even absent in HIV-infected patients. These findings indicate a low grade small bowel atrophy and a maturational defect of enterocytes in HIV-infection which may also be caused by a loss of activated regulatory T cells in the mucosa of HIV-infected patients.

Introduction:

The gastrointestinal (GI) tract is a major target organ for opportunistic infections and secondary malignancies in human immunodeficiency virus (HIV) infection, and gastrointestinal symptoms such as weight loss, diarrhea, and abdominal pain are frequent in HIV-infected patients [1]. There are indications that the GI mucosa itself may be affected by HIV [2]. Normal intestinal LP-T cells are predominantly of the helper phenotype and are more activated than lymphocytes from other tissue sites [3]. Since HIV predominantly infects CD4+ T lymphocytes and viral replication is dependent on cellular activation, LP-T cells may be an important site of primary infection and replication of HIV. Infection of LP-T cells with HIV may lead to a loss and/or functional impairment of these regulatory T cells in the intestinal mucosa with consequences for the local immune barrier and mucosal structure and function.

Methods:

PATIENTS:

60 HIV-infected patients, who underwent diagnostic upper endoscopy because of gastrointestinal symptoms, were studied. According to the classification of the Center for Disease Control (CDC)

4 were at stage II, 7 at stage III, and 49 at stage IV of the disease. All patients (3 women, 57 men) were whites, the age ranging from 21 to 61 years (median 37 years). There were no patients receiving parenteral nutrition included. Biopsy specimens of patients where routine gastroduodenoscopic and histologic examination did not reveal pathologic changes were used as controls.

DETECTION OF LYMPHOCYTE SURFACE ANTIGENS AND HIV ANTIGEN:

4-6 µm acetone/chloroform fixed cryostat sections of snap frozen biopsy specimens were incubated with monoclonal mouse antibodies to HIV antigen p24 (DuPont) or to lymphocyte surface antigens CD4, CD8, IL-2 receptor, HML-1, Ki67, or EBM11. The reaction was visualized by the alkaline-phosphatase-anti-alkaline-phosphatase(APAAP)-technique [2]. Controls were performed by substituting primary antibody with non-immune mouse immunoglobulin and with sections of HIV seronegative patients. Slides were evaluated microscopically in a blinded manner; subpopulations were determined as number of stained cells per 100 mononuclear cells of the lamina propria.

QUANTITATIVE HISTOCHEMISTRY:

Brush border enzyme activities of lactase/ß-glucosidase and alkaline phosphatase were measured by enzyme histochemistry on unfixed 10 µm cryostat sections of snap frozen distal duodenal biopsy specimens [4]. For the detection of lactase/ß-glucosidase activity, 5Br-4Cl-3indoxyl-ß-D-fucoside was used as substrate with an ironII/ironIII redox system as additional reagent for the development of the indigo-dye deposits in the brush border membrane. Alkaline phosphatase was measured with Naphthol-As-Bi-phosphate as substrate with Fast Blue B as coupling reagent. Absorbance measurement was performed on a Leitz MPV2 microdensitometer. The transition zone between lower and medium third of the villus was defined as "basal" measuring point, the transition zone between medium and apical third of the villus as "apical" measuring point. Uncolored tissue served as blank (100% transmission). Ten measurements per measuring position were averaged.

MORPHOMETRY:

Morphometry of the duodenal mucosa was performed by microdissection of distal duodenal biopsy specimens [5]. From each sample 10 random isolated villi were chosen for the determination of their height, width, and breadth both at the base and at the apex. From these values the surface area of an individual villus was calculated. Subsequently, the individual crypts were dissected and their lengths were recorded. The total number of mitotic figures per crypt was counted. The means of the morphometric data for the individual specimen were compared.

Results:

DETECTION OF HIV ANTIGEN P24:

HIV antigen p24 was detected by immunohistology in the intestinal mucosa in 22 of all 57 investigated patients (in 4 of 11 patients at stages II or III and in 16 of 40 patients at stage IV). Four of 12 patients receiving zidovudine had detectable mucosal HIV-infected cells. In 11 patients with detectable mucosal HIV antigen p24 no enteric pathogen could be identified. Detection of HIV-infected cells did not correlate with the number of CD4 positive cells in the peripheral blood or with the presence of gastrointestinal symptoms. Infected cells were located in the lamina propria and morphologically identified as lymphocytes and occasionally macrophages. Positive cells were also found on rare occasions above the basal membrane between epithelial cells, therefore

suspected to be infected intraepithelial lymphocytes. There were no positive cells in control sections of HIV-seronegative patients.

LYMPHOCYTE SUBPOPULATIONS IN THE INTESTINAL LAMINA PROPRIA:

Using immunohistology, the number of CD3+, CD4+, CD8+, IL-2R+, Leu8+, HML-1+, Ki67+, and EBM11+ cells was determined in intestinal biopsies of patients with HIV-infection (Table 1). The number of CD4+ cells in the lamina propria was only slightly decreased in patients with HIV-infection compared to controls. However, the number of CD8+ cells was significantly increased resulting in a decreased CD4/CD8 ratio in the intestinal lamina proria in HIV-infection. There was no clear correlation to the stage of the disease or to the number of circulating CD4+ cells. The number of IL-2R bearing cells in the mucosa was significantly decreased in HIV-infection compared to controls. No differences were found for the number of Leu8+ or HML-1+ cells, or the number of macrophages (EBM11+) in the intestinal lamina propria of controls and HIV-infected patients. In both, controls and HIV-infection, no Ki67+ cells (a marker expressed in proliferating cells) were observed.

Table 1. Abnormalities of lymphocyte subpopulations in the intestinal mucosa of patients with HIV-infection.

	CD3	CD4	CD8	IL-2R	Leu8	HML-1	EBM11
Control	29 (15-52)	25 (8-40)	12 (4-28)	5 (2-17)	5 (0-9)	12 (4-23)	12 (7-30)
Total HIV	35 (12-47)	24 (2-49)	19 (7-41)	2 (1-27)	4 (0-15)	15 (6-29)	16 (2-30)
I-	34 (15-47)	25 (2-49)	19 (7-41)	3 (1-27)	5 (0-15)	18 (6-29)	16 (7-30)
I+	40 (23-45)	21 (3-34)	14 (10-24)	2 (1-11)	2 (1-10)	15 (6-27)	17 (2-30)
p24-	35 (19-45)	21 (3-49)	19 (10-41)	2 (1-10)	3 (0-10)	15 (6-29)	17 (2-30)
p24+	36 (12-47)	26 (3-44)	15 (10-32)	2 (1-15)	5 (1-15)	15 (6-27)	16 (2-24)
CDC II/III	35 (23-47)	31 (19-49)	19 (10-34)	4 (2-27)	5 (0-15)	14 (6-27)	16 (9-30)
CDC IV	37 (15-45)	24 (2-44)	17 (7-41)	2 (1-12)	2 (1-10)	16 (6-29)	17 (2-30)
DWL-	34 (12-47)	24 (2-49)	21 (7-41)	3 (1-16)	3 (0-9)	15 (7-29)	12 (2-30)
DWL+	37 (19-45)	24 (3-44)	17 (10-35)	2 (1-13)	5 (1-15)	15 (6-27)	16 (2-30)

Values are given as median (minimum - maximum) of the numbers of stained cells per 100 lamina propria mononuclear cells; I-/I+: Absence or presence of an intestinal pathogen; DWL: Diarrhoea-and/or weight loss.

MORPHOMETRY:

Morphometric data of distal duodenal biopsies were assessed by three-dimensional measurement of microdissected specimens. Median villus surface in HIV-infected patients was decreased to 0.207 mm^2 (0.000-0.623 mm^2), compared to 249 mm^2 (0.081-0.415 mm^2) in 42 controls ($p<0.05$). Crypt depth was significantly increased in HIV-infected patients versus controls, the number of mitotic figures per crypt in HIV-infected patients was normal (Fig. 1). When comparing the different groups of HIV-infected patients defined by the presence or absence of intestinal infection or mucosal HIV antigen p24, and controls, patients with intestinal infection showed normal numbers of mitotic figures per crypt and increased crypt depth. Patients without intestinal infection and patients with detectable mucosal HIV antigen p24 showed decreased numbers of mitotic figures per crypt ($P<0.05$) and normal crypt depth compared to controls. Patients receiving antiviral drugs or co-trimoxazole and patients who did not receive these drugs were not different with regard to morphologic parameters.

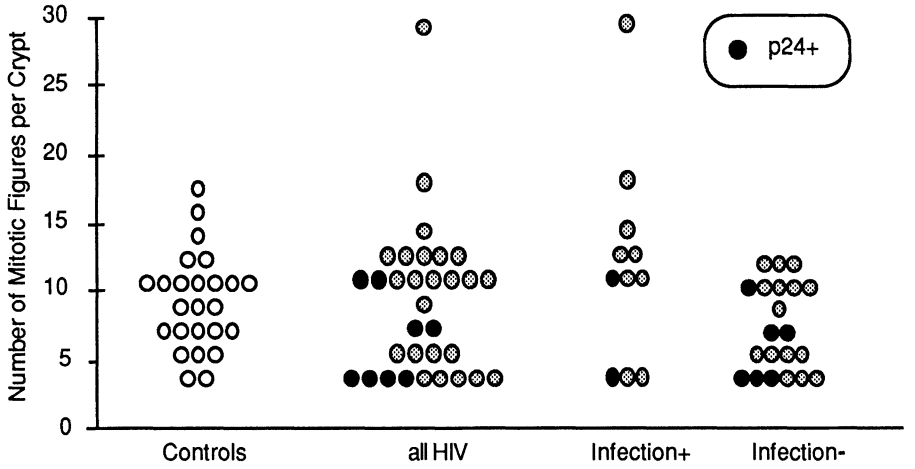

Figure 1. Number of mitotic figures in the crypts of the small intestinal mucosa of HIV-infected patients and controls.

ENZYME HISTOCHEMISTRY:

Fifteen of 25 patients had no detectable lactase/ß-glucosidase activity in the duodenal brush border membrane (Fig. 2). When lactase/ß-glucosidase activity was measureable, activity was significantly decreased compared to 7 control biopsies at the basal part of the villus in all HIV-infected patients and in the subgroups without intestinal infection and with detectable mucosal HIV antigen p24. Compared to controls, measurable lactase/ß-glucosidase activity was significantly decreased in patients without intestinal infection at the apical part of villus.

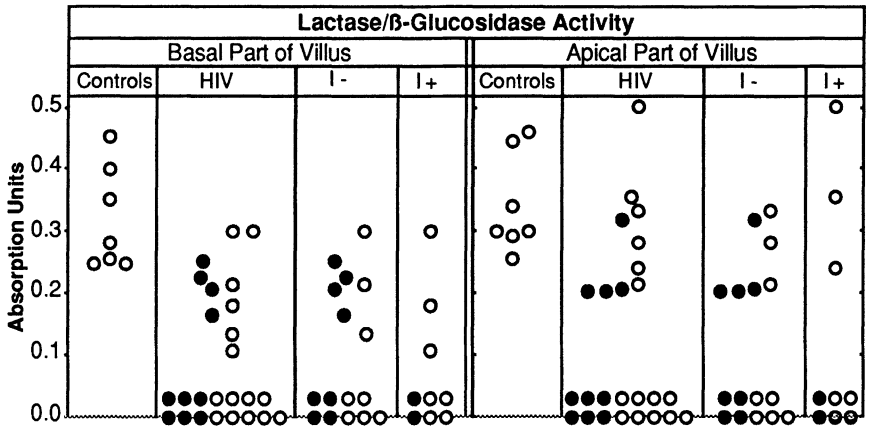

Figure 2. Activity of brush border lactase/ß-glucosidase in the small intestinal mucosa of HIV-infected patients and controls; • HIV-antigen p24+.

Activities of alkaline phosphatase at the basal and apical part of the villus were not statistically different in HIV-infected patients compared to controls. However, median activities at the apical part of the villus tended to be lower in the group with HIV antigen p24 in the mucosa vs. the group without, especially in the absence of intestinal infection. In both enzymes activities did

not correlate with the number of CD4 positive cells in the peripheral blood. In both enzymes, there were no significant differences between patients receiving antiviral drugs or co-trimoxazole and patients who did not receive these drugs.

Discussion: This study shows that HIV infects mononuclear cells in the intestinal lamina propria. Most likely caused by mucosal HIV-infection a decrease of the CD4/CD8 ratio and a loss of IL-2 receptor bearing cells in the lamina propria occurs. In contrast to earlier studies [6],we have found no decrease in CD4+ LP-T-cells in HIV-infection. Recent studies have shown that intestinal LP-T cells do not proliferate after antigenic stimulation [7]. Thus, HIV-infection of these T cells may not cause cell death, because HIV-replication and its cytopathic effect depends on cellular proliferation. HIV-infection of LP-T cells rather may cause functional impairment with consequences for the local immune barrier leading to the high number of opportunistic infections and secondary malignancies of the GI tract in HIV-infection.

Moreover, there are data highly indicative of functional interrelations between mucosal structure and the mucosal immune system. [8] Our studies showed that a high percentage of HIV-infected patients had decreased or absent lactase/ß-glucosidase activity in the brush border membrane. In addition, HIV-infected patients without intestinal infections had a low number of mitotic figures in the crypts, especially in those patients with demonstrable HIV antigen p24 in the mucosa. These findings are indicative of a low grade small bowel atrophy with hyporegeneration and a maturational defect of enterocytes. These changes may be caused directly by HIV-infection of the mucosal T cells. Functional impairment of LP-T cells with loss of IL-2 receptors by HIV infection could be responsible for these hypoproliferative changes seen in the patients with HIV-infected cells in the mucosa.

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In vitro infection of human intestinal mucosal mononuclear and epithelial cells by the human immunodeficiency virus (HIV). A preliminary report

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ABSTRACT. A method for infecting intestinal lamina propria mononuclear cells with HIV in vitro is described. These cells appear to be more susceptible to HIV than peripheral blood mononuclear cells, and allow replication and release of infectious virus. Evidence that the HIV envelope protein gp120 binds to cultured epithelial cells is also presented, including preliminary data that this binding might be specific. These studies provide additional insight into the mechanisms of AIDS enteropathy.

The acquired immunodeficiency syndrome (AIDS) is caused by infection with the human immunodeficiency virus (HIV), a retrovirus of the lentivirus family. As a consequence of this infection, a series of complex pathogenic events takes place, ultimately leading to systemic spread of HIV and multiorgan failure (1). Gastrointestinal involvement is frequent in HIV-infected individuals. All segments of the digestive tract can be affected, the most common clinical manifestations being diarrhea, malabsorption, opportunistic infection and malignacy (2). The existence of and "AIDS enteropathy" has been postulated (3), but the mechanisms underlying the gastrointestinal involvement in AIDS are poorly understood, and there has been only limited investigation of its pathophysiology. In AIDS mucosal T cells are depleted of the CD4 subset (4). The virus has been found in the small and large bowel of HIV-infected individuals (5), and HIV RNA has been detected in the intestinal lamina propria (6). In addition, there is evidence that gut epithelial cells are susceptible to HIV infection in vitro (7). In spite of these findings, it is still uncertain whether HIV infects both mucosal epithelial and immune cells in vivo, and how its presence is linked to the gastrointestinal manifestations. The intestinal mucosa is likely to offer an excellent environment for HIV establishment and replication, considering the predominance of CD4+ lymphocytes and the enhanced state of activation of mucosal immune cells, two factors that are essential for a productive HIV infection (8). Unfortunately, access to these cells in AIDS patients is limited, restricting the opportunity to study their activities and related abnormalities. An alternate approach to the study of in vivo HIV-infected cells would be to infect relevant cells in vitro with HIV, and then study them under controlled experimental conditions. Therefore, the present study was designed to investigate the conditions and mechanisms for infecting gut epithelial and immune cells in vitro with HIV.

Materials and Methods

Isolation of intestinal mucosal mononuclear cells. Purified intestinal lamina propria mononuclear cells (LPMC) were obtained from surgically resected bowel specimens using an enzymatic technique. LPMC were isolated from histologically normal gut mucosa and from intestine affected by Crohn's disease and ulcerative colitis.

In vitro HIV infection. Freshly isolated LPMC were infected with HIV by incubating them with cell-free supernatants from the H9/HIV-1_B cell line (9). Bulk cultures of LPMC were exposed to supernatants containing a known amount of p24 core antigen at 37°C in the presence of polybrene (2 μg/ml), and with or without phytohemagglutinin (PHA, 2-3 μg/ml). After 2-3 hours, LPMC were washed 3-4 times to eliminate non adherent viral particles, counted, suspended in RPMI 1640 containing 20% fetal calf serum (FCS), 2% penicillin-streptomycin, 10% interleukin 2, sodium hepes, sodium bicarbonate and PHA, and distributed at $2X10^6$/ml/well of a 24 well cluster plate. At various time intervals (1 to 15 days), LPMC culture supernatants were collected for HIV antigen detection, while LPMC were harvested, washed, and fixed (with acetone at room temperature) on slides for demonstration of HIV-infection by immunofluorescence.

HIV antigen capture. Detection of HIV in the LPMC supernatants was accomplished by measuring the concentration of p24 core antigen using a commercial kit (E.I. Du Pont de Nemours). Supernatants from HIV-exposed and control LPMC cultures were treated with Triton X (to disrupt intact viral particles) and incubated overnight in microtiter plate wells pre-coated with rabbit polyclonal anti-HIV p24. After incubation, wells were washed several times and incubated with a biotin-labeled rabbit anti-HIV p24 to form a sandwich. After thorough washing of the wells, streptavidin-horseradish peroxidase conjugate was added to increase test sensitivity, and color was developed with O-phenylene diamine as substrate. The reaction was stopped by placing 50 μl of 4N H_2S0_4 in each well, and plates were read at wave length of 490-492 nm with a reference filter at 620 nm. In this assay, the intensity of the color developed is proportional to the quantity of HIV p24 antigen captured. For each assay, dilutions of a known amount of p24 were used to generate a standard curve to quantify the experimental samples.

Indirect immunofluorescence for HIV. Aliquots (15 μl of a $6\text{-}7X10^6$ cells/ml suspension) of LPMC acetone-fixed on a slide were covered by biotin-labeled anti-HIV human polyvalent antibody (Cellular Products) and control biotin-labeled immunoglobulin, and incubated for 45 minutes at 37°C in a humidified chamber. Slides were washed 3 times with phosphate-buffered saline (PBS), after which a FITC-streptavidin solution was added followed by incubation for 45 minutes at anti-HIV 37°C in a humidified chamber. Slides were washed again, air-dried, and examined under ultraviolet light.

Epithelial cell lines and microvillous membrane preparation. The human carcinoma cell lines HT29 and CaCO2 were maintained in RPMI 1640 with 10% FCS. Microvillous membranes (MVM) were isolated from cells grown at confluency by the calcium precipitation method of Kessler (10) and stored at -20°C until utilized.

Binding of HIV protein to intestinal epithelial cells. Recombinant (r) HIV envelope protein gp120 (MicroGeneSys) was dialysed with PBS-Tween 20 and reacted with sodium cyanobromohydride and 5 μCi[^{14}C] formaldehyde overnight at 4°C according to the method of Jentoft (11) and dialysed again with PBS-Tween 20 (0.005%). Labelling achieved 14,250-29700 cpm/μg of protein. Microtiter plate wells were incubated with PBS-1% bovine serum albumin (BSA) for 1 hour at 37°C to inhibit non specific binding sites and subsequently coated with MVM of HT29 and CaCO2 cells. After an overnight incubation, wells were washed and dried and 10 μl of 1 mg/ml PBS-BSA were added to each well. Various amounts of [^{14}C] rgp120 were then added to a total volume of 100 μl/well and the plate was incubated at room temperature for 1 hour. Aliquots (50 μl) of supernatants were removed for counting unbound ligand. To determine bound rgp 120, wells were washed with PBS, 200 μl of 0.75% sodium dodecly sulfate (SDS) were added to each well and the plate incubated overnight. Contents of the wells were collected, and subsequently wells were washed 3 times with 75 μl SDS for 5 minutes. These washes were also collected and added to the original contents for counting of total bound ligand.

Results

Initial experiments were performed using inocula of HIV with high p24 concentrations and freshly isolated LPMC from normal or inflamed bowel. All LPMC cultures became infected, as shown by a progressive increase of p24 levels in the supernatants with time. Both PHA-treated and - untreated LPMC were infected, although mitogen pretreatment resulted in higher levels of p24 antigen in the cultures (Table 1). When LPMC were cultured in parallel with normal peripheral blood mononuclear cells (PBMC), LPMC became HIV-infected earlier and to a higher degree than PBMC, as demonstrated by immunofluorescence at day 3 and considerably higher concentrations of p24 in the supernatants at day 8 (Table 2). The amount of p24 antigen present in the supernatants at any given time was directly proportional to its original concentration in the inoculum used to infect LPMC or PBMC (Table 2). Infected LPMC produced actively infectious

Table 1. Levels (pg/ml) of p24 core antigen in supernatants of PHA-stimulated and - unstimulated LPMC infected with HIV

Cells + supernatants	days in culture	
	1	6
LPMC + HIV(-)/H9	0	0
LPMC + HIV(-)/H9 + PHA	0	0
LPMC + HIV(+)/H9	1150	27100
LPMC + HIV(+)/H9 + PHA	1158	71944

Table 2. Levels of p24 (pg/ml) core antigen in supernatants of LPMC, PBMC and H9 cells infected with HIV and respective immunofluorescence

Cells	HIV dose(pg/ml)	days in culture					
		1		3		8	
		p24	IF	p24	IF	p24	IF
LPMC	10,000	154	±	257	+	20000	+++
PBMC		156	-	191	-	4050	++
H9		141	-	349	+	600	++
LPMC	5,000	106		193		12500	
PBMC		57		83		6500	
LPMC	2,500	57		87		1400	
PBMC		31		47		2000	
LPMC	1,250	15		38		180	
PBMC		11		16		1450	
LPMC	0	0		0		0	
PMBC		0		0		0	

Table 3. Levels (pg/ml) of p24 core antigen in supernatants of PHA-stimulated LPMC, H9 cells and PHA-induced PBMC blasts infected with LPMC-derived HIV(+) culture supernatants

Cells + supernatants	days in culture		
	1	9	15
LPMC + HIV(+) sup	0	0	0
LPMC + HIV(-)/H9	0	0	0
H9 cells + HIV(+) sup	7.3	10.2	11.1
H9 cells + HIV(-)/H9	0	0	0
PBMC blasts + HIV(+) sup	9.9	11.3	-
PBMC blasts + HIV(-)/H9	0	0	-

HIV, as demonstrated by the ability of p24-positive LPMC supernatants to further infect H9 cells and PHA-induced PBMC blasts (Table 3). The isolation method used to obtain LPMC did not contribute to these cells' susceptibility to HIV, since PBMC exposed to the same enzymatic treatment failed to show any increment in p24 antigen production.

Additional experiments were carried out to investigate binding of HIV to intestinal epithelial cells. Using [^{14}C] rgp120, binding was observed with MVM preparations derived from HT29 cells. The amount of protein bound increased with the quantity added, attaining an apparent plateau at 4-6 μg. In initial studies to evaluate binding specificity, a 20 fold excess of cold (unlabeled) rgp120 was used to compete with the radiolabeled ligand, resulting in approximately 90% inhibition of HIV envelope protein binding to HT29 MVM. Similar studies examined binding of rgp120 to CaCO2 MVM. A low level of binding was observed and its specificity is being evaluated in further experiments. Preliminary investigation also showed evidence for binding of radiolabelled rgp120 to freshly isolated LPMC.

Summary and Conclusions

This study demonstrates that human intestinal mucosal mononuclear cells can be infected in vitro by HIV. This process is enhanced by activation with PHA, and the degree of infection appears to be proportional to the dose of HIV used to infect the target cells. Without pre-activation by PHA, HIV infection of LPMC also occurs, but large doses of virus appear to be required. LPMC may be more susceptible than PBMC to HIV infection. HIV-infected LPMC are able to produce viable virus which can infect other cells. In addition, our study offers initial evidence that the gp120 HIV envelope protein binds to colon carcinoma epithelial cell lines, and that this binding can be inhibited by cold gp20. Binding of gp120 to LPMC needs further investigation.

These results provide additional evidence that the gastrointestinal tract is an organ susceptible to HIV infection. Our data indicate that LPMC not only become infected, but produce and release viable HIV in culture, suggesting that a similar phenomenon is likely to occur in vivo. Whether gastrointestinal infection by HIV is transmitted by hematogenous spread or directly through the mucosa, the virus is likely to find an environment favorable for its growth and replication. In the mucosa CD4 lymphocytes are abundant and they are in an activated state (12), which probably facilitates HIV entry (13) and may explain why LPMC may be more susceptible to HIV infection than PBMC. Furthermore, our results on HIV binding to transformed colonocytes provide a

possible mechanism as to how gut epithelial cells become infected. The consequences of this mucosal infection are still not clear. However, a recent report suggests that HIV may be directly responsible for low-grade bowel atrophy and a maturational defect of enterocytes, leading to clinical symptoms even in the absence of superimposed opportunistic microorganisms (14).

The development of in vitro systems to study HIV infection of intestinal cells should circumvent the problem of obtaining cells directly from AIDS patients. More importantly, such systems should offer an opportunity to further investigate the pathogenic events following HIV colonization in the gut. With the availability of large numbers of HIV-infected intestinal mucosal mononuclear cells, a variety of mucosal immune phenomena can be investigated, including cytopathic effect, consequences of deficiency of CD4 lymphocytes, cytotoxic phenomena and B cell abnormalities. Consequently, the effect of HIV on mucosal mononuclear and epithelial cells will be better understood as will be the overall impact of AIDS on the gastrointestinal tract.

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Secretory and serum IgA are inversely altered in AIDS patients

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ABSTRACT. It is well-documented that levels of total serum IgA as well as IgA-containing immune complexes are often markedly and selectively increased in patients with AIDS. IgA antibodies specific for HIV have been demonstrated in mucosal secretions. All of these findings indicate a profound involvement of both the systemic and mucosal IgA systems in this disease. The present experiments were initiated to determine if alterations of the secretory component of the IgA system similar to those of its systemic counterpart could be identified. In order to determine if the secretory IgA system was quantitatively affected in a manner similar to that of its serum analogue, whole saliva samples obtained from normal volunteers and from patients with AIDS were assayed by quantitative ELISA for levels of total IgA and IgA subclasses. Total salivary IgA was decreased in the AIDS patients. Levels of salivary IgA1 did not differ significantly in the 2 populations. On the other hand, IgA2 levels were markedly diminished in patients with AIDS. Since a major mucosal (but not serum) IgA isotype is selectively diminished in AIDS (as opposed to often extreme elevations in total serum IgA), we conclude that the systemic and mucosal IgA systems appear to be inversely affected in this disease.

1. Introduction

The acquired immune deficiency syndrome (AIDS) is well-characterized with respect to multiple immune abnormalities, including a variety of aberrancies of the B lymphocyte compartment of the immune system. In this regard, it is well-documented that levels of total serum IgA in AIDS patients are often markedly and selectively increased (1). We have further shown that elevated levels of immune complexes in this disease can primarily be attributed to IgA that is restricted to the IgA1 subclass (2). Others have demonstrated the presence of IgA antibodies to the human immunodeficiency virus (HIV) in saliva, cervical secretions and milk (3-5); however, the levels of total IgA in external secretions have apparently not been reported. In this paper, we report data from preliminary studies aimed at characterization of total secretory IgA in AIDS in order to determine if the mucosal IgA system is affected in this disease in the same manner as its systemic counterpart.

2. Materials and Methods

2.1 SAMPLE POPULATIONS

Thirty-three patients with AIDS (diagnosed according to accepted criteria of the Centers for Disease Control) and 20 normal laboratory volunteers donated samples for use in this study. Stimulated whole saliva was collected from these individuals by allowing them to drool into a large conical centrifuge tube after placing a drop of lemon juice on the tongue. The saliva was immediately alliquotted and frozen.

2.2 ASSAY SYSTEM

Levels of total IgA in saliva were measured by quantitative ELISA on plates coated with a rabbit $F(ab')_2$ affinity-purified reagent specific for human IgA (Pel-Freeze, Rogers, AR). After incubation of the samples, the plates were subjected to consecutive incubations with biotin-labeled $F(ab')_2$ affinity-purified goat anti-IgA (Tago, Burlingame, CA), avidin-conjugated peroxidase, and enzyme substrate. Levels of IgA subclasses were determined similarly, except that after incubation of the samples, the plates were subjected to incubations with monoclonal reagents specific for IgA1 or IgA2 (6), followed by biotinylated goat anti-mouse immunoglobulins (Southern Biotechnology, Birmingham, AL). Standard curves were set up using purified colostral IgA, assuming an IgA1:IgA2 ratio of 60:40 in the subclass-specific determinations. All samples from patients and controls were run simultaneously using identical reagents and incubation times. The plates were read on an automated reader and the data then calculated on an Apple Macintosh computer using a Logit-log program.

3. Results

3.1 TOTAL SALIVARY IgA LEVELS

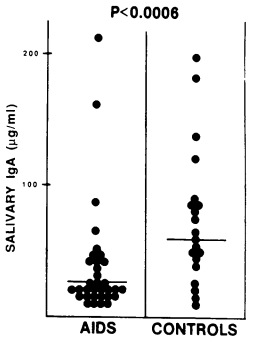

Figure 1. Total salivary IgA levels were significantly depressed in the AIDS patients (range=7-210 µg/ml, geometric mean=28 when compared to normal controls (range=7-201 µg/ml, g.m. = 58). These findings prompted us to examine the subclass composition of salivary IgA in the same patient population.

3.2 SALIVARY IgA1 AND IgA2 LEVELS

Saliva samples of 28 AIDS patients and 15 controls were used for these studies. As seen in Table 1, there were no statistical differences in IgA1 levels when the two populations were compared. On the other hand, salivary IgA2 levels were significantly lower in the AIDS patients.

TABLE 1. Salivary IgA1 and IgA2 levels in AIDS patients and laboratory controls

	IgA1	IgA2
AIDS subjects (N = 20)	6-96 (21)	1-72 (6)[a]
Normal controls (N = 15)	9-82 (23)	8-72 (28)

Range of values in μg/ml (geometric mean). Determined by ELISA; all samples run simultaneously in each assay.

[a] $p < 0.0001$.

4. Discussion

Most antigen encounters, including microbial infections (and sexual contacts with HIV-infected individuals) occur at mucosal surfaces, where secretory IgA antibodies can effectively exclude microbes or neutralize viral particles. HIV has been found in cells and fluids from a variety of mucosal sites including the oral cavity, the intestinal epithelium, semen, colostrum and milk, as well as the uterine cervix and cervical secretions (reviewed in 7). Secretory IgA antibodies normally mediate protection of the mucosal surface in all of these areas.

Since transmission of the AIDS virus so often occurs via mucous membranes, it is timely to study the immune response to HIV infection in external secretions. In this regard, it has been documented that saliva, cervical secretions and milk of seropositive individuals contain anti-HIV antibodies, as discussed above. With respect to serum or systemic IgA, it is clear that serum IgA levels are often significantly elevated in AIDS. The data regarding serum IgA anti-HIV antibodies have been somewhat contradictory, and will not be discussed here due to space limitations. However, it is pertinent to suggest that since IgA antibodies do not fix complement via classical mechanisms, they might actually protect HIV from opsonic anti-HIV isotypes (8).

IgA is composed of 2 subclasses (IgA1 and IgA2), which are present in serum and external secretions in different proportions. IgA2 constitutes at least 40% of total secretory IgA; in contrast, it is a relatively minor component of serum IgA. Using quantitative ELISA to measure secretory IgA in saliva, we have shown that salivary IgA levels are decreased in AIDS, and that this decrease appears to result from selective depletion of one IgA subclass. These findings are in oppositon to those previously reported by many investigators with respect to markedly increased levels of serum IgA. It is not known at present if IgA subclass ratios are altered in the systemic component of the IgA system in AIDS.

Our data imply that the systemic and mucosal IgA systems are affected inversely in HIV infection, although additional studies on external secretions other than saliva are required

to completely define this phenomenon. It is important to remember that individuals with AIDS do not succumb to HIV infection but to a wide variety of opportunistic infections and malignancies. Most of these opportunistic infections are initiated at mucosal surfaces such as the lung or gastrointestinal tract. This is perhaps a more important reason to study the secretory immune system in AIDS than to assess mucosal protection against HIV itself. IgA2 is quantitatively a more important component of the secretory IgA system than of its serum counterpart, and our experiments indicate that AIDS patients may have deficient mechanisms of protection at mucosal surfaces. This deficiency in turn may contribute to many of the commonly observed secondary infections to which these individuals are susceptible.

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A quantitative histological study of enteropathy associated with HIV infection

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1. Introduction

Diarrhoea is a common problem in Acquired Immunodeficiency Syndrome (AIDS) or AIDS-Related Complex (ARC) from Human Immunodeficiency Viral (HIV) infection. Previous studies have described common or exotic enteric infections [1], or have described a primary enteropathy of the small intestine [2]. As enteropathy with villous atrophy and crypt hyperplasia is often, if not exclusively, associated with immunological reactions [3], it is important to define quantitatively the enteropathy associated with HIV infection.

2. Subjects and Methods

Eight ambulatory HIV subjects (CD4 cells in blood: 61±43/ μL, mean±SD): normal range: 405-2205) had AIDS (n=5) or ARC (n=3). Two had enteric infections (Isospora belli, Cryptosporidium) and two had Kaposi's sarcoma of the oesophagus and stomach or of the skin. Seven subjects had oral and/or oesophageal candidiasis and were receiving zidovudine (AZT) at the time of study. CD4 lymphocytes and CD4/CD8 ratio values were determined in peripheral blood by flow cytometry within 6 weeks of intestinal biopsy. Sixteen 'normal', three coeliac, and these HIV subjects had endoscopy and duodenal biopsy. Informed consent was obtained for endoscopy from each patient and the protocol for this study was approved by the Human Ethics' Committee of the Royal Adelaide Hospital. Duodenal biopsies were microdissected after Feulgen staining [4]. Villus area was calculated by a trapezoid geometric approximation from measurements of apical and basal widths and villus length, and crypt length and mitotic count were measured. Another biopsy was fixed in Carnoy's fixative, and histological sections stained with H & E and Alcian blue (pH 0.3)/safranin for intraepithelial lymphocyte (IEL), mucosal mast cell and goblet cell counts [4]. All results are expressed as mean±SD.

3. Results

TABLE. Intestinal morphology and duodenal cell counts.

	Control (n=16)	HIV (n=8)	Coeliac (n=3)	
Villus area	0.500 ± 0.064^{ab}	0.363 ± 0.081^{a}	0.182 ± 0.161^{b}	mm^2
Crypt length	225 ± 28^{c}	239 ± 36	503 ± 102^{c}	μm
Mitotic count	2.4 ± 0.82^{de}	3.7 ± 1.2^{d}	6.9 ± 0.1^{e}	/crypt
IEL	47 ± 21	37 ± 18	55*	/mm
MMC	36 ± 15	41 ± 18	44 ± 12	/mm
Goblet cells	70 ± 17	71 ± 15	51*	/mm

*One biopsy from coeliac subjects was unsuitable for these counts. $^{a-e}p<0.0001$.

4. Discussion

Our study has demonstrated an unusual pattern of enteropathy in AIDS/ARC with villous atrophy and impaired crypt hyperplasia but with normal duodenal counts of MMC, IEL and goblet cells. The discrepancy between a slightly (but impaired) increase in mitotic count but normal crypt length suggested that firstly immunodeficiency reduced the "drive" for crypt proliferation and secondly that there was possible direct viral destruction of crypt cells.

5. Acknowledgements

We thank Dr D. Gillis for allowing us to study two of his patients and to Mr A. Bishop who performed the flow cytometry (Division of Human Immunology, Institute of Medical and Veterinary Science).

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Mucosal and systemic immune parameters in SIV-infected and normal Rhesus monkeys

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Simian immunodeficiency virus (SIV) induces an AIDS-like disease in asian primate species such as *Macaca mulatta* [1]. The significant similarities in the *in vitro* and *in vivo* biological properties of SIV virus and HIV-1 induced human AIDS, provides an excellent animal model to study the mucosal immune system in AIDS. Our preliminary studies have involved the characterization of peripheral blood mononuclear cells (PBMC) and mucosal tissues from the Rhesus macaque.

- *Animals*: Heparinized blood and tissues from normal and SIV infected *Macaca mulatta* monkeys were provided from the California Primate Research Center, Davis, CA.
- *Isolation of cells*: PBMC were isolated by Ficoll-Hypaque gradient centrifugation and the mononuclear cells were cultured for 6 days with pokeweed mitogen (PWM).
- *Tissues*: Specimens of small and large intestine, parotid salivary glands and spleen tissues obtained from SIV-infected and non-infected monkeys were fixed in acid (5%)-ethanol (95%), paraffin-embedded [2] and cut in ~4 μ sections.
- *Enzyme-linked immunospot assay* (ELISPOT) [3] was used to enumerate IgA, IgG, IgM, and IgG and IgA subclass secreting cells.
- *Levels of immunoglobulin* isotypes in monkey serum were determined by ELISA.
- *Immunofluorescence*: a) Surface: isolated PBMC were incubated with fluorochrome labeled anti-human T or B cell reagents (Becton-Dickinson). Optimal results were obtained with phycoerythrin (PE) labeled monoclonal anti-T cytotoxic/suppressor cells (anti-Leu 2a) and anti-T helper/inducer (anti-Leu 3a). The percentage of positive cells was determined by Flow cytometry (FACSstar®; b) Cytoplasmic: acid-alcohol fixed cytocentrifuge preparations of PWM-stimulated PBMC or paraffin embedded tissue sections were stained with fluorochrome labeled anti-human immunoglobulin (Ig) antibodies (H chain specific) (Southern Biotechnology Assoc, Tago and Jackson) or with anti-monkey IgA (Nordic).

Due to the lack of commercial anti-primate monkey reagents, we determined the cross-reactivity of anti-human reagents with monkey immunoglobulins and ascertained their specificity. We found, by ELISA using human standards, ~57% recognition for IgM, 45% for IgG, and only ~20% for IgA. The mouse monoclonal anti-human IgA and IgG subclass antibodies were found to have a very poor cross-reactivity. Therefore, in our studies we used anti-human IgG and IgM reagents, and biotinylated anti-monkey IgA.

STUDIES WITH NORMAL RHESUS PBMC

a) *T cells*. Following surface staining and flow cytometry analysis, we found that Leu-1 and Leu-4 T cell epitopes were not detectable, and that the ratio of $CD4^+/CD8^+$ T cells present in

PBMC varied from 0.55 to 0.78. In contrast to the distribution of human T cell subsets, we observed the predominance of T cytotoxic/suppressor cells, which agrees with another study on primate lymphocytes [4].

b) *Immunoglobulin synthesis*. The results of cytoplasmic immunofluorescence analysis are presented in the following table.

	Isotype/Subclass								
	IgA[a]	IgM[a]	IgG[a]	IgA1[a]	IgA2[a]	IgG1[b]	IgG2[b]	IgG3[b]	IgG4[b]
% IF^+ cells	1.5-7%	8-16%	60-79%	<1%	<1%	72-95%	4-24%	3-24%	0-10%

[a]from total Ig^+ cells; [b]from IgG^+ cells

c) *Immunoglobulin secretions*. The number of cells secreting IgA, IgG, IgM, and IgA and IgG subclasses from PWM stimulated monkey PBMC are presented below:

	Isotype/Subclass								
	IgA	IgM	IgG	IgA1	IgA2	IgG1	IgG2	IgG3	IgG4
SFC/10^6cells	20-200	320-4,500	500-8,100	0-70	0-54	0-1,100	0-280	0-60	0-50

The range is given to indicate that PWM does not always induce B cell differentiation and also to indicate individual variation that occurred.

After systemic immunization with influenza virus vaccine, cells secreting antigen-specific antibodies were detected in isolated PBMC.

	Influenza-specific antibodies		
	IgA	IgG	IgM
SFC/10^6 cells	45	50	540

MUCOSAL TISSUES IN SIV INFECTED AND NONINFECTED MONKEYS

Despite low numbers of plasma cells present in spleens (and lymph nodes) of SIV-infected monkeys, the homologous intestine and salivary glands sections showed similar or increased numbers of Ig positive cells when compared with non SIV-infected macaques. As with normal animals, the predominant isotype varied between individual donors.

Interestingly, the small intestine and salivary glands of SIV-infected macaques showed increased IgM and decreased IgA plasma cell numbers. These results show that polyclonal and antigen-specific B cell responses can be assessed in macaques and the aberrancy in Ig-producing cells in mucosal sites would suggest that the IgA system may be centrally important in this disease.

Using this primate model, some questions regarding the relationship between the mucosal immune system and HIV-1 pathogenesis may be addressed, and the information obtained can lead to the possible development of a mucosal vaccine.

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SECTION U: INFLAMMATORY BOWEL DISEASES

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Activation of human lamina propria mononuclear cells in inflammatory bowel disease

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ABSTRACT The state of activation of human lamina propria mononuclear cells (LPMnc) obtained from either organ transplant donors or from operation specimens from inflammatory bowel disease (IBD) patients were studied by three-color fluorescence activated cell sorting (FACS). Freshly isolated LPMnc from normal donors as well as from IBD patients were autofluoresent. The autofluorescence could be subtracted from the FACS data on a cell-by-cell basis by a mathematical post processing step. Normal LPMnc are *in vivo* activated and express increased levels of 4F2-antigen, transferrin and interleukin-2 receptor. The number of lymphocytes, which express these early activation antigens was markedly increased in both Ulcerative Colitis (U.C.) and Crohn's disease (C.D.). This underlines the hypothesis of a major upregulation of the mucosal immune system due to the disease process. B cells in particular were activated, suggesting they may be of particular importance in the disease process of IBD.

INTRODUCTION

There is increasing evidence that an altered intestinal immunoregulation may play an important role in inflammatory bowel disease (1). These diseases seem to lead to an increased *in vivo* activation of intestinal (2,12) as well as peripheral lymphocytes (2-4) as measured by expression of early activation associated antigens. These antigens are 4F2 (5), the transferrin receptor (5E9, (6)) and the interleukin-2 receptors (7). However, a systematic study of the activation of lymphocyte subpopulations in IBD has not yet been performed on isolated LPMnc. A reason for this might have been the high levels of autofluorescence these cells display, requiring a complex methodological approach.

METHODS

Cells were isolated from the lamina propria by collagenase digestion and density centrifugation as previously described (8). Specimens were obtained from organ transplant donors (n=4) or patients undergoing bowel resections for bleeding cecal haemangioma (n=1), atonic colon (n=1), ulcerative colitis (U.C., n=8) or Crohn's disease (C.D., n=5). Intestinal tissue from IBD patients was from involved sites.

Three Color Immunofluorescence

Lamina propria mononuclear cells (LPMnc) were stained with phycoerythrin coupled monoclonal antibodies: Leu4 (T cells), Leu12 (B cells), Leu3a ($CD4^+$ T cells), Leu2a ($CD8^+$ T cells), Leu 11/Leu 19 (NK cells), and LeuM3

(macrophages/monocytes). Antibodies against 4F2, the IL2- and the Transferrin-Receptor (5E9) were used in a two-step labeling procedure employing Texas Red coupled sheep-anti-Mouse-IgG antibodies. Detailed procedures were described previously (9). Data were collected as listmode files on an EPICS-753 flow cytometer. The green channel was used to monitor and to mathematically eliminate autofluorescence on a cell by cell basis in a post-processing step, which was performed on an IBM PS/2 (Figures 1,2). Either 25000 or 50000 cells were processed for each sample. Data are presented as mean of percent activation marker positive cells of each lymphocyte subpopulation ±STD.

Immunofluorescence in Frozen Tissue Sections

Staining was performed using a modified technique as published previously (10,11). The same primary and secondary labeled antibodies as in the studies using isolated mononuclear cells were employed. In addition a monoclonal antibody recognizing leucocyte common antigen was used (Dako). Intestines used for tissue sections were different from those used to isolate mononuclear cells. Data are presented as mean of percent activation marker positive cells of total leukocytes ±SEM.

RESULTS

Calculation and Subtraction of Autofluorescence:

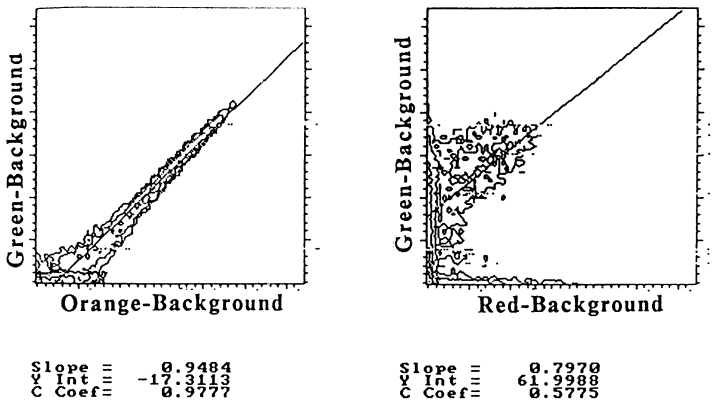

Figure 1
Dual parameter histograms in an unstained sample display a fixed correlation between the amounts of green, orange and red autofluorescence respectively. Linear regressions are used to calculate the amounts of orange and red autofluoresence related to the green signal. By postprocessing the data it is possible to subtract the amount of autofluorescence from the orange and red signal on a cell by cell basis.

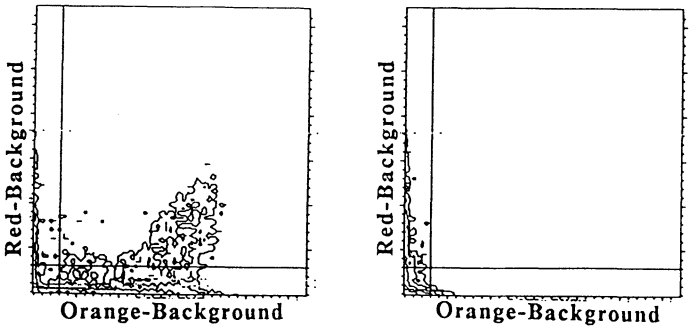

Figure 2
This sample was not stained. Histograms show red and orange autofluorescence before *(left)* and after subtraction of autofluoresence *(right).*

Table 1
Lymphocyte subpopulations (mean percent of positive cells ± STD)

	Leu4 T cells	*Leu12 B cells*	*LeuM3 MØ*	*Leu11/19 NK cells*	*Leu2a $CD8^+T$*	*Leu 3a $CD4^+T$*
normal	*45±15*	*29±14*	*12±7*	*8.7±5*	*17±7*	*33±7*
U.C.	*44±11*	*23±9*	*15±7*	*7.4±4*	*16±5*	*23±4*
C.D.	*36±14*	*19±7*	*19±8*	*6.4±2*	*10±5*	*25±10*

As shown in *Table 1* there are no differences in the representation of lymphocyte subpopulations between the disease groups and normal controls. The disease process therefore does not affect the composition of LPMnc in regard to lymphocyte subsets.

Table 2
Transferrin receptor expression (mean percent of positive cells ± STD)

	Leu12 Bcells	*Leu4 Tcells*	*Leu2a $CD8^+T$*	*Leu3a $CD4^+$Tcells*
normal	*13±6*	*3.8±2*	*11±4*	*11±6*
U.C.	*43±19*	*34±15*	*60±15*	*40±18*
C.D.	*43±23*	*26±9*	*24±3*	*29±13*

The number of transferrin-receptor (5E9) coexpressing cells in U.C. and C.D. are increased in all lymphocyte subpopulations *(Table 2, $p<0.05$ for all values)*. In the T cell population this increase appears to be greater in U.C. than in C.D..

Table 3
Interleukin 2-receptors expression
(mean percent of positive lymphocytes ± STD)

	Leu12 Bcells	*Leu4 Tcells*	*Leu2a $CD8^+T$*	*Leu3a $CD4^+T$*
normal	*9.4±4*	*8.2±5*	*9.5±5*	*12±7*
U.C.	*49±8**	*31±12**	*37±9**	*29±8**
C.D.	*26±12**	*19±10*	*19±12*	*19±8*

Significantly more intestinal lymphocytes in Ulcerative Colitis (U.C.) and Crohn's Disease (C.D.) express Interleukin-2 (IL2) receptors than in normal intestine *(Table 3)*. A higher percentage of B cells expressed the IL2-Receptor than T-lymphocytes *(*=$p<0.05$ as compared to normal controls)*.

Table 4
4F2 expression (mean percent of positive lymphocytes ± STD)

	Leu12 Bcells	*Leu4 Tcells*	*Leu2a $CD8^+T$*	*Leu3a $CD4^+T$*
normal	*42±5*	*51±13*	*64±12*	*54±11*
U.C.	*80±5**	*63±17*	*82±14**	*75±21*
C.D.	*87±16**	*63±17*	*60±16*	*61±15*

Baseline expression of 4F2-antigen in isolated, normal intestinal mononuclear cells is high. A significant increase of 4F2 expression in the disease group can be seen only on B lymphocytes and $CD8^+T$ cells in U.C. *(Table 4, *=$p<0.05$ as compared to normal controls)*.

Table 5
Expression of activation associated antigens in frozen tissue sections (percent of leukocytes ± SEM)

	Transferrin Receptor	*Interleukin 2 Receptor*	*4F2 Antigen*
normal n=7	*9.7±3.1*	*10.4±3.7*	*4.9±2.5*
U.C. n=9	*16.5±1.6 **	*13.3±2.2*	*16.5±7.2*
C.D. n=7	*27.1±2.9 **	*25.2±4.2 **	*32.6±7.2 **

Immunohistologic data confirm the increase in lymphocytes exressing activation associated antigens in IBD as compared to normal intestine *(Table 5).* Data are given in percent of leukocyte common antigen expressing cells coexpressing activation antigens *(*=p<0.05 as compared to normal controls).*

DISCUSSION

Autofluorescence is a major phenomenon of intestinal mononuclear cells. For meaningful two color immunofluorescence it has to be eliminated. We show a way of subtracting it on a cell-by-cell basis.

Normal intestinal lymphocytes are an *in vivo* activated population as shown by 4F2, Transferrin- and Interleukin 2 receptor expression as well as previously shown by functional parameters (8,9). These data are in agreement with studies on non-human primates, showing increased expression of genes associated with T cell activation (13).

The number of activated cells in all subpopulations in U.C. and C.D. with respect to expression of Interleukin 2 and Transferrin-receptors is significantly higher than in lymphocytes isolated from normal intestine. This underlines the hypothesis of a major upregulation of the gut associated lymphoid tissue related to the disease process. These findings are confirmed in tissue sections by immunofluorescence analysis.

In accordance with the findings of Konttinen et al (12) and Fais et al (10) Crohn's Disease causes higher numbers of lymphocytes expressing 4F2 or the transferrin receptor than the Interleukin 2 receptor.

Interestingly, a high number of isolated mononuclear cells from normal controls expresses the 4F2 antigen. This differs from our immunohistologic study, which represents results obtained with a technique, which is very different in sensitivity

compared to FACS. However, these results confirm our previous studies in normal human intestine (9). High levels of 4F2 expressing cells may be characteristic for the state of *in vivo* activation in the normal intestinal immune system. A further increase caused by the disease process could be seen only in the B cell population.

B lymphocytes also show a particular increase in Interleukin 2-Receptor expression in IBD. This suggests that the inflammatory process is associated with a heightened state of activation in which B cells participate differently from T cells. A reason for this might be a different responsiveness to cytokines present in the inflamed lamina propria.

Activated B cells may be of particular importance in respect to the high levels of spontaneous antibody secretion of normal LPMnc and changes in the isotypes and subclasses of secreted antibodies have been shown to be specific events in both inflammatory bowel diseases (8,14). Therefore, the activation of B cells may play a pivotal role in the pathogenesis of IBD.

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Distribution of mononuclear cells in the mucosa of patients with Crohn's disease

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ABSTRACT. We present a method that is suitable to follow shifts in antigen distribution on gut associated lymphocytes in the time course of chronic inflammatory bowel disease. It may possibly lead to functional analysis of the gut associated lymphoid tissue in different stages of Crohn's disease.

Introduction

Morphological and functional characterization of gut associated lymphoid cells was predomiantly done on surgical specimens. We developed a method to isolate sufficient numbers of viable cells from biopsies taken under routine endoscopy. This allows us to characterize cells from the same patients at different points in time, and take samples from various locations from the colon.

Methods

Colonic biopsies and were taken from healthy individuals and patients with acute gastroenteritis, Crohn's disease and ulcerative colitis. The specimen was washed, mucus was removed with Dithiothreitol (1mM, 3', at room temperature) and epithelial cells and intraepithelial cells were separated with EDTA (0.75mM, 90', 4°C). The remaining biopsy was then digested in enzyme solution (48U/ml collagenase, 300U/ml DNAse, 37°C overnight) and the cells were washed repeatedly. 6.4 x 10^6 LPL could be harvested from 100mg biopsy tissue. Mononuclear cells were isolated by density gradient centrifugation and stained with a panel of monoclonal antibodies using standard immunofluorescence techniques.

Results

Compared to normal controls (9.7 ± 2.3%) and patients with acute enteritis (7.3 ± 2.5%) patients with Crohn's disease have increased numbers of monocytes (Leu M3) in noninflamed areas of the mucosa (14.0 ± 1.8%), and normal numbers of monocytes in inflamed areas (10.8 ± 1.6). The distribution of the B-cell marker CD20 on lamina propria lymphocytes isolated from normal individuals (6 ± 0.7) and patients with acute enteritis (9.4 ± 1.3) was similar as in biopsies taken from patients with Crohn's disease at noninflamed areas (6 ± 1.3). At the site of inflammation in patients with Crohn's disease (CD) and in ulcerative colitis (UC) CD20 was markedly increased (15.5 ± 1.4% in CD, 18.2 ± 2.3% in UC). T-cell marker CD3 was decreased in inflamed area of Crohn's disease as compared to noninflamed area, normal controls, ulcerative colitis, and enteritis (7 ± 0.8% as opposed to 12-18%). The CD4/CD8 ratio was similar in all biopsy specimens we looked at. Activation antigens T9 and CD25 were elevated in Crohn's disease (inflamed: 15.4 ± 2.2%, uninflamed: 13 ± 4.9%), ulcerative colitis (18.6 ± 3.7%), and, to some extent in acute enteritis (10.2 ±3%). Normal control: 7.0 ± 2.9%. Fc_{α}-receptor-positive cells were increased in inflamed areas of Crohn's disease (20 ± 4.4%) and in ulcerative colitis (18 ± 4%), control: 9.6 ± 2.5%. Vicia Villosa Agglutinin-receptor bearing cells were increased in chronic and acute inflammation of the colon from 28% in normal controls to approximately 38% in all other groups surveyed.

Discussion

Our method allows us to monitor patients with Crohn's disease at different stages in the disease process and include patients that do not undergo surgery as controls. Thereby the patient collective is greater and can be much better selected than in studies that rely on surgical specimens as a source of lamina propria lymphocytes. Our results show specific changes in the distribution of cell surface antigens on LPL in patients with Crohn's disease, that are not found in acute enteritis or ulcerative colitis. There is a sharp increase of B-cells and activated T-cells in inflamed areas. We will be able to characterize viable cells from biopsy specimens functionally which will lead to further understanding of the pathophysiological process involved in Crohn's disease.

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Activated T cells and macrophages in the intestinal mucosa of children with inflammatory bowel disease

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ABSTRACT. IL-2 receptor (CD25) bearing cells can be visualised by immunohistochemistry in the intestinal lamina propria of children with Crohn's disease or ulcerative colitis, but not in control bowel. The CD25+ cells occur as aggregates in the lamina propria below the epithelium, and scattered throughout the lamina propria. Double staining revealed that the CD25+ cells in Crohn's disease are 60-88% CD3+,CD4+,CD8-, indicating that they are T cells whereas in ulcerative colitis the CD25+ cells are mostly CD3-,CD4+,HLA-DR+, indicating that they are macrophages. Differential expression of CD25 on T cells and macrophages serves to distinguish the immunologic lesions in ulcerative colitis and Crohn's disease.

1. INTRODUCTION.

Immunohistochemical and immunofluorescence techniques have been used extensively in recent years to characterise the inflammatory cells in the diseased mucosa in inflammatory bowel disease. The most significant findings, which apply to both ulcerative colitis (UC) and Crohn's disease are a pronounced infiltrate of lymphocytes and macrophages into diseased mucosa (1,2), an increase in plasma cells of all isotypes, but especially those secreting IgG (3), and increased expression of Class 11 major histocompatability molecules on epithelial cells (4). Classical features which serve to distinguish Crohn's disease from ulcerative colitis are well known; including granulomata and transmural inflammation in Crohn's disease and epithelial cell damage and goblet cell depletion in ulcerative colitis (5). Immunohistologic features which differentiate Crohn's disease from ulcerative colitis are less well established. Increased IgA1 plasma cells are a feature of both conditions but their proportion is higher in ulcerative colitis than in Crohn's disease (6). No differences in the T cell infiltrate in UC

and Crohn's have been described (7). Recently it has been reported that there is a population of RFD1-,RFD7+ macrophages in the bowel in Crohn's colitis which is not present in normal bowel or UC (8).
Crohn's disease is generally considered to be due to a T cell mediated hypersensitivity reaction to some as yet undefined antigen. If this indeed is the case then it would be expected that phenotypic markers of T cell activation would be seen in disease bowel. In fact this has not been the case (7,9,10). Here we show using appropriate methodology that activated T cells are a prominent feature of Crohn's disease, but not ulcerative colitis.

2. METHODS

Patients. Normal small intestine was obtained from 4 patients aged 6 months to 65 yrs. Three pieces of histologically normal small bowel from patients with Crohn's disease was also studied. Colonic biopsies were obtained from 5 normal children. Diseased small bowel was obtained from 13 children aged between 10 and 17 yrs with Crohn's disease. Samples of diseased colon, if available were also taken. Colon from 5 patients with ulcerative colitis was also studied. All diseased tissue was from resected bowel.

Monoclonal Antibodies and Immunohistochemical staining
Frozen sections (8um) of intestine were cut and stained immunohistochemically using the indirect alkaline phosphatase anti-alkaline phosphatase (APAAP) technique (11). Fast red was used to visualise staining and the slides were counterstained with Mayer's haematoxylin. Endogenous brush border alkaline phosphatase served as a positive internal control of substrate activity for each staining run. Monoclonal antibodies used were anti-CD3, anti-CD4 and anti-CD8 (Becton-Dickinson, Mountain View, California); anti-CD25 (a gift from Dr T Waldmann, NIH, Bethesda, Maryland); and anti-HLA-DR (Dako Ltd, High Wycombe, Bucks).
In order to determine whether CD25 cells expressed CD3, CD4,CD8 or HLA-DR, a sequential staining technique was used as described elsewhere (12,13). Briefly, tissues were first stained with anti-CD3,or CD4, or CD8,or HLA-DR, or without primary antibody, by the indirect immunoperoxidase technique to give a brown reaction product. The sections were then washed and stained with anti-CD25 using fast red and the APAAP technique. Any cell expressing for example, CD3, would stain up brown from the immunoperoxidase. If that cell also expressed CD25 it would stain brownish/red (purple). Importantly, any cell expressing CD25 but not CD3 would stain red.

Quantitation. The number of lamina propria cells expressing CD25 in single stained slides was determined by making differential counts of cells staining red (CD25+) to unstained cells (identified by their blue nuclei). At least 500 cells were counted in different parts of the lamina propria. The CD25 cells are expressed as a percentage of the total number of cells counted. As will be shown below, CD25+ cells were frequently present as subepithelial aggregates which were noted but were impossible to quantitate. All differential counts were therefore made around the crypt region (small bowel) or at the base of the glands (large bowel) where CD25+ cells were not aggregated.
The extent to which immunoperoxidase staining with anti-CD3,CD4,CD8 or anti-HLA-DR blocked anti-CD25 staining with fast red was determined by first determining the percentage of red CD25+ cells in an area of lamina propria, followed by determining the percentage of cells remaining red in a serial section after blocking with a given monoclonal and the peroxidase technique. The percent reduction in CD25+ cells staining red prior staining with a given monoclonal antibody and the peroxidase technique was taken as the percentage of CD25+ cells also expressing the antigen recognised by the first monoclonal.

3.RESULTS

Expression of CD25 in normal intestine, Crohn's disease and ulcerative colitis

CD25+ cells were rare in normal small and large bowel lamina propria, but were seen in Peyer's patches and isolated lymphoid follicles. In small and large bowel affected with Crohn's disease and ulcerative colitis there were numerous CD25+ cells in the lamina propria. These occurred as subepithelial aggregates of large foamy cells and as single cells scattered throughout the lamina propria. It was impossible to quantify the aggregates, but deeper in the lamina propria, where the CD25+ cells were scattered throughout the lamina propria, differential counts were possible (Table 1)

Table 1. The percentage of CD25+ cells in the lamina propria of children with ulcerative colitis or Crohn's disease.

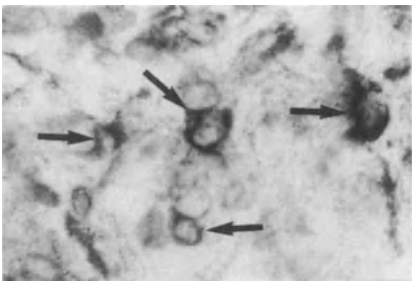
	%CD25+ cells ±1sd
Normal small bowel n=7	<1
Normal colon n=6	<1
Crohn's small bowel n=8	38±9
Crohn's colon n=6	25±8
Ulcerative colitis n=5	14±10

Nature of the CD25+ cells in the lamina propria

There was considerable morphologic heterogeneity in the CD25+ cells. Beneath the epithelium most were large cells with the morphologic appearance of macrophages wheres deeper

in the mucosa most of the cells were small and round (Fig1).

Figure 1. CD25+ cells (arrowed) in the lamina propria of a child with Crohn's disease.



A double staining procedure, involving blocking CD25 APAAP red staining by a prior treatment with another monoclonal and the peroxidase technique was sused to identify the CD25+ cells. In Crohn's ileitis and colitis CD3 blocked 60-90% of the CD25 staining, CD4 completely blocked it and CD8 had no effect. Thus in Crohn's disease most of the CD25+ cells are CD3+,CD4+ (T cells). In contrast in UC anti-CD3 had virtually no blocking effect on CD25 staining (10%), however CD4, and anti-HLA-DR competely blocked CD25 staining. Thus in UC most of the CD25+ cells have the phenotype CD3-,4+,DR+ (macrophages or dendritic cells). The morphology and location of these cells is however more consistent with these cells being macrophages.

4. Discussion.

There have been a number of studies on the lymphoid/myeloid infiltrate in the mucosa of patients with chronic inflammatory bowel disease. In both ulcerative colitis and Crohn's disease there is an increased lymphocytic infiltrate compared to normal bowel, but the ratio of CD4 to CD8 cells is unchanged (7). There is a pronounced macrophage infiltrate and changes in macrophage/dendritic cell populations in IBD (14-16), and there is also evidence that the macrophages in IBD are in an increased state of activation compared to normal lamina propria macrophages (17). Crohn's disease

bears the stigmata of a T cell mediated reaction, but surprisingly, a number of previous studies have failed to observe phenotypic markers of T cells activation, such as CD25 as a major feature of the lesion, both in tissue sections and on isolated cells (6-8).
The major observation reported here is that, contrary to previous reports, CD25+ cells are abundant in the gut of children with IBD. These cells were detected using alkaline phosphatase immunohistochemistry, whereas previous studies had used immunofluorescence which is less sensitive. Independent confirmation of the abundance of CD25+ cells in inflammatory bowel disease has also recently been published (18).
The subepithelial aggregates of large foamy CD25+ cells also expressed CD4 and HLA-DR. These probably correspond to the subepithelial macrophage aggregates and their CD25 expression is probably a consequence of immune stimulation. CD25+ macrophages are seen in the lung in sarcoidosis (19) and in vitro experiments have shown that interferon-gamma can induce CD25 on macrophages (19,20).
Double staining revealed that in ulcerative colitis most of the CD25+ cells were CD3-, CD4+ and were therefore likely to be macrophages. It is unlikely that they are dendritic cells because in ulcerative colitis these are stellate cells with long cytoplasmic processes (8). However in Crohn's disease most of the CD25+ cells were CD3+,CD4+,CD8- (T cells). In addition, in single stained specimens many of the CD25+ cells had the morphologic appearance of lymphocytes. CD25 is expressed very early after T cell activation; gene expression being detectable as early as 4-6 hours after stimulation (21). With continued activation however CD25 expression diminishes (22). Thus in Crohn's disease the CD25+ T cells are probably recently activated, perhaps having recently extravasated from the blood. The antigen(s) however to which these T cells are responding is unknown and the identification of these antigens would be a major step forward in our understanding of Crohn's disease. The T cell response is however likely to be polyclonal in response to a number of antigens since recent studies on the diversity of T cell receptors found on gut T cells in Crohn's disease gave no evidence of a monoclonal or oligoclonal response (23). In dealing with inflamed mucosa however there is always the problem that the T cells responding to antigen may be diluted out by a non-specific influx of T cells into the mucosa.
The results of this present study indicate that activated CD4+ T cells may be responsible for the lesion in Crohn's disease, presumably by the production of inflammatory mediators. That these cells are not as abundant in ulcerative colitis serves to emphasise the differences between Crohn's

disease and ulcerative colitis which are also evident histopathologically (5).

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Colonic mucosal T lymphocytes in ulcerative colitis: expression of the CD7 antigen in relation to MHC class II (HLA-D) antigens

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Introduction

Despite evidence of immune system involvement in Ulcerative Colitis (UC), no consistent picture of systemic immune abnormality has emerged. In the colonic mucosa, plasma cell numbers are increased although IgA production on a "per cell" basis is reduced [1], suggesting a subtle alteration of antigen presentation and/or immunoregulation. However, no major perturbation of T lymphocyte subsets has been reported [2]. The epithelium in inflamed colon is frequently HLA-DR+ [3]. The only known inducer of HLA-DR expression is Interferon-γ, a product of activated T cells, although mucosal T cells do not express "classical" activation markers in UC [2]. Nevertheless, there is evidence that CD7 is a marker of increased T cell activity in the intestinal mucosa [4].

The aims of this study were to further characterise the immunoregulatory T cells of the colonic mucosa with particular reference to CD7 antigen expression by T cell subsets, and its relationship with induction of MHC class II (HLA-D) antigen expression by epithelial cells.

Materials and Methods

30 controls and 21 UC patients in clinical remission (10 "total" colitis; 11 "distal" colitis) were studied. Double-label immunofluorescence was performed on cryostat sections with antibodies to T cells and activation markers using class-specific anti-IgG and IgM conjugates [5]. The antibodies used were RFT11 (CD2), UCHT1 (CD3), Dako and Calbiochem T4 (CD4), RFT1 (CD5), MBG6 (CD6), RFT2 (CD7), RFT8 (CD8), Leu 11b (CD16, NK cells), anti-Tac (CD25, IL-2 receptor), Leu 8 (suppressor-inducer subset of CD4+ cells), OKT9 (CD71, transferrin receptor), Ki67 (nuclear proliferation antigen), W1/36 (MHC class I), RFDR1, RFDR2 and Dako-DR (MHC class II). At least 200 cells per specimen were counted by two independent observers.

Results

INTRAEPITHELIAL T LYMPHOCYTES (IEL)

In controls, over half the T cells (54%) were CD8+, although very wide individual variations were observed. Approximately 60% of CD8+ cells were CD5+. 20-60% of IEL were CD7+, of which nearly half were CD8+. Essentially no Leu 8+ or Leu 11+ (NK) cells were observed. Too few cells expressed CD6 to permit reliable enumeration.

In UC patients, the mean CD4:CD8 ratio (0.82:1), percentage of CD8 cells co-expressing CD5 (56%) and T cell density were comparable with controls. However, of the CD7+ cells, an increased percentage (from 49% to 58%) were CD8+ (NS).

LAMINA PROPRIA T LYMPHOCYTES (LPL)

In the control group, the mean CD4:CD8 ratio was 2.4:1. The percentage and subset distribution of CD7+ cells was comparable to the IEL. However, nearly half (45%) of CD8+ LPL were $CD5^-$. Virtually no Leu 11+ or Leu 8+ T cells were found.

In the entire UC patient group, the CD4:CD8 ratio was increased from 2.55:1 to 2.9:1 (NS). There was essentially no difference in the percentage of CD8+ cells which were CD5+ CD6+, nor in the subset distribution of CD7+ cells. However, in a smaller patient group studied (n=6), there was a significant increase in the percentage of CD6+ cells which were CD7+ ($P<0.05$). As in controls, Leu 11+ or Leu 8+ cells were not observed.

MARKERS OF T CELL ACTIVATION AND MHC ANTIGEN EXPRESSION

T cells were not HLA-D+ nor CD25+. No identifiable T cells were OKT9+ or Ki67+. In these respects, T cells of UC patients did not differ from controls, although occasional CD3+ LPL were noted which appeared to be very weakly HLA-D+.

In control and patient groups, the epithelium was strongly positive for MHC class I antigens (HLA-A,B,C). In control patients, there was no expression of class II (HLA-D). In 11/19 patients, enterocytes were HLA-D+. HLA-D+ epithelium was observed in 7/8 patients with total colitis, but only in 4/11 patients with distal colitis, where the reaction was less intense. However, when grouped according to HLA-D expression, there was a significant difference ($P<0.02$) between HLA-D+ and $HLA\text{-}D^-$ patients with respect to expression of CD7 by LPL CD4+ T cells.

Discussion

Although mucosal T cells in UC did not display classical markers of activation (HLA-D, CD25), there was a correlation between expression of CD7 by CD4+ cells and induction of HLA-D expression in enterocytes. It would therefore appear that mucosal T cells need not undergo full "activation" to become immunologically active and secrete Interferon-γ.

If HLA-D+ epithelium has antigen-presenting capabilities, this could result in T cell stimulation in absence of any major perturbation of T cell subsets. The lack of CD4+ Leu 8+ suppressor-inducer cells in UC argues for defective immunosuppression, or for a failure of normal suppressive mechanisms following a local hypersensitivity reaction. This could lead to a perpetuation of the chronic inflammatory state, triggered by a normal response to dietary or microbial antigens.

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Antibody dependent cell-mediated cytotoxicity in culture supernatants of anticolon antibody producing cells from patients with ulcerative colitis

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ABSTRACT. Anticolon antibodies were synthesized by Epstein-Barr virus (EBV)-transformed B cells from patients with ulcerative colitis. The IgG subclasses of these anticolon antibodies were variable. Anticolon antibody positive culture supernatants demonstrated higher activity of anticolon antibody dependent cell-mediated cytotoxicity (ADCC) against colon epithelial cells than that of the nagative culture supernatants. There was a positive correlation between anticolon antibody activity detected by Enzyme-linked immunosorbent assay (ELISA) and ADCC activity. It is suggested that anticolon antibodies produced in the colonic mucosa may participate in the pathogenesis of ulcerative colitis.

INTRODUCTION. It is well known that anticolon antibody is present in the sera from patients with ulcerative colitis[1)2)]. The precise role of this autoantibody remains unknown. In the present study, we investigated in vitro anticolon antibody producion using EBV transformation, and ADCC activity of the culture supernatanuts of EBV-transformed cells against colon epithelial cells.

MATERIALS AND METHODS. ELISA for anticolon antibodies and IgG subclasses ; The isolated epithelial cells from Wistar rat colon were incubated overnight in 96-well plates and then fixed with 0.5% glutaraldehyde in phosphate buffered saline (PBS) for 5 min. After washed with PBS, fixed colon cells were incubated with 100µl of the culture medium from each well of EBV-transformed or 1:50 diluted serum. After 1 hr incubation at 37°C, the cells were washed with PBS and then incubated with goat anti-human IgG or mouse monoclonal anti-human IgG_1, IgG_2, IgG_3, and IgG_4 conjugated to alkaline phosphatase. The cells were then washed with PBS and incubated with 0.5mM p-nitrophenyl phosphate. The optical density was read at 419 nm with ELISA reader. Wells with optical densities greater than two standard deviations above the mean of the nagative controls were scored as positive for anticolon antibody activity. EBV-transformation method for anticolon antibody producing cells ; Lymphocytes isolated from colonic mucosa or peripheral blood were suspended in the culture supernatant of B95-8 cell line and then washed once with complete medium (RPMI 1640 medium containing antibiotics and 10% FCS). $1x10^4$

or lx105 EBV-exposed cells were seeded in each well of a 96-well plate. Three or four weeks after the cultures were initiated, supernatant from each well was harvested and tested for anticolon antibody production in an ELISA. ADCC assay ; ^{51}Cr labeled colo 205 (human colon cancer cell line) which has common antigenicity to rat or human colon epithelial cell were seeded in a 96-well microtest plate. Effector cells were prepared from heparinized peripheral blood from healthy control, and added in 50:1 ratio of effector to target cells. ^{51}Cr-labeled target cells were incubated with 50µl of duluted serum or culture supernatant from EBV-transformed cells and effector cells for four hours at 37°C in 5% CO_2. At the end of the incubation, 100µl of supernatant was collected from each well and the radioactivity was measured. ADCC activity was calculated as % cytotoxicity.

RESULTS AND DISCUSSION. The frequencies of anticolon antibodies in the sera from patients with ulcerative colitis were about 71% (n=41). The IgG subclasses of anticolon antibodies were tested in 11 patients. Nine of them were positive in IgG_1, 7 in IgG_2, 1 in IgG_3 and 11 in IgG_4 respectively. The estimated frequencies of anticolon antibody producing EBV-transformed cells were much higher in colonic mucosa than in peripheral blood. The IgG subclasses of the culture medium from the anticolon antibody positive wells of EBV transformed cells were similar to those of the sera. Although the precise property of each IgG subclass is unknown, single IgG subclass may not be related to this disease. ADCC activity of the culture supernatants from the anticolon antibody positive wells (%cytotoxicity= 8.3 ± 1.3) was significantly higher than that from the anticolon antibody negative wells (%cytotoxicity=2.1 ± 0.7) ($p<0.01$). There was a positive correlation between anticolon antibody activity detected by ELISA and ADCC activity of the culture supernatants ($r=0.52$, $p<0.05$). These results were compatible with the previous reports that ADCC mechanisms may have some role in the pathogenesis of ulcerative colitis[3),4)]. In consideration of the fact that anticolon antibody producing cells and IgG producing cells were numerous in colonic mucosa of patients with ulcerative colitis[5)], anticolon antibody produced by mucosal B cells may have an important role to induce the injury of colon epithelial cells in vivo.

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HML1-Positive cells in the gut-associated lyphoid tissue in patients with Crohn's disease

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ABSTRACT. HML1 defines a surface antigen on mucosa associated lymphocytes. In patients with Crohn's disease HML1-expression in peripheral blood is increased. In the lamina propria HML1-expression almost equal in normal controls, patients with acute enteritic infection and ulcerative colitis. In the lamina propria of patients with Crohn's disease the expression is normal in not affected areas, there is a marked decrease of HML1-expression in inflamed parts of the gut.

Introduction

The HML1 antibody was made by immunizing a mouse with human intraepithelial lymphocytes (IEL). In the human intestine 95% of the IEL and 30-40% of lamina propria lymphocytes (LPL) express the HML1 antigen. The antibody also binds lymphocytes in the mammary gland and the bronchial epithelium, and it has been found on T cell lymphoma derived from the gut.
In this paper we describe the distribution of HML1-positive cells in Crohn's disease and ulcerative colitis, and present evidence of regulatory functions of HML1-positive cells.

Methods

Peripheral blood mononuclear cells were isolated by density gradient centrifugation over Lymphoprep medium. Colonic biopsies and were taken from healthy individuals and patients with acute gastroenteritis, Crohn's disease and ulcerative colitis. The specimen was washed, mucus was removed with Dithiothreitol (1mM, 3', at room

temperature) and epithelial cells and intraepithelial cells were separated with EDTA (0.75mM, 90', 4°C). The remaining biopsy was then digested in enzyme solution (48U/ml collagenase, 300U/ml DNAse, 37°C overnight) and the cells were washed repeatedly. $6.4x10^6$ LPL could be harvested from 100mg biopsy tissue. Cells were stained with standard immunofluorescence techniques, autologous B-cell cultures were done in RPMI 1640 medium containing 10%FCS and 400µl/ml Pokeweed mitogen.

Results

In the peripheral blood only few cells express the HML1-antigen. However we found a slightly higher number in patients with Crohn's disease (6%) than in normal controls (4%). In the lamina propria of healthy controls 31.6 ± 2.7% of the mononuclear cells are HML1-positive, in patients with ulcerativ colitis 39 ± 2.9%, and in patients with Crohn's disease 28.4 ± 2.2% if the biopsy was taken from macroscopically uninvolved mucosa. If the biopsy was taken from an inflamed site however we found a drastic reduction of HML1-positive cells, merely 9.7 ± 1.8% were positive.
Looking at coexpression with other surface markers we found a striking correlation of HML1-expression and expression of Transferrin receptor.
Studies of the functional role of HML1-cells were done in autologous B-cell cultures from peripheral blood. When B- and T-cells were cocultured for 7 days we found 546ng immunoglobulin per culture in supernatants from healthy individuals. With the HML1 cells removed by panning only 236ngIg/culture was secreted. The same decrease was found in patients with Crohn's disease: 418ng/culture with the HML1 cells, 286ng/culture without HML1 cells. Similar results were found for the IgA-secretion. In healthy individuals removal of HML1 cells decreased the IgA-secretion from 107ng/culture to 61ng/culture. In patients with Crohn's disease IgA secretion was decreased from 247ng/culture to 149ng/culture.

Discussion

In inflamed mucosal areas of patients with Crohn's disease expression of HML1 on lymphoid cells is significantly lower than in normal controls. This is characteristic for Crohn's disease, and not found in ulcerative colitis or enteritic infection. In contrast HML1-expression in peripheral blood is increased in patients with Crohn's disease. This supports the hypothesis of disturbance of compartment borders in Crohn's disease. HML1-positive cells were found to play an important role in regulating immunoglobulin secretion.

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Cell adhesion molecules in autoimmune enteropathy

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INTRODUCTION

Adhesion to both haematopoetic and non-haematopoetic cells is an obligatory step in antigen presentation and effector cell function. Intercellular adhesion molecule-1 (ICAM-1) is a cell surface glcoprotein whose expression is up-regulated by cytokines *in vitro* and by inflammatory processes *in vivo*. It acts as a ligand for the lymphocyte function associated antigen (LFA-1), a molecule which is important in antigen dependent and antigen non-dependent T lymphocyte function. In addition, it plays a relevant role in the adhesion and function of essentially all leukocyte cell types (1). In the small intestine, ICAM-1 expression has been demonstrated in the lamina propria (LP) on most endothelial cells, on dendritic-like cells and macrophages, but it is generally absent on epithelial cells (2). Autoimmune enteropathy is clinically characterised by protracted diarrhoea, and the affected children have circulating autoantibodies to enterocytes. Examination of biopsy material has revealed aberrant expression of HLA Class II products on crypt enterocytes (3).

The aim of this study was to investigate ICAM-1 and LFA-1 molecule expression in the small intestinal mucosa from children with autoimmune enteropathy, compare it with histologically normal tissue from control children, and relate it to Class II product expression.

PATIENTS AND METHODS

Six children with autoimmune enteropathy (4 male, 2 female, aged 2-12 yrs, median 5) and 5 control children (3 male, 2 female, aged 1-10 yrs, median 3) were included in the study. All children had presented with chronic diarrhoea and/or failure to thrive, and underwent small intestinal mucosal biopsy. A portion of the tissue was used for conventional histological assessment, and another piece was snap frozen and stored at -70°C. 4μ cryostat sections were cut and stained using IFL with MoAbs to LFA-1 (TS1/22), ICAM-1 (RR/1) (gift from Prof. T Springer), HLA-DR non-polymorphic region (Mid 3) and thyroglobulin (P11) (as negative control).

RESULTS

In all specimens examined, lymphomononuclear cells expressing LFA-1 and ICAM-1 molecules were detected in the lamina propria. A proportion of dendritic and endothelial-like cell populations were documented amongst the ICAM-1 positive cells. In general, the number of positive cells, and their intensity of expression was increased in patients compared with controls. ICAM-1 expression was generally absent from control crypts, but in 2 of the 5 specimens it was expressed in isolated goblet cells. In contrast, ICAM-1 was positive in the crypt epithelium from all 6 patients with a stronger reactivity in the goblet cells. LFA-1 products were only detectable within the crypt epithelium of 3/6 pathological samples studied. Villous epithelial cells were invariably negative for the two specificities in both patients and control biopsies.

HLA-DR expression was positive in the crypt enterocytes of patients, and negative in control crypts, and P11 monoclonal antibody was negative throughout.

DISCUSSION

A 'de novo' expression of LFA-1 molecules was detected within the crypt epithelium from a proportion of jejunal biopsies of children with autoimmune enteropathy and, up-regulation of ICAM-1 molecules was found within the crypt epithelium in all the small intestinal samples of these children.

Autoimmune enteropathy is characterised by inappropriate HLA-DR expression in the crypt epithelium and this phenomenon has been shown to enable enterocytes to present autoantigens to activated T cells, thus initiating/perpetuating the autoimmune epithelial cell damage. Cell adhesion molecules are known to enhance the efficiency of T lymphocyte interactions wth both target and accessory cells. It is envisaged that in autoimmune enteropathy the inappropriate or enhanced expression of adhesion molecules and the 'de novo' appearance of HLA-DR on enterocytes might facilitate autoantigen presentation by increasing cell adhesion.

In a similar fashion, the lack of adhesion molecule expression on villous epithelium, both in physiological and pathological conditions in a location where HLA-DR products are physiologically expressed might have a protective role against hazardous autoimmune mechanisms against these cells.

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Leucocyte adhesion molecules in inflammatory bowel disease: expression by colonic macrophages

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Introduction

Recent interest has focused on intercellular adhesion molecules and their ligands (the "integrins"), which mediate cell-cell interactions (1). Three leukocyte adhesion glycoproteins share a common β-chain (CD18); the α-chains are LFA-1 ("Lymphocyte Function-associated Antigen-1"; CD11a), MAC-1 (CD11b) and p150,95 (CD11c), respectively. ICAM-1 ("Intercellular Adhesion Molecule-1"; CD54) is the ligand/receptor for LFA-1. The LFA-1/ICAM-1 interaction of T cells and mononuclear phagocytes is central for antigen presentation, antigen priming and for the mediation of inflammatory responses (2). CD11b and CD11c are receptors for complement C3bi and mediate phagocytosis of opsonized particles and cell binding to endothelium.

At present, the immune mechanisms which mediate the local reaction in inflammatory bowel disease (IBD) remain unclear. Although numbers of plasma cells (3) and macrophages (4) are increased, there is little apparent change in regulatory T cell populations. Nevertheless, there is some evidence for increased T cell activity (5) and for macrophage activation (6).

Expression of the leukocyte adhesion molecules has not been studied in IBD, although all available evidence suggests that they may be central in the mediation of the inflammatory response.

Materials and Methods

Biopsy tissues were obtained from 10 histologically normal controls, 11 patients with active Ulcerative Colitis (UC) and 9 patients with Crohn's Disease (CD). Cryostat sections were cut and double-labelled by immunofluorescence with a panel of monoclonal antibodies and class-specific second layers (anti-IgG/FITC and anti-IgM/TRITC). The antibodies used comprised MHM24 (anti-LFA-1, CD11a), OKM1 (anti-MAC-1, CD11b), 3.9 (anti-p150,95, CD11c), MHM23 (anti-common β chain, CD18), RR1/1 (anti-ICAM-1, CD54), all of IgG class, and RFDR1 (anti-HLA-DR, polymorphic determinant) of IgM class.

Macrophages were identified on the basis of morphology and expression of HLA-DR, and examined for expression of adhesion molecules. For each patient, a minimum of 300 cells were counted per tissue section by two independent observers. The Mann-Whitney U-test was used for statistical evaluation, figures quoted are mean $\pm$ SD.

Results

NORMAL COLONIC MUCOSA

In the normal colonic mucosa, expression of LFA-1 was extensive, but mainly associated with T cells. Of the HLA-DR+ macrophages, only a minority (12.5% ± 5.6) expressed LFA-1. Very few macrophages were OKM-1+ (2.7% ± 1.9), whereas a substantial percentage (35.6% ± 16.5) coexpressed CD11c. ICAM-1+ macrophages were rarely seen, amounting to only 6.9% (± 3.9) of the total. Vascular endothelium was also positive for ICAM-1.

ULCERATIVE COLITIS

There was a striking increase in the percentage of macrophages expressing ICAM-1 (69.8% ± 12.8) in all patients with Ulcerative Colitis ($P<0.001$). The proportion of macrophages which expressed LFA-1 was significantly higher in the UC patients (21.6% ± 8.1) than in normal controls ($P<0.01$). However, no significant differences were observed in the percentages of macrophages expressing CD11b (2.1% ± 1.2) and CD11c (43.87% ± 15.2). In 10/11 patients, the colonic epithelium was HLA-DR+. In all UC patients, there was a higher density of HLA-DR+ macrophages in the lamina propria.

CROHN'S DISEASE

The percentage of ICAM-1+ macrophages was also significantly higher (45.7% ± 22.8) in Crohn's Disease than in normal controls ($P<0.001$), showing a relation to disease activity. The percentage of macrophages expressing LFA-1 was also significantly increased to 18.2% (± 6.1) ($P<0.05$). Expression of CD11b was unaltered, but a slight increase of expression of CD11c was observed (50.3% ± 20.2) ($P<0.05$). The epithelium was HLA-DR+ in only two patients with Crohn's Disease.

Summary and Conclusions

Our data indicate that expression of leukocyte adhesion molecules by macrophages changes markedly in IBD. The changes relate to integrins involved in macrophage-T cell interactions (ICAM-1 and LFA-1), but not those involved in opsonisation and phagocytosis (CD11b and CD11c). These results suggest that antigen presentation by macrophages is considerably increased in active IBD, which could lead to a T cell-mediated perpetuation of the chronic inflammatory state.

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Respiratory burst capacity of human intestinal macrophage subpopulations

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ABSTRACT

We investigated the capacity of subpopulations of macrophages from normal and inflamed human colonic mucosa to undergo respiratory burst. This was studied by their ability to reduce nitroblue tetrazolium. Majority of cells labelled with monoclonal antibody RFD1 (which also labels dendritic cells) were able to phagocytose opsonised zymosan and median 40% were able to undergo respiratory burst. Significantly greater proportion of RFD9 positive macrophages (which are present only in mucosa of colons with active inflammatory bowel disease) were able to release oxygen radicals compared to macrophages labelled by "pan-macrophage" markers from the same specimens. Thus intestinal macrophages express a marker present on dendritic cells. RFD9 positive macrophages in inflamed mucosa are an activated population of cells.

Introduction

Morphological and histochemical heterogeneity of macrophages in normal human ileum and colon has been demonstrated (1). Recently this heterogeneity has been demonstrated using monoclonal antibodies (2-5). The majority of these cells are HLA-D positive and also stain with antibodies EBM11 and Y1/82A (CD68). A large proportion also stain with monoclonal antibodies RFD1 and RFD7. Antibody RFD1 has been shown to label peripheral blood dentritic cells (7) and also dendritic cells in lymph nodes (8).

In active inflammatory bowel disease (IBD), two new subpopulations of cells were seen, these stained with monoclonal antibodies RFD9 and 3G8(2).

Functional studies have shown that only a minority of macrophages isolated from normal ileum and colon are able to undergo a respiratory burst in response to various triggers. Significantly greater proportion of macrophages isolated from mucosa with active IBD release oxygen radicals and hence appear to be activated (6).

The aim of the present study was to investigate the capacity of RFD1 and RFD9 positive macrophages isolated

from normal and inflamed (active IBD) human intestinal mucosa to undergo respiratory burst.

Methods

TISSUE

Normal colonic mucosa was obtained from colons resected for carcinoma. It was obtained at least 5 cm from tumour and was histologically normal.

Inflamed ileal and colonic mucosa was obtained from intestine resected for active inflammatory bowel disease. All the patients were on intravenous corticosteroids at the time of the operation.

ISOLATION OF INTESTINAL MONONUCLEAR CELLS

Mononuclear cells (MNC) were obtained from normal and inflamed mucosa using an EDTA/collagenase technique as described before(2,6). In brief, after treatment with dithiothreitol (Sigma), epithelial cells were removed from strips of mucosa by shaking with EDTA (5mM) in three, half hour steps. After cutting into small pieces, the mucosa was then digested with collagenase from Clostridium histolyticum (Boeringher Mannheim) at a concentration of 1 mg/ml. Mononuclear cells were obtained by centrifugation on Ficoll-Paque. This population contained a median 13.5% (range of 7-22%) of macrophages (Y1/82A positive).

TRIGGERS

Phorbol 12-myristate 13-acetate and opsonised zymosan were used as triggers.

RESPIRATORY BURST ACTIVITY

This was performed as described before(6). Assays for respiratory burst activity were performed within two hours of isolation of the mononuclear cells.

1.5×10^6 mononuclear cells were incubated with 1 mg of nitroblue tetrazoium (NBT; Sigma), PMA (200 ng/ml) or opsonised zymosan (15 µg/ml). Cells with PMA were incubated for half an hour and those with opsonised zymosan for 1 hour. Cytocentrifuge preparation were then made, air dried and fixed in acetone. They were then stored at -20°C until used for immunohistochemistry.

IMMUNOHISTOCHESMISTRY

Cytocentrifuge preparations of mononuclear cells triggered with opsonised zymosan were stained using antibodies RFD1, RFD9, 201521 (anti-HLA-D). Preparation of mononuclear cells triggered with PMA were stained with antibodies Y1/82A and RFD9.

The peroxidase technique was used for staining (9) (endogenous peroxidase activity having been blocked) except

for RFD9, for which the APAAP technique (10) was used.
In each cytocentrifuge preparation, the proportion of cells (stained with the monoclonal antibody) undergoing respiratory burst (as shown by deep blue-black formazan 3staining) was determined. The slides were coded and analyses were performed blind.

STATISTICS
Analysis was performed using Wilcoxon Rank Sum Test for paired data and correlation using Rank Spearman Correlation Coefficient.

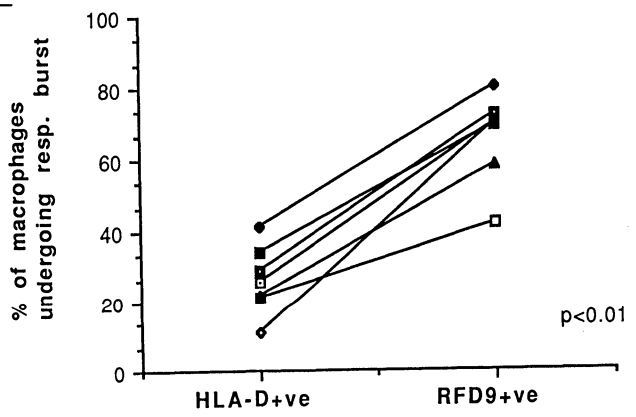
Results

Labelled cells (brown and red) undergoing respiratory burst (blue-black staining) were easy to identify.

RFD1 ANTIBODY
Mononuclear cells isolated from normal colonic mucosa (five) and mucosa with active ulcerative colitis (two) were studied.
Median 90 (range 74-98)% of RFD1 positive cells were able to phagocytose opsonised zymosan and of these 40 (7-45)% were able to undergo respiratory burst.
There was significant correlation between RFD1 positive and HLA-D positive macrophages, from the same specimens, undergoing respiratory burst ($r=0.83$; $p= <0.05$).

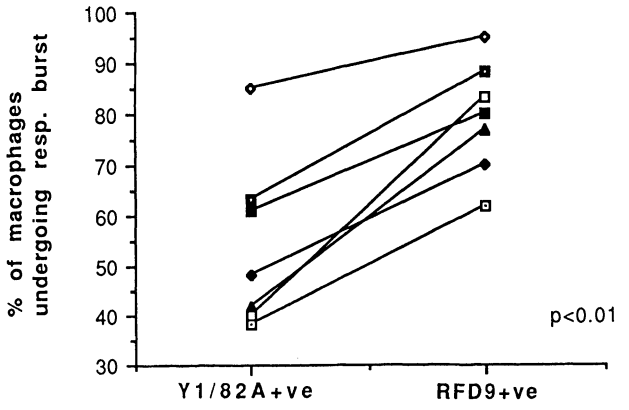
RFD9 ANTIBODY
For these experiments, mononuclear cells were isolated from mucosa with active ulcerative colitis (three) and Crohn's disease (four).
Median 70 (42-72)% of RFD9 positive macrophages (with phagocytosed zymosan) were able to undergo respiratory burst compared to 26 (11-41)% of HLA-D positive macrophages from the same specimens ($p<0.01$; Fig 1).

Fig 1



When PMA was used as a trigger 80 (62-95)% of RFD9 positive macrophages were able to release oxygen radicals compared to 48 (38-85)% of Y1/82A positive macrophage's from the same specimens (P <0.01; Fig 2).

Fig 2



Discussion

During a respiratory burst, oxygen is taken up by the cell and enzymatically converted by membrane associated NADPH oxidase to superoxide anion, hydrogen peroxide and hydroxyl radicals. NBT in the presence of these radicals gives a deep blue-black formazam reaction product, allowing identification of these cells (11,12). Any contaminating polymorphonuclear leucocytes can be ignored as they are not labelled by the antibodies.

Antibody RFD1 labels macrophages in normal ileal and colonic mucosa and also mucosa in active inflammatory bowel disease. RFD1 positive macrophages are predominantly large and round in the superficial lamina propria and have a "dendritic" morphology in the deep lamina propria (2). RFD1 labels a product of HLA-D locus which is preferentially expressed on dendritic cells. It has been shown to label dendritic cells in T cell zones and those in circulation(7,8). Our previous studies have found significant numbers of dendritic RFD1 positive (and HLA-D positive) cells in the inter follicular region of Peyer's patches(4). These cells did not label with macrophage specific monoclonal antibodies. In the present study we have shown that the majority of RFD1 positive cells isolated from normal and inflamed mucosa are macrophages as shown by their capacity to phagocytose opsonised zymosan. A proportion of these cells were able to undergo respiratory burst. This suggests that human intestinal

mononuclear phagocyte system cells have features of both macrophages and dendritic cells. This has been confirmed by our previous studies on antigen presenting activities of isolated human intestinal mononuclear cells(11).

The second part of the study examined the capacity of RFD9 positive macrophages, which are found in inflamed (IBD) but not normal colonic mucosa, to release oxygen radicals. This antibody has been shown to label epithelioid cells and tingible body macrophages(14). Compared to "pan-macrophage" markers, significantly greater proportion of RFD9 positive macrophages were able to undergo respiratory burst. An enhanced or unregulated respiratory burst is a feature of activated macrophages. Thus it seems that RFD9 positive macrophages found in inflamed mucosa in active inflammatory bowel disease are activated. Using similar methods, we have shown that macrophages in active inflammatory bowel disease express interleukin 2 receptors and that the majority of these are able to undergo respiratory burst and hence also appear to be activated(15).

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T cell lymphokine mRNA expression, lymphokine utilization, and regulatory function in the intestinal mucosal immune system

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Abstract

The capacity of mucosal lymphocytes to produce and use lymphokines and their effects on regulation of immunoglobulin production were determined in normal nonhuman primates. In comparison to lymphocytes from peripheral sites, there was high expression of IL-4 and IL-5 mRNA in both mesenteric lymph node and lamina propria T cells. Northern blots of RNA from mitogen-activated lamina propria T cells contained more mRNA for IL-2 and IFN-γ than mesenteric lymph node T cells. T cells from lamina propria had high IL-2 induced but no IL-4 induced proliferative responses. In contrast, mesenteric lymph node T cells had high IL-4 induced and lower IL-2 induced proliferative responses compared to lamina propria T cells. Both IL-2 and IFN-γ enhanced immunoglobulin production, whereas IL-4 inhibited immunoglobulin production. IFN-γ reversed the inhibitory effect of IL-4. Lamina propria T cells had higher helper activity in pokeweed mitogen stimulated cultures and exhibited less inhibition by IL-4 than mesenteric lymph node T cells. These data and previous studies suggest that T cells in an inductive site such as the mesenteric lymph node are primarily "naive" cells whereas T cells in the effector compartment of the lamina propria are comprised primarily of differentiated "memory" cells.

Introduction

In the gastrointestinal tract, lymphocytes in organized lymphoid sites such as mesenteric lymph nodes are thought to carry out specialized tasks necessary for initiating responses to antigens derived from the intestinal lumen. In contrast, lymphocytes located diffusely throughout the intestinal lamina propria and epithelial cell layer are thought to carry out specialized effector functions which are important for host defense. The molecular basis for differences in the function of T cells in the inductive and effector compartments of the mucosal immune system may be due in part to differences in their capacities to produce and use lymphokines. In this regard, we have shown that T cells in the lamina propria are in a more activated state than T cells in other sites (1), that they have the capacity to produce high levels of IL-2 (2) and that they release helper factors in response to specific antigens (3). The aim of the present study was to investigate the capacity of mesenteric lymph node and lamina propria T cells to express mRNA for the lymphokines IL-2, IL-4, IL-5 and IFN-γ, to determine the ability of T cells to proliferate in response to IL-2 and IL-4, and to determine the effect of lymphokines on T cell dependent immunoglobulin synthesis.

Materials and Methods

Blood and tissues were obtained from normal, healthy Rhesus monkeys. Human blood was obtained from normal healthy laboratory workers. Lymphocyte populations were isolated using previously described methods. Mitogen and lymphokine induced proliferation were determined by incorporation of 3H-thymidine in 96 well plates. Regulation of immunoglobulin production was determined by assay of PWM stimulated culture supernatants by ELISA. Lymphokine mRNA expression was assessed in Northern blots of total cellular RNA hybridized with the indicated cDNA or RNA probes.

Results

EXPRESSION OF LYMPHOKINE mRNA BY MITOGEN-STIMULATED T CELLS.

Initially experiments were carried out to determine whether there are differences in the steady-state levels of lymphokine mRNA following activation of lymphocytes isolated from different sites of normal nonhuman primates. No specific hybridization was detected in unactivated lymphocytes obtained from any site. When lymphocytes from different sites were activated with a combination of ionomycin and PMA, both mesenteric lymph node and lamina propria T cells were found to express high levels of mRNA for IL-4 and IL-5 in comparison to cells from peripheral blood, spleen, or peripheral lymph nodes. When the same blot was hybridized with IL-2 cDNA, lamina propria lymphocytes had the highest levels of IL-2 mRNA, whereas mesenteric lymph node mRNA was lower. In contrast, IFN-γ mRNA was low in mesenteric lymph nodes compared to all other sites. Thus, intestinal lamina propria lymphocytes have high capacity to express IL-2, IFN-γ, IL-4 and IL-5 mRNA while mesenteric lymph node cells have much lower capacity to express IL-2 and IFN-γ but have a high capacity to express IL-4 and IL-5 mRNA after activation.

PROLIFERATIVE RESPONSES OF T CELL POPULATIONS.

Next, studies were carried out to examine the capacity of intestinal T cell populations to use IL2 or IL-4 as growth factors. The proliferative responses of T cells isolated from either mesenteric lymph node or lamina propria were compared to those of T cells from peripheral blood or spleen of normal nonhuman primates. Background proliferative responses and responses induced by ConA were not significantly different among the different cell populations. Human recombinant IL-2 induced dose dependent proliferative responses of nonhuman primate T cells, which were highest in T cells isolated from the intestinal lamina propria. In contrast, when T cells were cultured with recombinant human IL-4 alone, significant dose-dependent proliferative responses were not observed in T cells isolated from any site . When T cells from PBL, spleen or mesenteric lymph node were cultured with IL-4 and PMA, dose-dependent proliferative responses were observed, and responses were highest in T cells isolated from the mesenteric lymph node. In contrast, T cells from the lamina propria did not exhibit a significant proliferative response to IL-4 + PMA at any dose, even though they had a high response to Con A and had the highest responses to IL-2 alone.

EFFECT OF IL-2, IFN-γ AND IL-4 ON PWM-STIMULATED IMMUNOGLOBULIN SYNTHESIS.

Since the studies above indicated significant differences in the production and utilization of lymphokines by mesenteric lymph node and lamina propria T cells, studies were

carried out to determine whether these differences might result in differences in the regulatory function of these T cell populations. First, to evaluate this question, the effect of exogenous lymphokines on the capacity of pokeweed mitogen-stimulated human lymphocytes to produce immunoglobulin was determined. Both rIL-2 and rIFN-γ enhanced immunoglobulin synthesis in a dose-dependent fashion. In contrast, recombinant human IL-4 caused a dose-dependent inhibition of immunoglobulin synthesis. This inhibitory effect was reversed by addition of recombinant human IFN-γ. The reversal of the IL-4 inhibitory effect by IFN-γ was observed only at low doses of IL-4, and was not observed at high doses of IL-4 (1000 U/ml).

These results suggested that lamina propria T cells, which express higher levels of IL-2 and IFN-γ mRNA, would have greater helper function for immunoglobulin synthesis than mesenteric lymph node T cells. To test this possibility, nonhuman primate mesenteric lymph node and lamina propria T cells were cultured with autologous spleen B cells alone or in the presence of increasing doses of recombinant human IL-4. Significantly more immunoglobulin was produced in cultures containing lamina propria T cells than mesenteric lymph node T cells, and the helper effect of lamina propria T cells was less inhibitable by exogenous IL-4.

Discussion

The results of the present investigation provide several new insights into the function of T cells in the inductive and effector limbs of the gastrointestinal immune system and provide further evidence that T cells in these compartments carry out specialized roles. First, it was shown that the T cells from mesenteric lymph nodes and lamina propria differ in expression of lymphokine mRNA: compared to mesenteric lymph node T cells, lamina propria T cells have significantly higher expression of IL-2 and IFN-γ mRNA; interestingly, both cell populations express high levels of IL-4 and IL-5 mRNA following activation in comparison to T cells from other sites. Previously we showed that activated lamina propria T cells secrete significantly higher levels of IL-2 than T cells from other sites of nonhuman primates, which correlates well with the IL-2 mRNA levels in the present study. We presume, but have not yet proved, that the levels of IFN-γ, IL-4 and IL-5 mRNA also will correlate with lymphokine secretion.

In parallel studies, it was shown that there are significant differences in the potential for utilization of lymphokines for proliferation by mesenteric lymph node and lamina propria T cells: whereas T cells in mesenteric lymph nodes can use IL-4 as a growth co-factor with PMA, T cells in the lamina propria do not. The lack of responsiveness of lamina propria T cells to IL-4 is in marked contrast to their high response to IL-2.

Experiments were also carried out to determine whether the differences in lymphokine mRNA expression and utilization by intestinal lymphocytes might have important effects on regulation of immunoglobulin synthesis in the gastrointestinal immune system. In agreement with previous publications, both IL-2 and IFN-γ enhanced immunoglobulin synthesis, and IL-4 inhibited immunoglobulin synthesis in a T cell dependent system. Since lamina propria T cells had higher capacity to express IL-2 and IFN-γ mRNA, it was therefore not surprising that lamina propria T cells had higher helper activity than mesenteric lymph node T cells for pokeweed mitogen stimulated immunoglobulin synthesis. Although both mesenteric lymph node and lamina propria T cells had a high capacity to express IL-4 mRNA, addition of exogenous IL-4 had less of an inhibitory effect on immunoglobulin production in cultures containing lamina propria T cells than mesenteric lymph node T cells. It is possibile that the lower inhibition of immunoglobulin production by IL-4 in cultures containing lamina propria T cells is associated with the low capacity of these cells to respond to IL-4. However, since it has been suggested that the inhibitory effect of rIL-4 on immunoglobulin

production is a direct effect on B cells, the differences in immunoglobulin production are more simply explained as a reflection of higher synthesis of IL-2 and IFN-γ by lamina propria T cells.

As shown in this study, lymphocytes associated with both the inductive and effector compartments of the mucosal immune system have high capacity to express IL-4 and IL-5 mRNA in comparison to lymphocytes from peripheral sites such as the peripheral blood, spleen and peripheral lymph nodes. Although the present investigation has focused primarily on the possible role of IL-4 in regulation of immunoglobulin synthesis, IL-4 and IL-5 have multiple effects on different cell lineages in mouse and man. In particular, IL-4 and IL-5 may have synergistic effects on IgA B cell differentiation in the mouse and IL-5 has been implicated as an IgA enhancing factor in humans. Thus the preferential production of IL-4 and IL-5 by mucosal T cells may play a key role in regulating B cell IgA differentiation in the mucosal immune system. In addition to its effects on IgA B cell differentiation, IL-5 also functions as an eosinophil colony stimulating factor in vitro, and thus it may play an important role in the maturation of intestinal eosinophils, which are a major component of the normal intestinal mucosa.

Table 1.
Evidence for Specialized T cell Function in the Mucosal Immune System

	Mesenteric Node	Lamina Propria
Proliferation:		
Specific antigen	++	-
Mitogens	++++	+++
Surface glycoproteins:		
CD45R	++++	±
Leu-8	++++	±
Activation:		
IL-2R	±	++
Lymphokine production:		
IL-2	++	++++
IFN-γ	++	++++
IL-4	++++	++++
IL-5	++++	++++
Lymphokine utilization		
IL-2	++	++++
IL-4	++++	-
Helper activity:	++	++++

An hypothesis which has been proposed to account for the different functional and phenotypic characteristics of human T cells is that their properties are a result of prior activation and differentiation by exposure to antigens (4-7). According to this thesis, so-called "naive" T cells, i.e., T cells which have not undergone prior activation by exposure to antigens, have low proliferative responses to recall antigens, have high proliferative responses to mitogens, have high expression of cell surface glycoproteins such as CD45R and Leu-8, have lower production of lymphokines such as IL-2 and IFN-γ, and have low helper activity. In contrast, so-called "memory" T cells have been suggested to comprise cells which have high proliferative responses to specific antigens, have low expression of CD45R and Leu8, have lower proliferative responses to mitogens, have high expression of lymphokines such as IL-2, IL-4 and IFN-γ, and have high helper activity. Table 1 shows a compilation of findings from this and previous studies of nonhuman primate and human intestinal lymphocytes. It can be seen that mesenteric lymph node T cells have the characteristics listed above which suggest that they are

comprised primarily of "naive" T cells, while most of the properties of lamina propria T cells are similar to those ascribed to "memory" T cells. An important exception is that in a previous study we found that lamina propria T cells failed to demonstrate proliferative responses to specific antigens, although they did manifest high helper activity in response to antigens. This finding indicates that "memory" T cells are present in the lamina propria, but that they are refractory to antigen-induced proliferation. Thus, the data suggests that T cells in the inductive and effector compartments of the intestinal immune system are comprised predominantly of "naive" and a specialized subset of "memory" T cells, respectively. Specifically, the mesenteric lymph node contains predominantly T cells which serve as a source of lymphocytes to expand the immune response to a novel antigen challenge; in this site the role of IL-4 may be to specifically enhance B and T cell proliferation while inhibiting B cell differentiation, and the relatively low capacity to produce IL-2 and IFN-γ may limit terminal B cell differentiation. On the other hand, the lamina propria contains predominantly differentiated memory T cells that have high helper activity for immunoglobulin synthesis and cytotoxic function due to high production of lymphokines. It is likely that the coordinate production of lymphokines by effector cells in the lamina propria is central for normal immunological defense in the intestinal environment.

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Interferons production by cultured lamina propria mononuclear cells in Crohn's disease and controls

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Introduction

IFNs are cytokine playing a central role in the regulation of immune response in humans. Production of IFN-γ is a specialized function of T lymphocytes which are capable of releasing IFN-γ in response to a variety of stimuli(1). IFN-γ is the most potent inducer of MHC-class II antigens on different cell types(2). CD is a disease characterized by a chronic active immune-mediated inflammatory response ongoing in the intestinal wall. LPMNC are activated in CD (3) and the intestinal epithelium from the inflamed areas in these conditions shows an increased expression of the MHC-class II antigens(4) . Class II antigens can be induced by recombinant IFN-γ on in vitro cultured normal human intestinal epithelial cells (5) . In this study, in order to examine whether LPMNC isolated from actively inflamed areas of CD patients are capable of releasing amounts of IFN, the spontaneous and induced release of IFN was measured in LPMNC cultures. The kinetics of the response of LPMNC to IFN inducers were also investigated.

Patients and methods

LPMNC were obtained by DTT-EDTA-Collagenase from surgical control samples of 7 C and 9 active CD.
Isolated LPMNC were checked for IL-2 receptor expression, using indirect immunofluorescence, and cultured in complete medium for 3 days with and without stimulation of the following:PHA(2ug/ml), Staphilococcal Enterotoxin B(SEB)(0.5 Ug/ml) as IFN-γ inducers, and the Newcastle Disease Virus(NDV) (10HA/millions cells) as IFN-α inducer. IFN was measured and characterized in culture supernatants at 12,24,48,and 72 hours. IFN was titred (log U/ml) by Sindbis virus haemagglutnin yield reduction after a single growth cicle. Antiviral activity was characterized by neutralization with specific antisera.

Results

The proportion of IL-2 receptor bearing cells were significantly higher in CD LPMNC than in C (21±6 and 9±4) ($p<0.01$).
No IFN was detected in control unstimulated cultures while in CD culture supernatants IFN-γ was found(0.9±0.3,1.1±0.3,1.1±0.4 and 1.3±0.3 respectively). Either PHA and SEB evoked a progressive increase of IFN-γ by control LPMNC (titers at 12,24,48 and 72 hours: SEB 1.2±0.2, 1.6±0.2,1.9±0.3,2.2±0.3 ; PHA 1.5±0.4,2.0±0.2,2.1±0.3,2.4±0.3) reaching the peak of IFN- γ releasing between 48 and 72 hours . Stimulated CD LPMNC released more IFN-γ than unstimulated CD LPMNC($p<0.01$) not significantly increasing over the culture period(titers at 12,24,48 and 72 hours: SEB 1.5±0.3,1.9±0.3, 1.9±0.3,2.0±0.2; PHA: 1.8±0.2, 2.1±0.3, 2.0±0.2,2.0±0.2). In these cultures the peak was reached between 24 and 48 hours.
NDV-induced IFN-α release was significantly higher ($p<0.01$) in C (1.5±0.3, 2.0±0.3, 2.0±0.3, 1.8±0.3) than in CD(0.8±0.2 and 1.0±0.3 at 24,48 and 72 hours).

Discussion

Activated LPMNC in CD are capable of spontaneously releasing IFN-γ as has been observed by alveolar mononuclear cell in lung sarcoidosis (6). The response to IFN-γ inducers of CD intestinal mononuclear cells differs from that of controls in terms of kinetics. CD mucosal lymphocytes may well be partially refractory to further stimulation in vitro. This observation may explain the decrease of IFN-γ mitogen induced production of LPMNC described in inflammatory bowel disease(7). The increased local release of IFN-γ by mucosal lymphocytes may account for the increased epithelial HLA-DR expression(4) and for the polyclonal B and T cell activation(3,8) observed in CD.
In CD the production of IFN-α by mucosal macrophages appeared to be defective. This finding suggest that a defective processing of viral particles by mucosal macrophages may have a role in the mucosal inflammatory response in CD.

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Interleukin 1β production by colonic mucosa from children with Crohn's disease

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INTRODUCTION

1. Introduction

Crohn's disease is a chronic relapsing inflammatory disorder involving the bowel, of unknown aetiology. The disease process involves abnormal cellular and humoral immunological mechanisms (1) and the affected mucosa is infiltrated by inflammatory cells. IL-1ß is important as a mediator of inflammation and initiator of the immune response (2). The aim of this study was to investigate IL-1ß production by colonic mucosa taken from children with CD, and compare this with production by control tissue both under basal culture conditions, and after stimulation with lipopolysaccharide (LPS).

2. Patients and Methods

2.1 PATIENTS

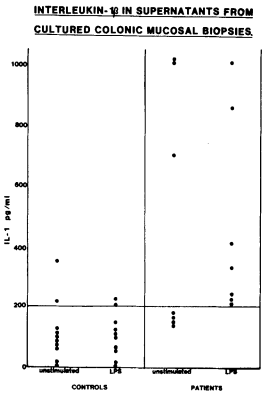
7 children with CD (5 male, 2 female; aged 8-17 years; median 13) and 10 control children (7 male, 3 female; aged 1-13 years; median 6) were studied. All children had chronic diarrhoea, the investigation of which included colonoscopy. Histology of colonic biopsies revealed active CD in all 7 patients, a mild non-specific colitis (NSC) in 4 controls, and normal mucosa in the other 6. Four patients were on steroid treatment.

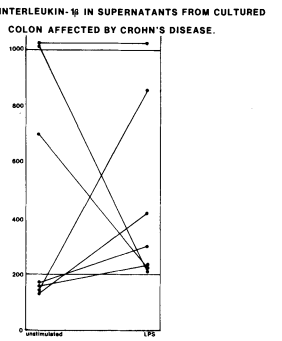
2.2 METHODS

Two extra colonic biopsies were taken at colonoscopy for this study. They were cultured for 24 hours in 2 ml of a culture medium RPMI (Gibco) with 10% fetal calf serum, glutamine (2mM), transferrin (5µg/ml) and antibiotics in multiwell tissue culture plates (Nunc). The supernatants, and tissue were collected separately and stored frozen. To measure IL-1 activity in supernatants, an ELISA assay (Cistron Biotechnology) was used. The detection range was 200-1000 pg/ml in our hands. The microtitration well plates were ready coated with monoclonal antibody specific for IL-1ß. Samples were incubated in duplicate, polyclonal rabbit anti IL-1 was added, then goat anti-rabbit IgG conjugated to horse radish peroxidase enzyme followed by the enzyme substrate. Colour intensity was measured using a microtitration plate reader. Frozen tissue sections

were stained with haematoxylin and eosin for histological assessment.

3. Results





IL-1ß concentration in supernatants is illustrated in Fig. 1-2. Three of 7 CD samples (with severe disease and on steroid treatment) produced enhanced concentrations of IL-1 under basal conditions and all 7 did so when stimulated. There was no detectable change in IL-1 production by control tissue when stimulated and the 2 positive results under basal conditions were obtained from patients with NSC. Histological examination revealed crypt attenuation in tissue from CD patients stimulated with LPS.

4. Discussion

IL1ß production by colonic mucosa was enhanced under basal conditions in children with severe, active CD, compared with controls. After stimulation ILIß production was found increased in all pathological samples. IL1ß is predominantly produced by activated macrophages but every cell with a potential accessory function (possibly including any epithelium in given circumstances) can be capable of secreting this lymphokine. It plays a pivotal role in the induction of lymphocyte activation, stimulation of inflammatory responses and in a variety of connective destructive process (2). It has been recently found to be cytotoxic for various tumour cell lines and suprisingly for ß cells of the pancreas (2) In our biopsies, an attenuation of crypt epithelium was seen in all samples after stimulation by LPS. Whether the IL1ß effect on colonic epithelial damage is direct or mediated by stimulation of immune/inflammatory mechanisms is not known.

We are interested in establishing whether autocrine production of IL1ß can occur by DR positive colonic enterocytes.

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Loss of vasoactive intestinal polypeptide immunoreactive nerve fibres in the colon of IBD patients

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The intestine is rich in peptidergic innervation, which modulates a variety of local phenomena, including mucosal immune reactivity (1). Among several gut neuropeptides, VIP has a well documented regulatory capacity over both cell-mediated and humoral immunity in the intestinal mucosa (2).

Abnormalities of intestinal VIP have been described in Crohn's disease (CD), including increased and decreased tissue concentrations of VIP in the mucosa, and morphological changes of VIP-positive nerve fibers in both involved and uninvolved segments (3,4). These studies were performed with a relatively small number of samples, without enumeration of total local nerve fibers, and without a systematic comparison between involved and uninvolved segments. Considering that CD may be caused by defects of intestinal immune reactivity (5), an abnormality of a potent immunoregulatory peptide such as VIP could be relevant to the pathogenesis of this condition.

The present study was designed to investigate possible defects of VIP immunoreactivity in all layers of bowel involved by CD, compare the number and distribution of VIP-positive nerves to the total number of local nerve fibers, determine the primary or secondary nature of the reported abnormalities, and to investigate whether such abnormalities are unique to CD or are present in other chronic inflammatory diseases of the gut, such as ulcerative colitis (UC).

Materials and Methods

Intestinal tissue was obtained from 34 disease control, 45 CD and 39 UC colonic specimens derived from surgical resections. Each tissue was evaluated for histological diagnosis and scored for degree of inflammation based on the density of inflammatory cells, presence of ulcers, cript abscesses and lymphoid follicles in the lamina propria.

Full thickness bowel tissue was fixed and nerve fibers were stained by the avidin-biotin peroxidase complex (ABC) technique using antibodies to VIP and S100 protein. Specificity of the anti-VIP antibodies was verified by complete elimination of staining by pre-incubation of the antiserum with VIP but not PYY. Quantitative analysis of VIP- and S100-immunoreactive nerve fivers was performed with a Bioquat II Digitizing Morphometry Program (Rand M Biometrics, Nashville, Tennessee). The total length of visible nerve fibers was measured and expressed as $\mu m/mm^2$, while the number of ganglion cells was measured in the submucosal and myenteric plexuses and expressed as number of cells/cm.

Statistical analysis was performed by one-way analysis of variance (ANOVA).

Results

Compared to controls, the lamina propria of CD and UC specimens contained significantly fewer nerve fibers immunoreactive for VIP (8969±763 vs 3273±442 and 1914±444 $\mu m/mm^2$, respectively; $p<0.0001$)) and S100 (9526±629 vs 5079±494 and 3775±571 $\mu m/mm^2$, respectively; $p<0.0001$). Similar findings were observed in the submucosa for VIP (714±65 vs 416±65 and 353±45 $\mu m/mm^2$, respectively; $p<0.0001$) but not S100 (969±63 vs 1016±90 and 994±106, $\mu m/mm^2$, respectively).

In the lamina propria and the submucosa, the linear density of VIP and S100-immunoreactive nerve fibers was significantly ($p<0.0001$) associated with the degree of inflammation, but not the type of inflammatory bowel disease. In uninvolved segments of CD and UC specimens the number and distribution of VIP- and S100-immunoreactive nerve fibers was comparable to those of controls.

Compared to control and CD, the number of submucosal ganglion cells immunoreactive with VIP was significantly decreased in UC tissues (44±4 and 54±4 vs 22±2 cells/cm, respectively; $p<0.0001$).

Measurements were not affected by the patient's sex, drug therapy, duration of disease, or region of the colon.

Summary and Conclusions

The results of our study show that the distribution and number of colonic nerve fibers in the lamina propria and submucosa of CD and UC tissues differ from those of histologically normal controls. The density of VIP- and S100-immunoreactive nerve fibers is related to the presence and the severity of inflammation, but not the type of inflammatory bowel disease (6). The effects of inflammation on the nerve fibers in the lamina propria differs from the effect in the submucosa. In addition, there is a decreased number of VIP-positive ganglion cells in the submucosal plexus of UC but not CD tissues.

Loss of VIP innervation in inflammatory bowel disease appears to represent a secondary and non disease-specific effect of inflammation. Thus, our study suggests that there are no primary abnormalities of VIP nerve fibers in the gut of CD patients. Although the encountered abnormalities are neither primary nor specific in nature, the intestinal immune events mediated by VIP-dependent effects are likely to be altered, and may contribute to the pathogenesis of inflammatory bowel disease (7).

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Characterisation of the anti-yeast *(S. cerevisiae)* antibodies associated with liver disease and inflammatory bowel disease

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Introduction

We have previously shown that the heat stable yeast opsonin associated with certain pathological sera was IgA anti-yeast mannan antibody (Yeaman & Kerr, Clin. exp. Immunol. (1987) 68, 200). These antibodies were characterised from the sera of 3 patients with liver disease. They provide a useful model system for study of the properties of serum IgA which has led to the isolation of an IgA receptor from human neutrophils. We now extend these studies to show that high levels of these antibodies are more common in Crohns disease than liver disease. We report their specificity and compare their activity with IgG anti-yeast mannan antibodies purified from the same sera.

Results and Discussion

We have assayed the levels of anti-yeast mannan antibodies in 600 pathological sera which showed some characteristics in common with those in which we first identified the heat stable opsonin. The results are summarised in Table 1

Disease group	n	FREQUENCY (%) IgG anti-mannan ELISA A405 normal	raised	high	IgA anti-mannan ELISA A405 normal	raised	high
Liver disease	312	77	22	1	89	8	1
Crohns disease	85	54	28	8*	78	15	7*
Ulcerative colitis	50	90	8	2*	96	2	2*
Immune complex	110	60	35	5	86	13	1
Controls	40	82	18	0	100	0	0

"Normal" = < 0.4 ; Raised = 0.4 - 1.4 ; High = >1.4

* The single patient with raised levels of IgG and IgA anti-mannan antibodies also had severe liver disease.

The results show that IgG but not IgA anti-mannan antibodies are frequently raised in normal sera. High levels of IgG anti-mannan antibodies can be detected in a range of pathological sera including liver disease, rheumatoid arthritis and infective endocarditis. High levels of IgA anti-mannan antibodies are less frequent but also found in a range of sera. Most noticeable are the high levels of IgG and IgA anti-mannan antibodies frequent in Crohn's disease which contrasts markedly with the low levels found in ulcerative colitis, except where there is accompanying liver disease.

On immunoblotting, the anti-mannan antibodies recognised purified yeast invertase and other high molecular weight mannoproteins of yeast cell wall. IgG and IgA antibodies showed similar specificity. They did not recognise other glycoproteins expressing high mannose structures such as ovalbumin, bovine ribonuclease, human IgM or complement component C3. Invertase is a dimeric mannoprotein of 270K with 50% of the weight being mannose attached as 18-20 asparagine linked units. The results suggest that the antigenic determinant includes the terminal alpha1-3 linked mannose residues, the major antigenic determinant identified from animal studies.

When six sera with high levels of anti-mannan antibodies were subjected to gel filtration on Superose 6 resin each was seen to contain both monomeric and dimeric IgA anti-mannan antibodies but in different proportions. IgG and IgA anti-mannan antibodies were purified from these sera by ammonium sulphate fractionation, ion exchange chromatography and affinity chromatography on Jacalin-Sepharose. No evidence has yet been found for the occurrence of IgA anti-mannan antibody which fails to bind to Jacalin.

IgA and IgG antibodies were both potent opsonins eliciting phagocytosis, respiratory burst and enzyme release from purified neutrophils. For assays where the neutrophils were in free suspension, IgA antibodies were the most potent; phagocytosis of yeast being stimulated only by those heat-treated sera with high levels of IgA antibody. Lucigenin enhanced chemiluminescence was induced to a similar amount from adherent neutrophils in the presence of yeast cell wall and either IgA or IgG antibodies.

For one patient where serial studies were made, there was a marked inverse correlation between heat stable opsonic activity (associated with IgA and IgG anti-mannan antibody) and the amount of IgG and IgA detected in circulating immune complexes. This does suggest the binding of the antibodies to antigen leading to complex formation. The occurrence of immune complex disease (fibrosing alveolitis and nephritis) during the period of the highest levels of immune complexes does suggest a possible pathological role for these complexes. The marked differences in the levels of anti-mannan antibodies in Crohn's disease and ulcerative colitis does suggest a possible use for their assay in the differential diagnosis of the two diseases.

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Involvement of the greater omentum of patients with Crohn's disease

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ABSTRACT. Mononuclear cells populating the greater omentum are morphologically characterized, their phenotype is closely related to mononuclear cells found in the lamina propria of the gut. Preliminar data on immunoglobulin secretion are presented.

Introduction

The greater omentum is populated by immunocompetent cells and plays an important role in inflammatory reactions in the peritoneal cavity. It can for instance relocate itself and cover intestinal perforation. The morphological and funcitonal properties of the lymphocytes present in the omentum are only marginally known. We isolated mononuclear cells from omentum specimens that have been won under surgery, and present data on the morphology and preliminar data on function of these cells.

Methods

Omentum specimens obtained surgically from patients with Crohn's disease or from patients undergoing elective cholecystectomy were washed and cut into small pieces with forceps. The fragments were digested enzymatically (48U/ml collagenase and 300U/ml DNAse at 37°C overnight) and the resulting cell suspension separated by density gradient centrifugation. The mononuclear cells were washed repeatedly and used for conventional immuno-fluorescence studies or cultured in RPMI 1640 medium containing 10% FCS and Pokeweed mitogen. After 7 days the supernatants were assayed for immunoglobulin content.

Results

The phenotype of mononuclear cells from the omentum differs from peripheral cells and is similar to that of cells derived from the intestinal mucosa. Monocytes are more frequent in the omentum (30 ± 4.5%) than in peripheral blood (10 ± 3%). CD4 cells are less frequent in the omentum (20 ± 2%) than in the peripheral blood (30 ±3.3%). CD8 cells are found as frequently in the blood as in the omentum. The CD4/CD8 ratio therefore is decreased in the omentum. Antigens associated with killer cells (Leu 7 and Leu 11) are more frequently present on mononuclear cells in the omentum. For all of the above antigens the pattern of distribution in the omentum versus the peripheral blood is the same in the normal controls as in patients undergoing surgery for Crohn's disease. A Crohn's disease-specific increase was found for the expression of HML1, T9-antigen and PNA-receptor in the affected omentum. 30 ± 5% of the cells were HML1 positive, only 2-4% of the peripheral cells and the cells from the normal omentum were positive. 30% of the Crohn's diseased omentum-cells were T9-positive, only 5-10% of peripheral cells and normal controls. PNA-receptor was elevated from 10% in the other groups to over 20% in the Crohn's diseased omentum. Looking at the antibody secretion of cells isolated from the omentum we found 5-fold higher levels of IgA and IgG in the supernatant of Crohn's diseased cells than in normal controls. IgM was only 2-fold increased in Crohn's disease.

Discussion

The phenotypical analysis of mononuclear cells populating the greater omentum shows the compartmental association between the omentum and the gut associated lymphoid tissue (GALT). Characteristic changes are found in the omentum of patients with Crohn's disease in that the expression of HML1, T9, and PNA-receptor is significantly elevated. This shows that the omentum takes part in the inflammatory process in Crohn's disease. The careful isolation procedure allows functional studies of omentum lymphocytes. We found markedly increased spontaneous secretion of IgA and IgG, as further evidence for the activation of omentum mononuclear cells in Crohn's disease.

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The effect of cigarette smoking on *in vitro* colonic immunoglobulin production in inflammatory bowel disease

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Introduction

Cigarette smoking is an important factor in the epidemiology of inflammatory bowel disease (IBD). Studies have shown that patients with ulcerative colitis (UC) tend to be non-smokers, while patients with Crohn's disease (CD) tend to contain more smokers than control groups [1]. Both peripheral and mucosal immunoglobulin secretion are altered in IBD, with the number and production capacity of the plasma cells responsible for IgG, IgM and, in particular, IgA production being altered [2]. Peripheral immunoglobulin production is also influenced by cigarette smoking, with smokers having a reduced serum level of IgA, G and M compared with non-smokers [3]. The aim of this study was to determine whether in vitro colonic mucosal immunoglobulin production is influenced by smoking habit in patients with IBD and in controls.

Patients and Methods

Details of smoking habit, and other social and clinical details, were obtained from patients attending a colonoscopy clinic. There were 26 patients with IBD (7 female), of whom 9 were smokers. A further group of 50 patients (22 females), of whom 17 were smokers, and were subsequently found to have a normal colon, acted as a control group.

Colonic biopsies were removed from the descending colon and preformed and post-cultural (48 hours) levels of immunoglobulins A, G and M were measured in both the biopsy tissue and in the tissue and culture medium. The immunoglobulins (ng/mg protein) were measured by an established ELISA technique [4]. The amounts of immunoglobulin measured in the tissue and culture medium were combined to give an absolute value, and the amount of newly synthesised immunoglobulin calculated by subtracting the preformed value from the absolute value.

Results

Newly synthesised IgA was increased in IBD, but IgG and IgM levels remained unchanged (Table). Analysis according to smoking habit showed that newly synthesised IgA was reduced in both groups of smokers, particularly in those with IBD. The effect of smoking on IgG was the reverse, with smokers having an increased IgG production, again especially in those with IBD. The picture for IgM was more complicated, with smokers with IBD having reduced levels of IgM compared with non-smokers, while smoking controls had a significantly increased level compared with non-smokers ($p<0.05$) (Table).

TABLE. Newly synthesised immunoglobulins (ng/mg total protein)

Patient group (n)	IgA Mean	IgA SEM	IgG Mean	IgG SEM	IgM Mean	IgM SEM
Controls (50)	12.6	3.1	15.8	3.0	1.1	0.4
IBD (26)	27.8	12.0	12.4	5.3	1.6	1.2
Controls						
Smokers (17)	6.4	1.7	17.9	5.8	2.2*	1.1
Non-smokers (33)	15.8	4.5	14.7	3.6	0.5	0.2
IBD						
Smokers (9)	7.0	4.0	25.9	13.4	0.4	1.1
Non-smokers (17)	38.8	17.9	5.3	3.1	2.2	1.8

* $p<0.05$

Discussion

The present results showed that smoking habit reduced IgA production, both in controls and especially in IBD, while the reverse was true for IgG production, and the effect on IgM production differed according to diagnosis. Cigarette smoking appears to influence local intestinal immunoglobulin production, which may be of importance in the pathogenesis of inflammatory bowel disease.

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Common antigenic determinant on ductular cells of normal pancreas, on mucosal cells of the gastrointestinal tract and on CEA

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1. Introduction

CEA is widely used as a human tumour marker and was first defined by Gold and Freedman in 1965 as an antigenic component in cancers derived from gastrointestinal tract epithelium. It is a member of a large family of immunologically related glycoproteins that vary in size and tissue distribution. Studies with c-DNA clones for CEA and NCA reveal that this family consist only of a limited number of different proteins with variable glycosylation, due to post-transcriptional modifications. The complete gene family includes about 10 closely related genes. Recently it was published that in contrast to mRNA coding for CEA, mRNA coding for NCA was expressed predominantly in cancerous tissues. A monoclonal antibody recognizing an epitope expressed on both CEA and NCA could therefore be a useful diagnostic reagent.

2. Materials and Methods

Monoclonal antibody 82-F19 was established after immunizing mice with human breast tumour cell line MCF-7 and fusion of spleen cells with mouse myeloma cell line P3-NS1/1-Ag 4-1. Antibody binding to different cultured human tumour cell lines and immunohistochemical studies on fresh frozen tissue sections were performed by the indirect immunoperoxidase method. For biochemical analysis proteins of 82-F19 positive tumour cells were solubilized with 1% CAPS, fractionated by SDS-gel electrophoresis of HPLC-separation according to size (column Du Pont GF-450, Zorbax). Antigen detection was performed by Western blot analysis or by coating fractionated proteins after molecular weight exclusion chromatography to microfluor W plates (Dynatech, Alexandria, VA) and screening fractions by ELISA technique as described earlier. For detection of 82-F10 antigen in the sera of patients with gastrointestinal tumours, 82-F19 antigen was purified from tumour cell line HT-29 (electroelution of the positive 100 kD band after SDS-gel-electrophoresis) and mAb 82-F19 reactively was tested after preincubation with sera of tumour patients and healthy donors, by an inhibition ELISA. Positive sera blocked mAb 82-F19 binding to the purified antigen.

3. Results

Immunohistological analysis performed on fresh frozen tissue sections show that mAb 82-F19 identifies a tumour-associated protein of the gastrointestinal tract. This antigen is expressed on numerous gastrointestinal tumours especially on human pancreatic adenocarcinoma. Most of the tumour cells in 5/5 pancreatic adenocarcinoma reacted with this antibody. In the normal pancreas only the ducts are detected by this mAb. The

antigen was also expressed on the cell surface of in vitro cultured gastroinestinal cell lines (pancreatic, stomach and colon cancer cell lines). Detergent solubilization of proteins iof these tumour cell lines, molecular weight exclusion chromatography, SDS-gel electrophoresus and Western blot analysis reveal that the molecules recognized by this mAb are proteins of about 200 and 100 kD. Using HT-29 colon cancer cells as antigen source the 100 kD band was detectable only. As shown by Western blot analysis and ELISA tests the mAb reacted with purified CEA. Therefore we tried to establish a sensitive inhibition ELISA test system to detect CEA in the sera of tumor patients. Our results were in good agreement with the results obtained by a commercial CEA-test-kit. In the group of 15 patients with pancreas carcinoma we were able to detect one additional and in the group of 15 patients with stomach carcinoma we detected two additional positive sera, which could not be assessed positivelywith the commercil CEA-test-kit.

4. Discussion

Based on the results obtined by Western blot analysis and positive staining of granulocytes, mAb 82-F19 presumably recognized an epitope which is expressed on both CEA and NCA. Therefore the antibody may be a useful reagent for clinical diagnostics. The epttope recognized by mAb 82-F19 may also be relevant in view of the close association between ductular epithelia in the normal pancreas and the tumour cells in pancreatic adenocarcinoma. These results emphasize the antigenic heterogeneity of CEA, which belongs to the immunoglobulin supergene family.

This CEA-epitope may therefore represent a common determinant of at least some members of the CEA-family. It also appears to be relevant in view of the close association between ductular epithelia in the normal pancreas and the tumour cells in pancreatic carcinoma. Supported by BMFT DI/KN O1GA 054/6.

SECTION V: FOOD ALLERGY AND INTESTINAL HYPERSENSITIVITY

(A) Studies in humans

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Cow's milk protein in human milk. Quantification, kinetics of appearance, size distribution and relation to atopy

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1. INTRODUCTION

Allergy to substances from cow's milk in the human breast milk has been recognized for about 70 years [1]. Also, egg antigens were detected in breast milk by a Prausnitz-Küstner test [2]. More recently, several authors have demonstrated dietary antigens such as ovalbumin from egg [3] and β-lactoglobulin (BLG) from cow's milk [4,5] in the breast milk. Increased levels of BLG were observed [4,6] in the mother's milk given to infants with cow's milk allergy (CMA).

We have [7,8] investigated the uptake of dietary antigens to the mother's milk in relation to CMA in breast-fed infants and atopy in their mothers. The kinetics for the appearance of the antigen in relation to the type of cow's milk consumed by the mother and the BLG size distribution in the mother's milk was also investigated.

2. MATERIALS AND METHODS

2.1. Study designs

Study 1: Milk from 9 mothers, with exclusively breast-fed infants suffering from CMA was obtained 4 h. after the intake of 500 ml of commercial cow's milk. The diagnosis of CMA was made by keeping the mother on a milk-free diet for 4 weeks followed by at least one challenge with cow's milk protein via the mother's milk. From 6 of the mothers a milk sample was additionally collected after 4 weeks on a diet free of cow's milk. Milk samples were also obtained from 10 mothers with healthy children.

Study 2: Ten healthy mothers and 10 mothers with atopy (asthma, rhinitis or atopic dermatitis) all of whom breast-fed their babies took part in the study with consecutive sampling of their breast milk. The women for one week had a daily intake of at least 1/2 l of cow's milk per day. After the intake of 500 ml of cow's milk the women abstained from cow's milk for 24 h. and breast milk was collected at

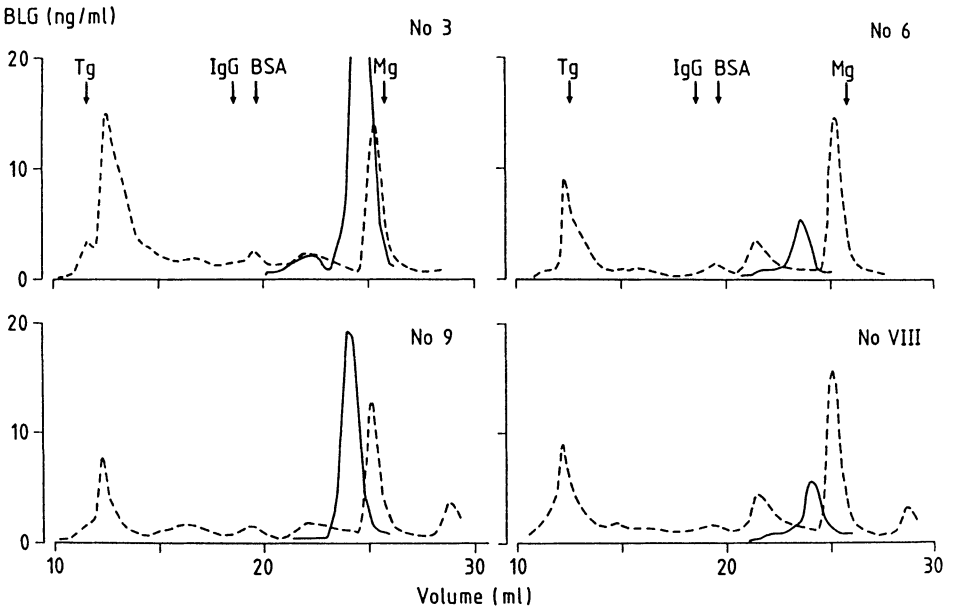

Figure 1. Estimation by HPLC-ELISA of the size distribution of β-lactoglobulin (BLG) in human milk. Samples obtained from three women with children allergic to cow's milk (nos. 3, 6 and 9) and one women (no. VII) with a healthy child. The elution volumes of thyroglobulin (Tg), IgG and myoglobin (mg).

0, 4, 8, 16, and 24 h. This scheme was repeated for two more weeks. The cow's milk consumed during these three consecutive weeks followed by the sampling of breast milk for 24 h. was either homogenized milk for two weeks and one week with un-homogenized milk or vice versa. The milk samples were frozen at -60°C and defatted before analysis.

2.2. ELISA analysis for BLG

The BLG concentrations in the defatted milk samples were determined by an ELISA described in detail previously [9]. All standards and controls were prepared in human milk devoid of BLG. The coefficient of variation (CV%) were in the present studies estimated from control samples at 10 μg/l and 3 or 1 μg/l of human milk. The between-days CV% were 14, 22 and 29, respectively, and the within-run CV% were 6, 7 and 7, respectively.

High performance liquid gel permeation chromatography was combined with ELISA analysis for BLG (HPLC-ELISA), as described [9]. A Toyo Soda TSK G 3000 SW size separation column was used for fractionation of the human milk samples. To determine elution volumes purified protein markers were used: thyroglobulin (Tg, M_r 6.6 kD) appearing in the void volume and IgG (M_r 1.5 kD) and myoglobin (Mg, M_r 1.7 kD).

3. RESULTS

3.1. Study 1: BLG was measurable by ELISA in 3 out of 9 milk samples from mothers with exclusively breast-fed infants with CMA, and in 1/10 samples from the mothers with healthy infants. The maximal concentration was 45 µg/l. After 4 weeks on a diet devoid of milk, the breast milk in one case still contained BLG, albeit at a lower concentration.

All samples were further investigated by HPLC-ELISA. The size of the BLG in the 4 milk samples with BLG detectable by direct application of ELISA (fig. 1) corresponded to intact monomeric BLG (18 kD). No BLG was revealed in the other samples.

3.2. Study 2: Five human milk samples were obtained during 24 h. at three consecutive weeks. The milk from 19/20 mothers contained measurable amounts of BLG. The maximal values (range 0.9-150 µg/l) were reached from 4-24 h. after the intake of 500 ml of cow's milk. The time course for the appearance of BLG in the breast milk is depicted (fig. 2) for two allergic (a and b) and two non-allergic mothers (c and d), showing one subject with a slow appearance of BLG in each group. BLG from the previous intake of milk was observed during the 24 h. sampling period in the women with a slow appearance of BLG in their milk. No differences were observed either after the consumption of homogenized or un-homogenized milk, or between the BLG levels in the breast milk from allergic and non-allergic mothers.

4. DISCUSSION

Our studies have focused on the characterization of the uptake of dietary antigen into the breast milk. BLG was demonstrable in the breast milk from virtually all (19/20) of the women. The kinetics of BLG uptake was seen to vary widely, with maximal levels at 4-24 h. after the last intake of cow's milk. In a number of cases with a slow uptake of BLG the 24 h. period of collecting the breast milk may have been too short to observe the peak levels of BLG. Also, the concentrations of BLG in the breast milk varied markedly, from 0.9-150 µg/l. During the 3 weeks of the study the maximal BLG levels were rather constant in the individual subject. No differences wer observed between women receiving homogenized or un-homogenized cow's milk, respectively, speaking aginst a major influence by the type of milk on the uptake of BLG into the blood.

We estimated the size distribution of BLG in the breast milk and demonstrated a uniform distribution in the four samples, corresponding to intact, monomeric BLG. This observation corresponds to the demonstration of intact ovalbumin in the breast milk, as estimated from column chromatography [3]. Thus, the antigens probably do not occur in the milk as antigen-antibody aggregates, in contrast to the findings of ovalbumin in serum with similar techniques [9,3]. Interestingly, antibodies to cow's milk proteins are frequently observed in the breast milk [10,5].

Previous work has mainly concentrated on the occurrence of dietary

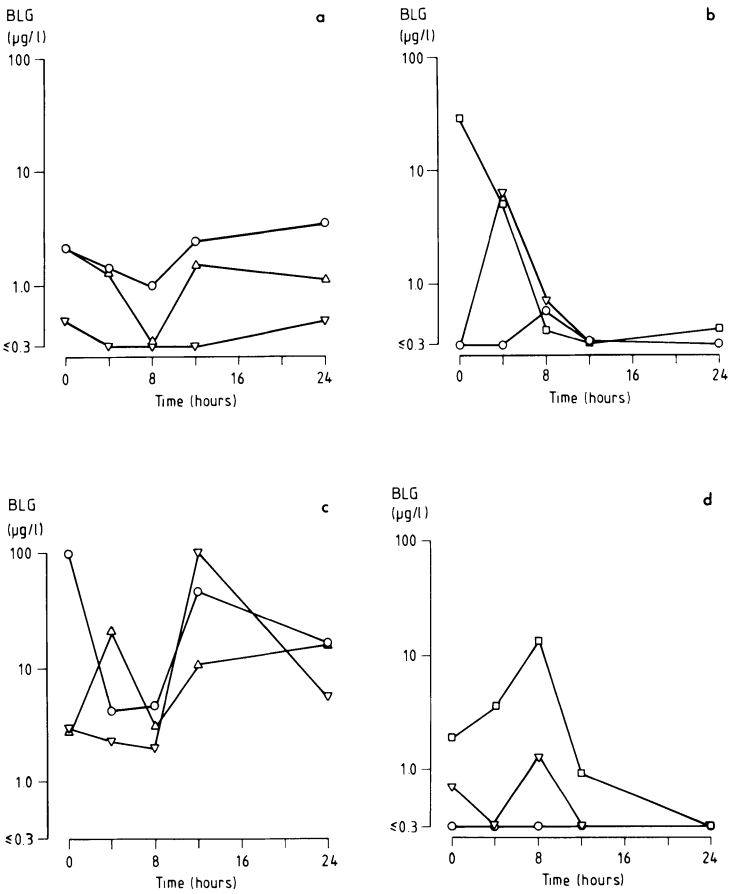

Figure 2. Typical examples of the time course for appearance of β-lactoglobulin (BLG) levels in the breast milk, obtained up to 24 h. after the intake of 500 ml of cow's milk. (a and b) atopic women, (c and d) healthy women. O and □ denotes the intake of homogenized milk, and ▽ and △ denotes unhomogenized milk.

antigens in breast milk in relation to allergy. A longitudinal study did show a certain constancy of the BLG levels in the milk from individual women during the whole lactation period [6]. A positive correlation between high levels of BLG in the breast milk and CMA and infant colic was indicated [6]. Our study of the milk from 9 mothers with infants suffering from CMA did not provide support for this hypothesis, since only 3 milk samples contained detectable BLG 4 h. after the intake of cow's milk. In another study [10] ovalbumin was detected at equal levels in about 75% of 19 milk samples given to fully breast fed infants with or without atopic eczema and positive prick tests to egg, speaking against a quantitative role of dietary antigen in this condition. Our study [8] of milk from 10 atopic and 10

healthy mothers showed no differences between the two groups, so atopic mothers do not per se deliver greater quantities of BLG antigen to their babies. However, all their infants remained healthy.

An obvious extension of the present work would be to compare directly the BLG concentrations obtained in the breast milk with the amounts in the circulation, to indicate whether secretion of dietary antigen occurs into the breast milk. Furthermore, in order to obtain reliable peak levels of antigen such as BLG in the breast milk a study with consecutive sampling of the breast milk given to babies with CMA and healthy controls seems warranted.

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Tri-phasic response of coeliac small intestinal mucosa to challenge with Frazer's digest (FF3) of gliadin

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ABSTRACT. In order to trace, in greater depth, the sequence of events involved in mucosal flattening, small groups of well-treated coeliac patients were challenged orally with FF3, in doses ranging from 0.1, 0.5, 1.0, 1.5, 3, 6 to 12G. Per-oral jejunal biopsies were performed with the Watson capsule pre-challenge, and at 12, 36, 60 and 84h post-challenge. Mucosae were processed to epon, and 1μm toluidine blue-stained sections were analyzed by computerized image analysis. Specimens were measured in terms of volumes of surface (V_{SE}) and crypt (V_{CR}) epithelium, and their corresponding populations ($N_{V,SE}$:$N_{V,CR}$) of intra-epithelial lymphocytes.

With doses of 0.1 through 1.5G FF3, a dose-related, time-dependent (12h) increase in small non-mitotic lymphocytes within villous epithelium occurred, but without any accompanying alteration in mucosal architecture. At a higher challenge dose (3G FF3) infiltration of villous epithelium occurred, together with lymphoid infiltrates into crypt epithelium. In addition, mucosal architecture was now altered by the appearance of crypt hyperplasia at 12 and 36h post-challenge. There was no reduction in villous height. Villous flattening only occurred with the two largest challenge doses of FF3 (6 and 12G) and was maximal at 60-84h with 6G, and at 12h with 12G FF3. In all cases, crypt hyperplasia preceded villous flattening when analyzed either in terms of (i) time-response per-challenge group or (ii) dose-response throughout the entire series of groups challenged.

Conclusions: 1. This sequence of changes (infiltrative -- hyperplastic -- destructive) is similar to that seen in experimental graft-versus-host reactions, in which crypt hyperplasia always precedes villous effacement. 2. It appears that in order to become flat, the mucosa must pass through the earlier phases of lymphocytic infiltration and crypt hyperplasia. 3. The lesions seen in coeliac disease, as revealed by these experimental changes, are fully consistent with a cell-mediated immune reaction within the small intestinal mucosa to dietary gluten.

1. Introduction

The manner, duration and mechanism(s) whereby coeliac jejunal mucosa becomes flattened are not well understood. That the evolution of the classic flat coeliac mucosa occurs over several years has only been recorded once (1). In becoming flat, it appears to be assumed that the mucosa becomes infiltrated with lymphocytes and plasma cells and that surface enterocytes are destroyed, resulting in progressive villous effacement. To compensate, there is progressive crypt hypertrophy and an increased rate of production, and transit, of enterocytes from the crypts to the now flattened surface epithelium (2).

This view forms the so-called 'haemolytic' model of mucosal damage, the crypt hypertrophy being due solely to increased enterocyte loss in much the same way that bone marrow hypertrophy compensates for increased destruction of circulating erythrocytes in haemolytic anemias (3).

One way to address the mechanism of mucosal flattening is by gluten-challenge. In order to approach this problem systematically, we challenged small groups of treated coeliac patients with a series of graded oral doses of gliadin (peptic-tryptic) digest (4). At each dose level, the effects on mucosal architecture were observed for five days, thus providing a fairly comprehensive view of the likely sequence by which mucosal flattening is brought about.

2. Methods

Crude commercial gliaden (Sigma) was sequentially digested with pepsin and trypsin, as originally described by Frazer (4). For control purposes, 500mg B-Lactoglobulin (BDH) was given as a single dose challenge, but neither control nor coeliac subjects showed any resulting immunopathologic response in jejunal mucosa: those data are not discussed further.

Challenge subjects were drawn from proven coeliac disease subjects who had responded well to a gluten-free diet. Mucosal biopsies were obtained by Crosby Capsule, rapidly fixed in cacodylate-buffered glutaraldehyde, processed to epon, sectioned at 1µm and stained with toluidine blue.

Appropriate sections were analysed by computerised image-analysis (5,6) as described in detail elsewhere. Briefly, mucosae were quantitated in terms of villous (or surface) epithelial volumes (V_{SE}) and crypt epithelial volumes (V_{CR}), with reference to a constant test area of muscularis mucosae ($10^4 \mu m^2$). The total number of surface (sIEL) and crypt (cIEL) per specimen was calculated from determinations of (i) mean nuclear lymphocytes diameter (D_N) per specimen (7) and (ii) counts of sectioned nuclear profiles contained within their respective volumes of surface, or crypt, epithelium.

3. Results

With small oral challenges (0.1 - 0.5G of peptic-tryptic digest) a dose-dependent accumulation of IEL into surface epithelium was seen within 12 hours of digest ingestion, and had largely waned thereafter. The influx of surface intraepithelial lymphocytes (sIEL) was unaccompanied by alterations in either surface (V_{SE}) or crypt (V_{CR}) epithelal volumes, ie the mucosa had not flattened. With 1.5g FF3, but not with any of the three smaller doses, an increase in crypt intraepithelial lymphocytes (cIEL), maximal at 12 hours post-challenge, occurred.

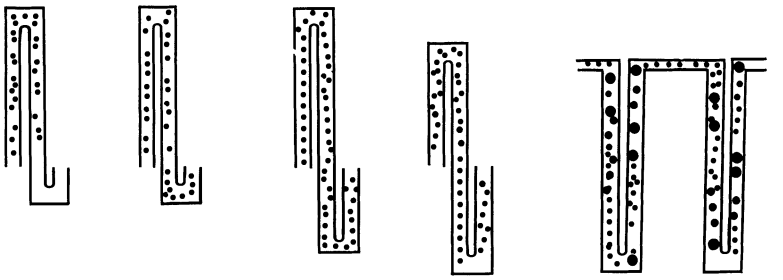
During the 3G challenge, there were no statistically significant alterations in villous height. Despite that, there was a consistent increase in crypt epithelial volumes to twice that of pre-challenge values, and which now lay within the reference range for flat (untreated) coeliac mucosae (8). At this dose, cIEL remained elevated throughout the challenge, while the dose response in sIEL peaked only at 12 hours.

With 6g challenge, the changes exaggerated those seen with 3G with V_{SE} falling below the lower range for normal villi at 60-84 hours post-challenge. The percentage rise in sIEL over pre-challenge control values was 95% (12-36hours) but at 60 & 84h when V_{SE} fell, sIEL also fell. On the contrary, crypt hypertrophy persisted throughout the challenge series (12-84h) accompanied by a substantial, and sustained, rise in cIEL.

After a 12G digest challenge, villus flattening into the coeliac range was evident by 12h post-challenge and was accompanied by a sharp fall in sIEL. This effect gradually reversed throughout the challenge series, as V_{SE} gradually increased to pre-challenge values by 84h. Crypt hypertrophy was evident at 12h and persisted throughout together with the highest and sustained increment in cIEL.

4. Discussion

It is clear, both from the use of computerized image-analysis, and the clinical approach in which 4 post-challenge mucosae were obtained in each of the 7 challenge-doses per group of treated coeliac patients, that there is a tri-phasic response of the intestinal mucosa to gluten challenge (Fig.1).



INFILTRATIVE ➡ HYPERPLASTIC ➡ DESTRUCTIVE

Figure 1. This diagram illustrates the tri-phasic evolution of a flat mucosa after gluten-challenge. Initially there is lymphoid infiltration of epithelium, followed by crypt hyperpasia which, in turn, is followed by loss of villous height and abnormalities in villous (surface) epithelium together with persisting crypt hypertrophy.

Initially, the mucosa exhibits an INFILTRATIVE phase in which small, essentially non-mitotic intraepithelial lymphocytes accumulated in a dose -, and time -, dependent fashion (doses 0.1 to 1.5G FF3) within the villous epithelium and, with 1.5G FF3, within crypt epithelium. With larger challenges, crypt hypertrophy became evident, and this second HYPERPLASTIC lesion of the crypts preceded any villous flattening: indeed, in the 3G challenge, no statistically significant loss of villous volume (V_{SE}) occurred, despite a doubling of V_{CR} at 12h post-challenge. Following 6G challenge, there was an initial hyperplastic lesion with enlarged, infiltrated crypts and normal villi whose epithelium also carried infiltrates of IEL. At later times (60-84h), while crypt hypertrophy and infiltration persisted, villi became smaller which coincided with a fall in sIEL. At 12G, complete villous flattening occurred 12h post-challenge, the lesion revealing all the features of the classic flat, or DESTRUCTIVE lesion (8).

There is a marked parallelism between this sequence of events, and those established in greater detail in experimental models of villous flattening, like allograft rejection and graft-versus-host reactions (GVHR) in which MHC class 2+ effector lymphocytes initiate similar immunopathologic phases. Such experiments have indicated that (a) intestinal damage is dose-related to the degree of MHC disparity (b) crypt hypertrophy is an early event, following close on the infiltration of normal-sized villi by IEL and that (c) villous flattening is late although T lymphocyte-dependent (9,10).

When viewed within the wider context of gluten-sensitivity (rather than untreated coeliac disease alone), the infiltrative lesion is also seen in some first degree coeliac relatives (11) and in patients with dermatitis herpetiformis with normal villi (12). However, some of the latter demonstrate crypt hypertrophy additional to infiltration of surface and crypt epithelium by IEL (13). Thus, the infiltrative and hyperplastic lesions are seen in other individuals in whom sensitivity to gluten is, or maybe, relevant. Thus, whether viewed statically, or dynamically during gluten challenge, it seems clear that to become flat (ie develop a destructive type lesion) the mucosa must initially and progressively evolve through the (i) infiltrative and (ii) hyperplastic

phases. The method of study, in relation to gluten challenge, is amenable to interpretation both in terms of a time-response, and dose-response. Furthermore, the challenge technique employed is equally useful in diagnosing gluten-sensitivity in circumstances in which there is, or has been, diagnostic uncertainty. The challenge technique, together with the proven method of computerised image-analysis of mucosal specimens, also provides the means of evaluating other enteropathies of presumed cell-mediated type that affect the human small-intestine (14).

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Soluble interleukin-2 receptor (sIL-2R) in coeliac disease

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Introduction

Activation of T lymphocytes with mitogens or specific antigen induces the synthesis of interleukin-2 (IL-2) and the expression of specific cell surface receptors (IL-2R) for this growth factor [1]. The IL-2 receptor consists of at least two IL-2 binding polypeptides with different binding affinities [2]. In vitro studies [3] have shown that the low affinity, 55 Kd chain of the IL-2R (the 'Tac' protein) is shed from activated T cells in a soluble form (sIL-2R). Activated macrophages [4] also express IL-2R and stimulated B cells will release small amounts of soluble IL-2R in vitro [5]. Soluble IL-2R is detectable in the serum of normal individuals and raised circulating sIL-2R concentrations occur in conditions characterised by increased immune activation [6 - 8]. In coeliac disease, the small intestinal mucosa of untreated patients contains lymphocytic and plasma cell infiltrates [9] and there is immunohistlogical [10] and functional evidence [11] of increased mucosal lymphocyte activity. As serum sIL-2R levels appear a useful marker of in vivo immune activation, we have examined patients with coeliac disease to establish whether gluten exclusion and gluten challenge affects serum sIL-2R concentrations.

Methods

33 patients with coeliac disease who were having jejunal biopsies for diagnostic purposes were studied. Sera were stored at -70°C until assayed. 15 patients were untreated and on normal diets at the time of investigation. 14 had subtotal villous atrophy and one severe partial villous atrophy of the jejunal mucosa. 14 of the untreated patients have, to date, shown histological improvement on gluten-free diets. 24 treated coeliac patients were studied, 10 of whom were

among those investigated whilst on a normal diet. The treated coeliac patients had been on gluten-free diets for a minimum period of two months.

Jejunal biopsies of 7 treated coeliac patients were normal, 15 had partial villous atrophy and 2 severe partial villous atrophy. A symptom control group of 15 patients age- and sex-matched to the untreated coeliac patients who underwent a jejunal biopsy to exclude coeliac disease was examined. The jejunal mucosa of patients in this group was histologically normal. Additionally, a non-symptomatic group of sex- and age-matched volunteers was studied.

16 patients with proven coeliac disease, who had been on a gluten-free diet for a minimum of six months, were given a gluten challenge consisting of 30 grams of gluten (BDH) per day for 7 days. Blood samples were taken for sIL-2R measurement immediately before gluten challenge, after 7 days' gluten ingestion and 4 weeks after reinstatement of a gluten-free diet. A control group of 13 healthy volunteers, 6 of whom were HLA-B8 positive, ingested 30 grams of gluten daily for 7 days in addition to their normal diets.

Serum soluble IL-2R concentrations were measured by an ELISA using two non-competing monoclonal antibodies to the 55Kd chain of the IL-2R as previously described [6].

Results

Serum levels of sIL-2R in untreated coeliac patients (Table 1) were significiantly greater than those of treated coeliac patients, symptomatic and healthy control groups ($p<0.001$). The sIL-2R concentrations in the treated coeliac patients, while lower than those of the untreated patients, were still significantly elevated relative to both control groups ($p<0.001$).

TABLE 1. Serum soluble IL-2R concentrations

	n	Units/ml (mean ± SEM)
Untreated coeliacs	15	1324 ± 223
Treated coeliacs	24	519 ± 60.3
Symptom controls	15	249 ± 22.4
Healthy controls	15	186 ± 25.9

The sIL-2R levels in the 10 coeliac patients examined both before (1590 ± 289) and after (727 ± 97) commencement of a gluten-free diet

were significantly lower ($p<0.01$) following treatment. The minimum length of gluten exclusion in this group was two months and the maximum 14 months. Examination of serum sIL-2R levels in four 'presumed' coeliac patients presenting with villous atrophy during the first four weeks of treatment (Table 2) showed that in 3 patients (A-C) with raised serum sIL-2R levels prior to dietary treatment, marked reductions in sIL-2R occurred within 2 weeks of gluten exclusion. (The fourth patient (D), whose untreated sIL-2R concentrations were unusually low relative to other untreated coeliac patients, was a patient with long-standing dermatitis herpetiformis and psoriasis.)

TABLE 2. Serum sIL-2R levels following commencement of a gluten-free diet (GFD) (Units/ml)

	PATIENTS			
Weeks GFD	A	B	C	D
0	850	430	750	50
1	690	340	380	50
2	170	170	-	100
4	90	-	240	50

The effect of one week's gluten ingestion on serum sIL-2R levels in treated coeliacs and controls is shown in Table 3. The control group, 6 of whom were HLA-B8-positive, showed no significant change in serum sIL-2R concentrations following gluten ingestion for one week. There was no significant difference in response between HLA-B8-positive and negative subjects. In contrast, gluten ingestion for one week significantly increased ($p<0.025$) serum sIL-2R levels in 16 patients with treated coeliac disease (Table 3) and reversion to a gluten-free diet for 4 weeks resulted in a significant reduction ($p<0.01$) in sIL-2R concentrations to below pre-challenge levels.

TABLE 3. Changes in serum sIL-2R levels in treated coeliac patients and controls on gluten challenge (Units/ml)

	pre-challenge	1 week gluten	4 weeks GFD
Coeliacs	423 $\pm$ 54.5	498 $\pm$ 64.7	367 $\pm$ 59
Controls	84.2 $\pm$ 11.3	68.4 $\pm$ 10.6	-

Discussion

A primary event in the antigen-induced activation of T lymphocytes is the secretion of IL-2 and the expression of cell surface IL-2 receptors. The release of the IL-2 receptor from activated cells [3] and the serological measurement of sIL-2R levels permits evaluation of in vivo immune activation. The serum sIL-2R concentrations in untreated coeliac patients were significantly higher than treated patients and both control groups. The reduction in serum sIL-2R following gluten exclusion occurred within one week in 3 patients (Table 2). The low sIL2R values in the patient with dermatitis herpetiformis, even prior to gluten exclusion, might be attributable to the immunomodulatory effects of Dapsone treatment [12].

The significant increase in serum sIL-2R levels in the treated coeliac patients following one week's gluten ingestion demonstrates a rapid immunological response to gluten. The sIL-2R values, however, did not reach those observed in untreated coeliac patients, suggesting that the high levels in these patients are a result of long-term immunostimulation. The serum sIL-2R levels in patients with coeliac disease therefore appear to reflect specific immunological activation in response to gluten ingestion and the results are supporting evidence for the role of T lymphocytes in the pathogenesis of the coeliac lesion.

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Lymphocyte activation as measured by IL-2 receptor expression to gluten fraction III in coeliac disease

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INTRODUCTION

Coeliac disease is characterised by a hypersensitivity reaction of the small intestine to cereals containing gluten, which results in intestinal damage. Peptic digests of gluten, such as gluten fraction 111, are toxic in coeliac patients. The mechanism causing damage to the small intestine in coeliac disease has not been fully elucidated, but is thought to involve an abnormal immune response to the gliadin component of cereals.

Evidence of systemic lymphocyte activation in the peripheral blood of coeliac subjects is very limited. Peripheral blood T lymphocytes from coeliac subjects are hyporesponsive to gluten stimulation as measured by either proliferation or skin testing for a delayed-type hypersensitivity (DTH) reaction. Despite systemic T lymphocytes hyporesponsiveness to gluten as measured above, the cells may nonetheless be sensitised to gluten, even in the absence of in vitro proliferation.

Activation antigens define the state of activation of human mature lymphocytes, they are not expressed on resting lymphocytes and can be detected using monoclonal antibodies on the cell surface. We have therefore investigated systemic lymphocyte activation using interleukin-2 receptor expression, as measured by an ELISA technique on lymphocytes from coeliac and normal subjects after sensitisation to gluten fraction 111.

SUBJECTS AND METHODS

A total of six adult coeliac patients were included in this study, along with six normal controls. All coeliac patients were on a gluten-free diet.

LYMPHOCYTE ACTIVATION BY ANTIGEN

Nylon wool enriched T lymphocytes adjusted to $1x10^7$/ml in complete medium were obtained from either coeliac patients or control subjects. The cells were cultured (0.1ml aliquots) in flat bottomed 96 well tissue culture plates. Experimental cultures were stimulated with 100ug/ml of F111. Control wells contained cells alone without antigen. Plates were incubated at 37°C in 5% CO_2 for 1 to 5 days before assaying for interleukin-2 receptor expression.

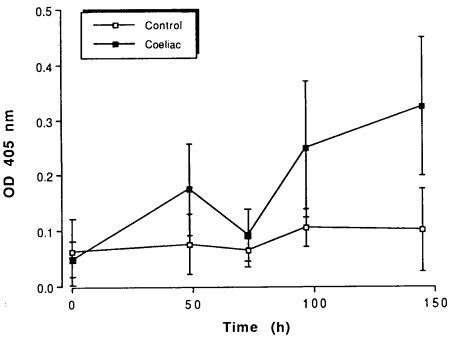
PROCEDURE FOR THE INTERLEUKIN-2 RECEPTOR CELL ELISA ASSAY

After incubation plates were centrifuged at 500g for 5 minutes. The cells were fixed with glutaraldehyde and an alkaline phosphatase ELISA was performed using a mouse anti human interleukin-2 receptor (Dakapatts, Glostrup, Denmark)

RESULTS

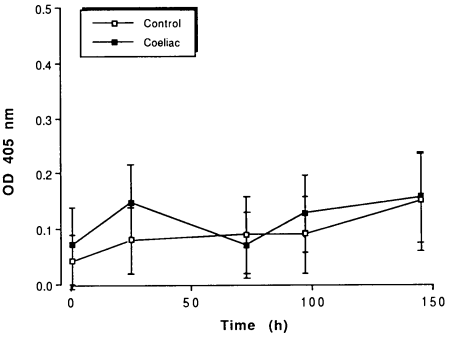
Measurement of T cell activation marker, interleukin-2 receptor expression by ELISA. Interleukin-2 receptor expression peaked on days 5-6 in response to gluten fraction 111 in lymphocyte cultures from coeliac subjects (Figure 1). In contrast, lymphocytes from normal controls when stimulated with F111, showed no detectable increase in interleukin-2 receptor expression. No increase in interleukin-2 receptor expression was detected on lymphocytes from unstimulated cultures from either coeliac or control subjects (Figure 2).

Figure 1.



Interleukin-2 receptor expression by T lymphocytes of coeliacs and normal subjects with 100 mg/ml gluten fraction 111.

Figure 2.



Interleukin-2 receptor expression by T lymphocytes of coeliacs and normal subjects after culture in media alone.

DISCUSSION

We have used an ELISA technique similar to that reported by Igiestseme and Herscowitz (1987) for mouse lymphocytes, to measure the activation of gluten-stimulated lymphocytes from coeliac patients, based on the change in expression of a T cell surface activation marker.

Quantitation of interleukin-2 receptors on lymphocytes from coeliac subjects after gliadin stimulation is a sensitive measure of lymphocyte activation in the peripheral blood. In this study, we have shown that in the peripheral blood of coeliac patients lymphocytes are specifically activated after exposure to gluten fraction 111 to show an increased expression of interleukin-2 receptors.

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Increased jejunal secretion of PGE_2 after local gliadin challenge in patients with coeliac disease

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Background

Prostaglandins have been implicated in the pathophysiology of diarrhoea in coeliac disease (1,2,3) . We have used a double-balloon catheter system, which allows the perfusion of a defined jejunal segment (4), to measure PGE_2 in jejunal secretions.

Material and methods

Thirteen patients with coeliac disease were studied and ten of them were challenged with gliadin in the perfused bowel segment. Eighteen healthy controls were also investigated with jejunal perfusion; 5 of them were challenged with gliadin. All perfusions were performed after fasting overnight . After cleansing the jejunal segment for 30 min with 0.9% saline, the bowel was perfused with a balanced phosphate buffer -solution containing aprotinin as a protease inhibitor and ^{14}C- PEG-4000 as an non-absorbable marker. The quality of the perfusions was acceptable with a mean recovery of 95% (83 - 106%) of ^{14}C-PEG 4000 in the celiacs and 93% , (88 -95 %)for controls .
In the challenge experiments an average of 12 mg±3 (SEM) gliadin was dissolved in 0.2 - 0.5 ml 70% ethanol, mixed with 30 ml of the perfusion fluid and then administered for 20 min after a 60 min basal perfusion period. Duplicates of PGE_2 were measured via a radioimmunoassay (NEN Du Pont, Dreieich, W Germany.). The variability was less than 8 %. The results are expressed as jejunal appearance rates calculated according to the formula ; concentration in perfusion fluid x 3 ml/min x 60 min / 10 cm = amount /cm intestine / hour).
Significance was tested by Students t test and analysis of variance Anova.

Results

Patients with active coeliac disease (n=7) had higher basal jejunal secretion of PGE2, 523± 229* pg/cm/h (mean±SEM), than both coeliac patients with normalised small bowel mucosa (n=6),160±44 pg/cm/h, and healthy controls (n=18), 84± 38 pg/cm/h, (* $p<0.05$).
Challenge with gliadin in the jejunal segment induced an significant increase in the jejunal secretion rate of PGE_2 ($p<0.05$)in coeliac patients (n=10), whereas in controls (n=5) no reaction could be detected.The relative increases among celiac patients varied between 1.5 to 15-fold and was on average 540 %.

Discussion

Our results suggests that gliadin in coeliac patients is a stimulus to jejunal mucosal release of arachidonic acid (AA) and the synthesis of prostaglandins.The observation of a higher basal secretion of PGE_2 in patients with histologically active celiac disease than in those with a normalised mucosal histology suggests that the inflammatory cells infiltrating the coeliac mucosa may be the source of prostaglandin synthesis.

Conclusion

Gliadin challenge induces jejunal PGE_2 secretion in coeliac patients.

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Lack of serological response to the E1B-58 kDa protein of adenovirus 12 (Ad12) in coeliac disease

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Introduction

The description of an amino acid sequence homology between the E1B-58kDa protein of adenovirus 12 and gliadin led to the suggestion that previous infection by this virus and subsequent exposure to gliadin could trigger the development of coeliac disease in susceptible individuals as a result of immunological cross-reactivity. Coeliac patients were subsequently shown to have elevated titres of neutralising antibodies to the whole virus and also a cell-mediated immune reaction to a synthetic peptide containing the sequence homology. There is no evidence yet available, however, for an immune response against the E1B-58kDa protein in coeliac patients. We have sought, therefore, to measure specific antibodies to this protein in coeliac patients and normal subjects.

Methods

Sera were obtained from 7 untreated and 16 treated adult coeliac patients and 10 normal subjects. They were analysed by radio-immunoprecipitation using metabolically-labelled adenovirus 12-transformed rat cells (which express the E1B-58kDa protein) followed by separation on polyacrylamide gels [1]. Appropriate transformed cells and sera as positive and negative controls were included. Antibodies to gliadin and to a synthetic gliadin peptide, identical to the amino acid sequence homology, were assayed by ELISA [1].

Results

None of the coeliac sera had detectable antibodies to the Ad12 E1B-58kDa protein, although one normal subject showed evidence of weak recognition of the 58kDa protein. Elevated titres of anti-gliadin antibodies were present in 8/23 coeliac patients (3 untreated and 5 treated patients). One normal and one treated coeliac patient had elevated titres of antibodies to the synthetic gliadin dodecapeptide, but there was no connection between titres of gliadin and synthetic peptide antibodies ($r = 0.34$, NS).

Discussion

These results show that none of the coeliac patients had antibodies to the E1B-58kDa protein of Ad12. It would be expected that such antibodies should be present in coeliac patients if humoral immunity displayed by cross-reactivity between the E1B-58kDa protein and gliadin was involved in the pathogenesis of coeliac disease. Lack of cross-reactivity is also suggested by the fact that 8/23 coeliac patients did have anti-gliadin antibodies but only one had a raised titre to the synthetic gliadin peptide. The lack of overall correlation between these antibodies is strongly suggestive that the antigenic epitopes of the shared homology region are either not immunodominant, not immunogenic or remain cryptic in the intact protein.

In the one normal subject where there was an elevated titre of antibodies to the synthetic peptide, there was also evidence of antibodies to the E1B-58kDa protein, although this subject had no elevated antigliadin antibody titre and no evidence of coeliac disease. This single, but important, result shows that there is antigenic cross-reactivity between the synthetic dodecapeptide and the E1B-58kDa protein, but not between the E1B-58kDa protein and gliadin. This further supports our contention that the antigenic epitopes within the sequence homology region are not immunodominant in man.

We conclude that there is a need for direct evidence to substantiate or refute the hypothesis that antigenic cross-reactivity between the Ad12 E1B-58kDa protein and gliadin, due to shared amino acid homology, is a causative factor in the aetiology of coeliac disease.

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Jejunal glycoprotein synthesis and secretion in coeliac disease

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Introduction

The jejunal mucosa of patients with untreated coeliac disease has been shown to exhibit several immunological differences from normal mucosa [1-3]. Whilst specific abnormalities associated with the epithelium, such as changes in HLA antigen expression, have been described [4], little is known about goblet cell function in coeliac disease. Alterations in mucus glycoprotein biosynthesis, however, have been associated with certain intestinal diseases [5]. Inflammatory mediators have been shown to influence pulmonary and gastrointestinal mucus glycoprotein secretion in animal models [6]. Since the enhanced mucosal immunological activity in coeliac disease [1-3] could influence intestinal glycoprotein production, we have investigated jejunal glycoprotein synthesis and secretion in patients with coeliac disease using in vitro organ culture systems.

Methods

Jejunal biopsies were obtained from 26 patients with coeliac disease (13 untreated with subtotal villous atrophy and 13 treated, of whom 8 had histologically normal mucosa and 5 partial villous atrophy) and from 19 patients with histologically normal mucosa. Biopsies were cultured in duplicate on sterile grids positioned over the central well of organ culture dishes (Falcon) in RPMI 1640 medium containing 10% FCS and 5μCi of D-[1-^{3}H]-glucosamine hydrochloride (specific activity 5.8Ci/mmol, Amersham) for 24 hours. Glycoprotein synthesis and secretion were measured as previously described [7] and the results expressed as dpm/mg biopsy protein.

Results

The total incorporation of ^{3}H-glucosamine into tissue and secreted glycoproteins by jejunal tissue from untreated coeliac patients (Table 1) was significantly greater than that of normal ($p<0.001$) and treated coeliac mucosa ($p<0.01$). The secretion of glycoproteins by untreated coeliac mucosa was also significantly greater than both normal ($p<0.001$) and treated coeliac tissue ($p<0.01$). In untreated coeliac mucosa 39.5% of the ^{3}H-glucosamine incorporated into acid-precipitable glycoproteins over a 24-hour period was secreted. Addition of Cyclosporin A (15μg/ml) did not significantly reduce *in vitro* glycoprotein biosynthesis by jejunal mucosa of untreated coeliac patients (463 ± 54; 423 ± 63 plus CyA).

TABLE 1. ^{3}H-glucosamine incorporation into tissue and secreted glycoproteins by normal, treated (TC) and untreated (UTC) coeliac mucosa.

	DPM/mg protein x 10^{-3} (Mean ± SEM)		
	Tissue	Secreted	Total
Control	188 ± 14	60 ± 9	248 ± 13
TC	192 ± 15	73 ± 13	265 ± 15.3
UTC	*258 ± 25	***169 ± 23	***427 ± 34

Mann-Whitney U test, *$p<0.05$, **$p<0.01$, ***$p<0.001$ from controls.

Discussion

These *in vitro* results show that intestinal glycoprotein biosynthesis is enhanced in patients with untreated coeliac disease, which may be a consequence of mucosal immune activation. Several immunological factors could contribute to these changes [6]. T lymphocytes have been implicated in intestinal goblet cell hyperplasia in helminth infected rats [8] and the increased T cell activation in untreated coeliac mucosa [2,3] could effect goblet cell maturation or differentiation. The observed changes in intestinal glycoprotein production may also be relevant to the increased intestinal permeability observed in patients with coeliac disease [9] and in the pathogenesis of the condition.

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Non-invasive tests in the diagnosis of coeliac disease in childhood

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INTRODUCTION. In order to find a non-invasive test for probing small intestinal structure and function, we used several tests in the same subject and compared the results with the morphology of the small-bowel biopsy. The tests were: gastrointestinal permeability assessments, disaccharidase activity estimation and determination of antibodies to gliadin, wheat germ agglutinin, beta-lactoglobulin and ovalbumin.

MATERIAL. All patients were children (0.6-16.8 ys) with suspected celiac disease. They were studied at presentation, after gluten-free diet and/or after gluten challenge. 'N' indicates the number of tests performed.

METHODS.
Mucosal barrier characteristics

The 6-hour urinary recovery of an oral load of different-sized polyethyleneglycols (PEG) (a mixture of PEG 400 and PEG 1000, size range 282-1250 dalton). The recovery ratio, described as the quotient between the recovery of 1074 dalton PEG and 370 dalton PEG, in percent, reflects the mucosal barrier properties (n=95).

The 5-hour urinary recovery of an oral load of a mono-disaccharide mixture (0.5 g L-rhamnose, 2.3 g lactulose). 10 g lactose and 10 g sucrose were added to make the solution hypertonic. The lactulose/rhamnose quotient describes the permeability (n=57).

The disaccharidase activity, indirectly estimated from the recovery of lactose and sucrose in the urine (n=47).

Antibodies to gluten peptides

Serum antibodies IgA and IgG towards gliadin at presentation (n=89), after gluten-free diet (n=29) and after gluten challenge (n=23).

Serum antibodies IgA and IgG to wheat germ agglutinin at presentation (n=63), after gluten-free diet (n=22) and after gluten challenge (n=21).

Serum antibodies IgA, IgG and IgM to ovalbumin and beta-lactoglobulin at presentation (n=89).

RESULTS.
Mucosal barrier characteristics

PEG recovery. The recovery ratio between a large and a small PEG molecule was significantly lower in children with villous atrophy than in children with normal small-bowel mucosa ($P<.005$ at presentation).

Lactulose/rhamnose quotient. The quotient between the recovery of a di- and a monosaccharide was significantly higher in children with villous atrophy ($P<.01$ at presentation).

Disaccharidase activity. The urinary recovery of lactose and sucrose was significantly higher in children with villous atrophy ($P<.001$).

Antibodies to food proteins

Children with villous atrophy had significantly higher levels of antibodies IgA and IgG to gliadin, wheat germ agglutinin, beta-lactoglobulin and ovalbumin. There was a positive correlation between levels of antibodies to gliadin and wheat germ agglutinin (coefficient of correlation 0.82 for IgA and 0.75 for IgG).

CONCLUSIONS. All tests used differed significantly between the group of children with normal small-bowel biopsy and those showing villous atrophy. From an individual point of view there was a considerable overlap and we found that no single test could safely substitute for the small-bowel biopsy at any stage of investigation for celiac disease.

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The secretory IgA system in coeliac disease characterized by cellular expression of IgA subclass and J chain

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INTRODUCTION

The frequency of coeliac disease (CD) is ten times increased in IgA-deficient individuals, and immunodeficiency states at the intestinal level are often associated with small-intestinal mucosal lesions. We therefore examined whether the functional capacity of jejunal immunocytes was altered in terms of IgA1 and IgA2 production and J ("joining")-chain expression in untreated and gluten-free diet (GFD) treated CD patients. The two IgA subclasses have different biological properties and J chain is required for epithelial polymeric immunoglobulin transport.

MATERIAL AND METHODS

The paired immunofluorescence staining methods used have been described previously, including the characteristics and working concentrations of the reagents (1). Briefly, jejunal biopsy specimens were prepared by extraction in cold isotonic saline, ethanol fixation, and paraffin embedding. For evaluation of IgA subclass-producing cells sections (6 µm) were first incubated with murine monoclonal antibody to IgA1 or IgA2, then with a mixture of tetramethylrhodamine isothiocyanate (TRITC)-labeled rabbit antihuman IgA and fluorescein isothiocyanate (FITC)-labeled antimouse IgG. To unmask antigenic determinants of the J chain, sections were first pre-treated with acid urea (6M, pH 3.2). The expression of IgA subclass and J chain was determined by an initial incubation step with a mixture of antibody to IgA1 or IgA2 and polyclonal TRITC-labelled anti-J chain, followed by a second incubation step including TRITC-labelled anti-J chain and FITC-labelled antimouse IgG.

RESULTS

A significantly increased proportion of IgA2 cells was found in both untreated ($p < 0.01$) and treated ($p < 0.05$) patients compared with controls (Table 1). Table 2 shows that only the median J-chain expression of IgA2 cells was marginally reduced in untreated CD patients.

TABLE 1. Distribution of IgA subclass-producing cells

	Median Percentage (range)	
	IgA1	IgA2
Controls (n=11)	72 (60-84)	28 (20-38)
Untreated CD (n=9)	53 (45-91)	47 (9-57)
GFD treated CD (n=11)	63 (35-84)	37 (15-69)

TABLE 2. J-chain positivity of IgA subclass-producing cells

	Median percentage (range)	
	IgA1	IgA2
Controls (n=11)	89 (68-96)	98 (90-100)
Untreated CD (n=9)	89 (50-95)	96 (87-100)
GFD treated CD (n=11)	89 (60-100)	98 (78-100)

DISCUSSION

We found that the J-chain expression of both IgA1- and IgA2-producing cells was fairely unchanged in CD compared with controls, and that the expanded IgA-cell population in CD (2) consisted mainly of J-chain-positive IgA2 plasma cells. The antimicrobial protective role of IgA2 seems to be superior to that of IgA1. Thus, our results indicated that the secretory immunity in CD was quantitatively and qualitatively enhanced in the proximal gut. In striking contrast to this finding we have recently observed that the increased colonic IgA-cell population in inflammatory bowel disease consists mainly of IgA1 cells with down-regulated J-chain expression of both IgA1 and IgA2 cells (1).

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Jejunal and serum anti-gliadin IgA in adult coeliac disease

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Elevated antibody (Ab) levels to gliadin (GL), the alcohol soluble fraction of gluten, are present in the serum of patients with coeliac disease (CD), an enteropathy caused by hypersensitivity to gluten. The site of production and role of these Abs in the pathogenesis of the disease are poorly understood. In order to evaluate in vivo the secretion of Ig by the jejunal mucosa in CD, we have analysed jejunal fluids collected during a perfusion of a 40cm jejunal segment under an occluding balloon which avoid contamination with other intestinal secretions (1). Diisopropylfluorophosphate (protease inhibitor) was added to the samples. Jejunal biopsies and serum were also collected.

We have studied samples from 6 untreated adult CD (group 3), 11 patients with other enteropathies and 7 cirrhotic patients (group 2), and 11 controls with a normal gastrointestinal status (group 1). Ig, Alb and secretory component (SC) concentrations were measured by immunoradiometric assay or immunonephelometry. Anti-GL IgA were determined by solid-phase RIA (crude wheat GL), expressing results in arbitrary units (2). Monomeric (m) and polymeric (p) IgA distributions were analysed by density gradient ultracentrfugations and correction factors were used for pIgA and sIgA (3).

In CD, jejunal secretion rates of mIgA, pIgA, IgM and SC are significantly higher than in both groups of controls (*p<0.02, **p<0.01, ***p<0.001) (Fig.1)

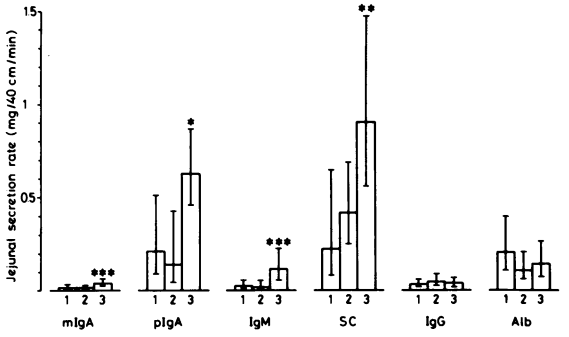

Fig.1 - Mean ± SD of the jejunal secretion rates in the 2 control groups (1 and 2), and in coeliac patients (3).

This is not the result of an increased passive diffusion from serum to the jejunal fluids as the Alb secretion rate is similar in the 3 groups of patients. In serum, mIgA and pIgA concentrations are not higher in CD, but sIgA concentration is more elevated in 4/6 CD patients. Numbers of IgA- and IgM- containing cells per mm^2 of jejunal lamina propria are higher than in controls ($p < 0.01$ and $p < 0.001$).
Anti-GL IgA are detected in all serum and jejunal fluids from CD patients, in 6/18 sera from non CD patients and 3/18 jejunal fluids from other non CD patients. In jejunal fluids from CD, Ab titers per ug of total IgA are higher than in serum. 95% are 11s IgA like total IgA whereas in serum 61% of anti-GL IgA are 10.2s dimeric IgA, not linked to SC, compared to only 14.5% total pIgA (Fig.2).

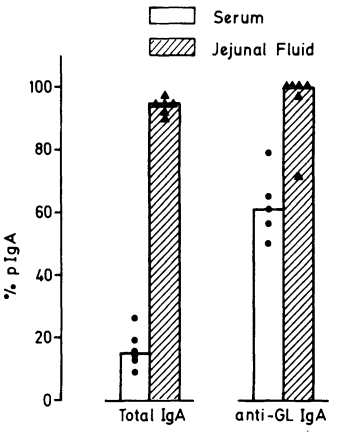

Fig.2 - Percentages of total IgA and of anti-GL IgA which are polymeric in serum and in jejunal fluids.

These results suggest that serum and intestinal anti-GL IgA in CD might be of different origin. Intestinal Abs are synthesized in the gut mucosa. The presence of Abs in serum is not the result of a reabsorption of intestinal Abs through a damaged mucosa since Ab size is different in serum and in secretions. It is also not secondary to an inadequate synthesis of SC as shown by the high SC jejunal secretion rate. Serum anti-GL IgA might be synthesized by blood circulating mononuclear cells (4). Alternatively, anti-GL pIgA might be of mucosal origin whereas anti-GL mIgA would be synthesized by memory cells which have migrated to the spleen, lymph nodes or bone marrow after a systemic exposure of CD patients to GL.

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Gliadin induces HLA-DR expression on crypt epithelium during organ culture of treated coeliac mucosa

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INTRODUCTION

In normal human small intestinal epithelium HLA-DR molecules are expressed by villus enterocytes, while epithelial cells in the crypts of Lieberkuhn are HLA-DR negative. Such a distribution is profoundly altered in untreated coeliac disease as, contrary to normals and treated patients, HLA-DR expression extend considerably into the crypt epithelium (Scott et al, Clin Exp Immunol 1988; 44: 233). These changes are thought to be the expression of activated cell-mediated immune mechanisms within the mucosa.

In this work we have used the organ culture technique to determine if, in the treated coeliac mucosa, these changes can be induced in vitro by gliadin peptides.

PATIENTS AND METHODS

Peroral jejunal biopsies were obtained from 9 coeliac patients who had been on a gluten-free diet for at least 2 years and from 4 controls. All subjects showed a normal mucosal morphology. Immediately after excision the biopsies were cultured in a serum containing medium, with or without added a peptic-tryptic digest of wheat gliadin (0.1 mg/ml). In some experiments the biopsies were cultured also in the presence of peptic-tryptic digest of maize prolamines at the same final concentration. After 30 hours in culture the tissue was snap frozen in liquid nitrogen. 5 u frozen section were cut and stained by immunofluorescence using a monoclonal antibody to a nonpolymorphic DR determinant (clone L 243, Becton-Dickinson, USA).

RESULTS

In all biopsy specimens from both controls and treated coeliac patients fluorescence was maximal at the top of villi, decreased towards the basis of villi, and was absent in the crypts. Within

the enterocytes fluorescence was more intense in the subapical and basolateral regions. A similar pattern was evident after in vitro culture without added gliadin peptides. When the biopsies of treated coeliac patients were cultured in the presence of gliadin peptides, in the majority of cases HLA-DR expression resulted enhanced in the crypt epithelium. At the same time the expression of HLA-DR in the surface epithelium resulted significantly reduced or even absent. Neither HLA-DR expression in crypt cells, or its disappearance from villous surface were observed in biopsies from control subjects cultured with gliadin, or in biopsies from coeliac patients cultured with maize peptides (Table).

Table

Proportion of biopsies showing expression of HLA-DR in jejunal surface and crypt epithelium after in vitro culture with or without gliadin peptides.

	Only medium		+ gliadin		+ maize	
	surface	crypts	surface	crypts	surface	crypts
Controls	3/3	0/3	4/4	0/4	ND	ND
Coeliacs	3/4	0/4	2*/9	5/9	2/3	0/3

* patchy distribution

DISCUSSION

Most data so far obtained in coeliac disease by the organ culture technique have been collected in patients on a gluten-containing diet. It has always proved difficult to reproduce in vitro the pathological features of the disease, using the organ culture of treated coeliac mucosa. Our preliminary data show that gliadin peptides do induce HLA-DR expression on crypt enterocytes in in vitro cultured mucosa from patients in remission. This observation provides indirect evidence for the activation of cell-mediated immune mechanisms within the mucosa. We are currently investigating if lymphokines are secreted in organ culture supernatants, as result of the presence of gliadin peptides.

Conflicting results have been reported in the literature on the expression of HLA-DR on the surface epithelium of untreated coeliac patients (Sarles et al, J Pediatr Gastroenterol Nutr 1987; 6: 51). We have shown the disappearance of this molecule from the mucosal surface during the organ culture, this finding being specific for the coeliac mucosa cultured in the presence of gliadin. The mechanisms underlying this phenomenon remain to be defined.

In perspective this model could prove useful for the identification of the gliadin epitopes involved in the activation of the specific mucosal cell-mediated immune response.

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Secretory antibodies to gliadin in coeliac disease

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INTRODUCTION

Humoral immunity in coeliac disease has been the subject of intensive investigation; numerous studies have established that patients with untreated coeliac disease have high levels of circulating antibodies to wheat-derived proteins such as gliadin, and that antibody levels fall following a period of treatment with gluten free diet. In contrast, information on intestinal humoral immunity is limited; this may be due to the relative difficulty in obtaining intestinal secretions for study. Saliva is a more easily obtained intestinal secretion; we were thus prompted to study salivary antibody response to determine whether it mirrored intestinal antibody response.

MATERIALS AND METHODS

Jejunal aspirate, serum and pure parotid saliva were collected at the time of jejunal biopsy in 26 untreated coeliacs, 22 treated coeliacs and 28 controls. Intestinal secretions were obtained by gut lavage in 15 untreated coeliacs, 19 treated coeliacs and 25 controls. Intestinal secretions were centrifuged, treated with protease inhibitors, aliquoted and stored at -70^{o} until assay. IgA, IgM and IgG antigliadin antibodies were measured by enzyme-linked immunosorbent assay (ELISA) using crude gliadin as an antigen.

RESULTS

1. Jejunal aspirate: high levels of IgA antibody were found in both untreated ($p<0.00001$) and treated ($p<0.002$) coeliacs, though levels were significantly higher ($p<0.0002$) in the untreated group. High levels of IgM antibody were also found in both untreated ($p<0.00001$) and treated ($p<0.00001$) coeliacs, with higher levels ($p<0.005$) in the untreated patients. Aspirate antibodies were predominantly in the IgA and IgM classes, but high levels of IgG antibody were also found in both untreated ($p<0.00001$) and treated ($p<0.02$) coeliacs with significantly higher levels ($p<0.05$) in the treated group.
2. Gut lavage fluid: as in jejunal aspirate, antibodies were mainly in the IgA and IgM classes. High levels of IgA antibody were found in untreated ($p<0.002$) and treated ($p<0.05$) coeliacs, with no

statistical difference between the two groups. High levels of IgM antibody were also found in both untreated ($p<0.0002$) and treated ($p<0.005$) coeliacs, with higher levels ($p<0.05$) in the untreated group. Both gut lavage fluid and jejunal aspirate were collected from a total of 20 patients (from the 3 groups); a positive correlation ($r=0.68$, $p<0.0001$) was found between IgA antibody levels in aspirate and gut lavage fluid.

3. Saliva: untreated coeliacs had higher levels of IgA ($p<0.005$) and IgG ($p<0.05$) antibodies compared to controls. Salivary antibody levels, however, were generally low, with a large overlap between patient groups. No correlation was found between salivary and aspirate antibody levels.

4. Serum: our findings mirrored those of other workers. Untreated coeliacs had high levels of IgA antibody ($p<0.00001$) with levels in treated patients similar to controls. High levels of IgG antibody were found in both untreated ($p<0.00001$) and treated ($p<0.02$) coeliacs, with higher levels ($p<0.0001$) in the untreated group. IgM antibody levels were similar in the 3 groups.

DISCUSSION

We have demonstrated a dissociation between systemic and intestinal humoral immunity in coeliac disease: firstly, intestinal antibodies are mainly in the IgA and IgM classes, whereas serum antibodies are in the IgA and IgG classes; secondly, intestinal antibody persists in coeliacs taking a gluten-free diet, whereas serum antibody falls. Persistent intestinal antibody could be due to continued ingestion of minute amounts of gluten; alternatively, there may be a longer immunological 'memory' at the intestinal level.

Salivary antibody levels do not reflect intestinal immune response; gut lavage, on the other hand, seems to offer a non-invasive alternative to intubation for the collection of intestinal secretions for antibody studies.

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Antibody dependent cell-mediated cytotoxicity in infants with cows milk protein intolerance and coeliac disease

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1. INTRODUCTION

ADCC (antibody dependent cell-mediated cytotoxicity) is an immune mechanism, which may participate in host defense as well as in immunopathological phenomena in the gastrointestinal tract. ADCC is usually mediated via IgG-antibodies and the K subset of mononuclear cells. Besides, an ADCC-reaction against bacteria mediated through secretory IgA-antibodies and intestinal lymphoid cells has been demonstrated (1).
It is likely that various specific humoral and cellular mechanisms may participate in food intolerances and that more than one type of immunological reaction may operate within the mucosa at one time. Hypothetically an ADCC-mechanism can be involved in the pathogenesis of food intolerances.

2. AIM

The aim of the study was to evaluate the role of an ADCC-reaction in infants with cow´s milk protein intolerance (CMPI) and coeliac disease (CD). We focused on the ADCC-mediating capacity of serum antibodies to betalactoglobulin (BLG) and to gliadin.

3. MATERIALS AND METHODS

3.1. Patients

Six groups of infants were analysed in an ADCC-assay with BLG-coated target cells; delayed onset CMPI with gastrointestinal symptoms (n=8), immediate onset CMPI with gastrointestinal and skin symptoms (n=8), immediate onset CMPI with skin symptoms only (n=8), untreated CD (n=10), treated CD (n=8) and controls (n=22).
Three groups of infants were tested in an ADCC-system with α-gliadin-coated target cells; untreated CD (n=18), treated CD (n=8) and controls (n=12).

3.2. ADCC-assay

Sera from the infants were tested with lymphocytes from healthy adults as effector cells and as target cells radiolabelled BLG-coated or α-gliadin-coated erytrocytes from the same donor were used. The cytotoxic activity was measured by release of radioactive chromium and the percentage of specific cytotoxicity was determined.
In addition an ELISA for the estimation of serum antibodies to BLG and gliadin was performed as previously described (2).

4. RESULTS

4.1. ADCC-assay with BLG-coated target cells

Sera from the delayed onset CMPI-group as well as the immediate onset CMPI-group with gastrointestinal symptoms showed significantly ($p < 0,05$) increased ADCC-reactivity as compared with controls. In contrast immediate onset CMPI with skin symptoms only had ADCC-values within the same range as controls. Despite the fact that the CD-groups had very high levels of antibodies against BLG, sera from the infants with CD showed, with a few exceptions, low ADCC-reactivity and did not differ from controls.

4.2. ADCC-assay with α-gliadin coated target cells

All patient groups showed a low but distinct ADCC-reactivity. Infants with CD did not differ significantly from controls.

5. DISCUSSION

In CMPI with gastrointestinal symptoms variable degrees of mucosal changes, similar to those in CD, are frequently seen. An ADCC-reaction might take place in the mucosa, where an epithelial cell absorbing food antigens such as BLG becomes the target cell and the lymphocyte in the intestinal mucosa the effector cell via antibodies to e.g. BLG present in the area.
Our results give rise to the assumption that ADCC may be an immunopathologic mechanism in CMPI with gastrointestinal symptomatology. In contrast the evaluation of antibodies to gliadin and their ADCC-mediating efficacy does not support the presence of an ADCC-mechanism in CD.

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IgA and IgG antibodies against gliadin in healthy blood donors and its relation to gluten enteropathy

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Introduction
The prevalence of coeliac disease (CD) in Sweden is about 1/1000. Recently, CD has been shown in other clinical situations without typical gastrointestinal symptoms which makes it necesserary to use laboratory markers to select patients for biopsy. It has been established that untreated CD patients have circulating antibodies against subfractions of gliadin. Several immuno-assays have been developed to detect class-specific antibodies against gliadin but some controversy exist about the value of the antibody as a marker for CD. Since most people are exposed to this complex food antigen it is important to analyse the prescens of IgG- and IgA- antibodies against the antigen in a healthy population. We have developed a modified micro-ELISA assay for detection of class-specific anti-gliadin antibodies. Most studies are based on patients and a small group of apperently healthy blood donors. We have analysed the prescens of IgG- and IgA antibodies against the antigen in a large population of apperently healthy blood donors and compared the results with analyses of sera from 40 untreated CD-patients with clinical symptoms.

Materials and methods
Subjects:
Blood from 1866 healthy blood donors (18-64y) were collected and sera were frozen for a period up to 3 months until analysed.
Patients with coeliac disease:
Sera from forthy untreated adults with CD were obtained at the Department of Medical Gastroenterology.

Micro-ELISA assay for IgA- and IgG anti-gliadin antibodies:
Microtiter plates were coated with 50 ug/ml ethanol-dissolved gliadin (Sigma,St.Louis,VA,USA). The plates were incubated for 60 minutes with serum diluted 1 to 10 in

phosphate buffered saline, and then washed. Peroxidase-conjugated anti-Ig antibodies (Dakopatts, Glostrup, Denmark) were used to detect class-specific anti-gliadin antibodies. The reaction was read spectrophoto-metrically and dilutions of a positive serum was used as a standard.
Jejunal biopsy:
A singel intestinal mucosa biopsy was taken from the duodeno-jejunal junction using a Watson capsule.

Results
IgG- and IgA-reactivity to gliadin:
The frequency distribution of the IgG- and IgA-activity was assymetric with a long tail to the right. As a cutoff point for positive tests the 97.5th percentile were set. The frequency of IgG-antibodies showed an U-loop distribution with the highest percentage of positive sera in the youngest (18-24y) and the oldest (55-64y) group. The distribution of IgA reactivity showed a similar pattern but not so pronounced.
Selection of donors for jejunal biopsy:
Blood donors with positive test were selected for further analyses and jejunal biopsy. Of 34 donors with IgA antibodies (>97.5p) 7 showed typical mucosal lesions as in CD. None of the 28 donors with IgG-antibodies and without IgA-antibodies (>97.5p) showed mucosal lesions. There were no differences in clinical symptoms and laboratory investigations between donors with and without mucosal lesions. Six of the blood donors with gut lesions accepted gluten-free diet and were all improved with mucosal remission within 12 months. All six had normal IgA- and IgG anti-gliadin activity at the time for repeat jejunal biopsy.
These results indicate a positive predictive value (PV) of 21% (7/34) for IgA-antibodies and a PV of 0% (0/28) for IgG-antibodies over the 97.5th percentile in this population. Comparing this with the population of untreated coeliac disease the sensitivity for the test was 72% (29/40) using IgA-antibodies and 40% (16/40) using IgG-antibodies.

Discussion
In the clinical situation the PV is probably the most important information of the test since CD has been shown in situations without typical gastrointestinal symptoms. Regarding an apperently healthy population we found that if we selected subjects for jejunal biopsy according to only high IgA-antigliadin activity we have a PV of 21%. If we selected according to only high IgG-antigliadin activity we found no donor with mucosal lesions.
The present study indicate that IgA-antibodies against gliadin are primarily a diagnostic marker of CD whereas IgG-antibodies might be more associated with the clinical symptoms.

SECTION V: FOOD ALLERGY AND INTESTINAL HYPERSENSITIVITY

(B) Studies in animals

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Interaction of circulating radiolabelled IgG antibody and fed protein antigen in the rat

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ABSTRACT. We tested the effect of fed protein antigen on circulating IgG antibody in rats passively supplied with a limited dose of antibody. Rats were given 1.0 g BSA or buffer by gavage and were injected i.v. with trace amounts of ^{125}I-rat anti-BSA antibodies plus 35 ug 'cold' anti-BSA antibodies. Rats were exsanguinated under anesthesia and perfused with buffer 1 or 18-22 h after feeding. Radioactivity in serum and liver was measured. At 1 h, there was less radioactivity in serum of BSA-fed compared to control animals ($p < 0.05$); this difference was greater at 18-22 h ($p < 0.001$). At 1 h, the content of radioactivity in the liver was greater in BSA-fed than control rats ($p < 0.005$); there was no significant difference at 18-22 h. On A5m gel permeation of serum obtained at 1 h from BSA-fed rats, radioactivity was detected in fractions expected to contain high and intermediate molecular size immune complexes; serum obtained from BSA-fed rats at 18-22 h contained no such fractions. Based on a comparison with the decrease in concentration of labeled antibody resulting from i.v. injection of BSA, BSA-fed rats appeared to have taken up 16.7 to 23.1 ug BSA of the 1.0 gram dose administered. These experiments confirm that immunoreactive protein is taken up from the gut into the circulation of fed animals, that considerable uptake occurs during the first hour but persists thereafter, and that IgG antibody-fed antigen complexes forming early after ingestion are suitable for clearance by the liver.

Introduction

Using sensitive immunoassays, several investigators have shown that minute amounts of food protein antigen are taken up from the lumen of the normal gastrointestinal tract and transferred to the systemic circulation [1]. Lim and Rowley showed that uptake of unlabeled food protein could also be detected in mice on the basis of its combination with radioiodinated antibodies injected intravenously into the animal [2]. In the present study, we tested the effect of fed antigen on circulating radiolabeled IgG antibody, studied the complexes formed and their clearance via the liver.

Methods

To elicit IgG antibodies, rats were immunized with bovine serum albumin emulsified in complete Freund's adjuvant. Booster injections were given 2 and 4 weeks later and antiserum was obtained 6-8 weeks after the start of immunization. A single pool of anti-BSA antiserum was used throughout; this pool contained 1.4 mg antibody protein per ml. The antibodies were of the IgG2a and IgG1 isotypes. Antibodies were purified by adsorption to and subsequent elution from a BSA-Sepharose 6B immunoadsorbent. Purified antibodies were labeled with ^{125}I using the lactoperoxidase method; unbound ^{125}I was removed by gel permeation. Labeled antibodies were freed of aggregates or breakdown products by gel permeation on acrylamide A5m columns prior to each experiment. The labeled antibodies were tested for their ability to bind to antigen and were used if they showed $\sim$ 80% specific binding.

Sprague-Dawley rats weighing 150-175 gm and not previously exposed to BSA, were fasted overnight and then gavage-fed, without anesthesia, using a silastic feeding tube. Controls received 0.1 M phosphate buffer; experimental rats received 1.0 gram BSA dissolved in buffer. Thereafter, rats were injected with ^{125}I-rat anti-BSA antibodies ($\sim$ 250 ng antibody protein, $\sim$ 300,000 cpm) together with 25 uL of antiserum containing 35 ug anti-BSA antibody. The amount of cold antibody selected was based on preliminary experiments which showed that this dose optimally enhanced the deposition of radioactivity in the liver of BSA-fed rats. One hour or 18-22 hours after the injection of antiserum, the rats were anesthetized with ether, the chest was opened, blood was removed via the left ventricle and the animals were perfused with 130 ml buffer to remove blood from the organs. Thereafter, the liver was weighed and its content of radioactivity was measured. To facilitate comparison among animals, we determined the percentage of the injected dose of radioactivity present in 1.0 ml serum and 1.0 gram of liver. Statistical analysis was performed with the Wilcoxon paired sample test [3].

Results and Discussion

In 10 experiments, we compared the amount of injected radioactivity (i.e. labeled rat anti-BSA antibody) remaining at one hour, in the serum of rats fed buffer or BSA. In 8 of the 10 experiments, less radioactivity remained in the serum of BSA-fed compared to buffer-fed rats. The difference was significant, $p < 0.05$. Examination of the thoroughly perfused liver showed that the liver of BSA-fed rats contained a greater percentage of the injected dose of labeled antibody than did the liver of buffer-fed rats. Enhanced deposition was seen in all BSA-fed rats; the difference from controls was significant, $p < 0.005$.

Serum obtained at 1 h from BSA- and buffer-fed rats was applied to acrylamide A5m gel permeation columns. The radioactivity in the

control serum had the same elution profile as the radiolabeled rat anti-BSA antibody injected at the start of the experiment. The radioactivity in the experimental serum eluted in fractions that are expected to contain high molecular weight, as well as intermediate molecular weight, moieties. In addition, residual free antibody was present. We believe that the first peak, as well as intermediate fractions, constitute antigen-antibody complexes. Because high molecular weight complexes are normally cleared very rapidly in the rat, we assume that their presence in serum one hour after feeding indicates the continued formation of such complexes by antigen taken up from the gut and combining with circulating antibody.

This series of experiments suggested that immunoreactive BSA enters the tissues and circulation of the BSA-fed rats, and combines with labeled (as well as unlabeled) anti-BSA antibodies so as to reduce their blood level. The BSA-anti-BSA antibody complexes formed appear to be deposited in the liver; there was no consistent evidence of deposition in other organs.

In 12 experiments, we compared the amount of injected labeled antibody remaining at 18-22 h in serum of rats fed buffer or BSA. In all 12 experiments, less radioactivity remained in the serum of BSA-fed compared to buffer-fed rats ($p < 0.001$). As expected, the difference tended to be greater than that seen after 1 h. There was considerably less radioactivity in the liver of buffer- or BSA-fed rats at 18-22 h compared to 1 h. There was no significant difference in the percentage of the injected dose of radioactivity remaining in the liver of BSA-fed compared to buffer-fed rats.

Serum obtained at 18-22 h from BSA- and buffer-fed rats was also examined by gel permeation. The radioactivity in control and experimental serum had the same elution profile as the radiolabeled rat anti-BSA antibody initially injected into the animals. There was no evidence of high molecular or intermediate molecular weight complexes in the serum from BSA-fed animals, suggesting that complexes present at 1 h, or forming thereafter, had been cleared completely by 18-22 h after feeding.

The combination of findings in these experiments suggest that immunoreactive BSA continues to enter the tissues and circulation of BSA-fed rats after the first hour, that it combines with labeled antibody and is, presumably, also deposited in the liver. By 18-22 h, however, both the circulation and the liver appears to be cleared of immune complexes.

The specificity of the reaction between circulating radiolabeled antibody and fed antigen was tested in rats that were fed either buffer, egg albumin (EA) or BSA; all animals were injected with labeled and 'cold' rat anti-BSA antibodies. The percentage of the injected dose of radioactivity remaining 22 h later in 1.0 ml serum from EA-fed rats was similar to that of the buffer-fed controls. The

level of labeled antibody remaining in the serum of BSA-fed rats was decreased in all 3 experiments.

The interaction between labeled antibody and fed antigen may be used to estimate the total amount of immunoreactive protein taken up from the intestine. In three experiments, each of which involved four animals, all were injected with labeled and unlabeled anti-BSA antibodies. In experiment LJ6, one rat was injected with buffer, one with 25 ug BSA and two were fed 1.0 gram BSA. After 22 h, the residual serum radioactivity (reflecting the blood level of labeled antibody) was determined. Intravenous injection of BSA lowered the level of circulating antibody, as did feeding. The extent to which the level of serum antibody was reduced by feeding corresponded to the level expected from an injection of 16.7 and 21.4 ug of BSA. Comparable results for experiments LJ7 and LJ5 were 20.6, 23.1 18.9 and 20 ug. To the extent that these experiments provide a valid measure, they suggest that the intact rat takes up about 20 ug iBSA from a fed dose of 1.0 gram or 0.002%. In mice, using a comparable method, Lim and Rowley estimated the uptake of a bacterial antigen as 0.08-0.09% of the dose administered by feeding [2].

In summary, we showed that (1) in rats, fed food protein antigen combined with labeled IgG rat antibody forming complexes which are cleared by the liver; (2) uptake of food protein and complex formation continued beyond the first hour after feeding, but (3) complexes were completely cleared from serum and liver by 18-22 h after feeding; (4) the effect of fed protein antigen on circulating rat antibody was antigen specific; (5) the interaction between labeled antibody and fed antigen may be used to estimate the total amount of immunoreactive protein taken up from the intestine. Thus, we have extended the observation initially made by Lim and Rowley to an additional species, the rat, and have shown that radiolabeled antibody may indeed be used to study the intestinal uptake of food protein.

Acknowledgements

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A rat model of intestinal hypersensitivity: mucosal mast cell activation following repeated feeding of antigen

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We have developed a rat model of food allergy in which repeated gavage challenges in anaphylactically sensitised animals were undertaken in order to study both mediator release and "bystander" protein uptake. Wistar rats were sensitised to ovalbumin (OVA) by an i.p. injection of 100ug protein in alum. In one experimental series animals sensitised on day 1 received a gavage feed on days 14-18 of OVA with or without BSA. Some controls received buffer alone, others were unsensitised. Blood samples were obtained before gavage and at various times thereafter. In a second experimental series animals were sensitised as before, gavaged with OVA on days 14 and 16, rested for 9 days and then re-challenged with OVA on days 26-28. Blood samples were obtained pre- gavage and 6 hours post-gavage. The rat serine protease RMCPII, a mediator specifically released by the mucosal mast cell, was measured in all serum samples. The bystander protein BSA was measured in selected groups as appropriate.

When animals pre-sensitised to ovalbumin were gavage challenged with this protein on day 14 markedly elevated levels of RMCPII were detected in the serum 6 hours after challenge compared to unprimed controls or controls not challenged with OVA. These results show that the release of RMCPII was antigen specific and only occurred in animals both primed and gavaged with OVA. Repeated daily gavage challenges of such animals over days 15-18 released significantly more RMCPII into the serum on each occasion than did similar challenges of control animals ($p<0.001$). However, the levels of mediator released following these later challenges were significantly lower than the day 14 level of mediator ($p<0.001$).

Animals in one OVA challenge group and one sham challenge control group also received BSA in the gavage and with one exception the level of this bystander protein was always higher in the blood samples taken 6 hours post-gavage from OVA challenged animals than in the corresponding samples taken from sham challenged animals. However, the BSA uptake did not correlate with RMCPII release.

In the second experimental series the animals challenged on days 14 and 16, rested for 9 days and then gavage challenged again on days 26-28, showed a pattern of RMCPII release similar to that observed on day 16 (see Figure).

Figure 1.

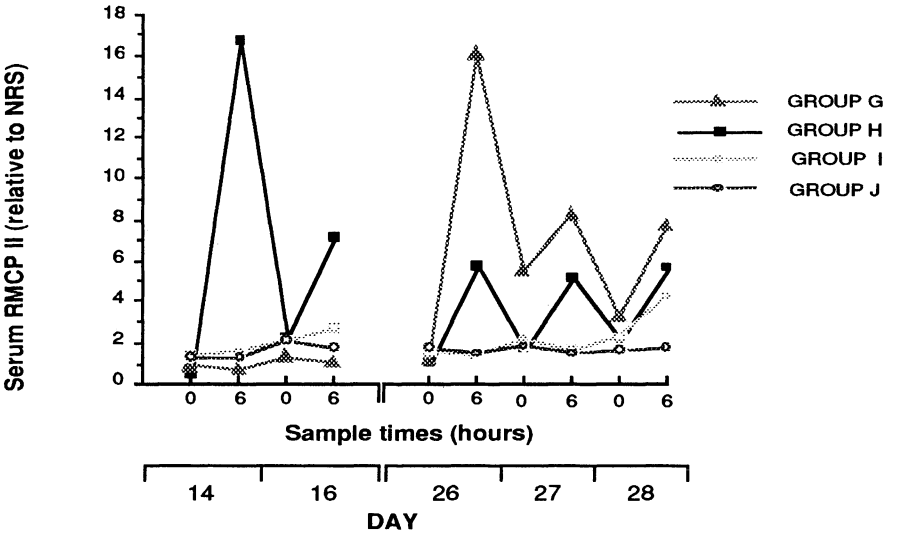

Figure 1. Mean serum levels of RMCPII in groups of rats treated as follows:

Group	*Ova sensitisation*	*Ova challenge (Day 14-16)*	*Ova Challenge (Day26-28)*
G	+	-	+
H	+	+	+
I	+	-	-
J	-	+	-

There was no evidence that the mucosal mast cells could recover their original (day 14) capacity to release RMCPII.

The levels of RMCPII detected in the systemic circulation following the protein challenges were lower than have been reported in pre-sensitised rats challenged with parasite extracts or in rats with primary nematode infections [1].

The present chronic gavage challenge model mimics the human intestinal hypersensitivity states more closely than our earlier acute challenge model [2] and should provide a basis for further studies.

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A novel experimental model of gut immediate hypersensitivity reactions to food antigens

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Introduction

It has been suggested by A. Walker (1) that local hyperpermeability of the gut due to a reaction of immediate hypersensitivity can lead to the development of allergy against "bystander" antigens.

In a first step, we elaborated a mouse model of experimental allergy by sensitizing mice with a monoclonal IgE antibody against cow's milk β-lactoglobulin, and by subsequently feeding them with this protein (2). In a second step, we fed the mice at the time of hyperpermeability with irrelevant antigens, cod fish parvalbumin, a well-known allergen, and edestin, a protein from indian hemp, thus unknown to the mice. The aim of this study was to verify whether these antigens, which if given orally normally lead to a tolerance, could stimulate the immunological system to secrete antibodies of different classes.

Material and methods

Antibodies of each isotype against beta-lactoglobulin (βLG), cod fish parvalbumin and edestin, were detected by RIA. For the measure of total IgE antibodies, polyvinyl wells were coated with anti antibodies (2), incubated with the sera, and then with labelled anti antibodies. Alternatively, mice sera were incubated with RBL 2H3.cells (3) and subsequently with labelled anti antibodies.

Experimental protocol BDF_1 mice were bled at day 0 and sensitized i.v. with 0.5 mg of pure IgE monoclonal antibody against βLG. They were then fasted two days and challenged orally on day 2 with 4 mg of polymerized βLG mixed with 4 mg of the two "bystander" antigens. Mice were then bled at day 7 and sensitized again with the same amount of IgE antibody (group I). This procedure was repeated for three weeks. Controls included mice sensitized but fed only with the "bystander" antigens (group II), and mice not sensitized but fed with βLG and the "bystander" antigens (group III).

Results and discussion

The aim of this work was to verify whether ingestion of food antigens during local hyperpermeability of the gut could lead to the production of IgE antibodies. We used our mouse model of gut experimental allergy to create a reaction of immediate hypersensitivity (2), which resulted in increased permeability of venulae of the serosa and submucosa. Absorption of the antigens at that time stimulated the synthesis of IgM antibodies specific for the three antigens, as soon as five days after the first feeding. A switch to IgG synthesis was observed after three weeks. IgE specific antibodies were difficult to detect, probably due to the competition with antibodies of the same specificity, but of other isotype. Therefore, we looked for total IgE antibodies. Like IgM antibodies, they appeared five days after the first feeding. Mice of group II, which had not been challenged with βLG, showed however high titers of antibody against this protein. This could be explained by the triggering of idiotype-specific B-lymphocytes which have an ability to enhance idiotype-specific anti-βLG production (5). This hypothesis was confirmed by the measure in RIA and in "in vitro" binding studies of high amounts of anti IgE antibodies (some of them being anti-idiotypic), in groups I and II. An increase in IgE antibodies and specific antibodies against the two "bystander" antigens was also seen in group II. This fact would suggest that an hyperpermeability has also occurred in this group. Studies are in progress to verify whether injection of IgE monoclonal antibody alone and subsequent production of anti IgE antibodies can lead to this phenomenon. Mice of group III stayed in a state of tolerance toward βLG and cod fish parvalbumin, but displayed antibodies against edestin after the third week. In conclusion, it was shown that ingestion of food antigens during gut hyperpermeability can brake immunological tolerance and lead to the production of IgE and IgG antibodies against these antigens.

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Anti-gliadin antibodies in the induction of experimental enteropathy

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Introduction

Diet-induced enteropathies, such as coeliac disease are thought to be immunologically-mediated, principally by cell-mediated mechanisms, although there is evidence for involvement of humoral mechanisms in the pathogenesis [1, 2]. Our aim was to investigate the role of antibodies in the induction of experimental inflammation in response to dietary antigens, using wheat gliadin as the test antigen.

Materials and Methods

A colony of Balb/c mice was established and maintained through multiple generations on a cereal-free diet. Splenocytes from mice immunised with gliadin were fused with NS0 myeloma cells. Hybridoma clones secreting antibody specific to gliadin were isolated by repeated limiting dilution and screened by ELISA. For 'in vivo' experiments, antibody GD3 of IgG_1 class was produced in the form of ascites.

IN VIVO ASSAYS

Intradermal Antigenic Challenge: GD3 antibody (50μl) in saline was injected intravenously (I/V) into mice, followed by intradermal (I/D) inoculation of gliadin or ovalbumen into footpads. Control animals received saline I/V. Footpad thickness was measured before injections and at 4, 8 and 24 hours. The mean of 10 measurements were taken for each footpad; results are quoted as mean ± SEM and compared by the Mann Whitney U-Test.

Feeding Experiments: GD3 (50μl) was injected I/V as above. One group of mice was fed immediately with 25mg gliadin or BSA by intubation, then sacrificed at 4 hours and serum samples and intestinal tissue were collected. The remaining mice were fed daily with 25mg gliadin or BSA. After 4 days, mice were footpad challenged and thicknesses were measured as above. Serum samples and intestinal tissue were also collected.

Morphological studies of intestinal mucosa: samples of intestinal mucosa were collected from mice at 5, 10 and 20cm distance from the stomach. Tissues were formalin fixed, sectioned and stained with H&E. Villous height and crypt depth were measured, together with the number of intraepithelial lymphocytes (IEL) per 100 enterocytes and enterocyte height in the villi [3].

Results

The anti-gliadin monoclonal antibody GD3 reacted with a common determinant of wheat gliadin sub-units and with other cereal prolamins, as assessed by immunoblotting.

FOOTPAD ANTIGENIC CHALLENGE

After I/V administration of GD3 antibody, a significant increase ($P<0.001$) in footpad thickness was observed at 4 hours following challenge with gliadin (0.27mm ± 0.020), compared with controls (0.072mm ± 0.023). Footpad thickness returned to normal after 24 hours.

FEEDING EXPERIMENTS

In groups of mice injected with GD3 antibody and fed daily with gliadin, the footpad response to gliadin challenge was abolished at 4 days (0.0027mm ± 0.023). In control mice injected with GD3, but maintained on a diet without gliadin, or fed BSA daily, a significant footpad response was demonstrated to gliadin (0.187mm ± 0.019 and 0.155mm ± 0.031, respectively). This response was significantly reduced compared with immediate challenge after injection of antibody ($P<0.011$ and $P<0.005$).

Circulating GD3 antibody titres: measurement of serum anti-gliadin antibody titres confirmed the removal of circulating antibody in mice fed gliadin daily over 4 days, compared with those fed BSA. Even at 4 hours post-feeding, there was a significant reduction in anti-gliadin antibody titre between the two groups ($P<0.002$).

Measurements of Enteropathy: There were no alterations in the mean height of villi, depth of crypts, infiltration of IEL or enterocyte height in experimental or control groups after 4 hours or 4 days.

Discussion and Conclusions

A number of groups have studied the effects of antibody and immune complex administration on the regulation of B cell responses in mice [4, 5]. However, the direct effect of such complexes on the mediation of intestinal disease processes has not been studied. Circulating monoclonal anti-gliadin antibody GD3 was capable of mediating inflammatory responses 'in vivo' after footpad challenge. However, this antibody alone appeared insufficient to induce significant enteropathy after oral gliadin challenge, although the antibody was specifically removed from the circulation.

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Responses of antigen-specific long term murine T cell lines to gliadin components

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Introduction

We have been interested both in the "in vitro" testing of gluten toxicity and in the immunological responses evoked by different gliadin fractions. In order to study these areas further, we have developed long-term murine T-helper cell lines which were specifically sensitive to gliadin components.

Methods

Antigen-specific, long-term T cell lines [1] were established from gluten-free Balb/C mice, pre-immunised with purified unfractionated wheat gliadin, or with fractions α, β, γ and ω, or GFIII. Antigenic specificity and cross-reactivity were assessed by stimulation assays and ^{3}H-thymidine incorporation.

Results

More than 85% of cells were of the T helper phenotype (Thy.1^{+}, L3T4^{+}). The cell lines remained stable and antigen-specific for up to 12 months (C.V. = 9.8%). Dose-related responses occurred to all gliadins, peaking at 125-250μg/ml. Unfractionated and α gliadin sensitised cells showed the highest incorporation, whereas cells sensitised to ω gliadin showed the lowest responses. None of the gliadin-sensitive cell lines showed any significant responses to ovalbumen, cytochrome C or a synthetic gliadin dodecapeptide homologous to an amino acid sequence in the adenovirus 12 E1B-58kDa protein.

Unfractionated and α-sensitive cells were the most restricted in their response, and ω-sensitive cells the least restricted. Conversely, unfractionated and α gliadin were the most effective

stimuli, being able to stimulate all cell lines to a similar degree. Beta and γ gliadins were less effective and ω gliadin and GFIII the least effective, although ω was more specific, stimulating principally ω-sensitive cells (Fig.)

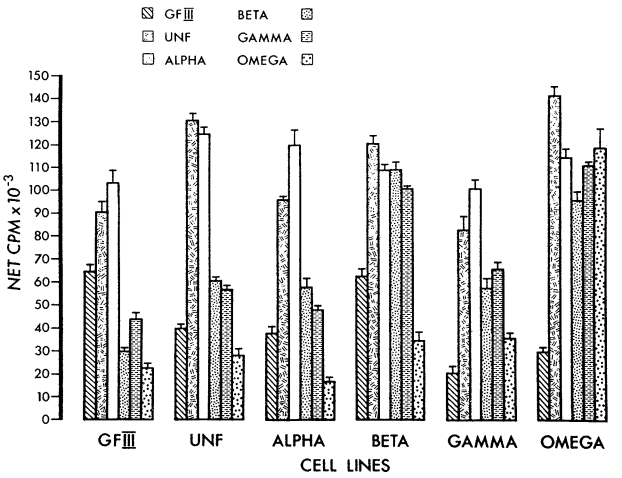

Figure. Peak ^{3}H-thymidine uptake (mean cpm ± SEM minus background 2335 ± 237 cpm) of each cell line to each gliadin preparation.

Discussion

These observations show that wheat gliadin and its major subfractions elicit specific T cell responses in mice, and that long-term T helper cell lines can be established and remain antigen-specific. The dose-response data suggest that α gliadin is the most antigenic, supported by its being the most effective stimulus in the cross-reactivity experiments. The lack of any response by gliadin-sensitive cells to the synthetic dodecapeptide of gliadin suggests that there is no antigenic epitope recognisable by the T cells and queries the proposed role of adenovirus 12 in the pathogenesis of coeliac disease. The cross-reactivity between gliadin fractions and the possible gradation of antigenicity are in accord with observations in coeliac disease, i.e. that there is both a mucosal and immunological response to all gliadin fractions, but that α gliadin is the most active in these respects.

Acknowledgments & Reference

We wish to thank Dr. P. J. Ciclitira (London), Dr. D. G. Hanson and Professor W. A. Walker (Boston) and Dr. D. P. Jewell (Oxford) for their help and gifts of various gliadin fractions.

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Disturbances in intestinal motility associated with soya-allergy in calves

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Introduction

Our earlier studies[1] showed that preruminant calves can develop gastrointestinal motor disturbances which apparently resulted from an allergic reaction to dietary soya protein. These disorders were accompanied by a rise in soya-specific IgG. The present study examined a possible link between systemic immune responses to soya and abberations in gut motility.

Methods

High titre bovine soya-specific antibody was passively transferred to 5 soya-naive calves. Three days later they were challenged with a feed containing heated soya flour. Further oral challenges with soya protein were given twice weekly. Gut motor disturbances were examined by recording myoelectic activity from electrodes implanted on the duodenum. Soya-specific IgG and IgG1 antibodies were measured in peripheral blood samples by ELISA.

Results

High titres of circulating soya-specific IgG and IgG1 antibodies were detected after passive transfer of soya-specific serum (Fig 1). No significant changes in titre occurred after repeated oral challenges with soya.

Fig 2. shows the changes in motor activity observed in the 5 calves during the experimental period. Despite the high level of soya-specific antibody, the number of propulsive contractions (periods of regular spiking activity, RSA) seen on the duodenum after the 1st soya feed (2) was similar to values after control casein-containing feeds (1 and 3). After many oral challenges with soya, there was an abnormal increase in number of RSAs and diarrhoea developed (4).

Fig 1

Serum soya-specific antibody following passive serum transfer & oral sensitisation with soya

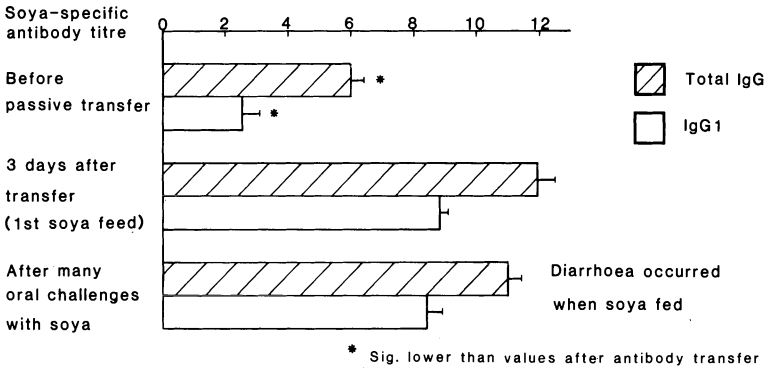
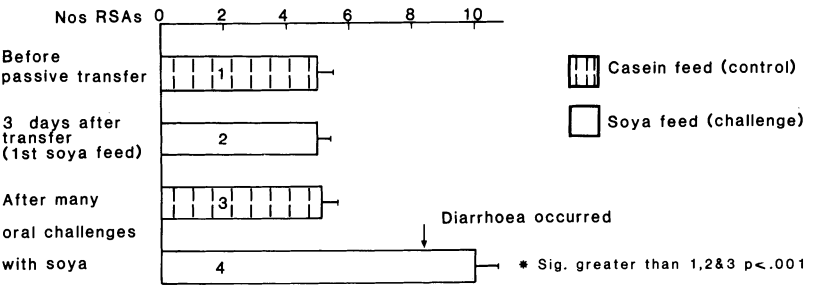


Fig 2

Propulsive contractions (RSAs) on duodenum during 360 mins after feeding



Discussion

There was no correlation between levels of serum soya-specific IgG or IgG1 and disturbances in gut motility after a soya feed. This suggests that soya-specific IgG and IgG1 may not be directly responsible for the disturbances in intestinal motility.

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Lymphocyte activation by gliadin in coeliac disease and in experimentally induced enteropathy of germfree rats

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The hypothesis of an immunologically mediated mechanism of mucosal damage by gluten has lately prevailed in the study of pathogenesis of coeliac disease (CD) /1-3/. We studied human lymphocyte response to gliadin and the behaviour of intraepithelial lymphocytes (IEL) in experimentally induced enteropathy after a repeated intragastric administration of gliadin to germfree (GF) rats early after birth.

MATERIALS AND METHODS

Patients. 24 adult patients with CD diagnosed upon biopsy were examined. Most of them (18) were on a gluten free diet. 24 healthy donors of the same age range were used as controls.

Lymphocyte stimulation in vitro. Mononuclear cells isolated from peripheral blood (MNC) were cultured for 7 d with PWM (5 µg/ml) or with gliadin (Frazer's III fraction) at 100, 10 and 1 µg/ml, and concentration of IgM, IgA and IgG was determined in the culture media by ELISA /4/.

Detection of antibodies. The repertoire of antibody specificities in culture media was determined by microELISA. Antigliadin antibodies were determined by the method previously used for diagnostic purposes /5,6/. Antibodies against haptens (TNP, FITC) were determined according to /7/. Incidence of autoantibodies was determined by using the following antigens: ssDNA, IgG and thyreoglobulin /6/.

Autoantibodies against enterocytes. A villus-to-crypt gradient of intestinal epithelial cells was prepared by 10-15 min successive incubations of everted segments of rat jejunum or parts of human duodenum obtained by surgery in PBS containing 1.5 mM EDTA and 0.5 mM dithiothreitol at

37 °C. After double washing in PBS the cells ($2x10^6$/ml PBS) were used for coating the wells of microplates. A 12-h incubation at 4 °C was followed by fixation with 0.5 % glutaraldehyde and the plates were blocked with 1 % bovine serum albumin.

Induction of enteropathy in GF rats with gliadin and the study of activity and IEL. GF rats fed after weaning on a diet containing mostly soybean flour were given, immediately after birth and then 3-times a week to the age of 2 months, intragastrically 0.5 % gliadin in increasing doses (0.1-1 ml) and the extent of damage to the intestinal mucosa was assessed on histological sections /8/. IEL isolated from the jejunum of 2-month old gliadin-treated and control rats perfused with PBS were purified on a nylon wool column /9/. Indirect immunofluorescence with the antibody MRC OX8 (Serotec) showed that the resulting suspension of IEL contained 80 % CD8+ lymphocytes. IEL were labelled with fluorescein /10/ and administered in a dose of $5x10^5$ into surgically prepared jejunal loops of 1-month old GF rats. The localization of the labelled lymphocytes and the degree of damage to the enterocytes were evaluated on histological sections.

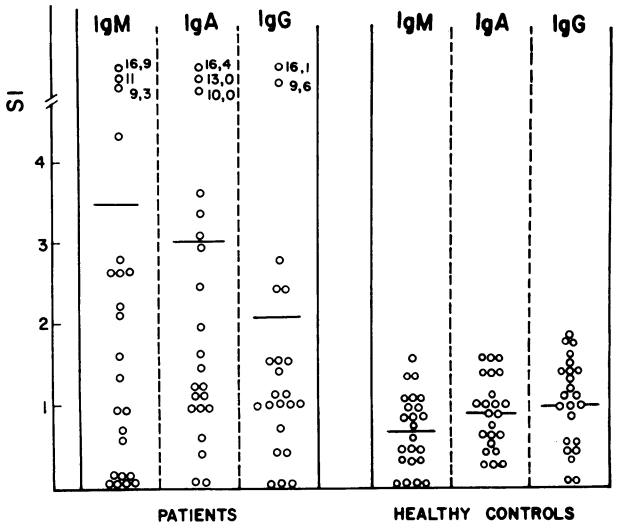

Fig. 1. Ig production by MNC of adults with CD after in vitro stimulation with gliadin. SI = stimulation index, ie. ratio of the amount of Ig produced in samples with gliadin to the amount of Ig in gliadin-free media.

RESULTS AND DISCUSSION

Polyclonal activation of lymphocytes from coeliacs by gliadin Production of immunoglobulins (Ig) determined in media after culturing MNC from patients and healthy controls with PWM was about the same. However, cultivation of MNC from patients with an optimum concentration of gliadin (10 µg/ml) caused a significant stimulation of production of Ig of all 3 isotypes (Fig. 1). Cultivation of separated cell populations showed that the increase in Ig production after gliadin stimulation occurs only in the presence of T cells and monocytes. Assessment of the antibody repertoire of Ig present in cultures of CD lymphocytes with gliadin indicated polyclonal activation of B cells: culture supernatants contained antibodies not only against gliadin but also against haptens (TNP, FITC) and autoantigens - ssDNA and IgG. Important was the finding of antibodies directed against enterocytes (Table 1). High levels of these antibodies were also recently found in a medium after a short cultivation of jejunal biopsies from CD patients and in the sera of CD patients (Table 1). Our results thus imply that in CD patients gliadin may act as a T dependent polyclonal B cell activator and so stimulate autoantibody production. The higher occurrence of B and T lymphocytes reacting with enterocyte antigens in CD patients points to the presence of an autoimmune process directed against intestinal mucosal cells.

TABLE 1. Anti-enterocyte IgG autoantibodies in sera of CD patients and in culture medium after stimulation of MNC with gliadin (means ± SEM in per cent of reference serum)

	Enterocytes used in ELISA		
Samples	Rat (villi)	Rat (crypts)	Human (pool)
CD patients/sera	154.5 ± 5.4	130.1 ± 3.8	150.6 ± 9.5
Controls/sera	102.6 ± 6.9	72.8 ± 4.0	96.2 ± 10.5
CD MNC/media	21.3 ± 2.1	34.2 ± 4.3	n.t.
Control MNC/media	3.4 ± 0.3	5.4 ± 0.2	n.t.

Participation of IEL in gliadin-induced enteropathy of GF rats. Repeated intragastric administration of 0.5 % gliadin from the birth to 2 months of age caused a shortening of jejunal villi, increase of lymphocyte infiltration and other morphological changes resembling those observed in CD patients (Table 2). Moreover, increased numbers of lymphocytes producing IgA and IgG in the lamina propria and a higher level of IgG and IgA anti-gliadin antibodies were found in these rats /8/. IEL isolated from gliadin treated rats and labelled with fluorescein, and then applied into jejunal segments of 1-month old untreated inbred GF rats,

passed within 1 h through the epithelial layer of the villi in contrast to lymphocytes from untreated GF controls which remained in the intestinal lumen. Six hours after application of IEL the jejunal segments displayed a marked damage.

TABLE 2. Changes in the jejunal mucosa of GF 2-month-old rats after repeated intragastric administration of gliadin (means ± SEM in/um)

Rats	Villus height	Crypt depth
Gliadin treated	326.2 ± 13.4 (17)	202.6 ± 6.0 (17)
Control	500.8 ± 18.1 (12)	115.7 ± 4.6 (12)

Our results point to the possible participation of B and T lymphocytes polyclonally activated by gliadin in the pathogenesis of human coeliac disease and gliadin-induced enteropathy. The role of intestinal lymphocytes polyclonally activated with PWM and anti-CD3 antibodies in the development of enteropathy has recently been elegantly proved by MacDonald and Spencer /11/ who pointed out the existence of a common effector mechanism causing damage of the intestine.

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Gastric mucosal barrier dysfunction during local and systemic anaphylaxis

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Introduction

Gastrointestinal anaphylaxis alters the integrity of the intestinal mucosal barrier in various ways. IgE mediated anaphylaxis increases intestinal permeability, may lead to mucosal damage and may trigger the release of intestinal globlet cell mucus.(1) Furthermore, there is enhancement of dietary antigen uptake in the intestine during anaphylactic reactions.(2) Such enhanced uptake of dietary antigens may stimulate the production of antibodies to these antigens. Macromolecular antigen uptake has only been considered to take place in the small intestine. We have developed a murine model to test the effect of systemic and local anaphylaxis on the gastric mucosal barrier and on the uptake of a bystander antigen from the stomach into the systemic circulation.

Methods

BDF_1 female mice were immunized with dinitrophenylated Ascaris suum extract. Immunized animals underwent laparotomy and non-traumatic pyloric occlusion using a microvascular clamp. Following wound closure, animals were gavage-fed 50 mg ovalbumin (OVA) together with 5µg, 10µg, or 50µg of the divalent hapten N,N'-di-2,4, dinitrophenyllysine (di-DNP-lysine) to induce a local anaphylactic response. Other mice were subjected to systemic anaphylaxis by intravenous injection of di-DNP-lysine given one minute after gavage feeding of OVA. Controls received saline intragastrically or intravenously. Blood was obtained by direct cardiac puncture 30 min. after challenge. Sera were assayed for immunoreactive OVA (iOVA) by a double antibody enzyme-linked immunosorbent assay (ELISA). Tissues were fixed in Karnovsky's fixative and 1 µm sections evaluated following Epon-embedding. Changes in vascular permeability during systemic anaphylaxis were assessed in separate experiments. Immunized mice which had undergone surgery were injected with 0.25 µCi ^{125}I-BSA and 5 µg di-DNP-lysine. 30 min. later, the animals were perfused with PBS via the right ventricle. Following homogenization of rinse fluid and organs, total and TCA-percipitable cpm were measured.

Results

Following intravenous administration of 5 µg di-DNP-lysine a ten-fold rise of immunoreactive ovalbumine serum levels (397 ± 106.1 ng/ml) above control values was seen (28.5 ± 4.7 ng/ml) (x ± SEM; $p < 0.01$). Intragastric administration of 50 µg di-DNP-lysine led to an over six-fold increase of iOVA levels (180 ± 26.3 ng/ml) ($p<0.05$). Unsensitized as well as sensitized control mice showed a consistent low level of ovalbumine uptake. Administration by gavage of 5 µg or 10 µg di-DNP-lysine together with 50 mg OVA did not lead to a significant rise in iOVA levels.

The total radioactivity in the stomach wall extract, as well as TCA-percipitable radioactivity of the gastric rinse fluid was significantly increased in animals undergoing anaphylaxis compared to controls ($23.6 \pm 3.9 \times 10^3$ cpm vs. $8.7 \pm 2.1 \times 10^3$ cpm in the gastric wall and $12.8 \pm 3.9 \times 10^3$ cpm vs. $9.7 \pm 1.2 \times 10^3$ cpm in the gastric rinse fluid; $p<0.05$).

Morphologic examinations revealed that mast cell counts in control mice immunized with DNP-Ascaris extract increased significantly by 75% when compared to non-immunized animals. Intravenous and intragastric challenge of immunized mice led to substantial mucosal injury. The superficial mucosa showed compactation of erythrocytes in superficial mucosal blood vessels, enhanced desquamation of necrotic cells, degranulation of mast cells and edema in the lamina propria mucosae. In immunized mice challenged by i.v. administration of 5 µg di-DNP-lysine, the submucosal mast cell count was reduced by 77% and the mucosal mast cells by 45% of that of immunized controls. This reduction in cell number presumbly reflects the complete release of granules from the cells.

Conclusions

Our findings suggest that the stomach plays a role in the uptake and possibly "processing" of food antigens in unprimed mice and mice which have undergone anaphylaxis. By which exact pathway such proteins may transverse the gastric mucosa is still under investigation. The stomach as the first digestive organ of the gastrointestinal tract to have prolonged contact with food antigen, may also be the first to sample macromolecular antigens and therefore initiate immunologic reactions towards these antigens. Active immunization elicitiing an IgE-mediated response can sensitize the gastric mucosa towards antigen. Challenge leads to gastric anaphylaxis and enhanced uptake of antigen. Such rapid influx of antigen may effect priming or tolerance reactions. Furthermore, hypersensitivity reactions taking place in the stomach may be an important mechanism of gastric mucosal damage.

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SECTION W: INFECTION AND MUCOSAL IMMUNITY

(A) Human

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Mucosal humoral responses in histologically normal gastric mucosa and *C. pylori* associated chronic gastritis

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Introduction

Chronic gastritis is a histological entity comprising a chronic inflammatory infiltrate with or without an active neutrophil infiltrate. Over the past 6 years the organism Campylobacter pylori (CP) has been found to be strongly associated with non-autoimmune chronic gastritis world-wide. Ingestion and treatment studies strongly suggest that the organism is the cause of the gastritis rather than a colonizer of already abnormal mucosa. Why the organism is pathogenic is unclear. Systemic humoral responses have been demonstrated by many groups and a previous study has suggested a local humoral response to CP with organism specific antibodies being identified in gastric juice [1]. The aim of the present study was to investigate possible gastric mucosal humoral responses to CP in bacteria positive and negative patients.

Methods

A total of 16 dyspeptic patients without ulceration were studied using endoscopic antral biopsies. One formalin fixed biopsy was used for histology (H&E) and CP identification (modified Giemsa). At endoscopy antral biopsies were taken. One was formalin fixed and routinely processed with the histological assessment being carried out on H & E stained sections and CP colonization judged on modified Giemsa stained sections [2]. Plasma cells were assessed by the indirect immunoperoxidase technique on formalin fixed sections. Plasma cell counts per unit area of mucosa were made for each antibody class, using an eye-piece graticule; at least 5 counts were made for each biopsy, and the mean value expressed per 0.25 mm^2 of mucosa. One biopsy (control) was immediately frozen in calcium chloride and stored at -70 °C. Two biopsies were separately divided and immersion cultured in RPMI supplemented with 10% FCS and glutamine. The media was changed daily for six days with the supernatents being stored at -70 °C.

The total immunoglobulins were assayed by indirect ELISA. The CP specific immunoglobulins were assayed by indirect ELISA using an ultracentrifuged sonicate as antigen.

Results

Seven patients were CP positive with 1 of the CP negative patients

having autoimmune chronic gastritis. In the culture studies the antibody content of the control biopsies was usually exceeded within 24 hours of culture. Maximal production occurred within 72 hours. Comparing the CP positive and negative patients the former had significantly higher IgG, IgA and IgM plasma cell counts per unit area of mucosa and produced significantly greater total IgA, sIgA and IgM. There was no significant difference in IgG production. CP specific immunoglobulins (IgG, IgA & IgM class) were only produced by colonized biopsies (Fig.1).

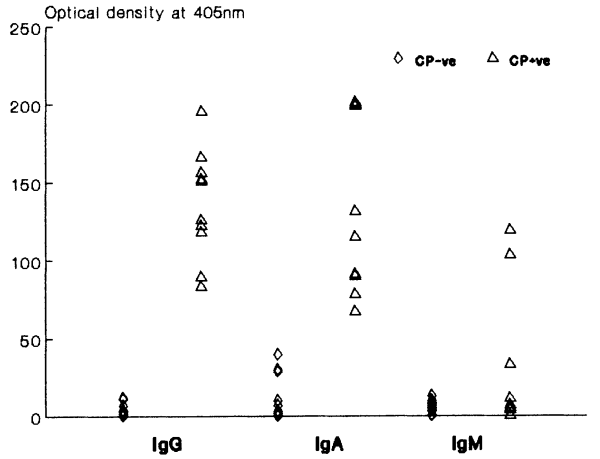

Fig.1. CP specific antibodies in pooled culture supernatents.

Discussion

These results demonstrate increased local production of total and CP specific immunoglobulins in colonized subjects. This local production correlates with the increased plasma cells found in CP associated chronic gastritis. The most marked antibody response was seen with IgA which is typical of mucosal humoral responses. The local CP specific humoral response indicates that a proportion of the inflammatory cell infiltrate seen in CP associated chronic gastritis is a response to CP antigens. This local response is of great interest as it is longstanding and does not appear to clear the organism. Whether this longstanding respons is in itself damaging is unclear. Further studies on this immune response may yield important information concerning host defences as the stomach is relatively accessible yet unlike the majority of the bowel uncontaminated by other bacteria.

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Local and systemic immune responses during *C. pylori* infections

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Campylobacter pylori is a Gram negative, flagellated microaerophilic bacterium which is characterised by its abundant urease production. This organism colonises the mucosa of the human gastric antrum where its presence is invariably associated with histologically defined chronic gastritis. The majority of patients colonised with C.pylori elicit a significant immune response detectable by ELISA techniques. Despite this immune response infection, once acquired, is apparently lifelong. We have investigated the ineffectiveness of this response by analysing the specificity of antibodies produced systemically and locally. Additionally we have analysed the types of immunocompetent cells infiltrating the gastric mucosa using immunohistological techniques.

MATERIALS AND METHODS:
C.pylori proteins were fractionated, on the basis of size exclusion, by FPLC. The urease containing fractions were identified by enzymic activity and the putative flagella containing fractions by cross-reaction with monoclonal antibodies against C.jejuni flagella. The fractions were analysed for antigenicity, using human sera, by ELISA and protein profile by SDS-PAGE.

Sera were collected from patients of known C.pylori status determined by culture and/or histology of endoscopy biopsies. Lymphnodes draining the stomach were obtained from patients, infected with C.pylori, undergoing vagotomy. The lymphnode lymphocytes were cultured overnight then stimulated by EBV transformation. Antibody secreting cells were enriched by dilution cloning or by rosetting with Dynabeads coupled to C.pylori antigens.

The specificities of antibodies against C.pylori antigens were identified by immunogold labelling at the ultrastructural level, western blotting of SDS-PAGE gels and radioimmunoprecipitiation of ^{125}I-labelled surface

proteins. The isotype of these antibodies was determined by ELISA using class specific goat antibodies coupled to peroxidase and subclass specific mouse monoclonal antibodies.

The cellular infiltrate into the gastric muscosa was identified using a panel of T and B cell markers in immunoperoxidase staining of cryostat sections of endoscopy biopsies.

RESULTS:

Immunogold labelling techniques clearly demonstrated that serum antibodies bound to both the bacterial surface and the sheathed flagella. The major immunoglobulin class of this response was IgG of the IgG1 and IgG4 subclasses. In most cases a rise in specific IgG2 and IgA was also seen but IgM antibodies were rarely detected.

The urease isolated by FPLC comprised 3 major polypeptides (61K, 56K and 31K). The putative flagellin protein had a 54K major polypeptide band. Both the urease and flagella containing fractions were highly antigenic when tested against patient sera by ELISA.

Serum antibodies were directed against a number of C.pylori antigens, as detected by western blotting and radioimmunoprecipitation. However, a remarkable variation in the antibody specificity to the protein antigens of this organism was observed.

Prior to transformation the lymphnode cells produced antibodies mainly of the IgA isotype and had a similar specificity to those found in the serum of the patient. Post EBV transformation the antibodies produced were IgM and these were primarily directed against the putative 54K flagella polypeptide.

Immunohistology of biopsy sections indicated that the cellular infiltrate comprised of mature B cells and CD3 positive T cells, mainly of the CD8 positive subset.

DISCUSSION:

Our results indicate that colonisation of the human gastric mucosa by C.pylori induces a considerable cellular and humoral immune response. The specificity of systemic antibodies for the surface proteins of C.pylori has been established. Similar specificities of locally produced antibodies were observed. It is evident that the reasons why these responses do not eliminate the infection, or even prevent recrudescence, requires further investigation. Moreover, the infiltration of CD8 positive T cells into the gastric mucosa, presumably in response to the bacterial antigen load, may be responsible for the gastroduodenal disease associated with this infection.

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False positive with *Campylobacter pylori* antibody ELISA?

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Introduction

A serological assay for *C. pylori* would be more convenient than gastric biopsy tests. A variety of ELISA assays for *C. pylori* have been reported, the sensitivities and specificities vary considerably [1]. Some groups have satisfactorily used crude antigen extracts of *C. pylori* while others report a high rate of false positive reactions. We have been impressed by the bimodal serology produced by crude antigen extracts and have undertaken an evaluation of an ELISA based on this extract taking into account the results of microbiological culture, microscopy, urease tests and the degree of inflammation in the the biopsy.

Materials and Methods

A total of 276 patients referred for investigation of upper-gastrointestinal symptoms were studied. Biopsies were taken from the gastric antrum. The presence of *C. pylori* was determined by microscopy, urease activity (CLO test, Auspharm, Sydney) and culture on modified Brucella agar. Inflammation in the biopsies was classified as active chronic, chronic or normal and further graded as severe, moderate, mild or minimal. ELISA plates were coated with a crude extract of *C. pylori* prepared by sonicating washed organisms and microcentrifuging the sonicate for 5 minutes at 10^3G. Sera were tested at a dilution of 1/50. A standard curve was used in each run in order to convert optical densities to ELISA units. Based on patients with normal histology, values of <5 units/ml were considered negative.

Results

The performance of the biopsy tests and ELISA for *C. pylori* in relation to the type of inflammation seen in the biopsy is shown in Table 1. The low frequency of ELISA positive reactions in patients with normal histology indicates that the ELISA is specific. A higher rate of positive reactions was observed among patients with only chronic inflammation (28.2%) and with active chronic inflammation (86%) compared to the CLO test, culture and microscopy. Of 14 patients with active chronic inflammation but negative ELISA only 4 had

evidence of *C. pylori* infection from the other biopsy assays. The ELISA results of patients with only chronic inflammation were analysed with respect to the severity of chronic inflammation. The frequency of positive ELISAs was high among patients with moderate chronic inflammation (75%) and, although lower in patients with mild or minimal chronic inflammation (25%), it was still higher than in patients with normal histology.

TABLE 1. Comparison of CLO, culture, microscopy and ELISA in patients with different degrees of gastric inflammation.

		GASTRIC HISTOLOGY		
		NORMAL	CHRONIC	ACTIVE/CHRONIC
	-	31	64	22
CLO	+	0	6	58
	-	30	68	24
CULTURE	+	0	2	49
	-	33	67	22
MICRO	+	0	4	58
	-	38	62	14
ELISA	+	1	26	86

Discussion

The serum ELISA using a crude antigen extract had a specificity of 97% based on patients with normal histology. The high degree of specificity suggests that positive reactions in subjects with chronic inflammation are true positives and therefore, that ELISA is more sensitive than the other tests. There are two possible reasons for the discordance between ELISA and the other tests in patients with only chronic inflammation. First, chronic inflammation may result from infection with numbers of organisms too low to be detected by CLO, culture or microscopy. Secondly, chronic inflammation may be a sequel to active inflammation in patients who have completely cleared their infection. The ELISA would still be positive due to the persistent nature of serology. If this second scenario is true then the specificity of ELISA for current infection would be low. The absence of a definitive assay for *C. pylori* infection makes it difficult to assess the performance of ELISAs, however it has been shown that urease test, culture and microscopy are insensitive compared to indirect immunofluorescence [2]. Our data suggest that crude extracts of *C. pylori* result in an ELISA which is specific for *C. pylori* antibody. Evaluation of ELISA tests for *C. pylori* should not be based on an assumption that any other single test is superior.

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The preparation of anti-*Campylobacter pylori* monoclonal antibodies and their clinical applications

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ABSTRACT. Three anti-Campylobacter pylori monoclonal antibodies were prepared. MoAb CP1 was suitable for the detection of C.pylori on tissue section by immunostaining, which was more sensitive than culture. The detection of circulating antibody to CP3 antigen defined by MoAb CP3 was useful to distinguish the disease groups from normal controls and to evaluate the grade of gastritis.

1. INTRODUCTION

Since Marshall and Warren's reports in 1983, the association of Campylobacter pylori(C.pylori) with gastritis and gastro-duodenal ulceration has renewed much interest. It has already reported that C.pylori was cultured from antral biopsy specimens in 60 to 70% of the patients with gastric and duodenal ulcers and also anti-C.pylori antibody was detected in sera of the patients. In order to detect C.pylori specifically on tissue section and detect a unique antibody against C.pylori in sera, monoclonal antibodies(MoAb) against C.pylori was prepared and tested for clinical significance.

2. MATERIALS AND METHODS

2.1. Monoclonal antibodies

Immortalized C.pylori(ATCC strain) was injected to Balb/c mouse and hybridomas of the splenocytes and mouse myeloma cells were screened by ELISA. Analysis of MoAb and detection of CP3 antibody in sera were performed by ELISA.

2.2. Immunoperoxidase staining

The indirect immunoperoxidase staining with anti-C.pylori MoAb was performed on tissue section. The histological grade of gastritis was classified according to Warren's grading.

2.3. Western blotting

Sonicated C.pylori antigen was electrophoresed in SDS-gel, and transfered to nitrocellulose membrane. The membrane was reacted with

each monoclonal antibody or the patient serum and then reacted with peroxidase labeled anti-mouse immunoglobulin (DAKO) or peroxidase labeled anti-human immunoglubulin (DAKO)and developed.

2.4. Affinity chromatography
Purified MoAb CP3 was coupled with CNB activated Sepharose 4B beads to make the affinity column and the corresponding antigen was purified from the sonicated C.pylori antigen.

3. RESULTS AND DISCUSSION

3.1. Monoclonal antibodies
Among 15 MoAb obtained, three MoAb CP1,CP2 and CP3 strongly reacted with sonicated C.pylori, while they did not react with C.jejuni, C.coli,C.fetus and E.coli at all. By western blotting analysis, MoAb CP1 reacted with a 36K dalton molecule of sonicated C.pylori, CP2 reacted with 58K as well as 180K dalton molecules and CP3 reacted with 25K and weakly with 58K dalton molecules[1].

3.2. Immunostaining of C.pylori on tissue section
Out of 25 specimens of chronic gastritis, 18 were positive (72%) for CP1 immunostaining and 14(56%) for culture. In gastric ulcer, 22/26(92%) were positive for CP1 immunostaining and 16/26(62%) for culture. Thus immunostaining method was more sensitive than culture method.

3.3. Profile of the serum antibody against C.pylori
Serum antibody in patients and control subjects against sonicated C.pylori antigen were analyzed by western blotting. Interestingly, the titers of the antibodies against 110K, 60K, 36K and 25K dalton molecules of the antigen were remarkably different between sera from patients and from controls. Firstly, we have decided to focus on the 25K dalton molecule antibody in sera.

3.4. Detection of CP3 antibody in sera
By western blotting, the patient serum clearly reacted with affinity purified CP3 antigen. Using ELISA consisting of purified CP3 antigen, the sera of gastric ulcer and chronic gastritis patients showed significantly higher titer(mean O.D.; 1072, 984, respectively) of CP3 antibody than controls(mean O.D.; 177) and the titer was related to the histological grade of gastritis. Thus the detection of CP3 antibody is considered useful for sero-diagnosis and evaluation of the grade of gastritis.

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Duodenal secretion of *Campylobacter pylori*-specific antibodies: relationship to gastroduodenal inflammation

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ABSTRACT. **Campylobacter pylori** is a recently discovered micro-organism found in the stomach of most patients suffering from chronic active gastritis. It is also found on gastric epithelium in the duodenum in the majority of those with duodenitis and also duodenal ulcer disease. Although gastric colonisation has been shown to produce a specific systemic and local humoral immune response, the results of duodenal infection are unknown. We have investigated the duodenal humoral immune response to **C.pylori** colonisation in 32 patients with dyspepsia. In vitro production of **C.pylori** -specific antibodies in cultured duodenal biopsies was assessed by ELISA. The duodenal secretion of **C.pylori** -specific IgG and IgA was elevated in patients with **C.pylori** gastritis, higher titres of IgA being secreted by the first part of the duodenum than the second. In the first part of the duodenum patients with duodenitis secreted significantly higher levels of **C.pylori** -specific IgA than patients with gastric colonisation but normal duodenal histology ($p<0.05$). This study suggests that the mucosal immune response to antral **C.pylori** colonisation is not confined solely to gastric tissue. The raised titres of duodenal **C.pylori** -specific IgA secreted by patients with active duodenitis suggest an enhanced local response to **C.pylori** which may have relevance to the pathogenesis of this condition.

INTRODUCTION

Colonisation of the gastric mucosa with **Campylobacter pylori** has been shown to result in both a specific systemic (1,2) and local immune response (2) to the bacterium. Mucosal anti- **C.pylori** antibodies have been found in gastric juice of fasted patients and short term organ culture of antral biopsies has confirmed the gastric origin of this humoral response (3).

In addition to colonising the stomach, **C.pylori** has been identified on gastric metaplastic epithelium in the duodenum (4,5). The duodenal colonisation with **C.pylori** is invariably associated with active inflammation.

Little is known of local duodenal immune responses, although T lymphocyte regulation of humoral responses has been demonstrated (6). The aims of this study were to investigate whether there is a specific humoral response to **C.pylori** in the duodenal mucosa of colonised subjects.

METHODS

Patients

Thirty two dyspeptic patients were studied. Endoscopic biopsies were taken from the first and second parts of the duodenum and the gastric antrum. One biopsy from each site was formalin fixed for routine histology. Histological identification of **C.pylori** was carried out using a modified Giemsa stain. One biopsy from the first and second parts of the duodenum was frozen in 0.04% $CaCl_2$ for subsequent homogenisation and estimation of tissue **C.pylori** -specific antibodies.

Biopsy culture

Biopsies from the first and second parts of the duodenum were immersion cultured for three days at 37° C in RPMI 1640 additionally buffered with 20mM HEPES and supplemented with 40µg/ml gentamicin and 10% fetal calf serum. At the end of culture, supernatants were stored at -70° C prior to assay. The biopsies were homogenised in 2ml 0.04% $CaCl_2$ and total biopsy protein was determined by a modified Lowry method (7).

C.pylori ELISA

C.pylori -specific IgG and IgA antibodies were assayed by indirect ELISA using an ultracentrifuged sonicate from one strain of **C.pylori** as antigen. Positive and negative control sera were included in each assay.

RESULTS

Eleven patients had entirely normal antral and duodenal mucosa and twenty one patients had antral gastritis of whom six had active duodenitis in the first part of the duodenum (Table 1). The second part of the duodenum was histologically normal in all patients. No **C.pylori** were detected on histologically normal antral mucosa, whilst all patients with antral gastritis were colonised.

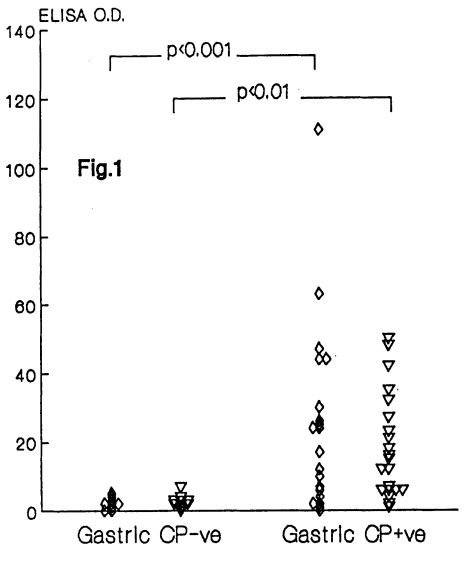
TABLE 1. Antral and Duodenal Histology

Antral Histology	Duodenal Bulb Histology	
	Normal	Active Duodenitis
Normal	11	0
Antral gastritis	15	6

The duodenal secretion of **C.pylori** -specific IgG (Fig. 1) and IgA (Fig. 2) in patients with **C.pylori** -associated antral gastritis was significantly greater than that of patients with normal antral mucosa. Higher titres of **C.pylori** -specific IgA were secreted by the first part of the duodenum than the second part (Fig. 2). Comparison of duodenal secretion of **C.pylori** -specific IgG and IgA in subjects with antral gastritis in relationship to their duodenal pathology showed no difference in the **C.pylori** -specific IgG secretion in patients with histologically normal duodenal mucosa and those with active duodenitis. However, in the first part of the duodenum, patients with active duodenitis secreted significantly higher ($p<0.05$) levels of **C.pylori** -specific IgA than patients with gastritis but normal duodenal histology (Table 2).

TABLE 2. Duodenal **C.Pylori** -specific IgA in patients with antral gastritis with or without active duodenitis

Duodenal Histology	ELISA O.D. x 100 (mean ± SEM)	
Normal	27 ± 6.1	
Active Duodenitis	65.3 ± 19.6	$p<0.05$



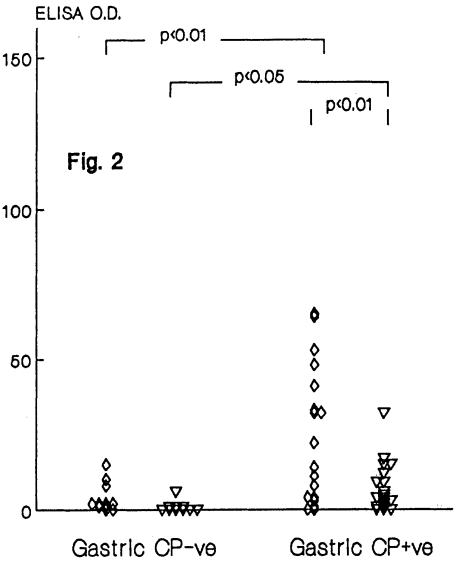


Figure 1 and Figure 2. ELISA titres for C.pylori-specific IgG (Fig. 1) and IgA (Fig. 2) secreted by the first (◊) and second (▽) parts of the duodenum in patients with and without C.pylori-associated antral gastritis.

DISCUSSION

Whilst serological responses to **C.pylori** have been well characterised (1,2), relatively little is known about mucosal immune responses to the bacteria. The extent of local immune responses within the gastrointestinal mucosa will reflect not only regional variations in antigen presentation, but also antigen localisation and antigen concentration. The present results demonstrate that specific **C.pylori** IgG and IgA antibodies are secreted during **in vitro** organ culture of duodenal mucosa of patients with antral gastritis; the IgA response being greater in the first part of the duodenum. As the majority of these patients had histologically normal duodenal mucosa, the results suggest that the mucosal immune response to antral **C.pylori** colonisation is not confined solely to gastric tissue. The local response in gastric tissue is characterised by increased numbers of activated T helper cells. Whether similar activation of duodenal T lymphocytes occurs in the duodenal mucosa is unknown.

A strong association between acid-induced duodenal gastric metaplasia, **C.pylori** -associated antral gastritis and active duodenitis has been reported (5). A synergistic role for **C.pylori** in the pathogenesis of duodenitis and duodenal ulceration has been proposed. The raised titres of duodenal **C.pylori** -specific IgA secreted by patients with active duodenitis suggest that the inflammation characteristic of duodenitis is at least, in part, an enhanced local response to **C.pylori** . This may be of relevance in the pathogenesis of duodenitis.

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Bacterial antigens in synovial fluid cells from patients with reactive arthritis

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ABSTRACT. Samples of synovial fluid leukocytes from 21 patients with reactive arthritis triggered by Yersinia enterocolitica O:3 infection were studied for the presence of bacterial antigens. Immunofluorescence with a rabbit antiserum to Yersinia enterocolitica O:3 and a monoclonal antibody to the lipopolysaccharide of the same bacteria was applied. Synovial fluid cells of 15 Yersinia patients stained positively in immunofluorescence. All the control samples from the 41 patients with other rheumatic diseases were negative. Synovial fluid cell deposits from 7 patients with Yersinia-triggered reactive arthritis and of 10 patients with other rheumatic diseases were studied also by Western blotting after electrophoresis using the same antibodies. Four samples of Yersinia patients gave positive results. All the control samples were negative. Our results indicate importance of bacterial structures in initiation and maintenance of reactive arthritis.

1. Introduction

Acute enteric infections caused by Yersiniae, Salmonellae, Shigellae and Campylobacters are sometimes complicated by development of reactive arthritis. These arthritides have been considered sterile in the sense that no microbes or microbial antigens enter the joints (Dumonde 1976). However, several immunological characteristics of patients developing reactive arthritis after Yersinia infection have suggested indirectly that bacterial antigens persist for long periods after the initial infection in these patients (Granfors et al. 1988, Toivanen et al. 1985). The most striking characteristic is especially strong and long persisting IgA response against Yersinia bacteria, seen particularly in antibodies of IgA2 subclass and those carrying the secretory component. These features have suggested the bacteria to persist in mucosal areas. Recently, also direct evidence for the persistence of Yersinia bacteria or antigenic structures in gastrointestinal mucosa of patients with arthritis has been obtained in immunofluorescence studies (Hoogkamp-Korstanje et al. 1988). We have found Yersinia antigens within immune complexes in circulation of patients with arthritis for long periods after acute Yersinia enterocolitica infection (Lahesmaa-Rantala et al. 1987a).

Recently, evidence has been obtained that microbial antigens also enter

the joints in reactive arthritides. Chlamydia antigens have been detected in synovial tissue and in synovial fluid cells after Chlamydia trachomatis urogenital infection (Keat et al. 1987, Schumacher et al. 1988). We have found Yersinia antigens within immune complexes in synovial fluid (Lahesmaa-Rantala et al. 1987b) and in synovial fluid cells (Granfors et al. 1989a) from patients with arthritis after Yersinia infection. In addition, Salmonella antigens were demonstrated in synovial fluid cells from a patient with Salmonella typhimurium-triggered arthritis (Granfors et al. 1989b).

2. Materials and Methods

2.1. THE PATIENTS AND THE SPECIMENS

Samples of synovial fluid leukocytes from 21 adult patients with reactive arthritis triggered by Yersinia enterocolitica O:3 were studied. The diagnosis of Yersinia-triggered reactive arthritis was made on the basis of serologic (serotype-specific enzyme-linked immunosorbent assay), bacteriologic, and clinical findings. All 21 patients had a serum antibody response to the causative microorganism that exceeded the mean response in 100 healthy blood donors by 4 SD. In 12 patients Yersinia enterocolitica was isolated from stool. Typical clinical signs of acute Yersinia infection (i.e., fever, diarrhea, or gastrointestinal pain) and of acute reactive arthritis was seen in all 21 patients.

Samples of synovial fluid leukocytes from 41 patients with other inflammatory joint diseases (20 of them fulfilling the American Rheumatism Association's criteria for rheumatoid arthritis) were studied as controls.

Synovial fluid was aspirated from the knee and mixed with heparin (50 IU/ml). Part of the cells were cytocentrifuged onto slides at 1000 rpm for five minutes (200 000 cells/slide), fixed in ethanol-acetic acid at -20°C, and stored at -70°C. Another part was frozen and stored in liquid nitrogen. The slides were used for immunofluorescence, and the solubilized cells were used for Western blotting.

2.2. BACTERIAL CULTURES OF THE SAMPLES

Samples of synovial fluid from the patients with reactive arthritis were cultured for viable bacteria as described recently (Granfors et al. 1989a). They were inoculated into three different media in an attempt to detect even minute amounts of bacteria, including L-forms. No viable organisms were recovered.

2.3. DETECTION OF BACTERIAL ANTIGENS

The presence of bacterial antigens in synovial fluid cells was sought by indirect immunofluorescence and by Western blotting after electrophoresis as described recently (Granfors et al. 1989a). Rabbit antiserum against Yersinia enterocolitica O:3 (640) obtained by immunization with live Yersinia enterocolitica O:3 bacteria expressing plasmid-encoded proteins and a mouse monoclonal antibody (A6) specific for the O-polysaccharide chain of Yersinia enterocolitica O:3 lipopolysaccharide (LPS) were used in both techniques.

3. Results

Yersinia antigens were detected by immunofluorescence in the synovial fluid cells from 15 of the 21 patients with Yersinia-triggered reactive arthritis. Rabbit antiserum and monoclonal antibody to the O-polysaccharide chain of the same bacteria gave identical results. The staining pattern varied from patient to patient; large granular and fine granular patterns were observed both in polymorphonuclear and mononuclear cells. Yersinia antigens were not detected by immunofluorescence in any synovial fluid cell samples from the 41 control patients.

Synovial fluid cells from nine patients with Yersinia-triggered reactive arthritis were studied also by Western blotting after electrophoresis. With use of rabbit antiserum four samples, and with use of monoclonal antibody three samples were positive. Synovial fluid cells from ten patients with rheumatoid arthritis studied simultaneously were negative with both antibodies used.

4. Discussion

During gastrointestinal infection and inflammation microbial antigens enter the body. How they are transported to the joints, independently, as immune complexes or in cells, is not known. We have found immune complexes containing Yersinia antigen in circulation as well as in synovial fluid from patients with Yersinia-triggered reactive arthritis (Lahesmaa-Rantala et al. 1987a,b). Occurrence of degradation products of Yersinia in mononuclear and polymorphonuclear cells in the synovial fluid (Granfors et al. 1989a) is in concert with the idea that bacterial structures are transported intracellularly to the joint. Processed microbial structures may function as a potent inflammatory mediators enhancing lymphocyte entrance to the joint at the level of vascular endothelium (Jalkanen et al. 1986, Jalkanen 1989).

Persons with HLA B27 antigen have greatly increased risk to develop reactive arthritis. How the presence of this antigen actually renders individuals susceptible to joint inflammation is not known exactly (Brewerton 1988, Keat 1983, Repo et al. 1984). It is apparent that the interaction between host and microbe is somehow inefficient in these patients and results in persistence of microorganisms for long a time and also in transportation of microbial components into joints.

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Interaction of bacteria and the epithelial surface in chronic bronchitis

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Introduction

Subjects with chronic lung disease are prone to recurrent episodes of acute bronchitis due to a disturbance in the normal host-parasite relationship involving colonizing bacteria. Non-serotypable *Haemophilus influenzae* (NTHI) has been commonly associated with acute exacerbations of bronchitis [1]. A prospective study of a population with chronic bronchitis showed that while about one third of subjects have repeated episodes of acute bronchitis, others with a similar degree of chronic lung disease develop few or no episodes of infection (unpublished data). Parameters of both colonization by bacteria and indices of host resistance have been studied to investigate possible mechanisms that determine infection susceptibility in subjects with chronic bronchitis.

Materials and Methods

109 subjects with established chronic bronchitis were recruited from a hospital clinic. An age matched control group of 37 subjects, who had no recognised lung disease were also studied. Chronic bronchitic subjects were classed as infection prone (IP) (≥2 infections per year) and infection resistant (IR) (≤1 infections per year). The 2 chronic bronchitic populations' demographic profiles were similar: age mean 66 years (range 37-86); FEV_1 mean 0.60 (range 0.12-1.29); current smokers 32%. Samples were taken from a subgroup of randomly selected subjects when they had been infection free for at least 21 days. A bacterial adhesion assay was performed using buccal epithelial cells from the study population and an adhesive NTHI (biotype I). Buccal cells were Gram stained and 25 cells counted for both the *in vitro* assay and quantitation of indigenous microorganisms attached *in vivo*, using a semi-quantitative scoring system for the latter study. Viable cell counts were performed by homogenizing calcium alginate oropharyngeal swabs and plating serial dilutions on chocolate and blood agar. Stimulated whole saliva was clarified and used in measuring aggregating activity by monitoring NTHI sedimentation, lysozyme concentration by the turbidimetric technique and IgA anti-*H. influenzae* antibody levels by an ELISA.

Results

Significant differences were shown to exist between IP and IR subjects:
a) Increased adherence of NTHI *in vitro* to buccal cells from IP subjects was observed ($p<0.05$). No difference was observed between the control and IR group.
b) There was increased adherence of indigenous microflora *in vivo* to buccal cells from IP subjects with Gram positive cocci ($p<0.01$). IP buccal cells adhered more Gram negative coccobacilli compared to control group ($p<0.05$). There was no difference between the control and IR group in the distribution or number of bacterial species attached to cells.
c) More bacteria were cultured from the oropharynx of the IP subjects ($p<0.05$). The control subjects viable bacterial cell count was less compared to IR ($p<0.001$) and IP subjects ($p<0.0001$). No difference was observed between groups in the bacterial species isolated.
d) Saliva aggregating activity of the IR group was twice that of the IP group. Aggregating activity of the control saliva was less than that of IR and IP subjects ($p<0.01$).
e) Free lysozyme activity was higher in IR saliva ($p<0.05$). Lysozyme concentration of control saliva was less than that of IR and IP subjects ($p<0.001$). The level of salivary lysozyme correlated with aggregation of NTHI ($r=0.68$).
f) IgA anti-*H. influenzae* antibody levels decreased ($p<0.05$) during the autumn and winter months in the IP group at a time when NTHI carriage was highest. IgA anti-*H. influenzae* antibody correlated with inhibition of adherence of NTHI ($r=0.92$).

Discussion

An increase in polybacterial carriage in the upper respiratory tract (URT) was common to chronic bronchitic subjects, irrespective of whether they had a history of recurrent bronchitis. It is probable, therefore, that the increase in bacteria reflects some basic defect in mucosal resistance. The essential difference between the IP and IR groups of chronic bronchitics determined in this study was the ability of buccal cells to bind bacteria, which correlated with indigenous bacterial colonization of the URT. There was no clear relationship between bacterial concentration and numbers attached to cells nor between the patterns of oropharyngeal isolates of IR subjects and normal controls. The most likely cause of increased bacterial attachment to buccal cells is an enhanced binding capacity of the cells and/or some factor(s) in the mucosal milieu which increases binding or increases the opportunity for attachment to occur. In this context, significant differences were observed in lysozyme activity and specific antibody responses in saliva. Lysozyme levels correlated with salivary aggregating activity of NTHI whilst specific IgA antibody inhibited the adherence of NTHI to buccal cells. Key differences in effector mechanisms within the population of chronic bronchitics have been identified. These may be consequential in modulating the microbial ecology of the respiratory tract, reflected in differences in bacterial colonization patterns and, hence, may determine the hosts' susceptibility to infection.

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Mucosal immunity of the middle ear

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ABSTRACT

To clarify mucosal immunity of the middle ear, the distribution of immunocompetent cells in the normal middle ear, the ability of secretory IgA in nasopharyngeal secretions to block the adherence between isolated epithelial cells of the nasopharynx and bacteria (*H. influenzae* and *S. pneumoniae*), and the effect of oral immunization on the prevention of otitis media, were investigated. The finding of the presence of few immunocompetent cells in the middle ear mucosa indicates that the normal middle ear does not receive antigenic stimuli. It was found that secretory IgA interferes with the bacterial adherence. Since oral immunization prevented the occurrence of both immune-mediated and bacterial otitis media in animal experiments of this study, oral vaccine for otitis media is proposed.

INTRODUCTION

Many studies have demonstrated that immunologic reactions are involved in the etiology and pathogenesis of otitis media. Evidence that secretory IgA is present in the middle ear effusion (MEE) suggested the presence of mucosal immunity in the middle ear, although the normal middle ear mucosa possesses few immunocompetent cells (Mogi et al. (1974) and Ogra et al. (1974)). It has been reported that antigen-antibody reactions or immune complex formation in the tympanic cavity, followed by complement activation, can be a pathogenic mechanism in otitis media with effusion (OME) (Ryan et al. (1986) and Suzuki et al. (1988)). This study was designed to investigate the distribution of immunocompetent cells in normal middle ear mucosa and to clarify the role of secretory IgA of nasopharyngeal secretion in the interference of bacterial infection of the middle ear. Immune regulatory mechanisms by which antigenic stimuli to the mucosa-associated lymphoid tissues enhance mucosal immunity and induce tolerance of the systemic immunity, were applied to the prevention of OME and bacterial infection of the middle ear in animal models.

MATERIALS AND METHODS

Analysis of Immunocompetent Cells in the Middle Ear Mucosa: A total of

15 ICR mice in germ-free (GF), specific-pathogen-free (SPF) and conventional (CV) conditions were employed. The method for quantitative analysis of immunocompetent cells was reported in a previous paper (Ichimiya et al. (1989)).

Interference of Bacterial Adherence to the Mucosal Cells by Secretory IgA: Nontypable *H. influenzae* and *S. pneumoniae* type III isolated from patients with OME were used. Specimens of nasopharyngeal secretion were obtained by aspiration from normal children and children with OME. The antibody activity of secretory IgA against *H. influenzae* and *S. pneumoniae* was determined by ELISA as reported by Yamaguchi et al. (1986). A mixturee of isolated nasopharyngeal cells' and bacterial suspensions was incubated, then the number of bacteria adhering to the epithelial cells counted using indirect immunofluorescent technique. An ELISA technique, elaborated by Ofek et al. (1986), was employed to investigate the blockage of adherence between epithelial cells and bacteria by secretory IgA in nasopharyngeal secretions.

Induction of Antigen-Specific IgA-Forming Cells in the Middle Ear Mucosa: Healthy male Hartley guinea pigs were used in this experiment. DNP-ovalbumin (DNP-OVA) was employed as soluble antigen and DNP-OVA conjugated with polyacrylamide gel beads as insoluble antigen (DNP-OVA-Pa). Animals were injected in the foot-pads with a mixture of DNP-OVA and Freund's complete adjuvant. One week after the systemic priming, DNP-OVA-Pa were inoculated into the duodenal lumen or into the tracheal lumen. To identify antigen-specific IgA-forming cells, a double-staining technique was used according to the method reported by Kawamura (1977).

Suppression of Immune-Mediated Otitis Media by Mucosa-Derived T Cells: Strain C_3H/HeN female mice (SPF) at 8 weeks of age were used for the experiments. The mice were given 40 mg of OVA by gastric intubation. Three days after the administration, Peyer's patches and spleen cells were harvested aseptically. As the control, the same strain of mice was fed with saline. To induce immune-mediated OME, mice received OVA into the tympanic cavity after the systemic immunization.

Oral Immunization for the Prevention of Bacterial Otitis Media: Healthy Hartley guinea pigs were injected in the foot-pads with 200 ug of formalin-killed *S. pneumoniae* type 19 mixed with Freund's complete adjuvant. One week after the systemic immunization, a gelatin capsule containing 200 ug of the killed bacteria was introduced into the stomach by gastric intubation using a capsule injector. The immunized animals received 10^6 live *S. pneumoniae* into the tympanic cavity through the eardrum.

RESULTS

Distribution of immunocompetent Cells in the Middle Ear Mucosa of Mice: As shown in Figure 1, the number of each lymphocyte subset is much smaller in the middle ear mucosa than in the nasal mucosa. Very few IgA+ and Lyt-1+ cells existed in the middle ear mucosa of CV mice, while IgM+ cells were found in all mice.

Bacterial Adherence to the Nasopharyngeal Epithelial Cells: The number of bacteria, both *H. influenzae* and *S. pneumoniae*, adhering to the epithelial cells was significantly smaller in patients having secretory

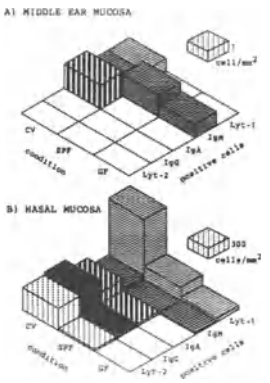

Figure 1. Distribution of lymphocyte subsets in the middle ear (A) and nasal mucosae (B).

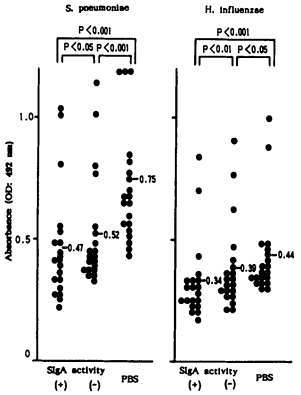

Figure 2. Inhibition of bacterial adherence by nasopharyngeal secretions.

IgA antibody activity in the nasopharyngeal secretion than that in patients having no antibody activity. Both mean ELISA values of the groups treated with nasopharyngeal secretions were significantly lower than that of the group treated with PBS (Figure 2).

Antigen-Specific IgA-Forming Cells: A considerable number of Antigen-specific IgA-forming cells were induced in various secretory sites, including the middle ear mucosa. Upon antigenic challenge in the tympanic cavity, 3 of 17 (17.6%) animals, which were immunized in the duodenal lumen, and 4 of 19 (21.1%) animals which received the antigen in the trachea, were found to have OME, while OME was present in all 18 animals without the mucosal immunization.

Suppression of Immune-Mediated otitis Media by Adoptive Transfer of Mucosa-Derived T Cells: As it has been demonstrated that T-suppressor cells in Peyer's patch cells migrate to the spleen (Richman et al., 1981), 2 X 10^7 fractionated spleen cells from OVA-fed donors were transferred to recipient mice. Spleen cells of OVA-fed donor mice were fractionated to T-cell and non-T-cell populations, then transferred to recipient mice one day before the systemic immunization. When splenic T cells from OVA-fed mice were transferred, anti-OVA IgG antibody response was suppressed. Immune-mediated OME was suppressed by adoptive transfer of mucosa-derived splenic T cells.

Prevention of Bacterial Otitis Media by Oral Immunization: Seven of 14 (50%) guinea pigs, which were orally administered the killed bacteria following systemic immunization, developed MEE, either purulent or serous, upon inoculation of live *S. pneumoniae* into the tympanic cavity. Otitis media occurred in 14 of 20 (70%) animals, which were systemically immunized twice. All 20 non-sensitized animals developed otitis media.

COMMENT

Although the tympanic cavity is exposed to the environment via the eustachian tube, the anatomical constitution and function of the tube protect the tympanic cavity from antigenic invasion, thereby keeping it sterile. Therefore, the almost total absence of immunocompetent cells in the middle ear mucosa is attributed to this situation. However, many

clinical and experimental studies have demonstrated the accumulation of immunocompetent cells, such as lymphocytes, macrophages, and plasma cells, in the inflamed middle ear (Mogi and Bernstein (1987)). It can said that the middle ear is immunologically a potential site.

Results of this study suggested that secretory IgA in nasopharyngeal secretions protect the middle ear from bacterial infection. our experiment successfully induced antigen-specific IgA-forming cells in the mucosa of the nasopharynx and middle ear. Mucosally immunized animals significantly suppressed the induction of immune-mediated OME. The results of animal experiments presented herein strongly suggest that mucosal immunization can be an effective treatment to prevent OME and bacterial otitis media because mucosa-derived regulatory T cells are beneficial not only to augment mucosal immunity (IgA response), but also to prevent IgG-mediated inflammatory reaction in the middle ear. Oral vaccine therapy is proposed for the prevention of otitis media.

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Entamoeba histolytica antigens recognized by IgA class human antibodies in serum and colostrum of puerperal women using immunoblotting techniques

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1. Introduction

The presence of specific IgA class antibodies against E.histolytica trophozoites has been demonstrated in colostra samples of Mexican women (Acosta et al 1985). Immunoblotting techniques have shown that IgG antibodies present in the sera patients with amebic hepatic abscess reacted with amebic antigens of a molecular weight of 150, 112, 85 and 17 kDa (Arroyo and Orozco 1987).

2. Materials and Methods

Twenty eight colostra and puerperal serum pairs were obtained from puerperal without a known clinical history of amebiasis admitted at Hospital de la Mujer, Secretaria de Salud in Mexico City. The 24 samples of sera from patients with hepatic amebic abscess came from Hospital General, Centro Medico "La Raza" IMSS in Mexico City.

Clone A trophozoites of HM1 IMSS strain were cultured axenically in TY1-S-33 media for 72 hours. Trophozoites were harvested and then broken by shaking and freeze-thawing in presence of 5 mm of p-hydroxy mercury benzoate in the running buffer. Gel electrophoresis and electrotransfer were done according to described techniques (Laemmli 1970 and Towbin 1979). Transferred nitrocellulose paper was blocked with bovine serum albumin one hour at 37°C, and then was exposed to the problem sample in a dilution of 1:400 for 1.5 hours at 37°C, the paper was washed three times with, and incubated with an anti human IgA peroxidase conjugate dilution of 1:500 overnight at 4°C, then it was washed three times with PBS-tween and exposed to 0.06% of 4-chloro-1-naphtol (Sigma) in PBS, plus 40 ul of hydrogen peroxide 30% per millilitre, for 30 minutes at room temperature. The blocking with lactose was done by adding 2% of this sugar to the problem sample dilution before incubation with the transferred paper.

3. Results

The IgA in the puerperal sera recognized with greater frequency the antigens with a molecular weight of 82.79, 52.14, 40.91, 37.75, 35.08, 33.52, 28.53, 27.53 and 25.32 kDa, IgA class antibodies present in colostrum reacted preferably with the antigens of a molecular weight of 1290, 82.79, 52.14, 40.91, 37.75,, 35.08, 33.52, 28.53 and 25.32 kDa. The IgA of sera from patients with amebic hepatic abscess diagnosis predominantly immunodetected the antigens of a molecular weight of 719, 118.45, 88.45, 69.45, 55.26, 52.14, 49.38, 42.72, 36.81, 35.08, 33.52, 25.32, 23,82, 23.47, 20.52 and 20.03 kDa. In four of nine cases the blocking with lactose produced different patterns with respect to normal control immunodetection.

4. Discussion

In this work the amebic antigens which are immunogenic for the human mucosal associated immune system were identified through immunoblotting with IgA. The lack of protective immune response against E. histolytica may be due, in part, to the infrequent recognition of the amebic lectin-adhesins (quoted in 5,6,7). Since, it was shown that lactose blocks - amebic lectins (Cano and Lopez 1987), we used this disaccharide to rule out non specific binding of IgA N-acetyl-D-galactosamine to the amebic lectins.

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SECTION W:
INFECTION AND
MUCOSAL IMMUNITY

(B) Animals

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Fimbrial adhesins and mucosal inflammation

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ABSTRACT. The bacterial components inducing the secretion of interleukin 6 (IL-6) and polymorphonuclear leucocytes (PMNL) were analyzed in a mouse model for urinary tract infection. Previous studies had shown that the lipid A moity of endotoxin and bacterial adhesins binding to Galα1-4Galβ-containing receptors synergistically activated mucosal inflammation. In this study the effect of each component was analyzed separately. Fimbrial proteins with the receptor binding adhesin activated the the secretion of IL-6; adhesin negative fimbriae did not. Isolated lipid A activated the PMNL response, but not the IL-6 response. The results suggest that the adhesin-receptor interactions are required to activate mucosal target cells to IL-6 secretion and that the endotoxin response mainly activates factors which recruit PMNL's to the mucosal surfaces.

1. Introduction

Escherichia coli bacteria induce a mucosal inflammatory response in the urinary tract (1). The mechanisms of this response have been analyzed in an ascending infection model in mice. Two bacterial components are the major activators of inflammation: bacterial adhesins binding to Galα1-4Galβ-containing glycolipid receptors on epithelial cells, mediating bacterial attachment and the lipid A moiety of lipopolysaccaride present in all E. coli strains. Earlier studies showed that the inflammatory response to whole E. coli bacteria was mimicked by adhesins and lipid A and that these components acted in synergy (2). Evidence that bacterial attachment increased mucosal inflammation was also obtained from studies in humans, where infections caused by attaching bacterial gave rise to a higher inflammatory response than other infections. Taken together, these results suggested that adhesins might promote mucosal inflammation not only by approaching endotoxin to the mucosal surface, but also by directly stimulating epithelial cells through ligand-receptor interaction.

Our studies have focused on two parametres of mucosal inflammation, IL-6 and urinary leucocyte excretion. Within minutes of mucosal challenge with whole attaching E. coli, IL-6 levels in the urine increase (3) and within hours a PMNL response can be measured as the influx of cells into the urine (1). The aim of the present study was to analyze the isolated actions of adhesins as well as lipid A in the triggering of the urinary secretion of IL-6 and PMNL's in mice.

2. Material and Methods

2.1. ANIMAL MODEL

Female C3H/HeJ (Lps ^{d}, Lps ^{d}) mice and C3H/HeN (Lps ^{n}, Lps ^{n}) mice were inoculated by urethral catheterization, as previously described (1, 2). After ether anesthesia, 0.1 ml of suspensions containing bacterial components (lipid A, fimbriae) were deposited in the bladder through a soft polyethylene catheter.

Urine samples were collected prior to and at various times after inoculation by gentle compression of the abdomen of the mice.

2.2 BACTERIAL FIMBRIAE

E. coli 21624 (serotype K12 [HB101]), used for the isolation of the Fimbriae Adhesin Complex (FAC), was a recombinant strain which had received the chromosomal pap DNA sequences encoding pili and adhesion from a wild-type uropathogenic E. coli strain (serotype O4:K12:H^-). The recombinant strain expressed the P-fimbriae of serotype $F7_1$ (4).

Bacteria were grown at 37°C for 18h on Loeb agar, suspended in PBS and heated for 30 min to 65°C. After cooling the suspension was centrifuged (15 000 x g, 30 min) to remove the defimbriated bacteria, glycine (20 mM) and EDTA (5mM) were added to the supernatant and the FAC precipitated with ammonium sulfate (10 % saturation). To remove contaminating lipids the FAC was suspended in ethanol and precipitated with LiCl (250 mM). The residual lipopolysaccaride was removed by deoxycholate (0.5 %) treatment at 60°C for 30 min.

To obtain fimbriae devoid of the adhesin the FAC solution was treated with Zwittergent 3-16 (0,5 %) at a temperature of 80°C for 30 min. This caused a dissociation of the FAC into fimbriae and adhesin. The fimbriae were collected by precipitation with LiCl (250 mM) and centrifugation.
The levels of endotoxin determined by the Limulus assay were 0,7 % in the adhesion positive and undetectable in the adhesion negative fimbriae.

2.3. QUANTITATION OF THE INFLAMMATORY RESPONSE

2.3.1. *IL-6 Assay*. The hybridoma cell line B9 which is dependant on IL-6 for growth was used for IL-6 determinations (3). The B9 cells were seeded into microtiter plates (5000 cells/well) and cultured in Iscoves Modified Dulbeccos Medium (IMDM), supplemented with $5x10^{-5}$ M β-Mercaptoethanol and 5% fetal calf serum. 3_H- Thymidine was added after 68 h of culture, and the cells were harvested 4 h later. The samples were tested in two-fold dilutions and compared to an IL-6 standard. One unit/ml is the concentration required for half-maximal proliferation of the B9 cells.

2.3.2. *Leucocyte excretion*. Urinary leucocytes were quanititated by light microscopy using a hemocytometer chamber. Mice with a preexisting leucocyte response or with bacteria were excluded.

3. Results

C3H/HeN mice were inoculated intravesically with adhesin positive or adhesin negative fimbriae (Table 2). A urinary IL-6 response occured within 30 minutes of inoculation, remained high for 2 hours and subsided 6 hours after inoculation. The adhesin positive fimbriae gave a significantly stronger reaction than the adhesin negative proteins.
Injection of adhesin positive fimbriae also elicited a urinary leucocyte response (Table 3). The response reached maximal levels at 6 hours after infection. The number of PMN cells in animals receiving adhesin negative fimbriae did not reach above background.
The adhesin positive fimbriae contained significantly more endotoxin than the adhesin negative proteins. To differentiate between the effect of endotoxin and the adhesin-receptor interaction, the experiments were performed in parallel in LPS non responder mice (C3H/HeJ). Mucosal administration of the adhesin positive fimbriae elicited IL-6 secretion also in LPS non responder mice. The levels were lower than these in LPS non responders, but not significantly different. The adhesin negative proteins did not elicit the IL-6 secretion. The adhesin positive fimbriae did not trigger a leucocyte response in the C3H/HeJ mice (Table 3).
The results were confirmed by mucosal challenge with isolated lipid A. IL-6 secretion was observed neither in LPS responder nor LPS non responder mice (Table 2). In contrast, a urinary PMN response to isolated lipid A occured in C3H/HeN mice (Table 3).

4. Discussion.

The mucosal inflammatory response to E. coli bacteria was shown to be induced by two bacterial components: adhesive proteins binding to Galα1-4Galβ-contining receptors and the lipid A moiety of endotoxin. The Galα1-4Galβ recognizing adhesins of E. coli are localized at the tip of fimbrial rods, which project out from the bacterial outer membrane. In this study, an IL-6 response was elicited by the intact fimbrial rod with the tip adhesin, but not by the fimbrial rod devoid of the adhesin. Furthermore, the activation if IL-6 was independent of endotoxin contaminating the fimbrial preparation, since it occured also in C3H/HeJ mice which are genetically defective in their response to lipid A.
Mucosal challenge with the lipid A moiety of endotoxin elicited a local PMNL response. This response was controlled at the level of the Lps gene, i.e. it occured only in C3H/HeN mice. Lipid A and the adhesins thus activated different aspects of the mucosal host response: the adhesins activated IL-6 secretion but not the PMNL response and lipid A activated the PMNL response but not IL-6 secretion. Endotoxin stimulation of the urinary tract may thus maintain the excretion of urinary leucocytes, charachteristic of patients with Gram negative bacteriuria but lacking a systemic response. In contrast, since IL-6 is an endogenous pyrogen, the activation of IL-6 secretion by the adhesins is consistent with the overrepresentation of attaching bacteria in patients with febrile infection .

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6. Examples

6.1. Tables

TABLE 1. Binding properties of bacteria and bacterial fimbriae

	Erythrocytes Hum p	Galα1-4Galβ latex beads
Wild type bacteria	+++	+++
Adh^+ fimbriae	+++	+++
Adh^- fimbriae	-	-

TABLE 2. IL-6 response at 2h to adh$^+$ fimbriae, adh$^-$ fimbriae and lipid A in HeN mice and HeJ mice.

C3H/HeN (mean (SEM))			C3H/HeJ (mean (SEM))		
Adh$^+$	Adh$^-$	lipid A	Adh$^+$	Adh$^-$	lipid A
269 (90)	66(38)	<10 (0)	174 (57)	36 (23)	<10 (0)

TABLE 3. Leucocyte response at 6h to adh$^+$ fimbriae, adh$^-$ fimbriae and lipid A in HeN mice and HeJ mice.

C3H/HeN (mean (SEM))			C3H/HeJ (mean (SEM))		
Adh$^+$	Adh$^-$	lipid A	Adh$^+$	Adh$^-$	lipid A
310 (69)	10 (5)	198 (45)	43 (8)	11 (8)	<10 (0)

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Cell-mediated immune kinetics in experimental bronchiectasis

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ABSTRACT. In a recently developed rat model of bronchiectasis (BX) generated by partial bronchial ligation and injection of Pseudomonas aeruginosa (Pa), the composition and distribution of immunocompetent cells were analysed at progressive timeintervals by immunohistochemical methods. Four groups of 25 animals were prepared: 1) Partial ligation and injection of Pa (Pa+LIG); 2) Injection of Pa without bronchial ligation (Pa+NOLIG); 3) Sham operated (S); 4) Age-matched normal controls (N). Bronchiectasis developed in 22/25 animals of the Pa+LIG group but in none of the 75 controls of the other 3 groups. This bronchial dilation began within 2 weeks and was accompanied by a T-lymphocyte proliferation beginning in the bronchus-associated lymphoid tissue (BALT). Up to week 4 this was composed mainly of CD4+ve cells. Large de novo lymphoid aggregates appeared in the lung parenchyma, with an initial (2-4 wks.) predominance of CD4+ cells. From week 8 onwards, a predominance of CD8+ cells was noted in both areas. The bronchial lamina propria of rats from the Pa+LIG group was infiltrated by significantly larger numbers of T-lymphocytes (mainly of CD8+ phenotype) and macrophages when compared with the other 3 groups, at all time points. The bronchial epithelium of 17/22 animals showing bronchial dilation in the Pa+LIG group expressed Ia antigen but that of the 75 animals in the other groups did not. These findings support the hypothesis that chronic inflammation in bronchiectasis is associated with a cell-mediated immune response developing as a consequence of bacterial infection in an airway where the clearance mechanisms have been impaired.

1. INTRODUCTION.

Bronchiectasis is a chronic lung disorder characterised by irreversible dilation of parts of the bronchial tree, usually associated with persistent inflammation which may lead to scarring and shrinkage of the lung and ultimately, in some cases, to cor pulmonale and death (1). The pathogenesis of bronchiectasis remains poorly understood and little work has been attempted in this area since that of Whitwell (2) in 1952. Recently, the "vicious circle" hypothesis for its pathogenesis proposed

by Cole (3) postulated that microbial colonisation of the bronchial tree subverts the host's normally protective inflammatory response into a tissue-damaging one resulting in progressive lung damage. One of the microorganisms frequently associated with chronic bronchial infection in bronchiectasis is Pseudomonas aeruginosa (1). The immunohistology of the inflammatory lesions which develop in a recently devised rat model of Pseudomonas-associated bronchiectasis has been shown (4) to resemble that previously found in resection specimens of human bronchiectasis (5). We have now examined the kinetics of development of this cell-mediated immune response using the animal model.

2. MATERIAL AND METHODS

2.1. Animal model. Bronchiectasis was induced in SPF Wistar outbred rats (Charles Rivers Ltd., Margate, UK) by a technique of apical lobe ligation. In brief, 160g animals were anaesthetised with an intramuscular injection of 0.02 ml of Hypnorm (Janssen Pharmaceutical Ltd., Marlow, U.K.), intubated with a 4FG cannula (Portex Ltd., Hythe, U.K.), ventilated with a small animal ventilator (Harvard Apparatus Ltd., Edenbridge, U.K.), and placed on their left side. Right thoracotomy through the 5th intercostal space was performed aseptically. The apical lobe was then retracted towards the diaphragm so as to expose the apical bronchus, which was partially ligated as near as possible to its orginin from the main bronchus, using surgical suture 7/0 prolene (Ethicon Ltd., Edinburgh, U.K.). A volume of 0.02 ml of bacterial suspension composed of 1×10^9 of per ml a clinical isolate of Pseudomonas aeruginosa (P455) was then injected into the ligated bronchus, the lungs inflated manually with a 5 ml syringe, and the chest wall closed in layers. Once spontaneous breathing was resumed, the animals were extubated and allowed to recover.

2.2. Experimental Design and Animals. Four groups of rats were included in the study, each containing 25 animals: Group 1 (Pa + LIG) - partial ligation of the apical lobe bronchus was followed by injection of P. aeruginosa as described above; Group 2 (Pa + NOLIG)- a similar amount of P.aeruginosa was injected without prior ligation of the bronchus; Group 3 (S) - sham operated animals; Group 4 (N) - age-matched normal controls. Five rats from each group were sacrificed after 2,4,8,12, and 16 weeks.

2.3. Immunohistochemical Procedures. Cryostat sections of 6 µm were obtained from the inflated apical lobes. An indirect immunoperoxidase method was used for the immunohistochemical studies using diaminobenzidine (Sigma Chemical Company, Inc., St. Louis, USA) development. The panel of monoclonal antibodies used was the following: MRC OX-52 (Serotec, Oxford, U.K.), present on rat T lymphocytes; MRC OX-19 (Serotec), rat T cells. MRC OX-19 and MRC OX-52 were used together to enhance the staining; MRC OX-8 (Serotec), rat cytotoxic/suppressor T cells; MRC OX-6 (Serotec), rat Ia antigen; ED1 (supplied by Dr. C. Dijkstra), rat macrophages and dendritic cells.

Three compartments of the lung tissue were assessed: epithelium, bronchus-associated lymphoid tissue (BALT) and other lymphoid aggregates, and peribronchial area. The positively stained cells were quantified using a graticule, which corresponded to a total

area of 0.024 mm at 400 x magnification. The results were expressed as the mean number of positive cells per unit area (0.024 mm^2) + standard deviation (SD). Student's t-test for non-paired data was used and the data considered statistically significant when $p \leq 0.05$.

3. RESULTS

3.1 T-Lymphocyte proliferation in BALT: Significant T-lymphocyte proliferation in BALT of animals of the Pa + LIG group was first observed at week 2 (p = 0.002), when compared with the N group. None of the animals in the Pa + NOLIG or S groups expressed such proliferation and a statistically significant difference was also seen when both control groups were compared with the Pa + LIG group (p =0.002, and p =0.003, respectively). Subsequently, T cell numbers decreased but were still significantly different from those in normal controls at weeks 4 (p = 008), 8 (p = 0.02), 12 (p = 0.01), and 16 (p = 0.04). Similar findings were seen in comparison with the Pa + NOLIG and S groups. This proliferation was due initially to increase in helper T-cells. A shift to proliferation of cytotoxic/ suppressor T-cells was seen from week 4 onwards. The macrophage-like cell populations in BALT did not change significantly in any group at any time point.

3.2. Composition of the newly formed lymphoid aggregates: T-lymphocyte proliferation was also seen in newly formed lymphoid aggregates in all animals of the Pa + LIG group. Extensive aggregates of T-lymphocytes were present in some animals but in others this phenomenon was less marked. No such aggregates were noticed however in the controls. This T cell accumulation was most marked at week 2 but began to decline from 8 weeks onwards. At first, the T-cells were almost exclusively putative helper cells (OX-8 negative) but the numbers of cytotoxic/ suppressor cells increased steadily up to week 8 and then began to decline. At week 12 almost all T cells were OX-8 positive. Many ED1 positive macrophages were also present in the aggregates.

3.3. Peribronchial infiltration: A less marked but statistically significant increase in the T-cell population was seen from week 2 in the Pa + LIG group when compared with the Pa + NOLIG, S, and N groups and this population remained significantly increased at all time points. The two control groups failed to show any difference when compared with normal rats. This T-cell increment was almost entirely due to the OX-8 positive subset at all time points. A statistically significant increase in the macrophage-like cells population at week 2 was seen in both Pa + LIG (p = 0.001), Pa + NOLIG (p = 0.01), and S groups (p = 0.0001) when compared with normal controls. This increase was not evident in the Pa + NOLIG and S groups from week 4 onwards, but in the Pa + LIG group macrophage infiltration remained significant compared with that in normal rats at all time points.

3.4. Expression of Ia by the bronchial epithelium: Ia antigen was expressed by the bronchial epithelium of 17/25 animals in the Pa + LIG group but not by the epithelium of the 75 rats of the three control groups. An association between the presence of bronchial dilatation and the epithelial expression of Ia was noticed: of the 22 rats of this group showing bronchiectasis, 17 expressed Ia antigen in the bronchial epithelium.

4. DISCUSSION

Introduction of Pseudomonas aeruginosa into the rat bronchus after partial ligation produces a model of bronchiectasis in which to test the "vicious circle" hypothesis of the pathogenesis of the condition. As a first step we have examined the kinetics of the cellular immunological inflammatory reactions associated with bronchiectasis and the results confirm the previous suggestion that this inflammation is associated with a cell-mediated immune response. Here we have shown that, following bronchial infection, failure to clear the bacterial load due to partial bronchial obstruction is associated with a massive cell-mediated immune response and imbalance between benefit and damage. The data produced here suggest that Pseudomonas aeruginosa may induce a local cell-mediated immune response in situations in which the mucociliary mechanisms of the bronchi are previously impaired. However, no direct evidence exists to confirm that the cell-mediated response observed in the bronchial wall is directed against antigens of the Pseudomonas. Other possibilities are that neo-antigens are released by the experimental procedure and that these promote auto-immune phenomena locally or, alternatively, that opportunistic viruses may take advantage of the bronchial damage and promote an immunological response. These possibilities could all explain the predominance of the CD8 positive subset in the bronchial wall.

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Immunity to *Bordetella pertussis* in a mouse model of respiratory infection

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Introduction
Pertussis (whooping cough) is a disease of the respiratory tract that may be characterized by episodes of paroxysmal coughing and can be fatal in children younger than one year of age. Filamentous hemagglutinin (FHA) is a 200 kDa protein thought to participate in the attachment of *B. pertussis* to the ciliated epithelium of the respiratory tract. We have used a mouse model of aerosol infection to evaluate purified FHA, administered either parenterally or mucosally, for its ability to protect against respiratory *B. pertussis* infection as well as to study the mechanism of protective immunity. This model is suitable in that certain aspects of the disease in humans, including bacterial attachment to the ciliated epithelium, leukocytosis and a greater severity of disease in the young, are also observed in mice.

Materials and Methods
FHA, free of contaminating pertussis toxin and endotoxin, was purified by the procedure of Sato et al. (1), and migrated as one band by SDS-PAGE. Mice were immunized and boosted intraperitoneally (ip) or intramuscularly (im) with 8 μg FHA adsorbed to aluminum hydroxide gel, and immunized intraduodenally (id) or subcutaneously (sc) with 100 μg unadsorbed FHA in PBS. In passive protection experiments, mice received normal rat globulin or rat anti-FHA intravenously, 24 hours prior to aerosol challenge.

Aerosol infection of BALB/c mice with *B. pertussis* was conducted as previously described (2). Neonatal mice were weighed and bled periodically after infection and deaths were noted. Lungs and tracheas from adult mice were aseptically removed, homogenized in sterile PBS, and dilutions of homogenates plated on Bordet-Gengou agar in order to determine the number of recoverable bacteria. Sera and bronchoalveolar lavage fluids (from exsanguinated mice receiving 0.5 ml PBS in the lungs) were analyzed by ELISA.

Results
Neonatal mice, immunized with 16 μg FHA on days 5 and 12 *postpartum* and challenged day 19 *postpartum* with an aerosol of *B. pertussis* 18323, had no increase in peripheral white blood cell count, gained weight and were alive 21 days after challenge in contrast to controls immunized with tetanus toxoid prior to challenge.

Parenteral immunization of 4 week BALB/c females with 8 μg FHA four weeks before challenge and a secondary dose of 8 μg one week before challenge resulted in a 1-2 log decrease in the number of *B. pertussis* recovered from the lungs and tracheas of animals one week after aerosol challenge, in comparison to controls immunized with tetanus toxoid (Table 1). High levels of IgM and IgG anti-FHA antibodies were detected in the serum and IgG anti-FHA was detected in the bronchoalveolar lavage fluid of animals one week after secondary ip or im immunization with 8 μg FHA (data not shown). Decreased colonization of the lungs and tracheas was also observed in mice administered intravenous rat anti-FHA globulin in comparison to mice receiving normal rat globulin twenty-four hours prior to

B. pertussis respiratory challenge .

Intraduodenal immunization of 8 week old BALB/c females with 100 μg unadsorbed FHA in PBS, 3 weeks prior to respiratory challenge with *B. pertussis* 18323, also resulted in a log reduction of bacteria recoverable from the lungs of mice in comparison to unimmunized controls (Table 1). Subcutaneous immunization with 100 μg FHA, however, resulted in a 3-4 log reduction of recoverable bacteria from the lungs and tracheas of challenged mice. IgA anti-FHA was detected in the lungs of id immunized mice, but not in unimmunized mice, two weeks after challenge.

Table 1 Immunization with FHA protects against *B. pertussis* colonization.

Bacterial recovery on day 9 after challenge
log cfu, mean ± s.d.

		FHA[A]			FHA[B,C]	
	Tetanus toxoid[A]	ip	im	unimmunized[B]	id	sc
LUNGS	6.88 (.47)	5.05 (.42)	5.05 (.29)	7.01 (.23)	5.53 (.52)	2.40 (.61)
TRACHEAS	4.77 (.53)	3.62 (.32)	3.80 (.24)	-	-	-

[A] immunized 4 weeks + boosted 1 week before challenge with 8 μg FHA or Tetanus toxoid

[B] immunized control or immunized with 100 μg FHA three weeks before challenge

[C] similar results were seen in a repeated experiment

Discussion

We demonstrate here that immunization with FHA prior to respiratory *B. pertussis* challenge, protects neonatal mice aginst leukocytosis and death and reduces the bacterial load in the lungs and tracheas of adult mice. Parenteral immunization with FHA resulted in an increase in IgG anti-FHA antibodies in the serum and bronchoalveolar lavage fluids. Passive adminstration of IgG anti-FHA also decreased the number of *B. pertussis* recovered from the lungs and tracheas of infected animals. As intravenously administered IgG has been shown to transude from the circulation to the lungs, we hypothesize that serum IgG anti-FHA that enters the lung via transudation is one mediator of protective immunity to *B.pertussis* .

We have also demonstrated that intraduodenal immunization with FHA, prior to aerosol challenge, is effective in reducing the number of *B. pertussis* recovered from the respiratory tract. Of note is the fact that subcutaneous immunization with FHA prior to challenge resulted in a decreased colonization of the respiratory tract in comparison to intraduodenal immunization, although both routes of immunization were effective in comparison to unimmunized controls. As purified FHA is easily degraded, one explanation for this phenomenon may be that FHA is more rapidly degraded in the acidic milieu of the digestive tract than when deposited subcutaneously. The detection of IgA anti-FHA antibodies in the lungs of id immunized mice suggests that this gut immunization regimen may disseminate FHA stimulable memory cells to the respiratory mucosa. Further studies are ongoing to define the role of IgA anti-FHA in protection against *B. pertussis* respiratory infection.

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Evaluation of the mitogenic activity of different fractions of *Entamoeba histolytica* - strain HM1 IMSS on lymphocytes of mesenteric lymph nodes and spleen of mice

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1. Introduction

Diverse investigators (1) (2) have demonstrated a mitogenic of amoebic extracts on circulating lymphocytes in humans and in the spleen of mice and it has been considered as a mechanism to evade the immunitary response; considering the importance of the lymphoid tissue associated to the intestine in amebiasis, the object of this work was to evaluate the mitogenic activity of different amoebic fractions on lymphocytes of mesenteric lymph nodes utilizing the method of blastoid transformation by the incorporation of 3H-Tdr.

2. Materials and Methods

12 week female mice were utilized in all experiments the lymphocytes of mesenteric lymph nodes were obtained according to the described method (3, the following amoebic fractions were studied: amoebic filtrate (FA) obtained according to the described method (4), total amoebic extract and the P-15, S-15, FN fractions in accordance to the reported procedure (5) and for the evaluation of the mitogenic activity of the different fractions the micromethod of blastoid transformation was utilized (6).

3. Results

Comparison of mitogenic activity of different amoebic fractions on lymphocytes from mesenteric lymph nodes and spleen of mice.

	Ug protein/100 ul maximum Stimulation	Lymphocytes Stimulation index (S.I.) M.L.N.	Spleen
Con A control positive	3 - 5	9.30	18.32
Amoebic filtrate	10	172.48	not done
P - 15 fraction	50	541.14	558.32
S - 15 fraction	50	2.03	2.14
F - N fraction	50	64.58	36.28
Total extract (tE)	100	675.69	349.86

$$S.I. = \frac{\text{c.p.m. of lymphocytes stimulated with amoebic fraction}}{\text{c.p.m. lymphocytes without stimulus}}$$

In each experiment 8 mice were utilized and each testing was by triplicate.

Results demonstrate mitogenic activity in different amoebic fractions; we still lack the definition of the composition and localization of the mitogenic proteins and to study if such activity is shared by the different amoebic strains, the described methodology will permit to evaluate in the near future the local cellular immunitary response against this parasite.

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Specific IgA response in small intestine during experimental toxoplasmosis

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144 Swiss Webster outbred mice were orally inoculated by 30 cysts of Toxoplasma gondii (PRUGNIAUD strain).
Intestinal washings and blood samples were obtained at different times post-infection (P.I.) from day 1 P.I. to 67 P.I. At day 67 P.I., 32 animals were reinfested in the same way and, then sacrificated at several days post-reinfection (P.R.). Serum samples and small intestine washings,first filtered, were analysed for IgA, IgG and IgM detection by a classic ELISA method.

The results are summarized in Figure 1 for toxoplasmic IgA coproantibodies. After the initial inoculation, IgA coproantibodies presented an early increase at day 1 P.I. and decreased to disappear at day 8 P.I. They showed a second peak at day 13 P.I. No IgA activity could be found after the third week P.I. After a first peak at day 4 P.I., seric specific IgA rose to their highest level after a month. Intestinal specific IgG response was more weak and slow. Two peaks were revealed 4 and 16 days P.I. As in previous work (2), we detected a transient specific IgG increase in serum followed by a regular rising. Maximum value were reached after 2 months. We did not found any noticeable variations in specific intestinal IgM levels. In serum, IgM antibodies rapidly increased and peaked between days 16 and 22 P.I.

After reinfestation, important and rapid increases of intestinal IgA were detected. Apparently, maximum levels took place about day 9 P.R. They could be revealed after 22 days.
Seric specific IgA gradually decreased without significant variation. No fluctuation was detected for IgG coproantibodies and seric IgG stood at their former level. Moreover, no specific IgM activity was found in intestinal washings.

Early intestinal and seric IgA and IgG increases could be explained by cross reactivity with other antigens in mice environment when they are first inoculated. Such IgA and IgG coproantibodies kinetics had been observed with other Coccidia in human and animals (1, 5). The second peak of intestinal IgA (13 days P.I.) can be compare to the

increase of biliary IgA in chicken with Eimeria tenella (3)and the recrudescence of IgA intestinal plasmocytes with Eimeria falciformis(4). As with Eimeria falciformis, reinfestation is followed by an important, rapid and long increase of specific intestinal IgA.

Further works have to precise mucosal immunity in toxoplasmosis and more specially, the exact part of specific IgA to limit reinfestation.

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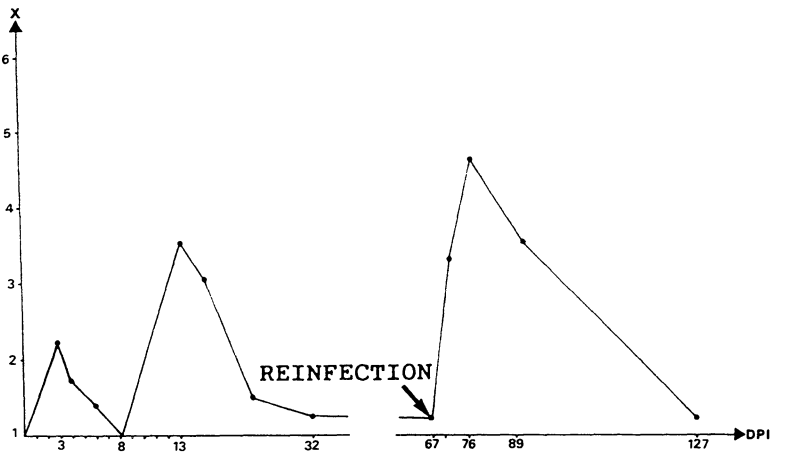

Figure 1 : IgA coproantibodies against T. gondii kinetic in orally inoculated mice (X= mean optical density of 8 different samples/mean optical density of 8 negative samples, DPi= days post infection).

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Serum and secretory IgA antibodies specific for *Toxoplasma gondii*: kinetics of the murine humoral response to infection and characterisation of target antigens

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INTRODUCTION. Infection with Toxoplasma gondii, a coccidian parasite, most commonly occurs via the oral route. Specific IgA antibodies are secreted in the gut following oral infection (Mc Leod et al., 1986). Such antibodies are considered to be protective against intestinal coccidiosis through inhibition of cell penetration by the parasite and of its subsequent intracellular development (Davis et al.,1979). Using an ELISA technique we have studied the kinetics of the IgA antibody response in serum and intestinal secretions from infected mice. Furthermore, we have characterized the toxoplasma antigens recognized by the IgA antibodies.

MATERIALS AND METHODS. Mice (OF1) were infected orally with 40 toxoplasma cysts (76K strain). Serum and intestinal samples were collected on day 0 of infection and thereafter once a week, with intestinal secretions obtained by the method of Elson et al (1984). A modified version of the ELISA described by Naot and Remington (1981) was used to detect anti-T.gondii IgA antibodies. Purified tachyzoïtes (RH strain) were separated by SDS-PAGE using 15% (W/V) acrylamide gels (Laemlli et al., 1970) under non reducing conditions and transferred to nitrocellulose (Towbin et al., 1978). The revelation was performed in an alkaline phosphatase system (Blake et al., 1984).

RESULTS. Serum anti-T.gondii IgA was first observed during the second week of infection and persisted throughout the experiment. In contrast IgA production in intestinal secretions began during the third week with both responses peaking during the sixth or seventh week of infection.The serum IgA antibodies were directed against eight major bands with molecular weights of 21000, 24000, 25000, 30000, 38000, 55000-60000, 70000. Two bands, 30 kDa and 38 kDa, were detected early on days 7 and 14 respectively whereas the others only became apparent on day 35. The intestinal IgA reacted strongly with the 30 kDa band and weakly with bands of 25 kDa, 40 kDa and 55-60 kDa. The 30 kDa band was first detected by intestinal IgA on day 14 post infection.

DISCUSSION. IgA antibodies may play a protective role against toxoplasmosis (Mc Leod et al., 1988). Following oral infection of mice with toxoplasma cysts of the 76K strain, serum and intestinal IgA reacting with T.gondii were detected. The IgA antibody response began slightly later in intestinal secretions than in serum but maximum production occurred at similar times. The serum results are in accordance with those of Le Fichoux (1988) who used an ISAGA technique for detection of human serum IgA. In contrast, mouse intestinal IgA was detected one week earlier than reported by Mc Leod et al (1986) also using an ELISA technique. The presence of this IgA antibody response to T.gondii was also demonstrated by western blotting. An early response in serum and in intestinal secretions was induced by a major protein band which migrates at 30 kDa. This band shows a migration pattern identical to that of the major surface antigen p30 as recognized by monoclonal antibodies. Two other protein bands recognized by intestinal IgA antibodies (25 kDa and a doublet of 55-60 kDa) migrate in identical fashion to the major T.gondii proteins p28 and p55-60. These proteins have been identified by monoclonal antibodies as respectively rhoptry proteins (Sadak et al., 1988) and dense granule protein. These specialized organelles seem to play an important role in host cell invasion. Toxplasma antigens recognized by IgA antibodies in the milk of infected lactating mice are currently under investigation. Characterization of the role of such antigens in local protective immunity is a prerequisite for future oral vaccination studies.

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Immunoregulation during infection with the nematode *Trichinella spiralis*: variation in immunity and cytokine production

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1. Introduction

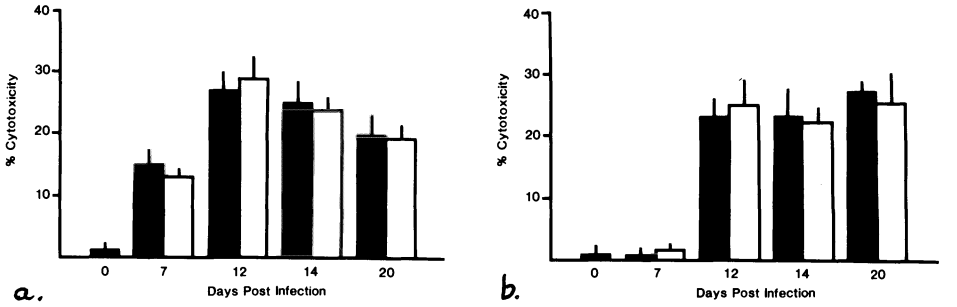
Host immunity to T. spiralis in the mouse is T cell dependent and directed primarily against the intestinal dwelling stages of infection. Release of lymphokines by $CD4^+$ T cells is believed to be involved in the acute inflammatory processes responsible for worm expulsion from the small intestine (see 1). Within inbred mice it can be shown that there is extensive strain dependent variation in worm expulsion, with both MHC (H-2) and background gene involvement. Concerning MHC influences, genes at the D end of the MHC complex have been shown to exert a marked influence upon the rate of worm expulsion. Ts-2, is a newly defined locus which maps to a position between the S and D regions, and profoundly influences the immune response to T. spiralis (see 2). Expression of the d allele at Ts-2 delays the expulsion of the parasite. Recently, the gene cluster encoding the Lymphokines Tumour Necrosis factor-($TNF-\alpha$) and Lymphotoxin (LT) have been mapped to the area occupied by the Ts-2 gene (3). The present study was designed to investigate the relationship between the expulsion of T. spiralis, the production of $LT/TNF-\alpha$ and the influence of the d allele expressed at D.

2. Methods

Inbred B10.G mice ($H-2^q$, q at D, Ts-2) and B10.T6R mice ($H-2^{Y2}$, d at D, Ts-2) were infected with T. spiralis on day 0. Mesenteric lymph node cell suspensions were prepared on days 0, 7, 12, 14 and 20 post infection, and cells (10^7/ml) stimulated in vitro with T. spiralis antigen (50ug/ml) at 37°C, 5% CO_2. After 24 hr supernatants were harvested and assayed (at 20% conc) for presence of $LT/TNF-\alpha$ using an L929 cytotoxicity assay. To differentiate between LT and $TNF-\alpha$ production replicate supernatants were incubated in the presence of a $TNF-\alpha$ specific neutralizing antibody. Levels of lymphokine were expressed as % cytotoxicity; the greater the toxicity, the greater the amount of lymphokine.

3. Results and Conclusions

Infection of both B10.G and B10T6R mice with T. spiralis generated cells (T cells) within the mesenteric lymph node capable of producing LT after specific stimulation with T. spiralis antigen. There was little evidence of TNF-α production. Cells from B10.G mice produced significant levels of LT earlier in infection than B10T6R (Fig 1). The results suggest that the d allele expressed at the D end or TS-2 region of the MHC may influence immunity to T. spiralis through production of LT. Moreover, from worm expulsion kinetics the results imply that faster expulsion by the B10.G mice relative to B10.T6R mice may be related to LT production.



□ = Assay in presence of anti- TNF-α antibody.

Figure 1. Production of TNF-α/LT by MLNC following infection with T. spiralis (a) B10.G mice (b) B10.T6R mice.

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The possible role of C-reactive protein in the immune expulsion of the tapeworm *Hymenolepis diminuta* from the rat

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INTRODUCTION

The tapeworm Hymenolepis diminuta provides a good model for the study of mucosal immune responses to adult cestodes. Few studies have been made on its natural host, the rat, and there is little evidence on the mechanisms involved in immune expulsion [1]. Our investigations have considered the role of the acute phase reactant C-reactive protein (CRP), both as an indicator of an inflammatory response in vivo and as a possible effector of complement mediated lysis of the parasite in vitro.

MATERIALS AND METHODS

Isolation of CRP

CRP was obtained by Ca^{2+} dependent affinity chromatography using ortho-phosphorylethanolamine linked to agarose. Serum was passed over a column with 0.05M TRIS/0.01M $CaCl_2$/0.15M NaCl (pH 7.4) and protein detected at 280nm. The CRP was then eluted using 0.05M TRIS/ 0.05M NaCl/0.05M EDTA (pH 7.4) and purity assessed using SDS-PAGE.

Infection Regime and Measurement of CRP in rat serum

Male Wistar rats were infected orally with 100 cysticercoids of H. diminuta. Microtitre plates were coated with a mouse anti-human CRP monoclonal antibody (4B4), blocked with 1% BSA and, after addition of 50 µl of the test serum, detection was carried out using rabbit anti-rat CRP polyclonal antibody.

In vitro culture

Isolated CRP was used in an in vitro culture system where 4 day old adult worms were incubated for up to 24h (37°C; 5% CO_2) in either normal rat serum or heat-inactivated serum (56°C for 30 min) in the presence or absence of CRP.

RESULTS AND DISCUSSION

Preliminary investigations using an *in vitro* culture system showed that within 2-3 hours 92-95% of 4 day old worms incubated in normal or heat inactivated serum with CRP showed a reduction in motility and produced local swollen opaque areas over their entire length. These results suggest that CRP alone mediates damage and may also be a possible effector of complement mediated lysis, since activation of complement by *H. diminuta* has been shown to be antibody independent [2]. However, De Beer *et al.* [3] showed that rat CRP was unable to activate C3 conversion. The opaque areas observed may be analagous to those previously reported *in vivo*, which were considered to be immunologically mediated [4].

An initial study of serum concentrations following affinity chromatography separation indicated an increase 4 days post infection with *H. diminuta*. This was confirmed using an ELISA technique and extended to show a peak elevation 7 days post infection, followed by a suppression at day 15 with a return towards control levels by day 35. In rats infected with *Schistosoma mansoni*, peak levels of CRP correlated with terminal worm rejection [5]. Our results may indicate the presence of an inflammatory response to the parasite; however, whether this contributes to immune expulsion remains to be elucidated.

ACKNOWLEDGEMENT

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Release of Fc receptors by culturing of *Actinobacillus actinomycetemcomitans* (A.a)

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Introduction

A. actinomycetemcomitans is a facultativ, Gram-negative coccobacillus implicated in oral and extraoral infections, particularly in periodontitis (Zambon, 1985). Increased levels of antibodies to *A.a.* was observed in juvenile periodontitis (Ebersole et al. 1983) and in adult periodontitis (Sandholm and Tolo, 1986). DNA/DNA reassociation experiments (Potts et al.1985) and RNA sequencing experiments (Paster, Dewhirst and Olsen, 1988) suggested that *A.a.* has strong similarities with *Hemophilius somnus* that recently was reported to express and release Fc-receptors (Widders et al. 1988). The present study was made to examine if also *A.a.* produce Fc-receptors.

Material and methods

A.a. 33384, type c, was cultured in broth for 48 hours. The cells were separated by centrifugation (8 000 xg for 30 min). Ammonium sulphate was added to the culture supernatant to 60% saturation and the solution was kept overnight at 4° C. The precipitate was collected by centrifugation (10 000 xg for 30 min), redissolved, dialysed against saline and stored at -20° C (Fraction I). The bacterial pellet was suspended in saline and the capsular fraction extracted by slow stirring for one hour at 4° C. The bacteria were separated by centrifugation at 20 000 xg for 30 min. The capsules were precipitated from the supernatant by addition of ammonium sulphate to 40% saturation, redissolved, dialysed in saline and stored at 4° C (Fraction II). The bacterial cells were suspended in saline and sonicated for 10 x 1 min. Cell remnants were removed by centrifugation at 12 000 xg for 10 min, and the supernatant was stored at-20° C (Fraction III). The protein content of each fraction was determined by the Lowry reaction, and the components were separated by SDS-PAGE using 10% gels. Samples of 4 ug (capsular material), 33 ug (bacterial sonicate) and 48 ug (medium components) were solubilized in buffer containing mercaptoethanol by heating at 100° C for 5 min. The components were transferred to nitrocellulose sheets and the sheets were incubated over night with biotinylated Fc-fragment of human IgG. Fc-fragments were purchased from Chemicon International (Los Angeles, CA), and biotinylated at our laboratory. In parallell experiments, separated A.a.- components were incubated overnight with human myeloma protein (IgG1), washed in saline/Tween 20 and incubated for one hour with biotin conjugated goat antiserum to human IgG. The sheets were washed, incubated for one hour with with avidin conjugated peroxidase and developed.

In a second series of experiments, we examined if Fc-fragments competed with binding of human IgG to *A.a.* components in ELISA. Polystyren plates were coated with Fraction I (1 ug/ml in PBS), stored overnight at 4° C and washed. Unlabelled Fc-fragments were dissolved (460 ug/ml) in PBS /Tween 20 and 100 ul of this solution was added per well. The plates were incubated at 37° C for one hour. Human serum was added to the wells to a final serum concentration of 1:500 in PBS/Tween 20 with 230 ug/ml Fc-fragment, and the plates were incubated for one hour. The plates were washed and incubated for one hour with alkaline phosphatase conjugated rabbit antiserum to the kappa chain of human IgG. After a final wash, the plates were incubated with the substrate and the optical density was read at 405 nm. Control wells that had not been exposed to human Fc-fragment were incubated with human serum in absence of Fc-fragment.

We also measured the binding of human IgG1 and IgG2 myeloma protein to *A.a.* fraction I in ELISA. The binding of myeloma proteins was compared with the binding of IgG in human sera positive for *A.a.* All experiments were run in 6 parallells, and the mean OD was calculated.

Results and Discussion

The immunoblots showed that sonicates and capsular material of *A.a.* included components that bind the Fc-fragment of human IgG as well as human myeloma proteins. Binding of human Fc-fragments were observed to sonicate components in the 39-50 , the 27-39 and the 17-29 kD range. In fraction II (capsules), one band in the 17-29 kD range showed binding of the Fc-fragment. The study showed that *A.a.* releases Fc-receptors that accumulate in the medium. The Fc-binding component in the medium migrated to the same position as protein A (41 kD). A 41 kD Fc-receptor was also observed in *Hemophilus somnus* (Yarnall et al.1988). Fc-receptors have been observed in streptococci (Kronwall 1977) and staphylococci (Langone1982) but to our knowledge this is the first observation of Fc-receptors in oral, Gram-negative, anaerobe bacteria. The observation of Fc-receptors in 3 oral bacteria is striking, and it is tempting to speculate if the capacity for synthesis of such components may be transferred by plasmids.

Pre-incubation of coated wells with human Fc-fragment reduced the binding of human IgG to medium components of *A.a.* in a dose dependent way. The contribution of nonspecific binding to *A.a.* components was verified by incubation with human myeloma proteins. The OD observed with myeloma protein (IgG1) corresponded to about 50% of the OD observed after incubation with reference serum. It is possible that the specific (Fab) activity of human serum to *A.a.* might have been overestimated in previous reports. The observation of Fc-receptors in *A.a.* also confounds the interpretation of immunofluorescent studies based on antibodies raised against *A.a.* since phagocytes expressing Fc-receptors may be positive.

The biological effect of bacterial Fc-receptors is not clear. Soluble Fc-receptors may interfere with complement activation, but this may be difficult to verify since alternative activation of complement probably occurs in presence of bacteria. Protein A promotes evasion of phagocytosis (Peterson et al.1977) presumably through interference with opsonizing antibodies, and release of Fc-receptors from A.a. may have a similar effect. We are presently examining this possibility in experiments with granulocytes.

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Effect of mucosally derived IgA monoclonal antibodies against *Salmonella typhimurium* in an *in vitro* adherence assay using Madin-Darby canine kidney cells

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Introduction. A primary function of secretory IgA (S-IgA) is thought to be the exclusion of infectious agents from mucosal surfaces. The present study aimed to investigate this by determining the effects of mucosally-derived monoclonal antibodies (Mabs) against lipopolysaccharide (LPS) on the surface properties (charge and hydrophobicity) of bacteria and their adherence to epithelial cells.

Methods and Results. 18 Mabs against Salmonella typhimurium (STM) LPS were produced by Peyer's patch immunisaition of rats using mesenteric lymph nodes and efferent mesenteric lymph as a source of primed lymphocytes (Dean et al. 1986) . 13 Mabs were IgA, 4 IgM and 1 IgG1. 5 were cross-reactive with S. minnesota LPS. The Mabs were divided into 4 groups based on isotype and cross-rectivity with S. minnesota LPS. All the Mabs except for the IgG1 antibody were capable of inhibiting adherence in an in vitro assay using STM cells and MDCK epithelial monolayers. Physico-chemical analysis of STM, treated and un-treated with Mabs, was carried out using two-phase aqueous polymer systems and hydrophobic interaction chromatography (Magnusson and Stjernstrom 1982) . IgA Mabs increased both the hydrophilicity of the bacteria and their net negative surface charge. IgM Mabs had no effect on bacterial hydrophobicity but did increase their net negative surface charge. The IgG1 Mab increased bacterial hydrophobicity but had no effect on their net negative surface charge. Inhibition of adherence of treated bacteria appeared to correlate with increase in their net negative surface charge. All IgA Mabs produced a 360 kD dimer. 9 produced two other forms (320 and 160 kD). The 160kD form corresponded to a monomer, whilst the 320 kD form could represent a dimeric IgA without J-chain. Each of the 3 molecular forms of the IgA Mabs were isolated and used in further adherence and physico-chemical studies. 160 kD forms had a reduced ability to inhibit adherence compared to both 320 and 360 kD forms, with 360 kD giving the greatest inhibition. All molecular forms gave a comparable increase in hydrophilicity. The 360 kD forms, and the 320 kD forms to a lesser extent, gave an increase in net negative surface charge whereas the 160 kD forms had no effect.

Adherence and surface properties of *Salmonella typhimurium* treated with monoclonal antibodies

	Monoclonal antibody	Isotype	Adherence*1 (%)	Hydrophobicity*2 (%)	Relative negative*3 surface charge
Controls	Bacteria alone	–	100	35	0.04
	Bacteria with non-specific Mab	–	99	33	0.03
Group 1 IgA cross reactive	6/8/13e	IgA	11	21	0.39
	6/8/81c	IgA	26	22	0.54
	6/8/5d	IgA	37	18	0.63
	6/81/13b	IgA	34	26	0.39
Group 2 non-IgA cross reactive	7/8/39c	IgM	36	33	0.65
Group 3 IgA non-cross reactive	6/8/16	IgA	32	15	0.74
	6/8/33c	IgA	32	17	0.89
	PIP18	IgA	21	18	0.45
	6/8/25	IgA	36	24	0.21
	PIP2	IgA	32	21	0.54
	6/8/42a	IgA	21	17	1.13
	6/8/81b	IgA	19	18	0.63
	6/8/54a	IgA	36	25	1.03
	6/8/52c	IgA	15	29	0.16
Group 4 non-IgA non-cross reactive	6/8/53b	IgM	32	42	0.64
	7/8/18	IgM	34	34	0.34
	PIP13	IgM	19	36	0.41
	7/8/64	IgG1	97	66	0.00
Molecular forms see Fig. 2	PIP 2 (160KD)	IgA	23	21	0.00
	PIP 2 (320KD)	IgA	17	19	0.21
	PIP 2 (360KD)	IgA	20	18	0.60
	PIP18 (160KD)	IgA	22	19	0.00
	PIP18 (320KD)	IgA	23	21	0.32
	PIP18 (360KD)	IgA	15	16	0.50

Conclusions. Most of the Mabs produced by mucosal immunisation with S.typhimurium were directed against LPS. All the IgA and IgM Mabs inhibited adherence of S. typhimurium to MDCK epithelial monolayers but the IgG1 Mab did not. Treatment of the bacteria with the Mabs had variable effects on their hydrophobicity but this did not correlate with their ability to inhibit adherence. In contrast, all the inhibiting Mabs increased bacterial surface negative charge. Increase in surface negative charge may therefore contribute to antibody-mediated inhibition of bacterial adherence in addition to Mab specificity.Three different molecular weight forms of IgA were found in hybridoma supernatants, 160 kD (monomeric) and 320 kD, 360 kD (dimeric). These were seperated in order to compare different molecular weight forms of IgA with the same idiotype. The dimeric forms were more efficient in inhibiting bacterial adherence than the monomeric forms from the same clone. There was a correlation between inhibition of bacterial adherence and increase in net negative surface charge.

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The development of Peyer's patches and a phosphocholine specific IgA antibody response following oral colonization of germ-free mice with *Morganella morganii*

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ABSTRACT. In order to analyze the differentiative processes and changes in functional potential among B cells in PP, especially in germinal centers (GC), fragment cultures were established using two halved PP in 2.0 ml supportive medium in an atmosphere of 90% O_2-10%CO_2. Both PP cultures from conventionally reared mice and formerly germ-free (GF) mice colonized with Morganella morganii (M.m.) could be maintained for >12 days with continued B cell division, especially among cells binding high levels of peanut agglutinin (PNA^{high}, a characteristic of GC cells). Over this period such cultures continued to secrete IgA and lesser amounts of IgM and, in the case of the PP from formerly germ-free mice, also showed net increases of IgA anti-phosphocholine (PC) Ab with avidities as high as the prototypic T15 Ab. Finally, 7-8 d fragment cultures yielded PNA^{high} B cells that could be restimulated in T dependent clonal microculture to secrete IgA, usually along with IgM but occasionally with other isotypes.

Introduction

The GC of PP contain a rapidly dividing subset of B cells which we believe has a role in the development of an IgA response (see Weinstein et al., this volume). Specifically, these cells include precursors of IgA secreting plasma cells found in lamina propria and exocrine tissue.

We sought to establish PP fragment cultures in order to define the events occurring during GC development and maintenance. In addition, we sought conditions where GC cells, defined as PNA^{high} B cells, continue to divide in vitro and tried to determine whether they differentiate functionally as they remain in culture without the possibility of emigration. We also wanted to determine whether the cultures could be used to detect a specific IgA response in PP. To this end, we have assayed for an antibody response to the phosphocholine (PC) antigenic determinant previously reported to be induced by M.m. given parenterally (1).

Materials and Methods

PP were recovered by dissection and placed in a cold, sterile RPMI medium containing 1% penicillin-streptomycin and 5% FCS. They were washed by five successive transfers through fresh medium, remaining in

each wash for 10 min before each transfer. Subsequently, 2 PP were halved with a sharp, sterile razor blade and placed into 2 ml conditioned HY-medium in an atmosphere of 90% O_2-10%CO_2 in a 24 well plate. Sampling the cultures involved dispersing the patches and removing dead cells and debris on a Ficoll gradient. A proportion of these cells were returned to culture and pulsed with ^{3}H-thymidine for 16 hrs.

Results and Discussion

Fragment cultures of PP from normal conventionally reared mice could be maintained for >12 d. and gave outputs of total IgA of about 2, 5, and 6.5 μg each at day 1, 5, and 10 respectively. The output of total IgM by these cultures increased over 10 d. but was significantly less than that of IgA. Little IgG was detected in culture fluid and this did not increase with time. There was no detectable anti-PC IgA Ab in these supernatants.

With time, large blast cells emerged from the fragments and eventually covered the bottom of the culture well. The $s\kappa^+$ B cell population increased over the 12 d from about 60% to 80%. The cell population maintained 15-20% PNA^{high} B cells. These were markedly enlarged and showed a conspicuous shift from $s\kappa^{low}$ to $s\kappa^{high}$. The proportion of cells bearing both the PNA^{high} and sIgA markers also increased in numbers. Radioautography of ^{3}H-thymidine pulsed cells revealed that the cell population continues to divide through d 12 in culture and that the PNA^{high} subpopulation obtained by FACS is slightly enriched in dividing cells (14%) at 5 d. Immunoperoxidase staining for cytoplasmic IgA showed that many of the large blast cells were positive.

Germ-free BALB/c mice were colonized orally with M.m. to determine whether GC developed in PP and whether a specific antibody response could be stimulated. There was little change in the surface phenotype (PNA vs. κ) of PP cells during the first 10 weeks although the PP somewhat enlarged. At 12, 15, and 20 weeks modest populations (10, 29, and 17% respectively) of PNA^{high} cells appeared and were mostly $s\kappa^{high}$. Although no anti-PC Ab was detected in the serum of these mice, in vivo fragments cultures of their PP taken at 12, 15, and 20 weeks after colonization showed incremental rises in IgA anti-PC Ab over 5-6 d of 9.5, 19.5, and 45 ng (vs. 400, 813, and 640 ng total IgA) from day 1 outputs of about 6.5 ng anti-PC IgA (vs. 200 ng total IgA). Surprisingly, IgM anti-PC Ab was barely detectable in these cultures and no IgG1 Ab was found. Hapten inhibition with PC showed that the Abs in the culture media had avidities similar to prototypic T15 IgA anti-PC.

The functional potential of conventional PP cells cultured for 7 d was examined by placing PNA^{high} B cells into secondary microculture (10 μl) with alloreactive D10 (T_H2) cells and dendritic cells (see Cebra et al., this volume).

Many of the positive clonal cultures made more than one isotype, usually including IgM. IgA was the next most prevalent isotype secreted by about 1/3 of the positive cultures.

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The use of heterohybridomas to produce human monoclonal antibodies against gonococcal outer membrane proteins

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Introduction

To achieve a better understanding of the immune response to gonococcal infection, we are interested in the production of antibodies at the mucosal level. Quantities of specific antibodies from these locations are hard to obtain making functional assays difficult. To produce these antibodies we attempted to make heterohybridomas secreting specific human monoclonal antibodies. Although our ultimate goal is to use lymphocytes isolated from human fallopian tubes as fusion partners we are reporting our success at fusing human peripheral blood mononuclear cells with murine myeloma cells for the production of specific antigonococcal monoclonal antibodies.

Materials and Methods

FUSION PARTNERS

Murine myelomas (SP-2, NS-1, and P63) were obtained from American Type Culture Collection, propagated and maintained using either RPMI 1640 or Delbeccos Minimal Essential Medium (DMEM) containing 10% newborn calf sera. Stock cultures were stored in cryoprotective media in liquid N_2. Peripheral blood lymphocytes were obtained from normal healthy volunteers, isolated over Histopaque, and depleted of T-cells by E-rosetting techniques. The resulting B-cell enriched populations were then used for immunization and subsequent fusion.

IN VITRO IMMUNIZATION

B-cell enriched populations were immunized with varying concentrations of Sarkosyl extracted outer membrane proteins [0.1, 1.0, 10, and 25 ug/ml of cells (1×10^5 cells/ml)] for 6 days in the presence of murine thymocyte conditioned DMEM, protein A (1×10^7 S. aureus /ml) and IL-1 (5 U/ml). On days 2 and 4 of immunization, additional complete medium containing autologous human sera was added. Following the immunization cells were harvested and used for fusions.

HETEROHYBRIDOMA PRODUCTION

Heterohybridomas were produced according to a modification of the procedure described by Oi and Herzenberg (4). Culture media from wells containing clones were assayed for specific gonococcal activity by both ELISA (3) and immunoblotting techniques (1). Specificity of the

monoclonals was determined by Western blotting techniques(2). Clones were propagated in tissue culture for production of quantities of antibody.

Results

Human lymphocytes immunized with varying concentrations of Sarkosyl extracted outer membrane proteins (OMP) were fused with each of three murine myelomas. The NS-1 myeloma was the most successful fusion partner with the human lymphocytes. At least forty-eight clones resulted from the fusions. All antigen concentrations (0.1 - 25 ug/ml) gave successful antibody producing clones. However, the 1 ug/ml concentration resulted in the only clones to be successfully propagated. Two clones, C6 and C8, from the limiting dilutions, were expanded to provide quantities of antibody. The culture media were positive for IgG antibody by both ELISA and Immunoblots. When these purified monoclonal antibodies were tested for specificity in the Western blot assay our C6 monoclonal antibody reacted with the PI OMP.

Discussion

Antibody has been implicated in the immune response to gonococcal infection for a number of years although its role in protection against infection has been elusive. Our efforts in these experiments have been to produce specific human antigonococcal monoclonal antibodies. Our initial results produced antibodies of an IgG isotype which were directed against PI. These positive results indicate that the production of human antibodies with specificity for the gonococcus from heterohybridoma fusion is possible. With this success fusions with lymphocytes from local sites such as the fallopian tube mucosa is now possible. Production of monoclonals from these lymphocytes would be advantageous to our understanding of immunological events involving antibody which occur at the mucosal level (i.e. antibody dependent cell mediated cytotoxicity).

Acknowledgement

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Lymphocyte proliferation in response to the antigens of colonisation factor antigen/I following primary *in vitro* immunisation

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1. Introduction

CFA/I is a pilus composed of repeating pilin protein subunits found on many serogroups of enterotoxigenic *Escherichia coli* which promotes attachment. Primary in vitro immunization of rabbit spleen cells with a synthetic peptide representing the n-terminal 1-13 amino acid fragment of the CFA/I subunit (CFA/I 1-13) produced anti-peptide antibodies which also recognized native CFA/I. We have developed a unique system of in vitro immunization followed by lymphocyte proliferation to determine that CFA/I 1-13 contains a T cell epitope.

2. Materials and Methods

2.1 *In vitro immunization.* Spleen cell suspensions were prepared from 1.5 kg New Zealand White rabbits (Hazleton, Denver PA) in DMEM supplemented with antibiotics, L-glutamine (2 mM), and 10 mM HEPES Buffer, 10% FCS (Hazleton), as well as MEM essential and non-essential amino acids (0.1 mM), $NaHCO_3$ (.06%), and 5 x 10^{-5} 2-ME (Sigma, St. Louis, MO). Cells (6 x 10^5 in 0.1 ml) were placed in round bottom culture plates with 0.1 ml antigen (Ag). At the eighth day of culture (37°C in 5% CO_2), cells were boosted with Ag in the presence of 2U per ml human rIL-2 (Boehringer Mannheim Biochemicals, Indianapolis, IN) or 4% (v/v) conditioned media of ConA stimulated rabbit spleen cells (ConA-CM) as a source of rabbit IL-2. Cultures were pulsed with 1 μCi [^{3}H]TdR on day 9 of culture and were harvested for scintillation counting 24 hours later. Highly purified CFA/1 was a generous gift from Dr. Britton (U. of Pittsburgh). CFA/1 1-13 (Val-Glu-Lys-Asn-Ile-Thr-Val-Thr-Ala-Ser-Val-Asp-Pro) was synthesized by Merrifield Solid Phase technology. KLH (Sigma) was employed as a standard of the immunization process.

3. Results

3.1 *In vitro immunization of rabbit spleen cells leads to a secondary response for protein or peptide antigens.* Spleen cells were immunized in vitro with KLH or the CFA/I 1-13 peptide and then challenged at day 8 (Table 1). The media control received IL-2 but not Ag. A source of IL-2 was required to support cellular proliferation in response to reintroduction of the Ag, and cultures did not respond to either Ag at day 0 indicating the rabbits were naive to these Ags at the time of sacrifice (data not shown).

TABLE 1. *Response to in vitro immunization of rabbit spleen cells with a protein or peptide antigen*[1]

Antigen	Human rIL-2 pulse		Rabbit ConA-CM pulse	
(ng/ml)	KLH	CFA/I 1-13	KLH	CFA/I 1-13
15	6135±728	4643±411[2]	4074±951[2]	4007±786
5	6584±998[2]	3317±308	2159±205	4647±200[2]
1.5	4614±612	3564±468	3881±738	1967±352
MEDIA	1368±336	1152±242	1227±249	1420±138

[1]In vitro immunization was conducted, and the mean of five replicates from a representative experiment ± standard error is shown. [2]significant (Ag versus Media) $p<.05$

3.2 *Cultures immunized with CFA/I 1-13 respond to the native protein.* Spleen cells were immunized in vitro with CFA/I, CFA/I 1-13, or media alone and then challenged with the related Ag or an unrelated Ag (KLH). Immunized cultures responded when the same Ag or the related Ag was reintroduced at day 8, but did not respond to the unrelated KLH (Table 2). Cultures given only media at day 0 did not respond to any Ag tested (Table 2).

TABLE 2. *Responses to challenge with a related antigen*[1]

Immunizing	Boosting Antigen			
Antigen	NONE	KLH	CFA/I	CFA/I 1-13
None	2440±398	2018±166	2842±258	2938±153
CFA/I	3501±411	2637±393	5627±753[2]	5457±354[2]
CFA/I 1-13	3562±845	4262±509	8444±643[2]	11037±1202[2]

[1]In vitro immunization was conducted using 1.5 ng/ml Ag. All cultures received human rIL-2 at day 8. Data shown is the mean of five replicates ± standard error. [2]$p<.02$

4. Discussion

Using a unique assay system, we have shown that immunization of T cells may be conducted in vitro against both protein and peptide antigens. Because IL-2 is required to observe the response, we believe the proliferation to represent primarily T cell activation; however, some B cell proliferation is also likely. This method may be an efficient way to locate and compare the immunogenicity of other T cell epitopes in CFA/I.

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Effect of intestinal mucosal environment on the hepatopathogenic potential of rotavirus in normal and immunocompromised hosts

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INTRODUCTION. The worldwide impact of rotaviruses (RV) as the leading cause of diarrhea in children has generated much interest in disease prevention by a specific vaccine. The Jennerian approach to RV vaccination of humans using orally administered heterologous (animal) strains has been the most promising [1]. The suckling mouse model has recently been shown to be applicable to study the disease induced by heterologous RVs [2]. Mice with severe combined immunodeficiency (SCID), which are known to suffer persistent murine RV infection, constitute a good model for the immunocompromised host. Recent studies in our laboratory have suggested that mucosal immunocompetence may play an important role in influencing heterologous RV infection in mice [3]. This was evidenced by extramucosal spread and hepatic virus replication of rhesus RV (RRV) in SCID mice. The aim of this study was to examine the effect of different routes of administration on the hepatopathogenic potential of RRV in SCID and normal mice.

MATERIALS AND METHODS. One to 3-day old CB17 mice with SCID and immunocompetent BALB/c mice were used in these studies. Heterologous rotavirus RRV strain MMU 18006, a candidate vaccine strain, had been plaque purified and grown in MA104 cells (1.2×10^7 PFU/mL). Homologous murine rotavirus (MRV) strain EDIM 5099 (2×10^7 ID_{50}/mL) was grown in suckling mice and the clarified intestinal homogenate was used as inoculum.

Groups of SCID and BALB/c mice were inoculated with 10-20 ul of live RRV or MRV by per oral (PO), intraperitoneal (IP) or intravenous (IV) routes. Infection was monitored by cell culture infectivity (CCI), immunofluorescence (IFA), light (LM) and electron microscopy (EM).

RESULTS. Homologous MRV produced acute diarrhea in BALB/c mice and persistent infection in SCID animals. No extramucosal spread of virus or hepatitis was observed in these mice. The outcome of RRV infection in SCID and BALB/c mice following PO, IP and IV inoculation is shown in Table 1. Surviving SCID mice in the group given RRV by PO route developed chronic liver disease. LM examination revealed an acute hepatitis with a temporal progression to foci of necrosis. The pattern of liver damage was similar in all groups of animals, but the severity was much more prominent in animals, especially SCID, given virus IP and IV. By

TABLE 1. Effect of different routes of RRV (10^5 PFU) administration on development of hepatitis in SCID and BALB/c mice

Route	Mouse strain	No. of animals	Icterus		Mortality	
			Frequency (%)	Incubation period (Mean days)	Frequency (%)	Average day of death
Oral	SCID	49	84	11.4	27	25.2
	BALB/c	92	21	8.6	0	-
IP	SCID	13	100	4.7	100	10.0
	BALB/c	24	100	5.6	100	12.9
IV	SCID	11	100	4.8	100	9.8
	BALB/c	20	100	5.8	94	12.4

EM, distinctive areas of virus replication were seen, with virus budding from membraneous structures in vacuolated cells resembling hepatocytes. RV was identified by CCI and/or IFA in 70 to 100% of symptomatic animals.

DISCUSSION. Recent studies in our laboratory have suggested altered tissue tropism of rotaviruses, previously shown to replicate exquisitely in the gut [3]. The findings of more severe and chronic hepatitis in SCID mice in comparison to normal mice indicated that an intact immune system is critical in reducing the virus load and subsequent spread of the virus to the liver. Herein we demonstrate that the route of virus administration is another important factor influencing the hepatopathogenic potential of RRV. All SCID and immunocompetent BALB/c mice given RRV by IP and IV routes exhibited hepatitis with 100% mortality. These results indicate that the hepatotropism of RRV appeared to be independent of processing of virus by intestinal environment.

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Prevention of murine Sendai virus infection by passive immmunisation with IgA and IgG monoclonal antibodies

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INTRODUCTION. Resistance to respiratory viral infections correlates with the presence of specific antibodies in the respiratory secretions. The secretory immune system with its predominant antibody, secretory IgA, poses the initial immunological barrier. Given that IgA is less phlogistic than IgG (reviewed in Ref. 1), and that IgA but not IgG is selectively transported via secretory component into respiratory secretions, if IgA and IgG are comparably protective in vivo, then immunization protocols designed to generate a predominately IgA response are theoretically advantageous since the airway would be protected against viral infections while lung inflammation and damage would be minimized. Therefore, this study seeks to compare the efficacy of passive immunization with IgA versus IgG monoclonal antibody.

MATERIALS AND METHODS. Anti-Sendai virus Mab were produced and characterized as previously reported (2,3). Equivalent ELISA and/or neutralization titers of IgA or IgG Mab were administered intranasally to etherized BALB/c mice. Control animals received either saline or an irrelevant Mab. One hour later the mice were challenged with 2.5×10^4 pfu of Sendai virus intranasally. After viral challenge, the mice received two additional intranasal doses of Mab at 4 and 24 hours. Three days later the animals were sacrificed and virus titers of lung homogenates were determined by plaque assay.

RESULTS. In 4 independent experiments (total of 132 mice) comparing virus hemagglutinin-neuraminidase (HN) specific IgA and IgG_{2a} neutralizing monoclonal antibodies (2 of each), there was no significant difference in the ability of IgA and IgG to protect virus-challenged mice (Fig. 1). Protection was dose dependent. One of the IgA Mab and one of the IgG Mab recognize the same HN epitope (data not shown). In contrast, an IgG_{2b} Mab specific for the virus fusion (F) protein did not protect mice but also did not neutralize virus in vitro (data not shown).

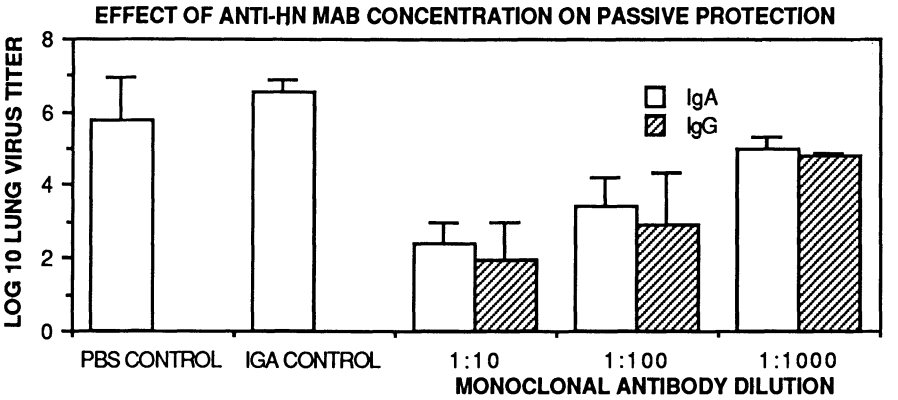

Fig. 1. Serial dilutions of IgA and IgG_{2a} virus neutralizing monoclonal antibodies reactive with Sendai virus HN protein were administered intranasally to virus challenged mice in 4 separate experiments (see Methods). Three days after infection lung virus titers were determined, and the mean $\log_{10}$ plaque forming units for each group were normalized to the PBS treated group. Means and SD of the pooled data from all 4 experiments are shown.

DISCUSSION. The effectiveness of an antiviral vaccine appears related to its ability to generate viral specific antibodies. IgA is the predominant antibody in the upper airway secretions, where most respiratory viruses initially invade the body. Anti-Sendai virus IgA and IgG Mab specifically protected mice equally well against subsequent viral challenge. Since IgA is as effective as IgG in protecting the respiratory tract and since a selective transport mechanism exists for transporting IgA but not IgG into the upper airway, these results support the rationale for developing mucosal immunization protocols designed to generate viral specific IgA responses.

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Secretory IgA oligosaccharide chains as receptors for bacterial lectins

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ABSTRACT. Type 1 fimbriae confer on *E. coli* and related species a capacity to adhere to, for example, colonic epithelial cells in a mannose-sensitive manner (1,2). The cellular receptors for type 1 fimbrial lectins are believed to be asparagine-linked oligosaccharide chains, but no mammalian receptor has yet been isolated. Immunoglobulin molecules are substituted with complex type asparagine-linked oligosaccharide chains (3). We here report that secretory IgA and IgA myelomas, especially of the IgA2 subclass, interact with type 1 fimbriated *E. coli* due to lectin/carbohydrate interactions, resulting in agglutination of the bacteria and interference with their adherence to human colonic epithelial cells.

1. MATERIALS AND METHODS

Myeloma proteins of the IgA isotype and secretory IgA were isolated as described (4). IgM myelomas were kindly supplied by Dr Schroenloher, Birmingham, Ala, and IgG myelomas by Dr Skvaril, Bern, Switzerland.

E. coli with either mannose-specific type 1 fimbriae (506MS), or Galα-1-4Galβ-specific P fimbriae (506MR) were obtained by transformation of a non-adhering fecal strain (2).

Immunoglobulin preparations (1g/l) were titrated by 1:2 steps and mixed with bacteria ($2x10^9$/ml) in microtitre plates in the absence or presence of 2.5% methyl-α-D-mannoside. The plates were incubated at 37°C for 30 min and at 4°C overnight and agglutination was read in a microscope.

Adherence to the HT-29 cell line was performed as earlier described (2) with immunoglobulins present at a final concentration of 0.6 g/l.

2. RESULTS

Half of the IgA myelomas of either IgA1 or IgA2 subclass agglutinated the mannose-specific *E. coli* strain 506MS, a reaction that was completely inhibited by mannose. No reaction was seen with the strain 506MR (Table 1). The IgG myelomas tested were all negative, while half of the IgM myelomas tested had agglutination titres similar to the IgA1 myelomas.

Most, but not all, of the agglutination induced by secretory IgA was reversed by mannose, suggesting that both lectin-carbohydrate interactions and antigen-antibody binding contributed to the agglutination. In contrast, gammaglobulin did not induce any mannose-reversible reaction with type 1-fimbriated *E. coli*. Secretory IgA, and IgA2 myeloma protein blocked the adherence of 506 MS to colonic epithelial cells from the HT-29 cell line, while gammaglobulin was inactive (Table 2).

TABLE 1. Mannose-reversible agglutination of type 1-fimbriated *E. coli* by IgA myelomas and secretory IgA.

Immunoglobulin preparation	n	Agglutination titre		
		506MS		506MR
		+ PBS	+ mannose	
IgA1 myelomas	6	<1-8	<1	<1
IgA2 myelomas	6	<1-512	<1	<1
Secretory IgA		128	2	<1
Gammaglobulin		2	4	<1

TABLE 2. Inhibition of adherence to colonic epithelial cells by secretory IgA and IgA2 myeloma protein.

Inhibitor	Adherence (bacteria/cell)
None	68
Mannose	<5
Secretory IgA	<5
IgA2 myeloma (Fel)	<5
Gammaglobulin	65

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Structure and accessibility of a protective synthetic peptide determinant derived from Pili of P-specific uropathogenic *Escherichia coli* strains

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ABSTRACT. The pilin protein of p-specific pili expressed by the uropathogenic *Escherichia coli* strain J96 carries a conserved, crossreactive linear B-cell determinant (R5-12). A synthetic peptide conjugate encompassing this determinant mediated homologous protection in a model of experimental pyelonephritis in mice. To study crossreactivity and to elucidate the fine-structure of this epitope, specific antisera directed at synthetic peptides scanning the N-terminal sequence were prepared. Recognition of heterologous peptides as well as heterologous pili varied considerably. Immunelectron-microscopy with anti-R1-12 antibodies demonstrated exclusive binding of antibodies at the tip of the pilus filament, indicating a hidden N-terminus and thus a possible role in filament polymerization. This further suggests that in addition to opsonization, inhibition of receptor binding by steric hindrance contributes to the protection observed.

1. Introduction

Uropathogenic *Escherichia coli* (UPEC) strains are a major source of ascending urinary tract infections and bacterial pyelonephritis. Attachment of UPEC to epithelial cells is mediated by pili-associated, p-specific adhesion systems. Pili consist of several hundred pilin subunits and carry at the tip a ternary protein complex (papE, F, and G) recognizing the αGal(1-4)ßGal carbohydrate receptor. A p-pilus vaccine prevented colonization and infection by the homologous strain in a model of experimental pyelonephritis in mice (1). The J96 p-pilus has been cloned and sequenced (HU849/pap5) (2,3). Linear immunogenic and antigenic determinants have been mapped with synthetic peptides in the pilin protein (4). Only antisera engendered by peptides R5-12 and R93-106 recognized also pili of heterologous p-specific strains. Synthetic peptides corresponding to antigenic epitopes have been studied in the murin pyelonephritis model (Figure 1) to determine their efficacy as vaccines and to identify linear "protective" determinants

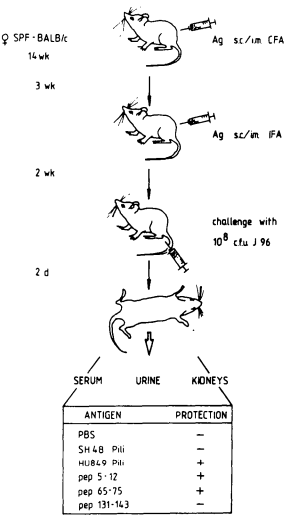

Figure 1: Protection study

(5,6). Immunization with synthetic peptides R5-12 and R65-75 provided efficient protection against homologous challenge. Here, overlapping segments of the conserved N-terminal region of the J96 pilin protein were prepared as synthetic peptides in an attempt to elucidate the fine-structure of the conserved determinant and to improve the crossreactivity of the antisera obtained. Access to the N-terminal pilin region was probed with antibodies directed at R1-12 using the immunogold technique.

2. Materials and Methods

Strain J96 (O4:K5:H51) is a hemolytic, colicin-V positive, motile, serum-resistant, p^+ and $MSHA^+$ human pyelonephritis isolate. Peptides have been selected and prepared as described (4-6). Antibody recognition of homologous and heterologous peptides was assessed by ELISA and crossreactivity with heterologous pilin proteins was assayed by Western blotting. For immunelectronmicroscopy the J96 derived recombinant strain HU849/pap5 (5) was used. Bacteria were reacted with anti-R1-12 antiserum and with Protein A adsorbed with 15 nm colloidal gold particles followed by negative staining.

3. Results

3.1. CROSSREACTIVITY OF PEPTIDE ANTISERA

Synthetic peptides corresponding to the J96 pilin sequences R1-12, R5-12, R5-15, R8-22, and to the 3669 sequence R1-12 were prepared (Table 1) and used to engender specific antipeptide antisera. All peptides were immunogenic and all antipeptide antisera except 3669 R1-12 recognized HU849(J96) pili by ELISA and Western blotting. Recognition of heterologous p-specific pili by Western blotting, however, varied considerably with the particular antipeptide antiserum used. Antiserum raised against the R5-12 conjugate recognized most of the p-specific pili tested. Thus, R5-12 corresponds to a highly conserved crossreactive determinant. Recognition of different synthetic peptides by heterologous antipeptide antisera was addressed by ELISA. Antibodies to R1-12

COMPARISON OF N-TERMINAL AMINO ACID SEQUENCES OF UTI-E.COLI PILINS AND ADHESINS

PROTEIN	ISOLATE	N-TERMINAL AMINO ACID SEQUENCE
afimbrial		1 24
AFA-I	KS52	N F T S S G T N G K V D L T I T E E C R V T V E...
AFA-V	MIR2194	A N Q G Q G V V N S K G T V I D A T C G I D P D...
fimbrial		1 22
F 71	C1212	A A T I P Q G Q G E V A F K G T V V D A P ...
F 72	AD110	- P - - - - - - - K - T - N - - - - - - - C G...
F 9	3669	- - S Q G S - - * * - N - - - - - I - - - C G I E T Q S A N Q..
F 11		- P - - - - - - - K - T - N - - - - - - - C S I S Q K S A D Q..
F 12	C1979	- P - - - E - - - K - T - N - - - -
F 13	J96 papA	- P - - - - - - - K - T - N - - - - - - - C S I S Q K S A D Q..
	papE	V D N L T F R G K L I I P A C T V S N T T V D W Q D...
	papF	D V Q I N I R G N V Y I P P C T I N N G Q N I V V D...
	papG	G W H N V M F Y A F N D Y L T T N A G N V K V I D Q...

Table 1: Comparison of N-terminal pilin sequences of *Escherichia coli*. The sequence corresponding to the crossreactive, protective determinant is underlined.

recognized the peptide R5-12 and *vice versa*. R5-12 antiserum additionally also bound to peptide R5-15 on solid phase which, however, was not at all recognized by anti-R1-12 antibodies. The R8-22 amino acid sequence in turn reacted with all other heterologous antipeptide antibodies. Antiserum directed at the 3669 N-terminal R1-12 recognized only the homologous peptide. These experiments indicated a common determinant to involve amino acids eight to twelve. Thus, this segment was synthesized as a trimer [R(8-12)3]. All heterologous antipeptide antisera as well as anti-HU849 pilus antiserum recognized this peptide on solid phase, indicating that indeed this sequence harboured the common epitope.

3.2 IMMUNOGOLDLABELING WITH ANTI-R1-12 ANTIBODIES

To probe the accessibility of the N-terminal domain of the pilin protein in the pilus filament, the recombinant HU849 strain expressing p-specific pili derived from J96 was reacted with anti-R1-12 antiserum, followed by Protein A labeled with colloidal gold. Pili were subsequently visualized by negative staining (Figure 2A). In contrast to antibodies raised against whole pili which labeled pili homogeneously (Figure 2B), antibodies directed at R1-12 bound exclusively near or at the tip of the filaments (Figure 2C, D).

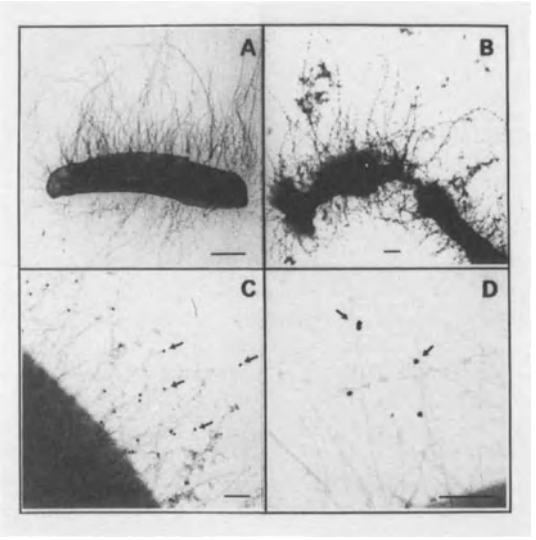

Figure 2: Immunelectronmicroscopy with anti-R1-12 antiserum using Protein A-colloidal gold particles. A: negative staining of recombinant HU849(J96) pili (bar: 5µm); B: labeling with anti-HU849 pili antiserum (bar: 0.5µm); C,D: labeling with anti-R1-12 antiserum (C bar: 180 nm, D bar: 150 nm).

4. Discussion

To elucidate the fine structure of the protective determinant identified in the N-terminal region of p-specific pilin derived from the pyelonephritogenic *Escherichia coli* strain J96, several segments of the N-terminus have been synthesized as overlapping peptides. As

expected, recognition of heterologous pilins in Western blotting and also of heterologous peptides by ELISA varied with the particular sequence used as immunogen, though all peptides shared a large part of their sequence. The R5-12 peptide-thyroglobulin conjugate induced antibodies which recognized most of the heterologous pili tested. Variations of this motif did not improve crossreactivity. The binding pattern of the synthetic peptides with heterologous antisera suggests that the immunodominant part of this determinant resides in amino acids 8-12. A synthetic peptide corresponding to this sequence will be assayed for the induction of crossreactive antibodies as well as for its potential as component of a synthetic vaccine.
Antibodies to R1-12 bound solely at the tip of the pilus filaments. This is consistent with the immunorecessive nature of the N-terminus of the structural pilin protein. One obvious explanation might be that the N-terminus is involved in polymerization and thus is not accessible to the immune system. The binding at the tip of the filament might in turn indicate a change in structure due to the adjacent ternary binding complex consisting of the papE, papF, and papG proteins rendering the N-termi-nus at this position accessible to antibodies. This might also offer an explanation for the protection seen in the animal model of experimental pyelonephritis as not only opsoni-zation but also steric hindrance of receptor recognition might be involved.

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6. Acknowledgments

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Kinetics of secretory and serum antibodies during intestinal colonisation by *Candida albicans*

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INTRODUCTION

Candida albicans , one of the most common opportunistic fungal pathogens, can cause both systemic and superficial infections (1).As the fungus most probably disseminates from the gastro-intestinal tract through mucosal surfaces, resistance to candidosis may be closely related to local immunity. In order to obtain more basic information concerning *C. albicans* -host interactions in the gastro-intestinal tract, we induced a transient colonization of the intestine in CD-1 mice and measured both secretory and serum antibody responses to a primary challenge with this opportunistic pathogen.

MATERIALS AND METHODS

Candida albicans LAM-1 was isolated and identified by Dr F. Auger (Hôpital St-Sacrement, Qué.). The microorganism was grown in Lee's medium and enumeration of colony forming units was carried out by plating samples on modified EMB agar containing 100µg/ml of chlortetracycline (EMBc)(2) .

CD-1 male mice were obtained from Charles-River Farm (St-Constant, Qué.) and used between 17 and 24 weeks of age.Mice were inoculated with 10^8 blastospores in sterile saline with a miniaturized intubation needle connected to a tuberculine seringe.Lentol (carbachol, MTC Pharmaceuticals, Ontario) was used to stimulate salivation by subcutaneous injections (200µg/mouse) (3). Sera, bile and intestinal contents were then sequentially collected from the mice. Pellets from centrifugation of the saliva as well as dilutions of weighed portions of the intestinal contents were plated on EMBc.

Specific antibodies to *C. albicans* were titrated on EIA plates coated with whole *C. albicans* (10^8 blastospores or hyphal elements per well) at the following dilutions : Serum samples were adjusted to 100, 10 and 1 µg/ml for IgG, IgM and IgA. Bile samples were used at 25, 5 and 1µg/ml IgA and saliva was diluted 1:6, 1:13 and 1:27 for the titration of anti-candidal antibodies. A total of 20 pre-infected mouse were used to assess basal immunoglobulins concentrations as well as specific antibody levels. The same mice were also screened for the presence of *C. albicans* in saliva and feces.

RESULTS

Candida albicans is not part of the normal intestinal flora in the CD-1 mouse. After intra-gastric

inoculation , very few organisms appear to succeed in colonizing the intestinal mucosa. However, the kinetics of a transient colonization is reproducible , showing a peak around day 4 ($7x10^4$CFU/g) followed by a sharp decrease in CFU's . A specific secretory IgA response,which can be detected in the bile, is rapidly induced upon colonization with a maximum titre on day 14. The secretory response measured against blastospores is somewhat delayed as compared to the one measured against the germinated form and remains lower throughout the kinetics. The microorganisms could also be recovered in the oral cavity of about half of the animals but without inducing any detectable specific salivary IgA antibodies .

The kinetics of serum antibodies can show two distinct patterns following intestinal colonization in individual animals : either rapid and high IgA (maximum at day 3) and IgG (maximun at day 4) antibody responses or a slow and weak IgA response (maximum at day 9) with no concommitant detection of specific IgG. When present, IgG antibodies react predominantly with the germinated forms in the early kinetics whereas IgA antibodies react equally well with both forms of the fungus. In no cases was specific IgM detected.

DISCUSSION

In our system, a transient intestinal colonization by *C. albicans* was obtained in normal adult animals without using any compromising agent. A recycling of *C. albicans* via feces brought about a low but quantifiable oral colonization .This colonization follows the intestinal outburst of the microorganism,but induces no detectable stimulation of a specific IgA salivary response. Systemic spread or persorbtion in the blood and lymph nodes was not detected in these experiments.

There appears to be a primary secretory immune response induced upon colonization of the intestinal mucosa starting as early as day 3 against germinated forms and on day 9 against blastospores. Pre-immune titres were very low and comparable to the one obtained with irrelevant IgA . However, a secondary systemic response appears to occur based on the following data : **1.** high IgM anti-candidal titres in the pre-immune sera with no further increase after challenge **2.** strong , rapid and persistent IgG response in some animals showing high pre-immune IgG titres **3.** a similar and concommitant serum IgA response in these animals. As in about half of the animals ,the absence of natural anti-candidal antibodies was associated with a slow and weak serum response (IgA), it is possible that a memory response can be occasionnally induced by related yeasts present in the gastro-intestinal tract (4).

The kinetics of biliary IgA antibodies is much slower than serum IgA antibodies ; moreover, secretory , but not serum antibodies, were predominantly directed against hyphal elements. The stimulation of both systemic and secretory immune systems probably reflects different host-parasite interactions in the context of intestinal colonization by *Candida albicans* .

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Haemophilus influenzae immunity in the respiratory tract

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Introduction

Acute exacerbations of chronic bronchitis have been linked to colonisation by nontypable *Haemophilus influenzae* (NTHI). An orally administered preparation of killed NTHI has been reported to provide protection against these acute episodes of infection [1]. To evaluate the mechanism of protection a rat model was established.

Materials and Methods

6 - 8 week old DA rats were maintained in a specific pathogen free environment. NTHI was grown overnight on chocolate agar, harvested, then formalin killed and prepared to a concentration of $2x10^{10}$ colony forming units (CFU). Rats were immunised by intra Peyer's patch (IPP), intratracheal (IT), oral (PO) and subuctaneous (SC) routes in regimes summarised in Table 1. At the end of each inoculation regime, rats were intratracheally administered a suspension of 10^8-10^9 CFU live NTHI. 4 hours later bronchial washings (BAL) were collected by lavage with phosphate buffered saline (PBS) containing heparin. Recovered bacteria were quantitated by plating serial dilutions on chocolate agar. Samples of serum, saliva, and BAL collected at the time of the clearance assay were assayed for presence of antibody directed at NTHI outer membrane protein. Antibody levels in serum and bronchial washings were standardised according to ELISA reactivity and assayed for *in vitro* bactericidal activity against NTHI in the presence of complement. Specificity of action of the vaccine was assessed by challenge of optimally immunised rats with *K. pneumoniae* and various NTHI biotypes.

Results

Dual GALT/BALT stimulation is necessary for protection (Table 1). Where IPP injection was substituted for 14 days of daily oral presentation of bacteria, clearance rate was accelerated (regime (2)). The action of the vaccine appears specific for NTHI, as the rate of clearance of *Klebsiella pneumoniae* was not enhanced. However, cross protection between different

biotypes of NTHI occured. The duration of protection was at least 40 days but less than 60 days. No correlation between ability to accelerate bacterial clearance, and presence of specific antibody of A, G or M isotypes in serum and secretions was observed. BAL antibody was not bactericidal and blocked the bactericidal effects of serum antibody.

Table 1. Assessment of Immunisation Regimes

Regime	Priming Dose#	Boost#	n	Clearance* (mean ± SEM)	p value
non immunised	PBS	PBS	38	100	
1	NTHI (IPP)	NTHI (IT)	6	4.6 ± 1.1	$p<0.05$
2	NTHI (PO 14d)	NTHI (IT)	6	1.0 ± 0.2	$p<0.05$
3	NTHI (IT)	NTHI (IT)	5	122 ± 14	$p>0.05$
4	NTHI (IP)	PBS (IT)	6	139 ± 21	$p>0.05$
5	NTHI (SC)	NTHI (IT)	5	92 ± 10	$p>0.05$

Priming dose 21 days and boosts 7 and 3 days before clearance assessment

*Clearance index = [number of bacteria harvested from immunised lungs / number of bacteria harvested from non-immunised lungs] x 100

SEM = standard error of mean

Discussion

A quantitative model of clearance of NTHI from the respiratory tract has been established. It was demonstrated: that both GALT and BALT required stimulation by antigen for significantly accelerated pulmonary bacterial clearance; that the effect of oral priming was specific for NTHI strains; and that protection lasted for about six weeks. There was no correlation between enhanced clearance of NTHI and presence of specific antibody in secretions and serum. Despite the limitations of the NTHI/rat clearance system as a model for acute bronchitis in man, several comments can be made. First, the model further consolidates the concept of a common mucosal system being activated through oral immunisation to modulate a colonising process of the bronchus mucosa. Second, it parallels the human disease in that antibody levels in serum and secretions do not appear to correlate with protection [1]. This feature of the response is in part explained by the observation that BAL antibody is not bactericidal for NTHI, and blocks the bactericidal effect of serum antibody. Third, the importance of dual gut-bronchus immunisation is emphasised. It is probable that in the human disease, colonisation itself provides sufficient stimulation of BALT in subjects with damaged airways, as most are colonised with NTHI.

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Endothelial cells, Kupffer cells and peritoneal macrophages activated by gamma interferon kill schistosomula *in vitro*

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INTRODUCTION. For a rational approach to the immunoprophylaxy of schistosomiasis, the immune defence mechanism against schistosomes must be known so that it can be selected and amplified for vaccination. Among the many immune mechanisms that may participate in immunity to schistosomiasis,that involving the cell-mediated immune response (CMI) appears to be the most relevant in the mouse, one of the closest models to humans. There is considerable evidence that activated macrophages are an important component in host resistance to schistosomiasis. Lymphokine (LK)-activated macrophages have been found to be schistosomulicidal in vitro and are probably involved both in resistance to reinfection by schistosomes and in protection following vaccination with irradiated cercariae. Interferon-gamma (IFN-γ) has been characterized as an important macrophage-activating factor, thus we decided to test its involvement in the activation of macrophages to become schistosomulicidal in different strains of mice. The liver has been shown to be an important site of attrition for juvenile schistosomes,thus we tested the schistosomulicidal capacity of LK or IFN-γ-activated Küpffer cells (KC) and endothelial cells (EC).

MATERIALS AND METHODS.

1) Lymphokines. Crude lymphokines (LK) were obtained from mouse spleen cells cultured with Con A (1). Pure mouse IFN-γ was supplied by Genentech Inc.

2) Preparation of Inflammatory Macrophages. Peritoneal cells were harvested from mice injected i.p with 1ml FCS before collection and prepared as previously described (1).

3) Isolation of Murine Küpffer Cells and Endothelial Liver Cells. These cells were isolated from mouse liver by collagenase perfusion, metrizamide density gradient separation and elutriation according to a previously described technique (3).

4) Cytotoxicity assay. Approximately $3x10^5$ adherent cells were

present in each well of 96-well, flat-bottomed plates. The effector to target ratio was 6000:1. Schistosomula killing was assessed after 48 hours by direct visual examination (1).

RESULTS. Inflammatory macrophages from C57 BL/6 mice injected i.p with FCS 24 hr before harvesting and activated in vitro by IFN-γ (50 U/ml) and LPS (1 ng/ml). for 5 hours were shown to kill an average of 80.5% (SE ± 5) of the parasites.
LK-activated macrophages killed an average of 55% (SE ± 1.3) of schistosomules. Similar results were obtained with cells from other strains of mice, namely A/J, P and 129 WEHI. with both LK and IFN-γ. Küpffer cells and endothelial cells from C 57 BL/6, activated by IFN-γ and LPS killed 100% and 89% (SE ± 3) of the schistosomula respectively.

DISCUSSION. In combination with LPS, gamma-IFN was able to activate MØ from different strains of mice (A/J, C 57 BL/6, 129 WEHI). In addition, küpffer cells and endothelial cells were shown to kill schistosomula in vitro when activated under the same conditions. The liver has been described as a major site of attrition of schistosomula during their migration from the skin to the hepatic portal system. The migration is entirely intravascular thus the parasite is in permanent and close contact with the endothelial wall. Our results suggest that, in addition to MØ (KC), activated endothelial cells (EC) may kill schistosomula in vivo and play an important role in resistance to schistosomiasis. FROM A GENERAL POINT OF VIEW, OUR DATA SUGGEST THAT EC ARE ALSO IMPORTANT EFFECTOR CELLS IN THE CELL-MEDIATED IMMUNE RESPONSE.

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SECTION X:
IgA AND HUMAN MUCOSAL DISEASES

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IgA degradation in human intestine

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ABSTRACT. The impairment of secretory IgA in human upper and lower intestine was studied. SDS-PAGE and immunoblot demonstrated that IgA peptide was cleaved, to more extent, in colonic fluid than in jejunal fluid, but IgA in mucous layer was almost intact. The level of specific IgA antibody to outer membrane antigen of *Bacteroides fragilis* was lower in colonic fluid than in jejunal fluid. We suggested that secretory IgA was considerably destroyed in intestinal intraluminal fluid but possibly exercised its power in mucus attaching epithelial surface.

INTRODUCTION

Intestinal mucosal membrane is a site where a host contacts with various antigens of microorganisms and foods, as well as a first line in the host defense mechanisms against pathogens. Secretory IgA plays an important role in immune defense and immune exclusion; inhibition of bacterial adherence to enterocytes, neutralizations of virus and toxin, inhibition of intestinal absorption of macromolecules, etc.. Intestinal lumen, however, is abundant with various proteolytic enzymes derived from bacterial production and intestinal secretion. Bacterial IgA proteases, specifically capable of cleaving human IgA peptide, have been considered as a virulent factor interfering with host mucosal immune system.

Mostly, IgA1 subclass is destroyed by IgA proteases.[1] IgA2 subclass, predominant in intestine, is generally resistant to IgA proteases. In many bacterial strains producing IgA proteases, only 3% of *Clostridium ramosum* degrades IgA2:A2m(1) allotype other than IgA1 subclass.[2,3]

Some *in vitro* studies showed that the activity of some IgA1 proteases was neutralized by secretory IgA and serum IgG and IgA.[4] Our question was to what extent secretory IgA was broken down or kept intact in *in vivo* intestinal lumen.

We studied the structural and functional impairment of secretory IgA in human upper and lower intestinal lumens and a role of the mucous layer on the mucosal membrane in the interruption of IgA protease activity.

MATERIALS and METHODS

Samples. All specimens were obtained from patients (Table 1) without gastrointestinal bleeding. Colonic fluid (CF) was collected from 50

patients passing clear yellow dirrhea a few hours after orally taking 4 litre of polyethylene glycol-electrolyte lavage solution (PEG-ELS) consisting of 236.00 g of polyethylene glycol 4000, 22.74 g of Na_2SO_4, 2.97 g of KCl, 5.86 g of NaCl and 6.74 g of $NaHCO_3$. Jejunal perfusate (JP) was obtained from 8 patients through naso-pharyngo-jejunal tubes by using 0.1 litre of physical saline. Mucous layer on mucosal membrane was taken from a normal part of a resected colon from a patient with sigmoid colon cancer. All of the samples were treated with EDTA, soybean trypsin inhibitor and phenyl-methylsulphonyl fluoride according to the method of Elson et al.[5] immediately after collecting samples and stored at -20°C.

Table 1. CF from 50 patients.

sex(man: 30. woman: 20.)	
age(range 31-82. mean 50.2.)	
colon polyp	19
colon cancer	3
gastric cancer	1
gastric ulcer	1
no disorder	26

JP from 8 patients.

sex(man: 8. woman: 0.)	
age(range 21-63. mean 46.8)	
colon polyp	2
diabetes mellitus	2
colon cancer	2
cirrhosis of the liver	1

Determination of protein and each class-specific immunoglobulin. Protein was determined by Lowry's method. Concentrations of IgA, IgM and IgG were measured by the double-antibody sandwich method of enzyme-linked immunosorbent assay (ELISA).

SDS-PAGE and immunoblotting. Eighty ng of IgA of each sample was electrophoresed in SDS-12% polyacrylamide gel slab under a reducing condition and transferred to a nitrocellulose sheet. The sheet was saturated with 3% bovine serum albumin and incubated with peroxidase-conjugated goat anti-human IgA. After washing the sheet, IgA peptide bands were visualized by 1-chrolo-4-naphtol. The distribution of IgA-peptide molecular weight was analysed on densitometer.

Preparation of outer membrane antigen (OMA) of *Bacteroides fragilis*. Eighteen mg of protein of *B. fragilis* was incubated in 0.02 M $NaHPO_4$ buffer (pH 7.4) with 1.5 mM EDTA and 2% laurylsarcosine and centrifuged at 150,000 g for 1.5 hr. The precipitate was washed and incubated with 0.02 mg DNase, 0.02 mg RNase and 10 mM $MgCl_2$. After wash and centrifugation, the precipitate was collected and stored at -20°C.

IgA antibody specific to OMA of *B. fragilis*. Specific IgA antibody was measured by the direct method of ELISA. Briefly, the ELISA plate was incubated with 500 ng of OMA of *B. fragilis* per each well and saturated with 1 mg/well of human albumin. Each sample was incubated with OMA of *B. ovatum* for absorption of non-specific substances before put on the coated plate. One μg of IgA of the sample was applyed to each well. Washing the plate, using alkaline phosphatase-conjugated anti-human IgA and p-nitrophenylphosphate, and optical density at 405 nm was read. One sample taken from jejunum was used as a control.

RESULTS

The percentages of IgA, IgM and IgG in total protein in JP was shown in Table 2. In CF, the protein determination was not accomplished because the reaction of Folin phenol reagent and

Table 2. Level of IgA, IgM & IgG in JP. mean±SEM(n=8)

	% in total protein
IgA	4.8±1.2
IgM	1.1±0.3
IgG	0.4±0.1

PEG-ELS developed precipitaiton. In Figure 1, the ratios of IgA/IgM, IgA/IgG and IgM IgG were compared between CF and JP. IgA/IgM, IgA/IgG and IgM/IgG were 9.1±0.6(mean± SEM), 40.2±6.9 and 5.3±0.9 in CF, and 5.1±1.0, 15.1±4.3 and 3.4±0.9 in JP, respectively. IgA/IgM and IgA/IgG in CF were significantly higher than those in JP ($p< 0.05$). In Figure 2, the analysis of reducing SDS-PAGE and immunoblotting revealed several IgA peptide fragments other than intact heavy and light chains in CF and JP. IgA in CF was degraded more than that in JP.

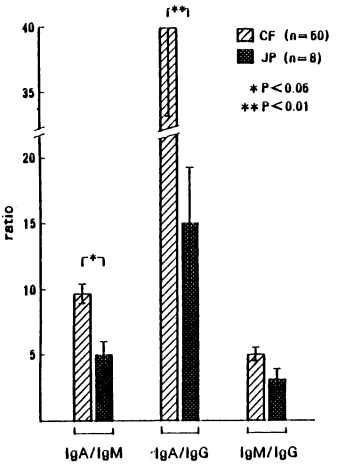

Figure 1. Ratios of IgA, IgM & IgG in CF 7 JP.

In the ELISA method for measuring IgA antibody specific to OMA of *B. fragilis*, the standard curve was almost linear. Figure 3 demonstrated that the level of specific IgA to OMA of *B. fragilis* in one μg of total IgA in CF (0.1±0.01 unit/1 μg IgA) was significantly lower than that in JP (0.42±0.11 unit/1 g IgA) ($p< 0.05$).

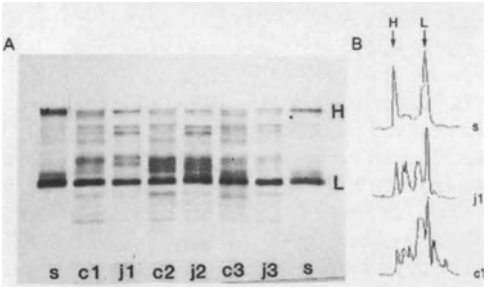

Figure 2. IgA in saliva(s), CF(c1-3) & JP(j1-3) analysed on SDS-PAGE(A) & densitometer(B). H: heavy chain. L: light chain.

The patterns of IgA-peptide bands on SDS-PAGE were compared between in CF and in mucous layer of normal sigmoid colon resected from a patient with colon cancer, as shown in Figure 4. IgA peptide was almost intact in mucous layer although IgA was considerably degraded in CF.

DISCUSSION

IgA, IgM and IgG constituted 4.8%, 1.1% and 0.4% of the protein in jejunal fluid from fasting human adults, respectively. IgA was apparently dominant in intestine although IgG was a major immunoglobulin in serum. The observation of increased ratios of IgA to IgM and IgG in lower intestine might result from IgA resistance to proteolysis. IgG and IgM were generally thought to be more susceptible to proteolytic enzymes than secretory IgA.[6]

Proteolytic enzymes produced by microflora were enriched in human intestine. More than twenty bacteria producing IgA proteases were reported, and sites of cleavage with those enzymes inclined to be in hinge region. The molecular weights of majority of cleaved IgA peptides ranged from 20,000 to 35,000 on reducing SDS-PAGE. The region around the junction of Fab and Fc was likely to be most fragile. The increase in lower levels of molecule of degraded IgA in colon indicated higher activity of proteolysis due to the increasing intestinal flora.

The activity of intestinal proteolysis naturally impaired the func-

tion of secretory IgA antibody. The level of specific IgA antibody to outer membrane antigen from *B. fragilis* was decreased in colon where cleavage of IgA peptide was increased.

Secretory IgA, however, appeared to be protected from proteolytic enzymes in mucous layer surfacing mucosal membrane, as demonstrated on SDS-PAGE. Presumably, secretory IgA antibody properly functioned on mucosa. As the antibody was released from mucus, it was degraded by enzymes. Consequently, it was more appropriate to investigate antibody in mucous layer than in intraluminal fluid for evaluation of intestinal humoral immunity.

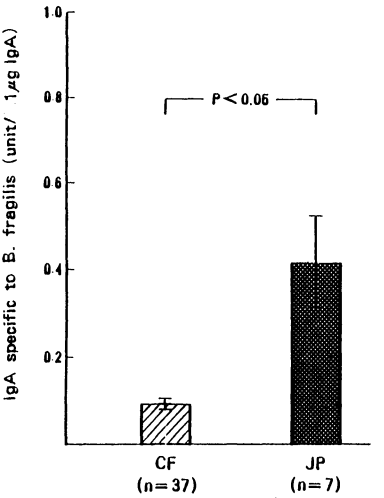

Figure 3. IgA specific to OMA of *B. fragilis* in CF & JP.

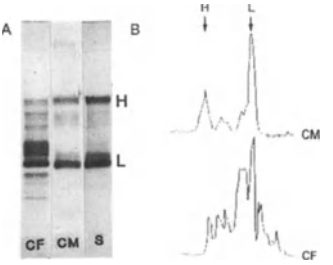

Figure 4. IgA in CF & colonic mucous layer(CM) analysed on SDS-PAGE(A) & densitometer(B). H: heavy chain. L: light chain.

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Inhibition of complement activation by human IgA antibodies and their Fab fragments

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ABSTRACT. The anti-inflammatory properties of human IgA antibodies have been investigated using IgA1 and IgG1 monoclonal antibodies and normal polyclonal IgG and IgA having antibody activity against staphylococcal α-toxin. IgA antibodies inhibited the classical pathway initiated either by polyclonal antibodies, or by monoclonal antibodies acting synergistically, and inhibition was independent of the ability of IgA antibodies to displace IgG. Furthermore, Fabα fragments of IgA1 antibodies, obtained by cleavage with bacterial IgA1 protease, possessed the same ability to inhibit complement activation as intact IgA1 antibodies. The results support the hypothesis that IgA1 proteases facilitate invasive infections by generating functionally defective Fabα fragments that protect the organisms against other immune defense mechanisms.

1. Introduction

Both plasma and secretory IgA are known to be incapable of activating complement by the classical pathway (CCP) [1], and are therefore regarded as non-inflammatory immunoglobulins [2]. The alternative complement pathway (ACP) can be activated by antigen-bound IgA antibodies from rodents [3,4], but not by human IgA antibodies [5-8]. As part of a study of the biological functions of human IgA, we have investigated monoclonal and polyclonal IgA and IgG antibodies physiologically complexed with antigen with respect to their effect on the complement system.

2. Materials and Methods

Human monoclonal anti-staphylococcal α-toxin (SAT) antibodies, IgA1.κ-Fug (monomeric), IgA1.λ-Tin (polymeric), and IgG1.κ-Fug, -JJF, and -Kal, were purified to >99.9% from myeloma sera by chromatography on DEAE-

Sepharose, protein A-Sepharose, and jacalin-agarose [8]. Polyclonal IgA and IgG having relatively high anti-SAT antibody activity were similarly purified from normal human serum. To obtain Fabα fragments, IgA1 preparations were treated with IgA1 protease from *Haemophilus influenzae* overnight at 37°C, and fractionated by HPLC on a TSK-G3000SW column [9]. IgA, IgG, and Fabα were assayed by ELISA [8].

CCP activation was determined by complement-fixation ELISA [10], on plates coated with avidin and biotinylated SAT [8]. Serial two-fold dilutions of IgA or Fabα were incubated in the plates overnight, followed by either polyclonal IgG at 10μg/ml of anti-SAT antibody, or an equal mixture of three monoclonal IgG1 anti-SAT antibodies at 3.3μg/ml each, for 4h. Alternatively, serial dilutions of IgA antibodies (or Fabα fragments) were incubated in 10μg/ml of IgG antibody in the plates overnight. Fresh human serum, diluted 1:25 in PBS pH 7.4 with 0.15mM Ca^{2+} and 0.5mM Mg^{2+}, was then added for 20 min at 37°C, to permit CCP activation. Bound C3b was detected by developing with peroxidase-conjugated anti-C3c (Dakopatts).

3. Results

IgA1-Tin and IgA1-Fug bound to SAT-coated plates inhibited polyclonal IgG antibody-mediated CCP activation, as revealed by the binding of C3b to the solid phase, in relation to the amount of IgA bound (Fig. 1). Polymeric IgA1-Tin was more inhibitory than monomeric IgA1-Fug, and 1-2μg/ml of IgA1 antibody caused ~50% inhibition of CCP activation. Polyclonal IgA anti-SAT antibody also demonstrated dose-dependent inhibition of the CCP, but myeloma IgA1 proteins devoid of anti-SAT activity were not inhibitory [11]. Similar results were obtained when the CCP was activated by an equimolar mixture of three monoclonal IgG1 antibodies acting synergistically [8]. There was no evidence in these experiments for the displacement of IgG antibodies from the SAT-coated plates. However, a competition ELISA showed that IgA1-Fug could displace IgG1-Fug (and *vice versa*), but not the other monoclonal IgG1 antibodies, and that IgA1-Tin could not compete against any of the IgG1 antibodies [11]. Thus inhibition of CCP activation did not require competitive displacement of IgG by IgA antibodies.

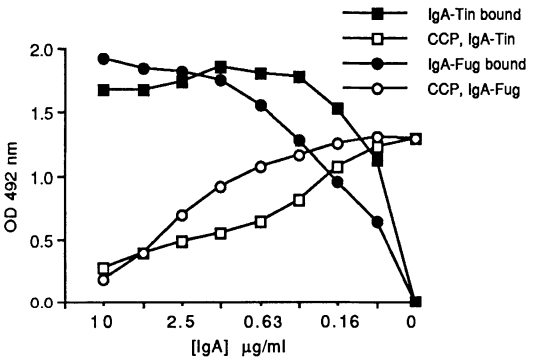

Fig. 1. Inhibition of CCP activation by IgA antibodies

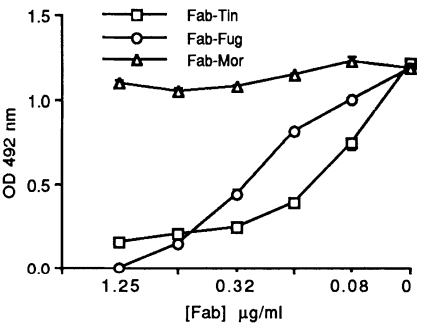

Fig. 2. Inhibition of CCP activation by Fab fragments of IgA

When Fabα fragments were tested in complement-fixation ELISA, CCP activation mediated by a mixture of three monoclonal IgG1 antibodies was inhibited to an extent similar to that shown by the intact parent IgA antibodies (Fig. 2). The Fabα fragment of an IgA1 myeloma protein (Mor) that does not recognize SAT was not inhibitory. Thus an intact Fcα region was not necessary for the inhibition of the CCP, and furthermore, the polymeric form of IgA1-Tin was not responsible for its inhibitory activity, since Fabα fragments are univalent. As might be expected, Fabγ fragments of IgG antibodies also inhibited CCP activation, but less effectively than IgA or Fabα fragments [11].

4. Discussion

Because it does not readily engage either of the two major effector mechanisms, complement and phagocytosis, whereby IgG and IgM antibodies exert their powerful anti-microbial effects, the the biological functions of plasma IgA have remained obscur. Although rat and mouse monoclonal IgA antibodies have been shown to activate the ACP [3,4], ACP activation by human IgA has only been demonstrated by IgA that has been aggregated, chemically modified, or otherwise denatured [8,12,13], but not by IgA antibodies bound to antigen [1,5-8]. We have confirmed this finding using unique IgA myeloma proteins that recognize a defined antigen, and have further shown that these human monoclonal IgA antibodies strongly inhibit the CCP mediated by IgG antibodies.

The mechanism of this inhibition is not entirely clear. The effect was shown by both polymeric and monomeric IgA, regardless of their ability to compete against IgG for binding to antigen. Since activation of the CCP by IgG antibodies requires a close proximity of adjacent Fcγ regions [14], we postulated that the insertion of IgA into the matrix of IgG antibodies would prevent the binding of C1q. However, we could demonstrate inhibition of C1q binding only by monoclonal IgA-Fug and only when C1q binding was assayed using a synergistic mixture of three monoclonal IgG's including IgG-Fug [11]. Thus another mechanism must account for the inhibition of the CCP by IgA-Tin.

There is a special significance to the findings with Fabα fragments, since these are produced by the action of uniquely specific bacterial IgA1 proteases on human IgA1 [15]. The production of IgA1 proteases has been associated with virulence in several important human mucosal pathogens, including the three principal causes of bacterial meningitis, *H. influenzae*, *Neissseria meningitidis*, and *Streptococcus pneumoniae* [16], and a novel hypothesis has been proposed to account for several features of these invasive infections [2,17]. It is postulated that in individuals who display pre-existing IgA antibodies to surface antigens of these bacteria, the action of IgA1 proteases results in the bacteria becoming coated with Fabα antibody fragments. These are defective in mucosal protection [2,9], but at the same time prevent the binding of other intact antibodies that would mediate protective immunity. Our present results indicate that Fabα fragments may indeed be able to inhibit IgG-mediated complement activation, which is thought to be important for systemic defense against these bacteria [18-20].

In a broader context, IgA antibodies have been found to inhibit several other immune effector mechanisms, including bacteriolysis [21], immediate

hypersensitivity and Arthus reactions [22], various forms of cell-mediated cytotoxicity [23-25], and chemotaxis or phagocytosis by neutrophils [26,27]. These all lead to the concept not only that IgA is *non*-inflammatory, but also that it can be *anti*-inflammatory. As such, it may have an important role in protecting the internal environment from the inflammatory consequences of other immune defense mechanisms [28].

5. Acknowledgements

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Characterisation of the structure of IgA molecules coating oral plaque bacteria

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1. INTRODUCTION

Oral bacteria collected in vivo are known to be coated with antibodies of the S-IgA class. The S-IgA1 subclass, constituting appr. 60% of S-IgA in saliva, is susceptible to IgA1 proteases which are produced by a number of oral bacterial species including the principal initiators of dental plaque formation Streptococcus sanguis and S.oralis. Cleavage of S-IgA1 by IgA1 proteases into FC_nSC and antigen-binding Fab_α fragments has been demonstrated to interfere with the adherence inhibiting capacity of S-IgA antibody. We have developed a method to examine the structure of IgA molecules present on bacterial surfaces. Using this method, we have examined the IgA molecules coating:

1.-IgA1 protease-producing and non-producing streptococci incubated in saliva,

2.-supragingival plaque bacteria from in vivo samples.

2. MATERIALS AND METHODS

S.sanguis ATCC 10556 (IgA1 protease-producer) and S.gordonii ATCC 10558 (non-IgA1 protease-producer) were grown for 24 hrs in trypticase peptone medium. The pelleted bacteria were resuspended in freshly collected submandibular/sublingual saliva ($5x10^8$/ml) and incubated at 33°C. During the incubation, samples were withdrawn and added to EDTA (50 mM final concentration), in order to arrest IgA1 protease activity, followed by two washes in EDTA containing buffer.

Incipient dental plaque was collected from a single individual showing no signs of gingivitis. After gentle sonication, sampled bacteria, mostly streptococci, appeared as single cells or short chains. They were washed in buffer containing 0.15% Tween 20.

IgA molecules coating saliva-incubated or plaque bacteria were analyzed by non-reducing SDS-PAGE and immunoblotting. Proteins solubilized by boiling the bacteria in sample buffer were separated by gradient PAGE (4-20%), electroblotted onto a strongly protein-binding Immobilon^R (polyvinylidene difluorid) membrane, and stained with affinity purified anti-alpha chain, anti-light chain, or monoclonal anti-CH1 antibodies. Isolated Fab_α fragments, produced by incubation with S.sanguis IgA1 protease, and purified colostral S-IgA, were used as references.

3. RESULTS

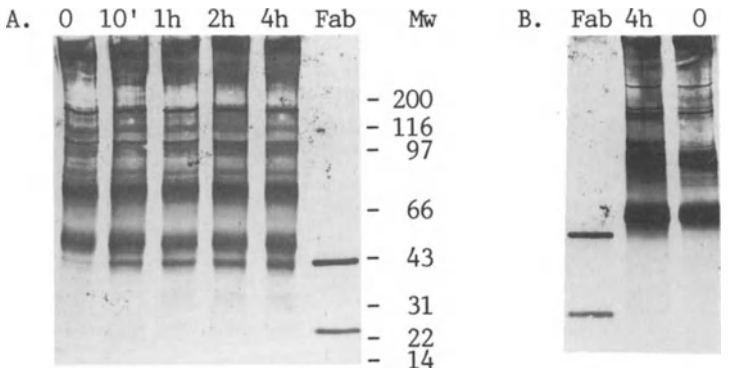

FIGURE 1. Anti-α stained immunoblots of IgA molecules eluted from saliva-incubated S.sanguis (A) and S.gordonii (B), at the indicated time points.

SDS-PAGE/immunoblotting analysis of S.sanguis or S.gordonii samples showed a complex band pattern. However, the proteins eluted from S.sanguis, but not from S.gordonii, also gave rise to a band that could be identified as Fab_{α} on grounds of molecular size and immunochemical staining.

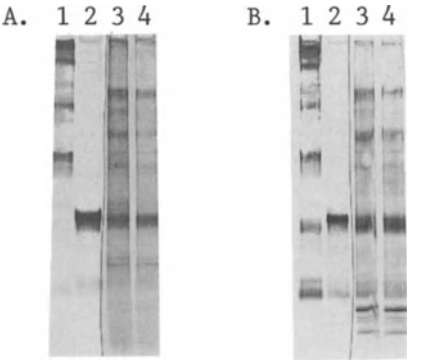

FIGURE 2. IgA molecules eluted from dental plaque bacteria. Immunoblots stained with anti-α chain (A) and anti-light chain (B) antibodies.
1: S-IgA reference; 2: Fab_{α} reference
3: Plaque bacteria 4: Plaque bacteria incubated with IgA1 protease.

Staining of the proteins eluted from plaque bacteria, disclosed a band with Fab_{α} characteristics. Incubation of plaque bacteria from the same sampling, with S.sanguis IgA1 protease did not significantly alter the band pattern or staining intensity of the bands indicating that the effect of IgA1 proteases had reached its maximum in vivo.

4. CONCLUSION

1. By a combination of non-reducing SDS-PAGE and immunoblotting it is possible to identify minute amounts of Fab_{α} eluted from the surface of $5x10^8$ bacteria grown in S-IgA containing media.
2. After incubation in pure saliva, IgA1 protease-producing as opposed to non-producing oral streptococci carry surface-bound Fab.
3. Bacteria in incipient dental plaque are coated with Fab_{α}.
4. The results of this study corroborates the hypothesis that IgA1 protease activity, by generating Fab_{α}-coated bacteria, may promote the formation of dental plaque.

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Molecular form and subclass of serum IgA in patients with IgA nephropathy

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1. Introduction

IgA nephropathy (IgAN) is considered to be the most common primary glomerulonephritis throughout the world. Elucidation of the origin of the IgA deposited in the glomerular mesangia may help determine whether IgAN is a disease of the secretory or the systemic IgA system. It is now generally accepted that the IgA found deposited in the glomeruli is almost exclusively of the IgA1 subclass [1], as is the IgA within circulating immune complexes in the sera of some IgAN patients [2]. However, the data in regard to the molecular form of IgA are not so absolute. The objective of this research was therefore to determine which form of IgA is elevated in the serum of IgAN patients, and to examine the relationship of molecular form with the state of the disease.

2. Materials and Methods

Sample Populations: Each sample population consisted of ten white males. Group N contained IgAN patients with normal kidney function (serum creatinine < 1.7 mg/dl), group A contained IgAN patients with abnormal kidney function (serum creatinine > 1.7 mg/dl) and group C contained healthy laboratory volunteers.

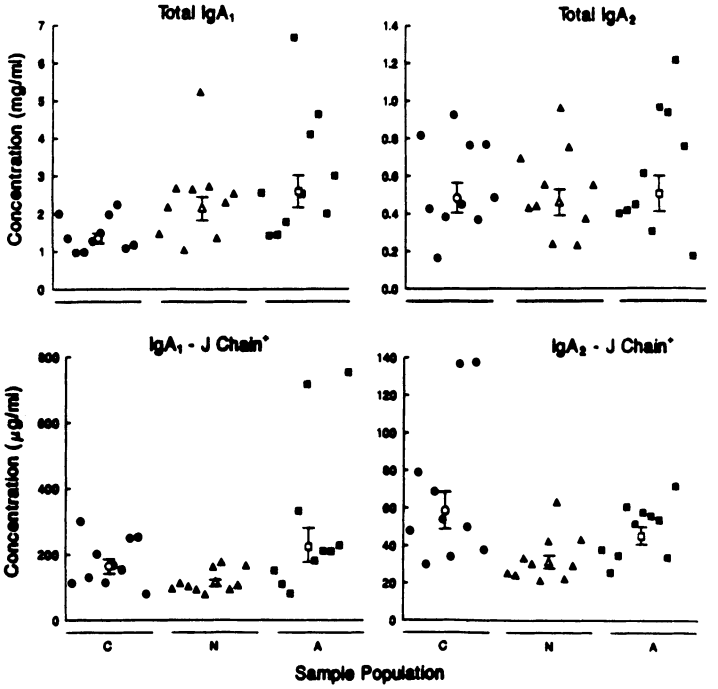
ELISA: Human polymeric IgA1 and IgA2 myeloma proteins, prepared as previously described [3], were used as the primary standards. Plates were coated with either the anti-IgA1 or anti-IgA2 monoclonal preparations (a gift from Dr. J. Radl). The plates were blocked with 5% fetal calf serum, washed, and dilutions of the samples and the standard were added in PBST containing 2% fetal calf serum. The preparations were incubated overnight at 4°C and the plates washed. For measuring total IgA1 or IgA2, the assay was completed by sequential addition of biotinylated goat anti-IgA, Streptavidin-phosphatase, and the substrate (Sigma 104) with a wash procedure between each step. For measuring J chain-containing IgA, the bound IgA was sequentially fixed with 0.2% glutaraldehyde, denatured with acid urea (5 M, pH 2.5), blocked, and then the assay completed with biotinylated anti-J chain (a gift from Dr. Mestecky), conjugate, and substrate.

Calculations: The sample concentration values were determined by the reference standard method using the Logit-Log option of the ELISANALYSIS software package [4]. Each sample was measured in at least four independent assays using independent dilution sequences. Statistical significance was determined using an ANOVA based on a logarithmic transformation of the mean values from the independent assays.

3. Results

Both patient groups had significantly more IgA1 than the control group ($p < 0.05$); the two patient groups had similar levels. The IgA2 levels were similar in all three groups. Both patient groups had similar levels of J chain-containing IgA1 in comparison to the control group; however, patient group A had

higher levels ($p < 0.05$) than did patient group N. Patient group A and the control group had similar levels of J chain-containing IgA2. However the levels in patient group N were significantly lower than those of patient group A ($p < 0.05$) and slightly lower than those of the control group ($p > 0.05 < 0.06$).



Open symbols represent the geometric means +/- the geometric standard errors.

4. Conclusions

The data confirm that the elevation in serum IgA found in IgAN is restricted to IgA1, and appears to be exclusively monomeric IgA1 at early stages of the disease; polymeric IgA1 may become elevated at later stages. The data further suggest that IgAN is a disease of the systemic IgA compartment. Further work is necessary to determine whether IgAN results from a defect in IgA1 catabolism or is the result of excessive IgA1 production due to a defect in regulation.

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B and T cell abnormalities in a large population of patients with IgA nephropathy

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During the past years several studies have been done on IgA immune regulation in patients with IgA nephropathy giving conflicting results (review in 1).In this study we report the in vitro production of IgA, IgG and IgM by peripheral mononuclear cells from a large number of patients with IgA nephropathy.Furthermore,we attempted to analyze whether the abnormalities in the Igs synthesis might be due to changes in the activity of B and/or T cells.

Material and Methods

Sixty six patients with histologically proven IgA nephropathy and 56 appropriate controls were studied.Peripheral blood mononuclear (PBM) cell suspensions were obtained by Ficoll-Hypaque gradient.B and T cells were obtained by rosetting with neuraminidase treated sheep erythrocytes.PBM($2x10^6$/ml)and B cells($1x10^6$)were maintained in culture for 7 days in the absence or in the presence of mitogens,PWM and Epstein-Barr virus(EBV)obtained from the supernate of the B 95-8 marmoset lymphoblastic line.Lymphocytes from patients and normals were cocultured in the presence of PWM(2).Concentration of Igs in the supernatant of cell cultures were measured by ELISA(2).

Results

Spontaneous production of IgA(345±40),IgG(616±59)e IgM(981±119)by PBM cells from patients was significantly greater than from controls(208±18;$p<0.005$;464±46;$p<0.05$;598±41;$p<0.01$,mean±SEM,respectively.These significant differences were maintained after the stimulation by PWM(not shown).Patients considered to be high producers of IgA,also simultaneously secreted more IgG(672±118)and IgM(1504±383)than patients producing IgA at normal rates(381±35;$p<0.005$;731±93;$p<0.01$ respectively).By contrast,isolated B cells from patients demonstrated a strikingly higher capacity to produce IgA compared with controls,while no difference was seen in the synthesis of IgG or IgM (Table 1).
T cells from patients were significantly more efficient in the synthesis of IgA(1359±104),IgG(1535±156) and IgM(2309±271) than those from

controls(675±82;1008±90;1433±115,respectively,p<0.01).

TABLE 1. Immunoglobulin synthesis by isolated B cells from controls and patients with IgA nephropathy.

	Spontaneous			EBV (100 ul/ml)		
	IgA	IgG	IgM	IgA	IgG	IgM
Controls(n=4)	168±16*	555±113	401±52	2558±202	20535±3607	3554±825
Patients(n=13)	566±153	583±135	950±476	4658±937	24257±741	3989±793
	p<0.0125	NS	NS	p<0.05	NS	NS

Cultures were maintained for 7 days;*mean±SEM; Student's t test

Discussion

The existence of IgA immune regulation abnormalities in patients with IgA nephropathy is controversial.Some reports described increased spontaneous or mitogen-induced synthesis,but normal rates of production have also been observed(review in 1).Differences in culture conditions,disease activity and the heterogeneity of the disease itself might account for these discrepancies,particularly since the number of patients in each series is relatively small.

In this paper we have observed that PBM from these patients,when cultured for 7 days,produced,as a group,more IgA,IgG and IgM than those of controls.By contrast,when isolated B cells were cultured,both unstimulated and stimulated with EB virus,mitogen that do not require T cells monocytes,only a significant increase in IgA was noted.Since altered T cell function might also explain the IgA abnormalities observed in these patients,we studied some functional parameters of these cells.As a group, T cells from patients were significantly more efficient than those from controls in the synthesis of IgA,IgG and IgM.These results suggest that a B cell overactivity specific for IgA might be a primary phenomenon.The further clonal expansion of T cells,with the subsequent increase in the synthesis of other Igs,could be of pathogenetic relevance since the presence of IgG-immune complexes in the circulation and/or in the mesangium is being considered important in the production of mesangial damage(3).

Acknowledgments:Supported by grants from CICYT,FISs and F.I.Alvarez Toledo

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The secretory IgA (sIgA) response in the mucosal urinary tract of malnourished children

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The effect of protein-energy malnutrition on the sIgA response has been recently studied in duodenal fluid, saliva, nasal secretions and tears of malnourished children[3,6,7,9] as well as in intestinal washes of mice[5]. Some, investigators have reported hight urinary sIgA levels in children with urinary tract infection[2,8].

The interaction between infection and nutrition is well established. Furthermore, urinary tract infection (UTI) must be one occult infection occurring in malnourished children.

The purposes of this work were to study the effect of the nutritional status on the urinary sIgA and the local immune response of malnourished children to the UTI.

We employed a Dip-Slide method to diagnostic the UTI, in 181 children (77 girls and 104 boys) that frequent day nurseries in the same community and have similar social economic conditions.

Four groups of children were considered, according to the nutritional status, as assessed by using the weight and the height percentiles in relation to the age[4]: Eutrophics (E), Severe Malnutrition (SM), Moderate Malnutrition (MM) and Normal Weight but Under Height (NW).

A midstream urine was obtained to detect the bacteriuria (Urotube, Roche) and the urinary abnormalities (Dipstick COMBUR 9, Boehringer). The rest was used for determination of sIgA, by ELISA, and creatinine.

Thirty nine children had UTI (table 01). The remaining 142 (control group) had no bacteriuria or any other clinical or urinary abnormalities.

The sIgA concentration (mg/l) and excretion rate (mg sIgA/g creatinine) of the SM and MM children were lower than the E and NW from the same age (Figures 1 and 2). Therefore only the excretion rate was significantly reduced (Mann-Withney "U" Test).

The sIgA concentration and excretion rate were significantly increased in the SM and MM children suffering of UTI, as compared to control group with the same nutritional status (Figures 3 and 4, Table 02). The total group with UTI (39 children) shows a significantly increased of sIgA's concentration and excretion rate.

Table 1 - Urinary findings and bacteriuria (detected by Dip-Slide Method) in 39 children (27,5% of the children that frequent day nurseries) with different nutritional status.

Age (years) NS	n	Sex boys	girls	Urinary Findings	Bacteriuria	n
0⊣ 1						
SM	3	1	2	LHA**(1); LA(1); A(1)	E. coli	3
MM	2	1	1	LHA(1)	E. coli	2
NW	2	2	-	-	E. coli	1
					Klebsiella	1
E	1	1	-	LA(1)	E. coli	1
1⊣ 2						
SM	3	2	1	LA(1); L(1)	E. coli	2
					Klebsiella	1
NW	5	3	2	LHA(2); LH(1)	E. coli	2
					Proteus mirabilis	3
E	2	-	2	-	Klebsiella	2
2⊣ 3						
SM	2	1	1	-	Proteus mirabilis	2
MM	7	3	4	LA(1); H(1)	E. coli	6
					Proteus mirabilis	1
NW	4	4	-	L(3)	E. coli	3
					Proteus mirabilis	1
E	1	1	-	-	Klebsiella	1
3⊣ 4						
MM	1	1	-	-	Proteus mirabilis	1
E	2	1	1	L(1)	E. coli	1
					Proteus mirabilis	1
4⊣ 5						
MM	3	-	3	LH(2)	E. coli	3
NW	1	-	1	-	Proteus morgani	1
0⊣ 5	39	21	18	L(16); H(8); A(9)	E. coli	24
					Klebsiella	5
					Proteus mirabilis	9
					Proteus morgani	1

( ) = cases number. NS = Nutritional Status; SM = Severe Malnutrition; MM = Moderate Malnutrition; NW = Normal Weight; E = Eutrophics.
**L = Leucocyturia; H = Hematuria; A = Albuminuria.

Table 2 - The urinary secretory IgA's concentration (mg/l) and excretion rate (mg sIgA/g Creatinine) in 39 children (age 3 months to 5 years) with urinary tract infection (UTI) compared to control groups of the same nutritional status.

NS	UTI GROUP			CONTROL GROUP		
	n	sIgA median (range)	Excretion Rate median (range)	n	sIgA median (range)	Excretion Rate median (range)
SM	08	1.07** (0.47-2.30)	3.98** (1.13-9.87)	15	0.31 (0.07-1.50)	0.97* (0.16-2.68)
MM	13	1.30** (0.33-9.70)	2.50** (1.44-6.87)	47	0.32 (0.03-1.90)	1.25* (0.15-2.64)
NW	12	1.10 (0.32-2.30)	2.83** (1.25-8.13)	40	0.70 (0.09-1.90)	1.36 (0.32-2.89)
E	06	1.10 (0.59-3.30)	2.34** (2.11-9.17)	40	0.66 (0.15-1.46)	1.50 (0.36-3.91)
TOTAL	39	1.20** (0.32-3.30)	2.87** (1.13-9.87)	142	0.67 (0.03-1.90)	1.44 (0.15-3.91)

Significantly different: * from the E and NW groups; ** from the controls groups.

NS = Nutritional Status; SM = Severe Malnutrition; MM = Moderate Malnutrition; NW = Normal Weight; E = Eutrophics.

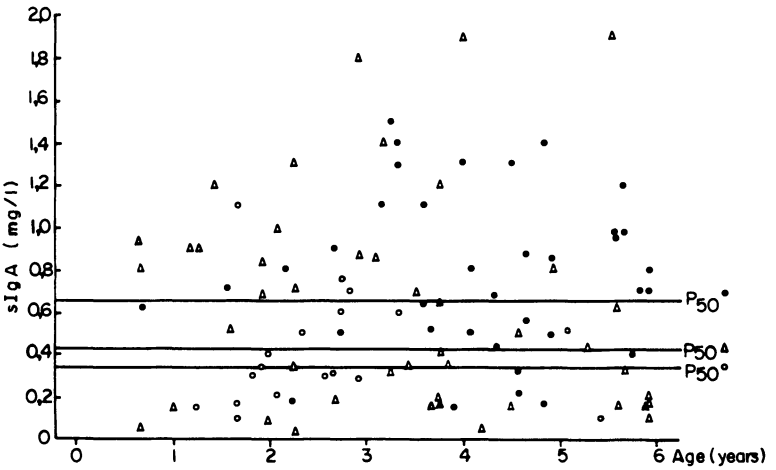

FIGURE 1 - URINARY SECRETORY Ig A (sIgA) IN 102 CHILDREN, ACCORDING TO THE NUTRITIONAL STATUS: 15 WITH SEVERE MALNUTRITION (σ), 47 WITH MODERATE MALNUTRITION (▲) AND 40 EUTROPHICS (•), THE HORIZONTAL LINES SHOW THE 50th PERCENTILE OF EACH NUTRITIONAL STATUS.

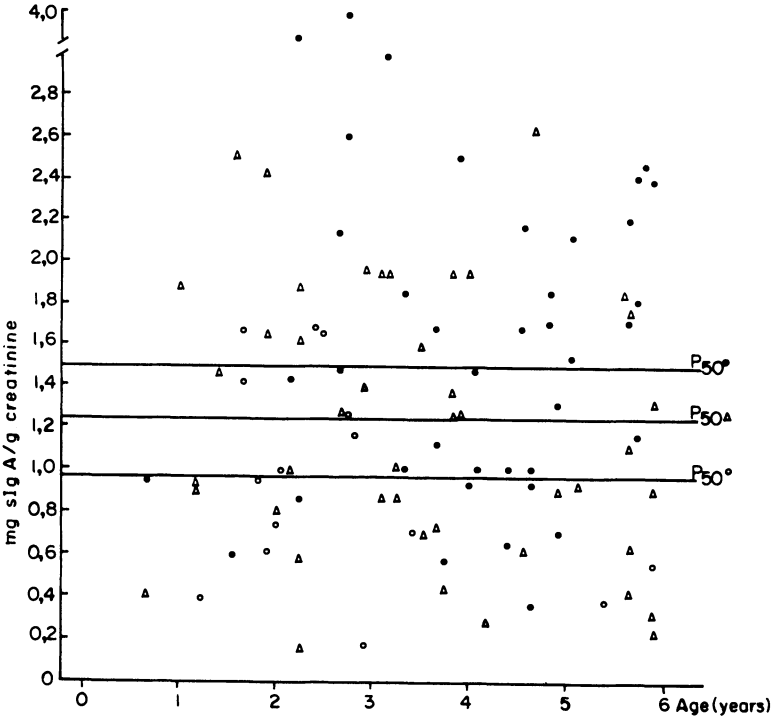

FIGURE 2 - EXCRETION RATE OF URINARY SECRETORY Ig A (sIgA) IN 102 CHILDREN ACCORDING TO THE NUTRITIONAL STATUS: 15 WITH SEVERE MALNUTRITION (•), 47 WITH MODERATE MALNUTRITION (▲) AND 40 EUTROPHICS (•). THE HORIZONTAL LINES SHOW THE 50th PERCENTILE OF EACH NUTRITIONAL STATE.

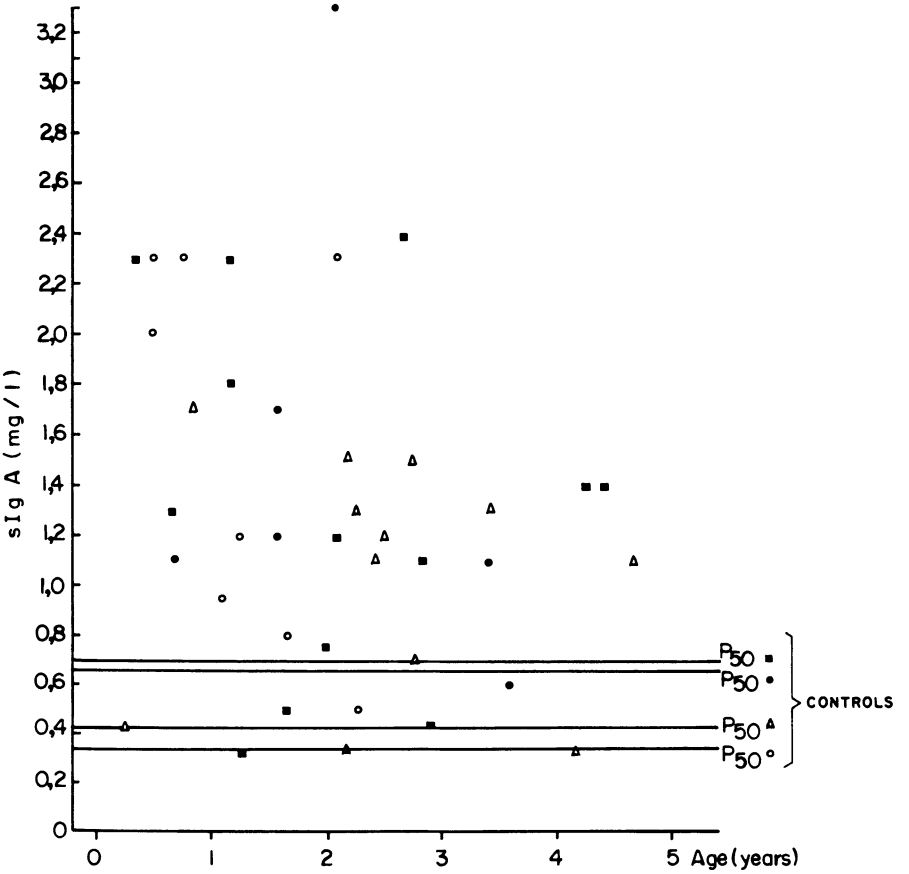

FIGURE 3 - CONCENTRATION OF URINARY SECRETORY Ig A (sIgA) IN 39 CHILDREN WITH URINARY TRACT INFECTION, DISTRIBUTED ACCORDING TO THE NUTRITIONAL STATUS: 8 WITH SEVERE MALNUTRITION (○), 13 WITH MODERATE MALNUTRITION (△), 12 WITH NORMAL WEIGHT (■) AND 6 EUTROPHICS (●). THE HORIZONTAL LINES SHOW THE 50th PERCENTILE FROM EACH NUTRITIONAL STATUS.

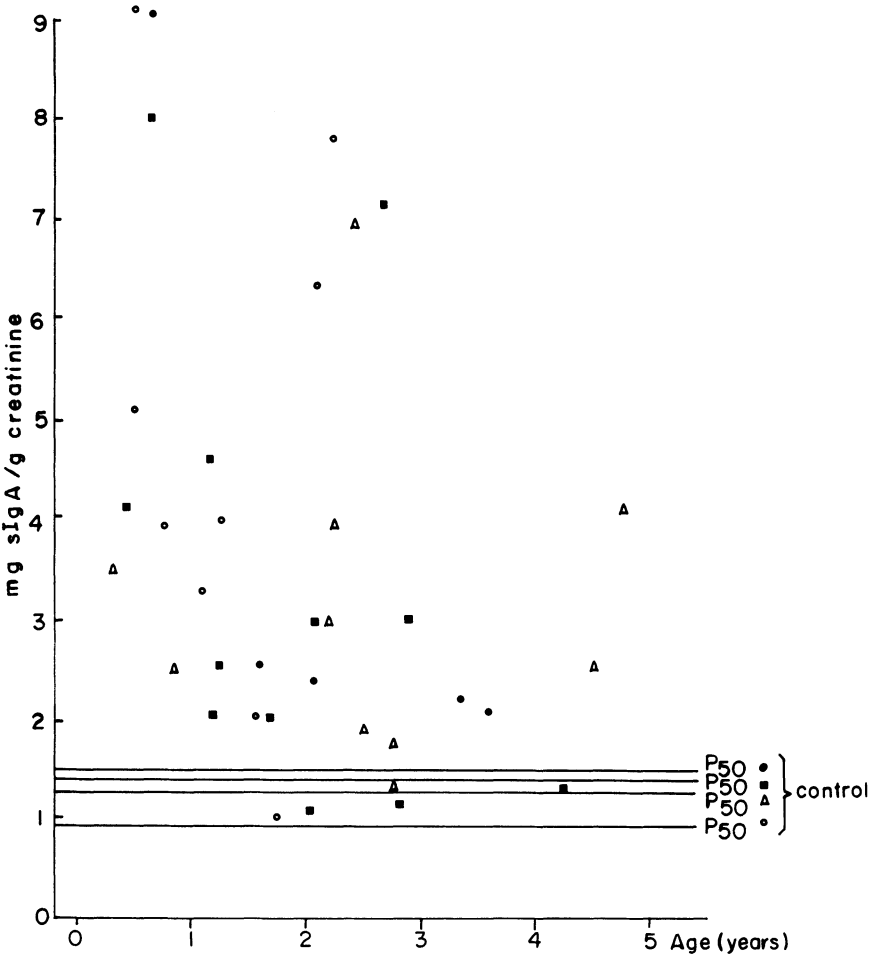

FIGURE 4 - EXCRETION RATE OF URINARY SECRETORY IgA (sIgA) IN 39 CHILDREN WITH URINARY TRACT INFECTION DISTRIBUTED ACCORDING TO THE NUTRITIONAL STATUS: 8 WITH SEVERE MALNUTRITION (°), 13 WITH MODERATE MALNUTRITION (△), 12 WITH NORMAL WEIGHT (▪) AND 6 EUTROPHICS (•). THE HORIZONTAL LINES SHOW THE 50th PERCENTILE OF THE CONTROL GROUP FROM EACH NUTRITIONAL STATUS.

The results about the local immune response in malnourished children are controverts. Bell et al.[1] detected elevated levels of intestinal immunoglobulins (IgG, sIgA and IgM) in malnourished children with enteric infections. More recently, Green and Heyworth [3] reported a significant decrease in IgA-containing cells in jejunal mucosa of the children with protein energy malnutrition, and gastroenteritis. By other hand, the sIgA response in the mucosal urinary tract is not well known.

Our results suggest that malnourished children have sIgA deficiency in the mucosal urinary tract. However, in the presence of UTI they are able to develop mucosal immune response with a significant increase of sIgA.

ACKNOWLEDGEMENTS

We thank the "LABORATÓRIO DE FISIOLOGIA DA NUTRIÇÃO" of the FEDERAL UNIVERSITY OF PERNAMBUCO, the "CRUZADA DE AÇÃO SOCIAL" of the Pernambuco's Government, and the ROCHE DIAGNOSTICA.

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The urinary secretory IgA (sIgA) concentration (mg/l) and excretion rate (mg sIgA/g creatinine) in children with recurrent urinary tract infection (rUTI)

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Urinary sIgA concentration (mg/l) was measured using ELISA, in 96 healthy children and 38 children with history of rUTI, from 2 to 10 years old.

Twenty one had no bacteriuria and 17 had positive urine cultures, at the time of study. A midstream specimen was obtained from each child for immediate determination of sIgA, and urinalysis. The creatinine was measured to determine the excretion rate (mg sIgA/g creatinine). In addition, urine culture was performed in those with history of rUTI. There were no significant differences of the sIgA concentration and excretion rate between healthy and rUTI children that had no bacteriuria.

The sIgA concentration and excretion rate were significantly higher in children with bacteriuria, as compared to healthy, as well as to children without bacteriuria (table 01).

The present study shows a significant elevation of urinary sIgA in children suffering rUTI with bacteriuria. The sIgA levels remain into the normal range in the intervals between the episodes of bacteriuria.

Some investigators refer a sIgA deficiency in children with rUTI[1]. Using a similar methodology to the present work Fliedner et al[1] (1986) encountered a significant redution of the excretion rate in children without bacteriuria that had history of rUTI but normal pyelogram and cystourethrogram. In our study 39% of the children had no radiologic abnormalities (tables 02 and 03). However the values of sIgA's concentration and excretion rate were similar to the normal children[4] (Figures 1 and 2). Furthermore, we didn't find significant difference between children with anatomic derangements and those without anomalies, what isn't according to Uehling et al[5]. Our results are similar to those of Riedasch and Ritz[2,3] that refer normal urinary sIgA in women with rUTI.

The present study do not supports the hypothesis of a sIgA deficiency as a factor of susceptibility for recurrence of the urinary tract infection.

Table 01 - The urinary secretory IgA concentration (mg/l) and excretion rate (mg sIgA/g creatinine) of children with recurrent urinary tract infection (rUTI) compared to normal children.

GROUP	n	sIgA median range	Excretion rate median range
rUTI with bacteriuria	17	4.71* 2.10-9.20	6.55* 2.50-23.82
rUTI without bacteriuria	21	1.10 0.09-3.20	1.64 0.21-5.71
Normal	96	0.83 0.21-2.50	0.89 0.14-2.80

* = Significant (Mann Whitney "U" test)

Table 02 - The urinary secretory IgA (sIgA) concentration (mg/l) and excretion rate (mg sIgA/g creatinine) of 17 children suffering recurrent urinary tract infection with bacteriuria.

Sex	Age	Urinary culture	Radiologic findings	sIgA	Excretion
boy	2y1m	E. coli	vesicoureteric reflux	2.1	11.7
girl	2y6m	E. coli	vesicoureteric reflux	3.3	3.2
girl	3y4m	E. coli	vesicoureteric reflux left renal agenesia	8.1	23.8
girl	3y9m	Proteus mirabilis	-	7.5	7.6
girl	4y2m	E. coli	left renal atrophy	3.8	8.6
girl	4y8m	E. coli	-	5.0	10.0
boy	4y8m	E. coli	lithiasis	5.2	4.4
girl	4y9m	E. coli	-	4.4	6.1
girl	5y1m	E. coli	-	5.5	6.5
girl	7y	Proteus morgani	vesicoureteric reflux	3.2	2.5
boy	7y7m	Enterococus Streptococus	vesicoureteric reflux	2.6	2.5
boy	8y2m	E. coli	vesicoureteric reflux	9.2	18.4
girl	8y7m	Klebsiella	-	3.6	6.0
girl	9y2m	E. coli	-	3.1	5.2
girl	9y3m	E. coli	ureteric duplication	6.9	6.8
boy	9y3m	Proteus mirabilis Klebsiella	-	2.7	9.6
boy	9y10m	E. coli	lithiasis	6.4	2.6

Table 03 - Urinary secretory IgA (sIgA) concentration (mg/l) and excretion rate (mg sIgA/g creatinine) of 21 children suffering recurrent urinary tract infection without bacteriuria.

Sex	Age	Radiologic findings	sIgA	Excretion
girl	1y	-	2.70	3.30
girl	1y7m	-	0.72	3.27
girl	2y1m	vesicoureteric reflux ureteric duplication	2.30	4.26
girl	3y1m	vesicoureteric reflux	1.60	5.71
girl	3y2m	vesicoureteric reflux	0.23	1.64
boy	3y5m	vesicoureteric reflux	0.80	1.33
girl	3y8m	renal lithiasis	2.10	1.25
girl	3y9m	vesicoureteric reflux	0.97	1.56
girl	3y11m	vesicoureteric reflux	1.90	3.39
girl	4y1m	-	1.20	0.94
girl	4y4m	-	0.09	0.45
girl	5y1m	vesicoureteric reflux	0.76	1.65
girl	5y10m	vesicoureteric reflux renal hypoplasia and ectopia	0.83	2.08
boy	5y11m	-	1.10	1.17
girl	6y	vesicoureteric reflux	1.00	0.91
boy	6y10m	-	1.90	2.71
girl	7y10m	renal ectopia	0.19	0.68
girl	8y1m	-	3.60	1.66
girl	8y11m	vesicoureteric reflux	0.34	2.61
boy	10y4m	-	0.62	0.21
girl	12y	-	2.20	1.10

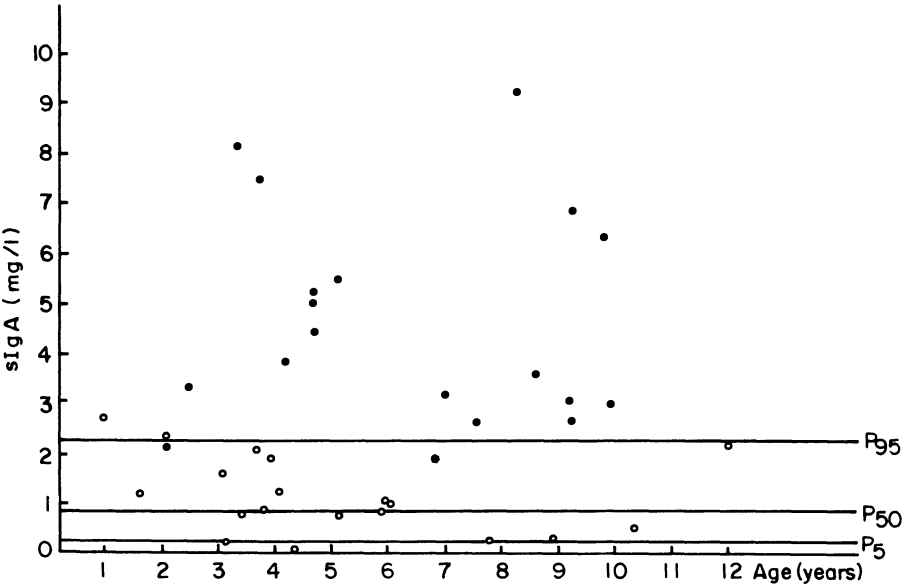

FIGURE 1 - THE CONCENTRATION OF THE URINARY SECRETORY Ig A (sIgA) IN 38 CHILDREN (1 TO 12 YEARS OLD) SUFFERING OF RECURRENT URINARY TRACT INFECTION, WITH BACTERIURIA (•) AND WITHOUT BACTERIURIA (∘). THE HORIZONTAL LINES SHOW THE PERCENTILES OF NORMAL CHILDREN.

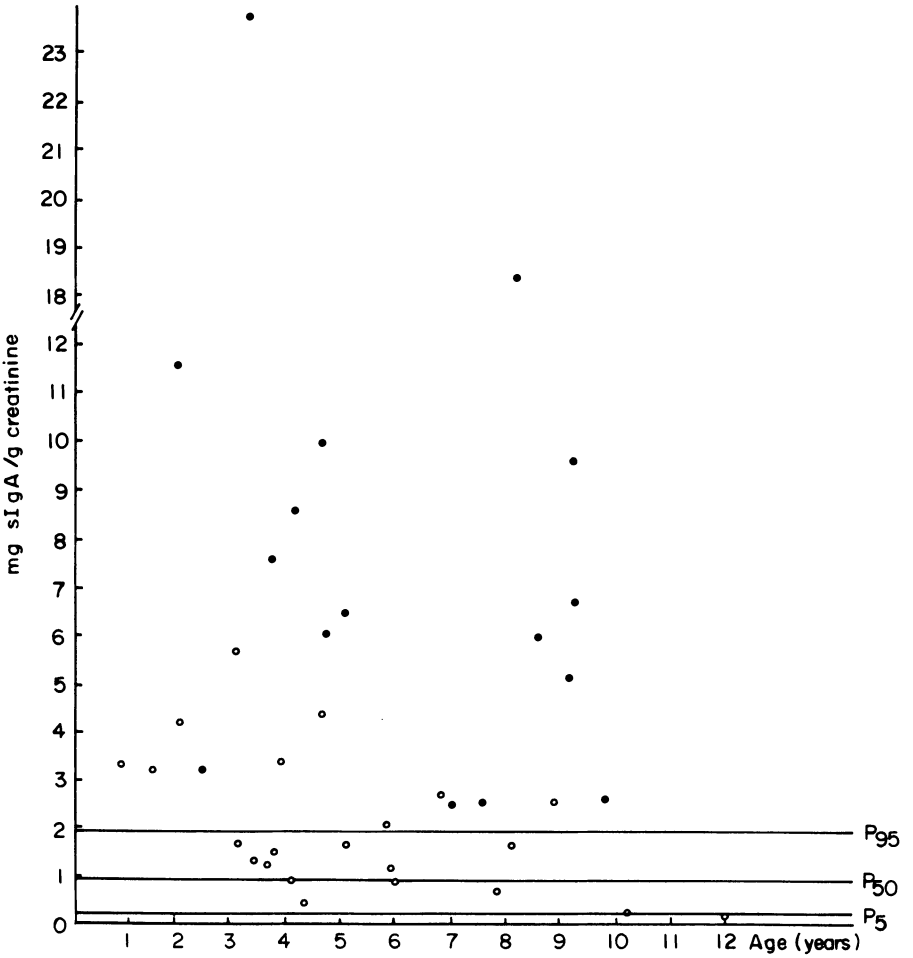

FIGURE 2 - THE EXCRETION RATE OF URINARY SECRETORY IgA IN 38 CHILDREN (1 TO 12 YEARS OLD) SUFFERING OF RECURRENT URINARY TRACT INFECTION. WITH BACTERIURIA (•) AND WITHOUT BACTERIURIA (○) THE HORIZONTAL LINES SHOW THE PERCENTILES OF THE NORMAL CHILDREN.

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Antibody secreting cells in acute urinary tract infection: indicator of local immune response possibly useful in localisation of infection

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1. INTRODUCTION

The mechanisms of host defence against urinary tract infections include local secretion of immunoglobulins through the mucosa into the urine. These antibodies, particularly of the secretory IgA isotype, bind to fimbriae and other virulence factors of the invading bacteria and thus prevent the adhesion of the microbes onto the epithelial cells. Therefore, in order to understand the pathogenesis of these diseases and the mechanisms important in the prevention of these infections, it is imperative to investigate local immune response.

This study was planned to do this in a new way, which we have previously applied to measure immune responses to infection or immunization of the gut (Kantele et al 1986, 1988). It is based on the cell circulation theory: lymphocytes get activated in the mucosa after contact with antigen, migrate to local lymphnodes to mature and return later via blood to the mucosa and secrete there antibodies (Bienenstock and Befus 1980, Tomasi 1983). We caught the homing lymphocytes in the blood and identified the antibody secreting cells (ASC) in vitro.

2. PATIENTS AND METHODS

The study included 36 patients of which 19 suffered from pyelonephritis and 17 from lower urinary tract infection (LUTI). The infecting bacteria were cultured from urine and the immune response against them was followed by determining ASC in the blood one week and again 4-7 weeks after the onset of the disease. This was done by separating lymphocytes and enumerating the ASCs among them with enzyme linked immunospot assay (ELISPOT, Sedgwick and Holt 1983, Czerkinsky et al 1983).

3. RESULTS AND DISCUSSION

Cells secreting antibodies specific to the pathogen were detected in 17/19 (89%) patients with acute pyelonephritis and in 12/17 (70%) patients with LUTI. The responses peaked 1 to 2 weeks after onset of the disease. Particularly in the patients with pyelonephritis ASC were still detectable at 4 to 6 weeks. This may indicate that, despite succesful treatment of the infection, bacterial antigens persisted and caused the prolonged response.

Although the proportion of the responders in the two groups of patients was quite similar, the vigour of the response differed statistically highly significantly in all Ig-classes. In acute pyelonephritis 79% of patients showed more than 100 IgA ASC/10^6 cells compared to only 6% in LUTI.

This method may be developed into a useful test for the localization of infection in the urinary tract.

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High polyclonal levels of IgA found in a patient with a severe malabsorption syndrome

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1. INTRODUCTION

We report on a patient with a severe malabsorption syndrome with high polyclonal levels of IgA that disappeared with the recovery from the gastrointestinal disorder.

2. MATERIALS AND METHODS

2. 1. Case report

A 73 year old man, who was a farmer bredding pigs, was admitted in April 1987 for a two months history of progressive diarrhea with weight loss. Neither lymphadenopathy nor hepatosplenomegaly were found. There was a steatorrhea with a malabsorption syndrome documented by an abnormal Schilling test without a vitamine B12 deficiency. The fiberoptic examination showed a morphologicaly normal mucosa with, histologicaly, an interstitial duodenitis with no villous atrophy and a polyclonal plasmocytosis (predominantly IgA). The colonoscopy was normal. All the culture and serologic tests were negative. A very high level of IgA was the only serum abnormality . The patient received cycline (2 g/day) during 4 months, that resulted in a dramatic improvement with a cessation of the malabsorption within a month, a gain in weight and a return to normal level of IgA within 6 months.

2. 2. Serum studies

Immunoglobulin (Ig) levels were determined by nephelometry. Patient's serum was analyzed by immunoelectrophoresis and immunofixation. IgA subclass levels were determined by RIA.

2. 3. Cellular studies

In June 1987, B lymphocytes were studied by direct immunofluorescence under microscope with a F(ab')2 anti-human Ig-FITC and with a F(ab')2

anti α chain-FITC. T cells were assayed by E rosetting. A lymphoblastoïd cell line (LCL) was obtained after EBV transformation.

2. 4. In vitro Ig synthesis

In september 1987 the IgA production by patient's PBL was studied spontaneously and with PWM (1 µg/ml) during a 7 days culture. The Ig levels in these supernatants and in the LCL supernatant were determined by ELISA.

2. 5. IgA purification and analysis

Patient's IgA was purifed by anionic exchange chromatography (DEAE Trisacryl*) and subsequent gel filtration (Sephadex G 200*). The whole serum and the IgA rich fraction were analyzed by density gradient ultracentrifugation and calibrated gel filtration (Sephacryl S 300*).

3. RESULTS

Transitory isolated very high polyclonal IgA levels with no pattern of α chain disease but a slight imbalance in the IgA subclass ratio (IgA 1 = 64 % ; IgA 2 = 36 %).

TABLE 1
Patient's Ig level in (g/l)

DATE	IgG	IgA	IgM
June 1985	12,8	2,47	1,3
April 1987	12,4	24,6	1,7
December 1987	14,9	2,78	1,14

B lymphocytes, sIgA B lymphocytes and T lymphocytes in the normal range (10, 1 and 58 % respectively).

Increased IgA response by PBL spontaneously (250 ng/ml) and after PWM stimulation (1 375 ng/ml), and by LCL (1 700 ng/ml).

Predominantly dimeric forms of IgA after analytical gel filtration and density gradient ultracentrifugation.

4. DISCUSSION

The clinical and biological evolution fo our patient is very unusual. Although all the cultures and serologic studies for numerous microorganisms were negative, the dramatic clinical improvement with cycline followed by a fall to the normal level of IgA suggests a link between the two abnormalities and an infectious unknown origin. Our preliminary results suggest that patient's IgA response is increased and that his IgA structure, at the time of the malabsorption syndrome, was at least a dimeric form. This would agree with the hypothesis of the polymeric forms of IgA as marker for recent activation by antigen.

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Disturbances of IgA immune response in patients with ankylosing spondylitis

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Ankylosing spondylitis (AS)has been associated with extraarticular inflammatory processes,especially at mucosal sites.In an attempt to detect possible abnormalities in the regulation of IgA responses,we have studied in the sera of patients with AS the presence of antibodies against endogenous antigens as F(ab')2 and Fc immunoglobulin portions, and exogenous antigens as bovine serum albumin (BSA) and dextran.

Material and Methods

We have studied 33 patients with AS according to New York diagnostic criteria,with a mean age of 36 years.The HLA-B27 antigen was present in 31 of 33 patients.Serum Igs were determined by the nephelometry technique.Circulating polymeric and monomeric IgA immune complexes(IC)and IgG IC were assessed by Raji cell assay as published(1).Detection of anti-dextran anti-BSA antibodies and IgA rheumatoid factor was carried out by ELISA.The measurement of shared idiotypes in serum was done by employing a polymeric antibody obtained in rabbits,as published (1).

Results

The percentage of patients with high serum levels of immunoglobulins,IC and antibodies against Fc and F(ab')2 portions of IgG is shown in Table 1.

TABLE 1. Percentage of patients with AS with elevated levels of immunoglobulins,immune complexes,IgA rheumatoid factor and shared idiotypes

Serum immunoglobulins	Patients with values above normal (%)
IgA	17/33 (52%)
IgG	5/33 (15%)
IgM	1/33 (3%)
Immune complexes	
Polymeric IgA	1/24 (4%)
Monomeric IgA	13/24 (54%)
IgG	6/24 (25%)
IgA rheumatoid factor	5/22 (23%)
Shared idiotypes	4/19 (21%)

When these data were correlated with laboratory and clinical activity the following results were obtained. The mean serum IgA concentration in patients having elevated erythrocyte sedimentation rate (ESR) was significantly higher (378±141 mg/dl),than that found in patients with normal ESR (260±148;$p<0.01$).The same findings were noted in patients with intense clinical activity (not shown). Although IgA-IC were more often found in patients with active disease than those in quiescent phases,the differences were not statistically different.No significant correlation was found between the presence and levels of IgA rheumatoid factor, as well as the shared idiotypes,and the laboratory and clinical activity. The levels of anti-BSA , IgG and IgA antibodies exceeded the normal upper limit in 32 and 14% of patients,respectively.Those of anti-dextran IgG and IgA in 41% and 32%.No significant correlation was found between the levels and occurrence of anti-BSA and anti-dextran antibodies,and clinical or analytical parameters.

Discussion

Our study confirms previous findings of elevated total serum IgA levels in a large proportion of patients with AS(2).Furthermore,of all parameters studied,serum IgA was the only with a good correlation with clinical and analytical activity.Recent data suggest that the increase in serum IgA is restricted to subclass IgA1(2).
Previous authors have not found any relation between activity of the disease and IC (3),but all of them determined only multimeric IgA-IC. In our study we have distinguished between polymeric and monomeric IgA-IC. The fact that patients with AS have chiefly monomeric IgA IC could explain the absence of more correlation with clinical activity. In an experimental model of IgA nephropathy,polymeric IgA was observed to be critical for renal deposition of complexes and induction of nephritic histological changes(4).It is possible that the existence of circulating IgA IC in patients with AS is only a consequence of an abnormal mucosal immune response but not related, or slightly,to the spondylitic process.However,those patients with polymeric IgA IC could have a risk of developing IgA nephropathy(5).The relatively elevated evidence of patients with IgA-rheumatoid factor could be relevant since these complexes seem to indicate a poor prognosis in early rheumatoid arthritis. The elevated frequency of patients with AS having shared idiotypes add another immunological resemblance with IgA nephropathy.The important incidence of anti-BSA and anti-dextran IgG and IgA antibodies is reminiscent of the presence of IgA antibodies to Klebsiella pneumoniae in AS patients,suggesting a defect in mucosal permeability to antigens.

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Characterisation of a proteolytic enzyme from *Proteus mirabilis* which cleaves immunoglobulins of the IgA1, IgA2 and IgG isotypes

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Introduction

Strains of Proteus mirabilis (PM) associated with human urinary tract infections have been found to secrete an extracellular protease with activity for immunoglobulins. We have purified the protease from culture supernates of P. mirabilis (strain 64676) by Sephacryl S-200 gel filtration and FPLC-Mono Q anion exchange chromatography followed by electrophoresis on non-denaturing polyacrylamide gels. The enzyme is a heat stable metallo-proteinase of Mol. wt. 50 K which is active over a broad range of pH values from pH 5-10 (optimum pH 8-9). In this report we describe the PM protease cleavage products of immunoglobulins of different isotypes.

Results and Discussion

COMPARISON OF IgA1 CLEAVAGE PRODUCTS OF PM, HI AND NG PROTEASES.

^{125}I-IgA1 was digested with proteases from P. mirabilis (PM), H. influenzae (HI) and N. gonorrhoeae (NG) and the cleavage products were analysed by SDS-PAGE and autoradiography (Fig. 1). The HI and NG proteases are classic microbial IgA1 proteases which cleave the heavy chain of only the IgA1 molecule in a proline-rich sequence of 13 amino acids in the hinge region (for review see Kornfeld and Plaut, 1981). Fragments of size 38 K (Fab) and 33 K (Fc) were obtained with the HI protease and 37 K (Fab) and 35 K (Fc) fragments with the NG protease. The sizes of these IgA1 cleavage products are consistent with cleavage of the heavy chain in the hinge region. However, the larger size of the Fab fragment (47 K) obtained with the PM protease is indicative of cleavage of the heavy chain outside the hinge region.

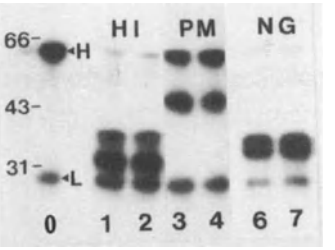

FIGURE 1 Lane 0, undigested ^{125}I-IgA1; lane 1, 3 and 6, protease digested ^{125}I-IgA1; lanes 2, 4 and 7, protease 1:2 digested ^{125}I-IgA1. (H = Heavy chain; L = Light chain)

CLEAVAGE OF DIFFERENT IMMUNOGLOBULIN ISOTYPES BY PM PROTEASE

Radioiodinated immunoglobulins of different isotypes were digested with PM protease and the cleavage products analyzed by SDS-PAGE and autoradiography (Fig. 2). The cleavage products of serum and secretory IgA1 and IgA2 isotypes were a 47 K Fab and a 34 K Fc fragment; the sIgA isotypes were degraded to a lesser degree perhaps through protection by the associated secretory component. Since the IgA2 isotype is resistant to the classic microbial IgA1-specific hinge-cleaving proteases because it lacks the proline-rich sequence of 13 amino acids found in the hinge region of IgA1, cleavage of IgA2 by the PM protease is further evidence that its cleavage site lies outside the hinge region of the α-chain. Free secretory component (SC) and SC bound to both the IgA1 and IgA2 isotypes appeared to be degraded to some extent by the PM protease. The heavy chain of IgG was degraded to a 31 K fragment slightly larger than the light chain but that of IgM was resistant to cleavage by the PM protease. The proteolysis of IgA1, IgA2 and IgG by the PM protease was inhibited by 5 mM EDTA.

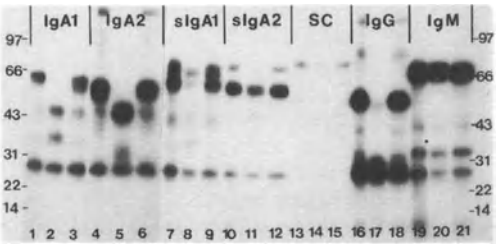

FIGURE 2 Lanes 1, 4, 7, 10, 13, 16, 19, undigested ^{125}I-Ig; lanes 2, 5, 8, 11, 14, 17, 20, PM digested ^{125}I-Ig; lanes 3, 6, 9, 12, 15, 18, 21, PM digested ^{125}I-Ig (+5 mM EDTA).

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Opsonisation of yeast and bacteria by human serum IgA antibodies

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Introduction

Most of the yeast opsonising activity of normal serum is EDTA sensitive and destroyed by heating at 56'. It is therefore considered to be complement dependent. Normal serum has in fact very little, detectable, heat stable opsonising activity for any microorganism, although hyperimmunised subjects can show considerable activity has been attributed to specific IgG antibodies. The levels of IgG anti-yeast antibodies detectable in normal serum are presumed to be insufficient for opsonisation in the absence of complement.

We have previously confirmed (Yeaman and Kerr, 1987) that the ability of most sera to opsonise yeast (S. cerevisiae) was decreased markedly by heating at 56' for 1h. However, we identified three sera from patients with liver disease whose opsonic activity was decreased only slightly on heating. These sera were characterised by their high levels of IgA anti-yeast mannan antibody. Fractionation of these sera by ammonium sulphate precipitation, ion exchange chromatography on FPLC monoQ resin and affinity chromatography on Jacalin-Sepharose showed that the opsonic activity copurified with IgA. Although the sera did also contain elevated levels of IgG anti-mannan antibodies, the purified IgG fractions were not opsonic in the assay used, which measured the phagocytosis of yeast by neutrophils in suspension. This was consistent with the observation that sera with elevated levels of IgG but not IgA anti-mannan antibody did not show heat stable opsonic activity.

This observation of a role for serum IgA in the stimulation of phagocytosis of microorganisms by neutrophils was contrary to earlier reports that IgA was not opsonic or indeed, inhibited phagocytosis. We have therefore extended these studies by characterising the ability of IgA and IgG purified from sera showing high levels of specific antibody to opsonise yeast and bacteria. We have also compared the ability of Sepharose-coupled IgG and IgA and heat aggregated immunoglobulins to stimulate neutrophils. The same IgA and IgG Sepharose preparations were used for the affinity purification of the IgG and IgA receptors from solubilised neutrophil membranes. The results show that IgA and IgG antibodies are of similar potency in the triggering of neutrophil function

and that the phagocytes express similar amounts of high affinity receptiors for the two classes of immunoglobulin

Results and Discussion

Opsonisation was measured by several assays, each developed to be applicable to the screening of large numbers of samples. Phagocytosis was measured by a radiometric assay which uses ^{125}I-labelled yeast cell wall; the phagocytosed yeast being separated from free yeast by centrifugation through Percoll in microfuge tubes. The neutrophil respiratory burst was assayed by lucigenin enhanced chemiluminescence measured in a 96 well luminometer as described by Blair et al.(1988). Degranulation was determined by the measurement of the release of enzymes, b glucuronidase, lysozyme and peroxidase into cell free supernatants.

We have now screened over 500 pathological sera from patients with liver, inflammatory bowel and autoimmune diseases for heat stable yeast opsonic activity and for IgA and IgG anti-yeast mannan antibodies. Using the assay for phagocytosis over 20 sera have now been identified with heat stable activity; all had elevated levels of IgA anti-mannan antibody whilst sera which had only elevated IgG anti-mannan did not. All of the sera with elevated IgA anti-mannan antibodies were shown to contain both monomeric and dimeric anti-mannan IgA although the relative proportions varied considerably. Greater than 98% of the total IgA in these sera was in each case monomeric.

IgG, monomeric IgA and dimeric IgA anti-yeast antibodies were purified as before. Using the phagocytosis assay in which both yeast and neutrophils are kept in suspension, heat stable opsonic activity was in each case associated with IgA anti-mannan antibody rather than IgG. If yeast and neutrophils were allowed to settle, then both IgA and IgG were equally efficient opsonins. Similarily, for assays in suspension, IgA antibodies were more efficient than IgG in causing the degranulation of neutrophils. However, the measurement of lucigenin enhanced chemiluminescence which is applicable only to adherent neutrophils showed both IgA and IgG to be opsonic. IgG and IgA preparations with similar protein concentrations and titres of anti-mannan antibody elicited a response of similar size. The chemiluminescent burst was less strong but more rapid than that elicited by complement opsonised yeast.

Nolan et al. (1986) have reported that raised levels of IgA antibodies against Lipid A (a major component of enteric bacterial endotoxin) are a common feature of liver disease. Since this offered a comparable model system for study of bacterial opsonisation, we assayed levels of these antibodies in the same sera. Although we were unable to confirm the original results we did identify several sera with high levels of anti-lipid A IgA , the highest being from a patient with liver disease and E. coli septicaemia. Purification of the immunoglobulins showed that the IgA anti-lipid A was all dimeric whilst anti-yeast mannan IgA from the same serum was both monomeric and dimeric. All of the IgA antibody against both lipid A and yeast mannan bound to Jacalin-Sepharose. E. coli opsonised using this serum or IgA purified from it,

produced a markedly more rapid chemiluminescent burst than the same bacteria opsonised in normal serum. IgA antibodies stimulated the phagocytosis of heat killed E. coli and the release of neutrophil granule enzymes. In view of the fact that these IgA antibodies were purified from a patient who had recently had E. coli septicaemia it is difficult to envisage that the antibodies would not be active in vivo as well as in vitro.

Since the neutrophil has been reported to possesss receptors for components of yeast and bacteria cell walls such as glucan and endotoxin which might enhance the phagocytosis, we have also studied the effects on neutrophils of IgA and IgG bound to Sepharose which represent targets devoid of the potential for other interactions. IgA-Sepharose and IgG Sepharose with a similar density of coupled antibody (range 0.2-2.0 mg /ml resin) elicited a similar size of repiratory burst although that for IgA-Sepharose was more rapid but less prolonged. The stimulation of release of lysozyme and b glucuronidase was greater for IgA Sepharose. Bovine serum albumin -Sepharose or underivatised Sepharose did not have any effect.

Affinity chromatography of detergent solubilised extracts of neutrophil membranes purified specific receptors for IgG and IgA. The amount of IgA receptor recovered being greater than that of the Fcr11, the higher affinity IgG Fc receptor.

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Influence of altered ELISA antigen-antibody reaction kinetics on measurement of IgA2 subclass levels in biological fluids

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Introduction

Specificity and affinity are the hallmarks of quantitative immunoassays. Both characteristics are a reflection of the "goodness of fit" of the antigen-antibody reaction. Many factors influence this association and therefore stringent physiochemical conditions are required for relating gravimetric amounts of analyte to the size of the immunoassay "signal". In order to investigate the biological significance of changes in IgA subclass levels in biological fluids "standardised" assay systems must be available for regulating published data in this area. In this study we report on some insights gained in this problem area while trying to establish an ELISA for quantifying IgA_2 levels in human biological fluids using widely available monoclonal antibodies.

Materials and Methods

(a) Colostrum: 25 samples, Day 1 - Day 10 post partum, from four healthy mothers; (b) Serum: 11 samples from healthy adult volunteers; (c) Standard serum for IgA_2 estimation (Binding Site Ltd.); (d) Antisera: Monoclonal anti-IgA2 antibodies clone NI512 (Becton Dickinson), clone 14-3-26 (Nordic), clone 2E2 (Janssen), peroxidase conjugated antihuman IgA antibody (Tago).

IgA2 levels in serum and milk samples were measured by ELISA previously described by Soppie et al (1987). All assays were carried out in triplicate and a reference serum control was included.

Results

We first investigated the reaction profile for IgA_2 levels in serum and colostrum samples, day 1 to day 4 post partum, using assay conditions described by Soppie et al. While serum IgA in the dilution range 10^{-6}-10^{-1} gave a sigmoidal curve appearance, it was clear that with increasing time post partum, the IgA2 profile was changing significantly. It was then decided to "optimise" the solid phase concentration of 3 anti-IgA2 monoclonal antibodies in a sandwich ELISA. The kinetic profile for the three antibodies was significantly different for each antibody tested within the dilution range 10^{-5} to 10^{-1} (Figure 1).

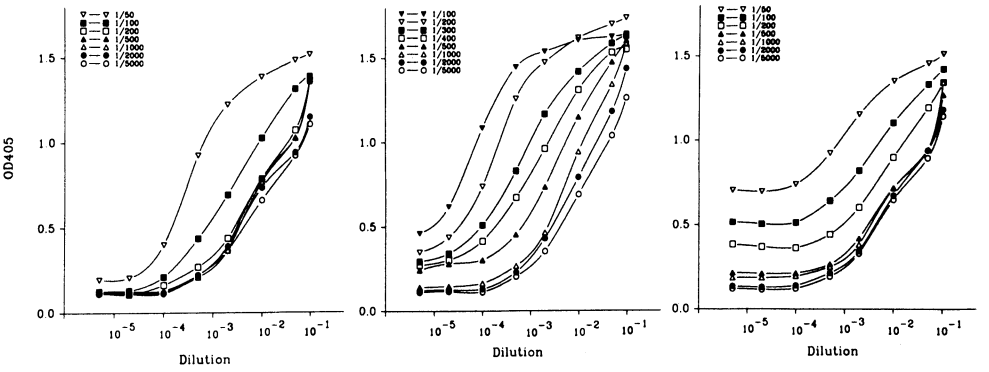

Figure 1. "Optimisation" of solid phase antibody concentration for quantitation of IgA_2 by ELISA.

Comparative analysis of these three monoclonal antibodies in a sandwich solid phase ELISA at optimal binding concentrations (clone 14-3-26 - 1/50 dil.; clone NI512 - 1/200 dil.; clone 2E2 -1/50 dil.) was carried out in serum and colostrum. Comparative data based on a gravimetric serum standard for serum IgA_2 levels was very poor between all three monoclonal antibodies (highest r value, r = 0.46). Measurement of colostrum IgA_2 levels over a very wide concentration range (0.01 to 80 mg/ml) indicated that two of the antibodies (clone 2E2 and clone NI512) had a high consistency in measuring IgA_2 levels (r = 0.98).

Discussion

IgA immunoglobulins in biological fluids and in particular IgA subclasses represent a very complex system with respect to the biophysical nature of the antigen being measured. The difficulty in quantifying a specific epitope using a monoclonal antibody in an immunoassay relative to a gravimetric standard is well demonstrated by the problems encountered in measuring IgA_2 levels in serum and milk in this study. If monoclonal antibodies are to be used for the quantitation of IgA subclass levels in biological fluids then the expression of the specific epitope relative to the biophysical configuration of the molecule must be established and this in turn related to gravimetric amounts of different molecular forms of the IgA subclass. The possibility that molecular separation techniques are required followed by quantitation of the individual molecular forms of the individual molecules in order to determine levels may be the answer to this problem.

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Determination of a standard to quantify the different molecular forms of human fecal IgA in local inflammatory and infectious diseases

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The molecular composition of fecal IgA is still poorly documented. These immunoglobulins (Ig) originate both from local synthesis and from serum, following increased desquamation of enterocytes (1). The respective proportions of these forms vary according to the status of the intestinal mucosa. It was thus of interest to investigate, and quantify, fecal IgA for diagnostic purposes. Indeed, monomers (mIgA) as well as colostral secretory IgA (SIgA), are unsuitable standards since they either overestimate, or underestimate, the total IgA (tIgA) results. We purified fecal polymeric IgA (pIgA) and used it as a standard to determine the fecal tIgA concentrations by a modified electro-immunodiffusion (mEID) assay, in inflammatory and in infectious intestinal diseases.

MATERIALS AND METHODS

• Preparation of a fecal standard. A pool of 4 stools, rich in IgA (300-556 mg/l) was 3-fold diluted in saline, stirred for 1 hr and centrifuged (30,000 g). Ig were precipitated by 50 % ammonium sulfate, and IgA were isolated by 2 successive ion-exchange chromatographies. After a Sephadex G200 gel-filtration, low molecular weight (M.W.) IgA were isolated by a Sephadex G100 column while pIgA were purified with a Sepharose CL6B column. The main IgA molecular forms were analysed by HPLC (Superose 12, Pharmacia) and by PAGE in a 2-16 % gradient while IgA and α1 antitrypsin (α1 AT) were assayed in the HPLC eluates by "sandwich" ELISA and EID, respectively.

• mEID assay of tIgA, mIgA and pIgA. Four groups were studied : a control group of 32 healthy subjects (13 females : 47 ± 16 yr and 19 males : 46 ± 11 yr) ; 12 adults with active Crohn's disease (8 females : 36 ± 15 yr and 4 males : 33 ± 12 yr) ; 10 AIDS with Cryptosporidiosis (1 female : 55 yr and 9 males : 37 ± 10 yr) and 11 adults with Giardiasis (2 females : 47 and 63 yr and 9 males : 39 ± 10 yr). The assay was performed as previously described (2). Briefly, 5 µl-deposits of a fecal extract and of the two standards (purified fecal pIgA and monoclonal mIgA) were introduced into wells on both sides of a gel barrier blocking the migration of heavy molecules. The quantities of tIgA and mIgA were derived from the upper and from the lower wells respectively and the difference between the two values corresponded to polymers. Results were expressed as daily fecal outputs and intestinal clearance according to BERNIER (3). Fecal outputs and clearances of the patients were compared to the controls using the Student's t-test.

RESULTS

As demonstrated by HPLC, PAGE and ELISA, the gel-filtrations separated two main molecular forms of fecal IgA. The first consisted in IgA of less than 200 kDa : 20 % eluting as pure IgA with an apparent M.W. similar to that of monomers ; while 80 % represented degraded IgA and α1 AT, eluting between 180 and 60 kDa, at the exclusion of any other protein. The second consisted in SIgA (94 % of dimers, M.W. ~ 370 kDa and 6 % of polymers, M.W. ~ 1000 kDa) dissociating into slightly lower M.W. molecules under the influence of an electric field. This heavy fraction was used as a standard to quantify tIgA by mEID in several intestinal diseases (table I).

Table I : mIgA and pIgA fecal output, and mIgA intestinal clearance (mIgA CL) in various gut diseases

	Fecal output (mg/day)			mIgA CL (ml/day)
	tIgA	pIgA	mIgA	
Active Crohn's diseases (n = 12)	268.4**	167.5**	100.9**	44.1**
AIDS with Cryptosporidiosis (n = 10)	443**	348**	100**	33.3**
Giardiasis (n = 11)	83**	71.3**	11.6*	2.1
Controls (n = 32)	21.2	16.5	4.4	3.2

(* $p < 0.01$; ** $p < 0.001$)

DISCUSSION

Fecal IgA mainly consisted of SIgA, which was partially protected from proteolysis by the secretory component. In addition, degradation products -either free or bound to α1 AT- were also observed. Thus, fecal IgA determinations require comparison to fecal standards, and this prompted us to use our SIgA-containing fraction.

We demonstrate that the mIgA fecal output and the intestinal clearance are significantly raised in active Crohn's disease, showing a large digestive protein-loss due to an increase in mucosal permeability. The high pIgA output also suggests a strong stimulation of the secretory immune system. In Giardiasis, and mainly in AIDS-associated Cryptosporidiosis, the high mIgA fecal output also indicates a passive protein transfer through the intestinal barrier as well as an increase in the pIgA output. Furthermore, our standard improves the accuracy of the fecal IgA assay, and thus can help in the biological diagnosis of various gastro-intestinal diseases.

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Distribution and semi-quantitative estimation of specific antibodies of the IgA subclass

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ABSTRACT. In order to further analyze the subclass restriction of antigen-specific IgA antibodies, we have studied serum antibodies from normal blood donors against protein and polysaccharide antigens. Human IgA hybridoma antibodies of the two subclasses, directed against PPS 8 and NP, respectively, were used as standards to estimate levels of antigen-specific antibodies. The results show an IgA1 dominance of naturally acquired antibodies against all the tested antigens.

1. Introduction.

The IgA subclass restriction in the antibody response is less investigated and understood as compared to the IgG subclass distribution. Serum IgG antibodies against protein antigens are mainly restricted to IgG1 with minor contributions by the IgG3 and IgG4 whereas polysaccharide antigens mainly induce IgG2 in adults.

2. Materials and Methods.

2.1 ANTIGENS.

Pneumococcal capsular polysaccharide (PPS) 1, PPS 6A, PPS 8, PPS 19F, PPS 23, Pneumolysin, (4-hydroxy-3-nitrophenacetyl)2 bovine serum albumine (NP2BSA), Haemophilus influenzae (H. I.) OMP, S. aureus α-toxin, teichoid acid from S. aureus, lipopolysaccharide from E. coli and dextran were used as antigens.

2.2 IMMUNOGLOBULINS.

Sera from healthy adult blood donors were used. A human IgA1 hybridoma antibody directed against PPS 8 was used in attempt to quantify IgA1 antibodies [1]. A human IgA2 hybridoma antibody directed against 4-hydroxy-3-nitrophenacetyl was generously donated by Dr. M.S. Neuberger, Medical Research Council Laboratory, Cambridge, U.K. and used in the semiquantitative estimations [2].

2.3 ELISA FOR ANTIGEN-SPECIFIC SUBCLASS DETERMINATIONS.

The levels were measured in single wells and all reagents were diluted in PBS with 0.05% Tween 20. Human IgA subclass hybridoma antibodies and sera were subsequently added and allowed to incubate overnight at room temperature. 0.15 M NaCl with 0.05% Tween 20 was used for washings. Monoclonal mouse anti-human IgA subclass antibodies used were anti-IgA1 from Becton Dickinson, Mechelen, Belgium, antibody code 1-155-1, diluted 1:150, and anti-IgA2 from Nordic Laboratories, Tilburg, The Netherlands, antibody code NI 512, diluted 1:2000. The determination in detail of IgA subclass distribution and the reactivity, sensitivity and specificity of the IgA subclass assay has been described elsewhere [3].

3. Results.

Distribution of naturally acquired IgA1 and IgA2 antibodies are given in Table I. Semiquantitative estimations of specific IgA subclass antibodies are shown in Table II.

TABLE I. IgA subclass distribution of specific antibodies.

Number of sera	Antigen	IgA1	IgA2
8	PPS 1	++	(+)
30	PPS 6A	++	(+)
8	PPS 8	++	(+)
30	PPS 19F	++	(+)
20	PPS 23	++	(+)
8	Pneumolysin	+	-
9	H. I. OMP	++	-
20	E. coli LPS	++	-
40	S. aureus α-toxin	++	(+)
40	S. aureus teichoid acid	++	(+)
20	Dextran	++	+ a)

a) in high titered sera.

TABLE II. Semiquantitative estimations of specific IgA subclass antibodies against PPS 6A and S. aureus α–toxin, given in ng/ml.

	Antigen PPS 6A		Antigen S. aureus α-toxin	
Donor	IgA1*	IgA2	IgA1*	IgA2
A	2006	0	1518	0
B	439	0	665	0
C	1097	115	1011	0
D	617	0	801	0
E	615	70	1776	75

* Corrected for low affinity [4].

4. Discussion.

In our assay the IgA subclass distribution of naturally acquired antibodies against a large number of antigens shows a predominance of IgA1 in normal individuals. This is in agreement with previous reports [5]. Our semiquantitative assay with human hybridoma Ig antibody shows lower amounts of specific IgA subclass antibodies than previous reports using solid-phase assays with myeloma proteins as standards [5]. However, the semiquantitated levels of specific IgA subclass antibodies may still be to high. Human chimeric IgM, IgG1, IgG2, IgG3, IgG4, IgE and IgA2 antibodies directed against 4-hydroxy-3-nitrophenacetyl exists [2]. An IgA1 hybridoma antibody with the same specificity would give an optimal set of reagents to quantify specific antibodies of different class and subclasses.

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IgA polymer levels in human sera: detection with a polymeric conformational monoclonal antibody (anti-P)

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ABSTRACT. A monoclonal antibody recognizing the IgA polymer (pIgA) conformation was used to estimate, by ELISA, the amount of true polymers in normal and in hepatitis sera. Normal sera contained $\leqslant$ 35 µg/ml of pIgA i.e. $\leqslant$ 1.5% of total IgA, these values being increased to 62 µg/ml and 2.2% for the hepatitis sera.

INTRODUCTION

Human sera IgA molecules mainly stand as 160 kDa monomers (mIgA) but J chain-containing dimers and polymers (pIgA) always coexist. Some of these polymers are SIgA since they contain an SC molecule. The problem of the respective amounts of these forms is still unsettled as the total polymer content estimations vary from less than 1 % up to 13 % according to the authors.

Using a monoclonal antibody (mAb) against the pIgA conformation (1), we established the concentration of these molecules in normal sera and in sera from patients suffering from chronic hepatitis B. These results, and the SIgA levels, were compared to the total IgA serum concentrations.

MATERIALS and METHODS

1) Normal sera were from 100 blood donors and pathological sera from 6 different subjects suffering from chronic hepatitis B (1-3 different bleedings per subject leading to 9 samples).

2) Estimations :

a) Total IgA was determined by single radial immunodiffusion, using the correction factors of Delacroix (2): 1.55 for dimer IgA and 2.24 for SIgA.

b) pIgA were determined by ELISA inhibition and by sandwich ELISA.

c) SIgA were determined by sandwich ELISA.

3) Standards :

- A unique pIgA1 myeloma protein was chosen because most IgA molecules in sera belong to this subclass.
- Colostral SIgA was chosen for SIgA determinations.

RESULTS

The figures in the table are the mean values of duplicate determinations at 3 different dilutions. The slopes of the standard curve (5 dilutions) and of individual sera (3 dilutions) were generally parallel.

Sera	pIgA in µg/ml	SIgA in µg/ml	tIgA in mg/ml	pIgA/tIgA in %	SIgA/pIgA in %
Normal (n=100)	35.7 ± 13.2 (med. 35)	15.2 ± 6.2 (med. 14.8)	2.4 ± 0.8 (med. 2.35)	1.5	42.6
Hepatitis (n=9)	62.2 ± 31.8 (med. 61)	38 ± 30.3 (med. 35)	2.79 ± 0.57 (med. 2.9)	2.2	61.3

The SIgA and pIgA mean concentrations in hepatitis sera are significantly increased ($P > 10^{-6}$ for SIgA, and $P > 2.6\ 10^{-6}$ for pIgA, by T-test) relatively to normal sera, while it is not the case ($P = 0.18$) for tIgA.

DISCUSSION

The sandwich ELISA had a 20-fold greater sensitivity than the inhibition ELISA, however both assays gave similar results.

The choice of the myeloma standard resulted from the high activity, between 1 and 5 ng, of this protein, in contrast with the partial loss of the P conformation in most purified polymeric preparations. Aggregates and native monomers were poorly revealed (in the 100 ng range). The interference of serum monomers in the ELISA was low since the samples were diluted 1:40,000 (or more) i.e. 2.4 mg/ 40,000 = 60 ng and the pIgA figures must be considered as maximal values.

- SIgA was also poorly detected by the anti-P mAb (sensitivity 50 ng), probably because of the presence of SC which must hide the P epitope, and thus does not interfere in the pIgA estimation .

Our pIgA values of $\leqslant$ 35 µg/ml, i.e. $\leqslant$ 1.5% of the total IgA in normal sera, are consistent with the results of Radl (3) who found that true polymers were below 1%, in contrast with the 10% commonly found by most authors using methods involving physical separations.

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SECTION Y: ANIMAL MODELS OF MUCOSAL DISEASES

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Inactivation of Peyer's patchs stimulates the formation of antibody containing cells in the spleen of the rat

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ABSTRACT. The effect of Peyer's patch (PP) inactivation on the development of antibody-containing cells (ACC) was studied in the spleen of the rat by immuno- and enzyme-histochemistry. Inactivation of PP was done by a burning method. More anti-TNP-ACC were detected in the spleen of PP-inactivated rats than in controls following intraperitoneal priming and intraintestinal booster immunization with TNP-KLH antigen. This significant difference indicates an important role of PP in the generation of T-suppressor cells (Ts).

1. INTRODUCTION

Besides their important role in the induction of local immune responses following oral immunization, PP were suggested to be responsible for the development of systemic suppression. These paradoxal functions of PP were believed to maintain appropriate reactions in the gut (Challacombe & Tomasi, 1980). It has been demonstrated that antigen-specific Ts were formed in the GALT following oral immunization (Mattingly & Waksman, 1978), and migrate to the spleen. We developed a method to inactivate PP and to study its effects on the immune response against TNP-KLH antigen in situ. In this study the effects of PP inactivation on the development of anti-TNP-ACC in the spleen were studied using immunohistochemistry (Claassen & Van Rooijen,1984).

2. MATERIALS AND METHODS

Adult Wistar rats (CPB, Zeist, The Netherlands) were used. Under Hypnorm (Duphar, Weesp, The Netherlands) anaesthesia laparotomy was performed; each visible PP was burnt by using a solder. One day before PP inactivation rats were primed intraperitoneally (i.p.) with 100 µg TNP-KLH with or without $AlPO_4$ adjuvant. Another group was primed with 1 mg TNP-KLH intragastrally (i.g.). Two weeks later all rats were boosted intraintestinally (i.i.) at the proximal jejunum. Spleen was collected 5 days after the booster immunization, and snap frozen in liquid nitrogen.

Cryostat sections were fixed in acetone and incubated with TNP-alkaline phosphatase (TNP-AlPh) overnight at 4°C. Sections were stained for AlPh using Fast blue BB base and levamisole (Sigma, USA). The morphology of PP was studied by immunohistochemistry at several time points (0,3,5,7, 10 and 14 days) after burning. Cryostat sections, fixed in acetone, and stained with His-14 McAb (State University, Groningen) for B cells and Ox-19 McAb (Serotec, France) for T cells.

3. RESULTS

Immediate after burning (day 0), there was a destruction of the serosal layer of the intestine beneath the follicle up to the external muscular sheath. At day 3 follicles had reduced in size, T cell areas became smaller and some parts had disappeared. B cells were recovered at day 3, whilst T cells started recovery at day 5. Numerous granulocytes and macrophages were present in the affected areas. Complete healing occurred at about day 14.

I.p. priming followed by i.i. booster immunization with TNP-KLH antigen gave rise to the formation of higher numbers of anti-TNP-ACC in the spleen of PP-inactivated rats than in controls. Similar results were also demonstrated in the spleen of PP-inactivated rats primed i.g. and boosted i.i. with the same antigen. When animals were immunized with TNP-KLH AlPO4 adjuvant, more anti-TNP-ACC were found in the spleen of PP-inactivated rats than in controls. The specific antibody containing cells occurred in small clusters.

In conclusion, this study strongly suggests that PP play a role in the induction of systemic immune suppression, because PP-inactivated rats develop significantly more anti-TNP-ACC in the spleen than controls. Most probably the increase in antibody formation in the spleen is due to the removal of suppressor cell populations by inactivation (burning) of PP.

4. ACKNOWLEDGEMENTS

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Mucosal immunoglobulin production during graft-versus-host reaction in mice

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INTRODUCTION

The induction of a semi-allogeneic Graft-versus-host reaction (GvHR) in non-irradiated F_1 hybrid mice can produce an acute and chronic form. The acute form of the reaction is characterised by a brief phase of immunostimulation, up to 14 days, followed by immunosuppression resulting in pancytopenia, aplastic anaemia and hypogammaglobulinaemia. The chronic form produces a stimulatory pathology with B lymphocyte hyperplasia leading to hypergammaglobulinaemia and the formation of autoantibodies (1).

During the initial 14 days of immunostimulation a local delayed-type hypersensitivity response (DTH) manifests in the small intestine, the peak of severity occurring between days 11 and 14 of the GvHR. An increase in IEL, and crypt cell turnover coincide with crypt hyperplasia (2). Activated T cells, of host origin, present in the mucosa, and are thought to exert their effect by the production of cytokines.

We have used this animal model of GvHR to study the influences of activated T cells and the local DTH response on the production of intestinal immunoglobulins. Simultaneously the systemic features, such as spleen index and immunoglobulin production were also studied.

METHODS

A semi-allogeneic GvHR was induced in (C57Bl/6JxDBA/2) BDF_1 non-irradiated adult mice by ip injection of 10^8 C57BL/6J spleen cells. At various times thereafter, serum, gut washings and jejunal sections were taken. Gut washings were obtained by initially feeding animals with a hypotonic lavage solution. After 30 mins the animals were sacrificed and the small intestine removed, washed through with a protease solution and ultraclarified. The immunoglobulins were further protected with protease inhibitors and stored at $-70^{o}C$.

The immunoglobulins both in the serum and gut washings were determined by ELISA. Jejunal sections were fixed in 4% buffered formalin and stained for IgA-, IgM- and IgG- containing plasma cells by an immunoperoxidase method.

RESULTS

A biphasic pattern of immunoglobulin production both systemically and in the intestine was observed. Mucosal IgA levels were increased on day 12 ($P<0.05$) and again on day 31 ($p<0.03$). Mucosal IgM remained elevated from day 11 until day 14 ($p<0.01$). A second dramatic increase occurred again in day 31 ($p<0.002$). These results were reflected in plasma cell counts in the lamina propria. No change in mucosal IgG levels were detected.

Serum (s)IgA and sIgM levels were increased from day 4 ($p<0.001$) reaching a peak on day 12 ($p<0.001$) and day 13 ($p<0.001$) respectively. While sIgM levels dropped below control levels ($p<0.05$) prior to a second small rise on day 32 ($p<0.001$), sIgA, after a phase of normal production, rose dramatically on day 32 ($p<0.001$). Levels of sIgG did not increase until day 13 ($p<0.001$), and again returned to normal (day 24) prior to a second rise on day 32 ($p<0.001$). This biphasic pattern was reflected in the spleen index.

DISCUSSION

This model of GvHR produced two phases of increased B cell activation and immunoglobulin production. The systemic and mucosal effects appear quite separate, as does the effect on each isotype. In the gut lumen, it would appear that while IgA concentrations are elevated for only one time point, IgM levels remain high for 4-5 days, which suggest a proliferation of IgM^+ cells but not IgA^+ cells. This dichotomy may be the result of lymphokines such as IL-4 and IL-5. The increase in serum immunoglobulins and spleen index reinforces the B cell hyperplasia in both the acute and chronic forms of the GvHR. The surge of immunoglobulins on days 31 and 32, may reflect the onset of the chronic GvHR. Whether the immunoglobulins, both systemic and mucosal, have autoantibody characteristics remains to be elucidated.

Importantly the presentation of activated T cells in the gut, and the local DTH response occurring during the GvHR does produce profound effects on intestinal immunoglobulin production, and as with the changes in gut morphology, the effects are possibly lymphokine mediated.

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CD4$^+$ T cells induce ductal changes of exocrine glands in MHC class 2- disparate GVHR

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ABSTRACT. Chronic nonsuppurative destructive cholangitis (CNSDC)-like lesions were induced in the exocrine glands as well as bile ducts in the major histocompatibility complex (MHC) class-II disparate graft-versus-host reaction (GVHR) postinjection of parental CD4$^+$ (L3T4$^+$) T cells.

1. INTRODUCTION

GVHR are caused in F1 hybrid mice injected with parental spleen cells. In F1 recipients either lymphoproliferative or immunosuppressive disorders are known to appear depending upon strain combinations. Recently, we have reported CNSDC-like bile duct lesions and the formation of epithelioid granulomas in the liver of (B6 x bm12)F1 mice with MHC class II-disparate GVHR induced by injection of parental spleen cells. In this study, we tried to reveal the lesions of ducts in other organs, including salivary glands and the pancreas.

2. MATERIALS AND METHODS

2.1. Mice

Strains of mice used in this study were indicated in TABLE 1.

TABLE 1. H-2 genotypes of strains of mice used

Strains	Alleles at H-2 regions			
	K	I-A	I-E	D
C57BL/6 (B6)	b	b	-	b
B6.C-H-2^{bm1} (bm1)	bm1	b	-	b
B6.C-H-2^{bm12} (bm12)	b	bm12	-	b
(B6 x bm12)F1	b	b/bm12	-	b

2.2. Induction of GVHR

These non-irradiated (B6 x bm12)F1 mice were injected with 1 x 10^7 $CD4^+$ or $CD8^+$ T cells of B6 mice via the tail vein, and sacrificed at the second week. Pancreas and salivary glands were resected for light microscopic and immunohistological analysis.

2.3. Immunoperoxidase staining procedure

For immunohistochemical studies we used the avidin-biotin-peroxidase method. The positive cells stained were counted in 0.25 x 0.25mm^2 of portal area at a magnification of x 400 under a microscope equipped with micrometer reticle.

2.4. Enumeration of immunoglobulin-producing cells (IgPC)

The number of IgPC in the spleen enumerated by the reverse plaque assay in a Cunningham chamber.

3. RESULTS

3.1. $CD4^+$ T cells induced CNSDC-like bile duct lesions and the formation of epithelioid granulomas and were summarized in TABLE 2.

TABLE 2. Numbers of IgMPC and IgGPC per spleen and hepatic lesions in (B6 x bm12)F1 recipient mice.

Donor	Liver			PFC ($x10^4$)	
	Granuloma	CNSDC	Cells	IgMPC	IgGPC
T_{NW}*	+	+	++	46.9	51.9
Lyt-2	-	+/-	+	9.4	11.0
L3T4	+	+	+++	51.5	69.9

*T_{NW}: whole T cells obtained from a nylon wool culumn.

3.2. This type of T cells also induced CNSDC-like ductal changes of salivary glands and the pancreas.

3.3. The same numbers of $CD4^+$ and $CD8^+$ T cells were observed around both ducts.

3.4. We could not detect the MHC class II antigen on the damaged epithelial cells by the second week postinjection.

4. DISCUSSION

CNSDC-like lesions were induced in the exocrine glands as well as bile ducts. We suppose that donor $CD4^+$ T cells are activated by Ia antigen on the cell surface and differentiate into the helper and/or cytotoxic cells, which inducing ductal lesions.

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Detection of TdT positive cells in the mesenteric lymph nodes of immunodeficient rats

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Introduction

Studies from several laboratories have shown that protein deficiency causes reversible impairment of mucosal immune responses to protein antigens in rat gut and a delay in the maturation of cell mediated immune function [1]. Besides, impaired T-lymphocyte transformation and changes in the absolute number of T-lymphocyte subsets have been associated with the presence of null cells containing TdT (terminal deoxynucleotidyl transferase) in the peripheral blood of malnourished children and in acute lymphoblastic leukemias.

The aim of the present study is to report the finding of TdT^+ cells in the MLNs of protein depleted immunodeficient rats, which under normal conditions do not present detectable amounts of these cells by comparative phenotypic analysis [2].

Materials and Methods

Cell markers were determined by labeling TdT^+ cells with W3/13, MRC-0X19 or MRC-0X6 mAbs by means of the indirect immunofluorescence technique. Animals and diets as well as suspensions were prepared as described in previous work [3].

Results

The presence of solely positive TdT cells and double positive $TdT^+W3/13^+$ was significantly increased in the PF group (rats fed a protein-free diet until loss of 25% of their initial body weight), as well as in casein refed groups R9 and R21, when compared to control groups in which no TdT^+ cells were detected (data not shown).

The comparison of the antigenic phenotype of TdT^+ cells is shown in Table 1. TdT^+0X19^+: thymic origin vs. TdT^+0X6^+: bone marrow (BM) procedence, did not render any statistical difference, implying that contribution from both organs was equal.

A kinetic study was undertaken by feeding weaning rats on a protein free diet for 3 and 10 days (PF3 and PF10). Results are presented as percentage in Table 2. The abrupt increase in all TdT subsets as soon as protein depletion begun, indicated that the thymus was the main provider in the first stages of protein deprivation.

TABLE 1. Absolute numbers of TdT^+ cells expressing the 0X19 and 0X6 phenotype (*).

	TdT^+	$0X19^+$	TdT^+0X19^+	TdT^+	$0X6^+$	$TdT^+ 0X6^+$
PF	1.1±0.1	0.33±0.06	0.61±0.06	0.93±0.16	0.34±0.04	0.62±0.08
R9	1.4±0.5	0.50±0.09	2.3±0.5	0.80±0.20	0.74±0.20	2.45±0.25
	- - -	18.3±1.6	- - -	- - -	17.6±2.9	- - -

(*) X±SE of 5-6 rats per group. (- - -): no cells detected as in control.

TABLE 2. TdT^+ CELLS IN MLNs EXPRESSING THE W3/13 AND 0X6 PHENOTYPE (**).

	TdT^+	$W3/13^+$	TdT^+W13^+	TdT^+	$0X6^+$	TdT^+0X6^+
Weaning	2.5±1.7	19.8±1.2	2.3±0.4[a]	1.0±0.3	29.2±1.9	1.2±0.7
PF3	10.3±2.0	8.6±0.3	20.0±09[b]	21.3±1.3	10.8±1.8	13.5±1.5
PF10	28.1±1.4	5.1±0.4	17.1±3.1	30.0±5.5	8.5±2.5	18.0±3.7
PF total	15.7±4.6	2.4±1.2	10.1±4.1[c]	12.0±3.5	9.2±0.3	16.0±1.0

(**) X±SE of 4 rats per group. [a]$p<0.02$; [b]$p<0.001$; [c]$p<0.005$ different from TdT^+0X6^+ of the same group.

Discussion

Increased levels of circulating glucocorticoids during nutritional stress (PF group) together with the lack of essential aminoacids, may be halting differentiation processes in the thymus and the BM at a very immature stage. The origin of MLN cells containing TdT^+ may be attributed to:
1- a 'leakage' of immature thymus processed cells into the bloodstream: TdT^+ cells presenting T-cell markers (TdT^+0X19^+) or;
2- BM deprived cells which may include B-cell precursors and prothymocytes destined for the thymus: TdT^+ cells bearing antigens encoded by class II MHC (Ia^+) (TdT^+0X6^+).

Comparative phenotypic analysis allowed us to ascertain that: these TdT^+ cells are originated in both the thymus and the BM.

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A monoclonal antibody which recognises a receptor for interleukin 2 on pig lymphocytes

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1. INTRODUCTION

Factors with functions similar to IL-2 (maintenance of blast cell proliferation) have been isolated from supernatants of activated pig lymphocytes (Gasbarre, Urban & Romanowski, 1984; English, Binns & Licence, 1985). Further, pig lymphoblasts are responsive to both native and recombinant human IL-2 (English et al, 1985; Fong & Doyle, 1986; Stott, Fenwick & Osburn, 1986). It appears, therefore, that pig lymphoblasts can express a receptor capable of IL-2-binding and signal transduction. We have attempted to raise a monoclonal antibody capable of recognising an IL-2 receptor on pig lymphocytes.

2. MATERIALS AND METHODS

Mice were immunised with 1 or 2 day pig lymphoblasts prepared by culturing pooled lymphocytes from six pigs in the presence of Concanavalin A (con A). Hybridoma supernatants were screened by their ability to inhibit pig-TCGF maintained proliferation of pig lymphoblasts.

3. RESULTS

3.1. Effect on IL-2 maintained proliferation

Pig lymphoblasts, prepared following 3 days culture with con A, continue to proliferate in the presence of recombinant human IL-2. In the presence of the monoclonal antibody 231 3B2 proliferation is inhibited. This inhibition can be reversed by the addition of excess IL-2.

3.2. Effect on binding of IL-2 to lymphoblasts

The effect of the monoclonal on binding of IL-2 to pig lymphoblasts was

determined using radiolabelled rhIL-2. Table 1 shows the amount of bound rhIL-2 in molecules per cell in the absence of any inhibitor and in the presence of 250u/ml of cold rhIL-2 or of dilutions of the monoclonal 231 3B2. The monoclonal antibody 230 3B2 almost completely inhibited binding of rhIL-2 at dilutions of 1/10 or 1/100.

Inhibitor	Concentration	Bound (molecules/cell)	% Specific Binding
None		167.6	100
rhIL-2	250 u/ml	24.7	0
231 3B2	1/10	32.6	5.5
	1/100	30.0	3.7
	1/1000	105.7	56.7

3.3. Expression of antigen in vitro

By fluorescent microscopy the antigen is present on the surface of both Con A- and PHA-activated cells but not on freshly isolated peripheral blood lymphocytes. Flow cytometry demonstrates the binding of the monoclonal antibody by activated lymphocytes between 6 and 12 hours after exposure to con A. Cells expressing the antigen are initially small but begin to enlarge (increase in forward angle light scatter) after 12 hours in culture.

3.4. Expression of antigen in vivo

Immunohistology on PLP fixed tissues using the monoclonal 231 3B2 demonstrates a population of positively staining cells within the lamina propria of three week old piglets. The evidence presented suggests that these are activated cells. We intend to extend this study to piglets of different ages and to the effects of dietary change.

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Effect of total parenteral nutrition (TPN) on gut associated lymphoid tissue

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ABSTRACT

Effect of total parenteral nutrition (TPN) on gut associated lymphoid tissue (GALT) was examined in rats. Rats were maintained with TPN by the method of Steiger. Control rats were allowed to have the same solution orally. The size of Peyer's patches in TPN rats was smaller than that in controls. The number of lymphocytes and the ratio of helper/non-helper T cells were decreased in intestinal lymph of TPN rats. The ratio of helper/non-helper T cells was decreased both in the intraepithelial space and in the lamina propria. These data indicate that oral food intake might play a role in the regulation of morphology and function of GALT.

INTRODUCTION

Gut associated lymphoid tissue (GALT) plays an important role in intestinal mucosal immunity. Our previous study revealed that dietary stimulation increased lymphocyte transport in intestinal lymph.[2,3] There is possibility that dietary stimulation plays a role in the maintenance of morphology and function of GALT.

In this study, we investigated that morphological changes of GALT induced by long term absence of oral food intake in rats maintained with total parenteral nutrition (TPN).

MATERIALS AND METHODS

Male Wistar rats weighing approximately 230g were maintained with TPN by the method of Steiger et al.[3] Control rats were allowed to have the same amount of nutrient solution orally.

After two weeks, GALT in control and TPN rats was examined as follows: 1. The area of Peyer's patches were determined. 2. Mesenteric lymphatic duct was cannulated and lymph flow was measured. Lymphocyte subsets were assessed in intestinal lymph using fluorescence activated cell sorter (FACS). 3. Lymphocyte subsets of isolated lymphocytes from Peyer's patches were assessed using FACS. 4. Lymphocyte subsets and immunoglobulin containing cells in the small intestinal mucosa were studied using an indirect immunoperoxidase technique. 5. Monoclonal antibodies against rat lympocytes W 3/13, W 3/25, OX-8 and OX-6 were regarded as pan T cells, helper T cells, non-helper T cells and Ia positive cells respectively.

RESULTS

There was no statistical difference in the weight gains between two groups. The area of Peyer's patches was decreased in TPN rats. Intestinal lymph flow and the number of lymphocytes transported in intestinal lymph were markedly reduced in TPN rats. The percentage of helper T cells in intestinal lymph was decreased in TPN rats. The ratio of helper/non-helper T cells in intestinal lymph was significantly decreased in TPN rats. The percentages of all subsets of Peyer's patch lymphocytes were not statistically different between two groups. The numbers of all T cell subsets in the intraepithelial space were decreased in TPN rats (Fig.1), and the number of non-helper T cells in the lamina propria was increased in TPN rats (Fig.2), resulting a reduction of the ratio of helper/non-helper T cells both in the intraepithelial space and in the lamina propria. The number of IgA containing cells in the lamina propria was significantly decreased in TPN rats.

SUMMARY AND CONCLUSION

In TPN rats; 1) The area of Peyer's patches was significantly decreased. 2) Reduction of intestinal lymph flow, the number of lymphocytes and the ratio of helper/non-helper T cells were observed in intestinal lympho. 3) The number of each T cell subset and the ratio of helper/non-helper T cells were decreased in the intraepithelial space of the small intestine. 4) The number of non-helper T cells was increased and the ratio of helper/non-helper T cells was decreased in the lamina propria of the small intestine. 5) Population of IgA containing cells was decreased in the lamina propria of the small intestine. These data suggest that oral food intake might play an important role in the regulation of morphology and function of GALT.

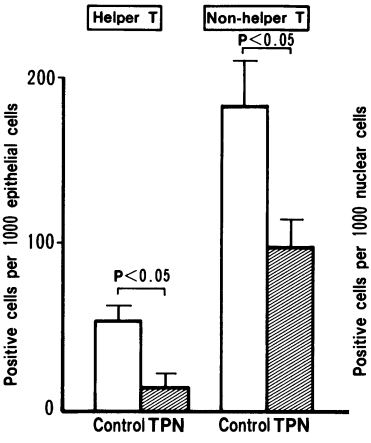

Fig. 1 T cell subsets in the intraepithelial space

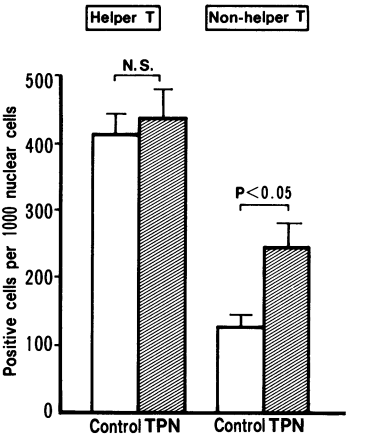

Fig. 2 T cell subsets in the lamina propria

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Immunological selection of related and unrelated microflora by germfree mice

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ABSTRACT. In this study the colonization pattern was investigated of a mouse-related (Colonization Resistance-)microflora (CRF-MF) and an unrelated human microflora (HUM-MF) in germfree isolated C3H/Rij mice. The composition of the HUM-MF appeared to change whereas the CRF-MF did not. HUM-MF associated animals also mounted a higher serum antibody level against 50-40% of the high concentrated obligate anaerobic bacteria, whereas this fraction was less than 10% in CRF-MF mice. We conclude that the colonization of the gut is determined immunologically by the antigenic composition of the microflora for which tolerance appears to exist.

1. Introduction

The colon contains numerous species of obligate anaerobic bacteria up to a concentration of 10E10 - 10E11 per g. faeces. This part is suggested to play a role in the pathogenesis of progressive wasting disease during immunological disorders like Graft versus Host Disease and in athymic mice [4]. Wasting disease is absent in immunocompromised germfree and in specified pathogen free mice e.g. associated with a Colonization Resistance microflora (CRF-MF) [2].

The intestinal microflora contains bacteria which are classified as indigenous (host-related, tolerogenic) or non-indigenous (host-unrelated, immunogenic). In mice, the immune response against intestinal bacteria from other (xenogeneic) animal species was found to be higher compared to intestinal bacteria from syngeneic or allogeneic mice. The level of the immune response against intestinal bacteria was found to be inversely correlated with the concentration in the faeces [1].

In this study the colonization pattern of CRF-MF and a Human-MF (HUM-MF) was investigated in germfree isolated C3H/Rij mice. Both MF's established in the gut of the mice.

2. Materials and Methods

Germfree isolated C3H/Rij mice monoassociated with *Staphylococcus epidermidis* were orally contaminated with 1:20 (w/v) (day 0) and 1:10 (w/v) (day 3) saline suspended faeces from murine CRF-MF associated Wistar rats or from a healthy human. Stools were cultured quantitatively for *St. epid.* and *Enterobacteriaceae* and Gram-stained for comparison.

Four and 6 weeks after the initial contamination, mice were intra-peritoneally (i.p.) injected with 0.5 ml. of a washed 1:50 (w/v) saline suspension of their association MF. Controls were injected with saline. Eight weeks after contamination all animals were exsanguinated. Sera and contents of caecum and colon were sampled individually.

Total serum anti-MF antibody titres as well as the ratio immunogenic/tolerogenic bacteria were detected by using an indirect immunofluorescence slide technique. The slides were stained with FITC conjugated Rabbit anti-mouse total Ig (Nordic), dilution 1:40.

Statistical evaluation was carried out with the Student's t-test at a significance level of $p < 0.05$.

3. Results

Within 3 days after the 1st contamination the stool cultures for *St. epid.* were all negative, which means that *St. epid.* were eliminated by the association MF's.

Gram-staining of association HUM-MF and 8th week-MF in HUM-MF mice showed that coccoid and small rod shaped bacteria,which were predominantly present in the former, had been replaced by long shaped fusiform bacteria 8 weeks later. No changes were found in CRF-MF mice

The faecal concentration of *Enterobacteriaceae* was only found to be increased significantly ($P < 0.05$) in HUM-MF animals during the first week after association

*TABLE 1. Total anti-association-MF and -8th-week-MF serum antibodies (10*2log #) in CRF-MF and HUM-MF associated C3H/Rij mice (SD between brackets)*

MICE			MICROFLORA	
ASSOC.MF	I.P.	N =	ASSOC.	8TH WEEK
CRF-MF	SALINE	4	5.3 (1.3)	2.0 (1.7)
CRF-MF	CRF-MF	8	6.6 (1.1)	5.6 (2.9)
HUM-MF	SALINE	6	8.7 (1.0)	4.0 (2.7)
HUM-MF	HUM-MF	5	10.0(0.7)	6.8 (0.8)

Except for the i.p. injected CRF-MF animals, anti association-MF antibody levels were significantly higher compared with the 8th week-MF (Table 1). HUM-MF animals all mounted higher anti-MF antibodies compared to CRF-MF animals. Moreover, the percentage of immunogenic highly concentrated obligate anaerobic bacteria in the association-MF and in the 8th week-MF was about 40-50% respectively in the HUM-MF mice, whereas these percentages were less than 10% in the CRF-MF mice.

4. Discussion

This study provides additional evidence that the composition of the intestinal microflora is determined by the specific immune reactivity of the host against colonizing bacterial species [3]. In addition to the requirement of proper enzymes for the digestion of nutrients provided by the host, the colonization in the gut may additionally also be determined by the antigenic composition of the indigenous microflora for which namely acquired tolerance exists.

Despite an initial low CR for *Enterobacteriaceae* in HUM-MF associated mice, the suspensions of CRF-MF and HUM-MF used for association both provided adequate CR to transient type bacteria. The HUM-MF, however, simplified obviously in the course of eight weeks. This perhaps antibody dependent process could be detected since the animals were maintained strictly isolated.

The present study makes likely that it is possible for a host organism to live in association with a persistent high ratio immunogenic/tolerogenic bacteria. This ratio may amoung else be important for the differentiation of the mucosal lining, which is known to be determined by the presence of microflora and submucosal lymphoid cells.

5. Acknowledgements

This study was financially supported by the Dutch Koningin Wilhelmina Fonds, grant 85-08.

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Deposition and short term clearance of radiolabelled bacteria and latex particles from rat organs

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Introduction

In-vivo kinetic studies of inhaled bacteria has been carried out in order to determine if the retention of deposited viable bacteria as an example for biologically active particles in comparison to inert dust particles is due to different short-term clearance mechanisms in the respiratory and gastrointestinal (GI) tract. An exposition model has been developed to study the elimination rates of Erysipelothrix rhusiopathiae and of inhaled polystyrene latex (PSL) microspheres in rats after inhalation compared with intratracheally application.

Previous investigators have reported pulmonary clearance and distribution of bacterial aerosols by evaluation of bacteria viability (for review see [1]) or by scanning radioactivity associated to bacteria [2] short-term kinetics of inhaled radiolabelled bacteria in rats has not been reported so far. Deposition and clearance rates of inhaled PSL microspheres in rats were described by [3] using an external thoracic and an excised lung technique.

Materials and Methods

In order to maintain viability of bacteria an external labelling using a homologous rabbit antiserum-, biotinylated protein A-, streptavidin-^{125}J complex was used. The conditions and stoichiometry of the labelling procedure were optimized using enzyme immunoassays (EIA) based on solid and fluid phase systems. Additionally a second internal labelling using ^{75}Se-methionine has been performed to determine the leaching rate of radioactivity in-vivo and the elimination following intratracheal application.

Trained female Fisher 344 rats were exposed one hour to aerosols made from a external ^{125}J labelled non virulent E.rh. strain (MMAD 1.13 µm, GSD 2.63, 5 x 10^8 bacteria/l air) and ^{51}Cr PSL microspheres (MMAD 0.46 µm, GSD 1.65) using a nose-only chamber and a jet nebulizer. 5 x 10^8 double labelled bacteria were given intratracheally to a third group. Up to 24 h serial external body measurement of gamma-activities in-vivo combined with excised organ radioactivity data determined by a gammacounter permit quantitative assessment of bacteria elimination in rats.

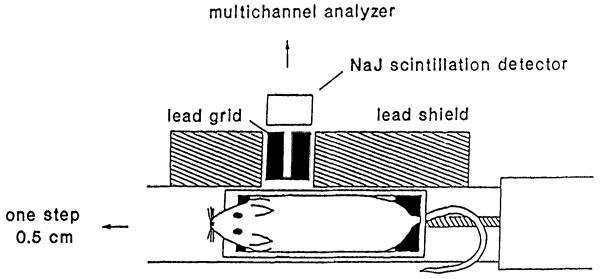

Fig.1 Experimental set-up for extracorporal measurement of ^{125}J and ^{51}Cr-activities in rats

Results

In-vivo 10 min after bacteria exposition 18 % of the measured radioactivity was within the head region, 16 % was within throat and neck, 22 % was within the lung region, 31 % was within the stomach and liver region, and 14 % within the lower intestine. The corresponding evaluated percentages for the exicised organs are 19, 4, 16, 21, and 40 %. The in-vivo percentages of PSL deposition are 14, 17, 34, 18, and 15 %. The PSL results from measuring excised organs are 21, 7, 47, 21, and 5 %. The percentages of ^{125}J and ^{75}Se given intratracheally and measured with a gammacounter are 7, 6, 63, 6, 2 % and 20, 7, 60, 1, 1 %, respectively. After 24 h these percentages had changed to 7, 9, 12, 11, 12 % and 4, 3, 8, 3, and 7 % for the deposited bacteria aerosols measured in-vivo and by gammacounter. Corresponding PSL rates are 11, 16, 25, 12, 14 % and 4, 1, 40, 2, and 5 %. For intratracheal application the percentages are 4, 6, 52, 9, 19 % and 8, 8, 34, 21, and 31 %. Immediately after exposure and up to 24 h we were able to isolate viable bacteria from lung, heart, liver, kidney, and spleen out of both groups.

Discussion

As an effect of the non-pulmonary radioactivity load transisting the GI tract the results of the extrathoracic measurements did not accurately reflect the data of organ measurements but minimizes the number of animals required for estimation of clearance parameters. To obtain data from exicsed organs is the most accurate way for determine clearance parameters. Immediately after inhalation more than half of the particles deposited are within the GI tract. GI clearance seems to be finished after 24 h. The results indicate that short-term bacteria elimination in the lung is faster than PSL clearance. Measuring bacterial lung clearance following intratracheal application depends upon the labelling method used but in comparison to aerosol application both clearances rates are diminished.

Acknowledgement - Supported by Fraunhofer-Gesellschaft, München, Germany

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Role of oxygen radicals in immune complex-induced enteropathy in rats

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ABSTRACT. In immune complex (IC)-induced enteropathy in rats, mucosal blood flow was decreased and thiobarbituric acid (TBA) reactants in the intestinal mucosa were increased. The injury score and TBA reactants in the intestinal mucosa were reduced in rats treated with superoxide dismutase (SOD) and catalase, polymorphonuclear leukocyte (PMN)-depleted rats and decomplemented rats. Generation of superoxide derived from PMN stimulated with IC was detected by the MCLA-dependent chemiluminescence method. Lipid peroxidation and PMN-derived oxygen radicals may play a pathogenetic role, and complement may be involved in lipid peroxidation in IC-induced enteropathy in rats.

1. Introduction

The small intestine is not usually considered to be a target of IC-induced tissue injury. A few studies have indicated, however, that the intestine is a site of IC deposition, which might be associated with tissue injury. Recently, oxygen radicals are considered to have a pathogenetic role in tissue injury. This study was designed to clarify the role of oxygen radicals in IC-induced enteropathy in rats.

2. Materials and Methods

2.1. PREPARATION OF IC

Antisera were obtained from rabbits immunized with bovine serum albumin (BSA) and Freund's complete adjuvant. The antibody level of antiserum was determined by Ouchterlony's analysis. IC was formed at five-fold antigen excess. Antiserum and antigen were mixed for 2 hr at 4 °C and then centrifuged at 1500 g for 20 min, and the supernate was used as soluble IC.

2.2. IC-INDUCED ENTEROPATHY

After an overnight fast, 3 ml of serum containing IC was injected i.v. into male Wistar rats. One hour after the injection, the rats were killed and their organs were examined. The intensity of serosal hyperemia was scored as descrived by Kirkham. TBA reactants in the small intestinal mucosa were determined by the method of Ohkawa. Mucosal blood flow was measured using a laser Doppler flowmeter. One hour before IC injection 50,000 U/kg of human SOD and 90,000 U/kg of

Table Injury score and TBA reactants in intestinal mucosa

Groups	n	injury score	TBA reactants(nmol/mg prot.)
Normal	5	0	0.38±0.04
BSA	5	0	0.39±0.05
Antiserum	5	0	0.39±0.05
BSA+Antiserum	5	3.4±1.1	0.57±0.09 **
Control	10	3.7±1.2	0.58±0.13
SOP	8	2.4±0.9 *	0.44±0.07 *
SOD+Catalase	8	2.1±0.6 **	0.43±0.09 *
Control	8	3.1±1.1	0.60±0.13
PMN-depletion	8	1.1±1.2 **	0.41±0.10 **
Control	8	3.9±1.2	0.67±0.17
Complement-depletion	8	1.8±1.0 **	0.46±0.09 **

Values are mean±S.D.. *$p<0.02$, **$p<0.01$

bovine catalase were injected s.c.. PMN-depleted rats were injected i.p. with 10 ml of anti-PMN antibody 18 hr before IC injection. Decomplemented rats were injected i.p. with 200 U/kg of purified cobra venom factor 6 hr before IC injection.

2.3. DETECTION OF SUPEROXIDE DERIVED FROM PMN

Superoxide generation by peritoneal PMN stimulated by purified IC was detected by the MCLA-dependent chemiluminescence method.

3. Results

3.1. MUCOSAL BLOOD FLOW AND TBA REACTANTS IN THE SMALL INTESTINAL MUCOSA

After IC injection, mucosal blood flow was significantly decreased, and hyperemic bands in the small intestine were observed. Histological findings showed congestion with accumulation of PMN and hemorrhage in the mucosa and submucosal lymphoid tissues. TBA reactants in the intestinal mucosa were significantly increased. After injection of BSA or antiserum alone, these pathological changes were not observed.

3.2. EFFECTS OF SOD, CATALASE, PMN-DEPLETION AND COMPLEMENT-DEPLETION

The injury score and TBA reactants in the intestinal mucosa were significantly reduced in the rats treated with SOD and SOD plus catalase, in PMN-depleted rats, and in decomplemented rats. In addition, superoxide generation from PMN stimulated with IC was detected and SOD (100U) scavenged the superoxide generation from PMN.

4. Discussion

Hemorrhagic enteropathy was observed after the i.v. injection of IC in rats. Mucosal blood flow was decreased and TBA reactants in the intestinal mucosa were increased. The treatment with SOD and SOD plus catalase, PMN-depletion, and complement-depletion decrease both the injury score and the increase in TBA reactants in the intestinal mucosa. Generation of superoxide from PMN stimulated with IC was detected in vitro. These results suggest that lipid peroxidation and PMN-derived oxygen radicals play an important role in the pathogenesis of IC-induced enteropathy in rats and that complement is involved in lipid peroxidation in this model.

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Effect of immunostimulation on different models of experimental gastric ulcer

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ABSTRACT. The influence of immunostimulation on rat experimental gastric ulcer was studied in different models: pylorus ligature, alcohol treatment, indomethacin application. Significant protection of the gastric wall was achieved by antigen-specific stimulation in pylorus ligated and alcohol treated animals. The missing protection in the indomethacin model gives indirect evidence for prostaglandins participating in antigen-specific protective reactions of gastric mucosa.

1. Introduction

Immunologic stimulation of rat and dog antral mucosa produces various effects on stomach functions: increased antral secretion of gastrin [1] and enhanced microcirculation in the mucosa of antrum and duodenum [2]. Production of gastrin with consecutive acid secretion as well as microcirculation are important factors in the genesis of gastro-duodenal ulcer disease. We have examined the influence of immunostimulation in experimental gastric ulcer models with different pathogenetic mechanisms.

2. Materials and Methods:

Subcutaneous immunization of male *Wistar*-rats was carried out with the synthetic antigen NIP (4-hydroxy-3-iodo-5-nitrophenylacetic acid). NIP was coupled to ovalbumin (NIP-OA) for immunization, to human gamma globulin (NIP-HGG) for intragastric challenge. The following ulcer models were used: pylorus ligature (*Shay*-ulcer, n=23), alcohol ulcer (intragastric application of 1 ml pure alcohol, n=12), and indomethacin ulcer (intraperitoneal injection of indomethacin, 2 mg/100 g b.w., n=11). Test groups were immunized rats (NIP-OA) which received the antigen NIP-HGG (10 mg) intragastrically. Controls were immunized animals receiving the control protein HGG or non-immunized animals receiving NIP-HGG or HGG. In the SHAY ulcer all transmural defects were counted 18 hours after pylorus ligature and application of NIP-HGG or HGG. The induction of alcohol ulcers was preceeded by the intragastric application of test substances 15 minutes prior to alcohol. Indomethacin was applied twice simultaneously with the antigen or HGG. Lesions were judged according to ulcer-index methods [3] 1 hour after alcohol and 4 respectively 14 hours after indomethacin treatment.

3. Results:

3.1. SHAY ULCER

In pylorus ligated rats mostly forestomach ulcers are induced under the conditions of stasis and distension by intraluminal acid [4]. The application of antigen reduced lesions of the gastric wall. In test animals (immunized with NIP-OA and challenged intragastrically with NIP-HGG) transmural

lesions (perforations, penetrations, transmural lesions) were significantly ($p<0.05$) lower (29 %) compared to control groups (61 %, 67 %). In previous experiments gastrin production was decreased in test animals corresponding to an increase in intraluminal gastric pH [5].

3.2. ALCOHOL ULCER

Pure alcohol leads to a characteristic pattern of mucosal damage, necrosis and hemorrhage, mainly along the mucosal folds in the glandular part of the rat stomach [3]. After alcohol treatment animals of the test group presented with a significantly ($p<0.02$) lower ulcer index. Antigen-specific stimulation with NIP produced an average index of 24 ± 6 mm in the test group whereas controls showed much higher index values (57 ± 10 mm, 61 ± 9 mm, 78 ± 19 mm). Mucus secretion in test animals was enhanced as judged by histomorphometric methods 30 and 60 minutes after application of alcohol.

3.3. INDOMETHACIN ULCER

The treatment with indomethacin produces dose dependent mucosal damage in corpus and antral mucosa of rat stomach. In contrast to the SHAY model and alcohol ulcer experiments *no* reduction of lesions (index method) was seen in the antigen-specific challenged animals. Controls showed higher (27 ± 9 mm, 37 ± 10 mm) and lower (17 ± 6 mm) index figures compared with the antigen-specific challenged animals of the test group (25 ± 7 mm).

4. Conclusions:

Antigenspecific stimulation of gastric mucosa significantly reduces mucosal damage in experimental gastric ulcer models (pylorus ligature and alcohol treatment). This ulceroprotective effect is not seen in indomethacin induced ulcers. Since prostaglandins (PG E_2, PG $F_{2\alpha}$) are known as protective agents [3] the inhibition of prostaglandin synthesis by indomethacin (inhibition of cyclooxygenase) may be responsible for this effect. Reduction of gastrin secretion followed by an increase of intragastric pH and enhanced mucus production are possible mechanisms in the immunologically mediated gastric reaction. Although detailed mechanisms are yet unknown these experiments clearly demonstrate that immune reactions may play an important role in the genesis of experimental gastric ulcers. The immunoprotective effect as demonstrated above could add a new dimension to ulcer treatment.

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Role of oxygen radicals and polymorphonuclear leukocytes in gastric mucosal injury

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ABSTRACT. In platelet activating factor (PAF) or compound 48/80 (C48/80)-induced gastric injury in rats, gastric mucosal blood flow was decreased and thiobarbituric acid (TBA) reactants in the gastric mucosa were increased. Gastric injury and the increase in TBA reactants in the gastric mucosa were inhibited in rats treated with superoxide dismutase (SOD) and/or catalase, and in polymorphonuclear leukocyte (PMN)-depleted rats. Oxygen radicals derived from PMN and lipid peroxidation may be involved in the pathogenesis of gastric injury in these models.

1.Introduction

Oxygen radicals and lipid peroxidation have been suggested to play a pathogenetic role in gastric injury. This study was designed to examine a role of PMN-derived oxygen radicals in gastric injury induced by PAF and C48/80, a mast cell degranulator.

2.Materials and Methods

2.1. PAF-INDUCED GASTRIC INJURY

PAF (3μg/kg) was i.v. injected to male Wistar rats (200g). One h after PAF injection, gastric mucosal blood flow, TBA reactants in gastric mucosa and gastric injury score (0: normal, 1: mild hyperemia, 2: moderate hyperemia and several erosions, 3: severe hyperemia and multiple erosions) were determined. SOD (50,000 U/kg) and/or catalase (92,400 U/kg) were s.c. injected 1.5 h before PAF injection. Allopurinol (50mg/kg) was orally administered 48 and 24 h before PAF injection. Anti-PMN antibody (10ml/kg) obtained from immunized rabbit serum was i.p. injected 18 h before PAF injection.

2.2. C48/80-INDUCED GASTRIC INJURY

C48/80 (0.75mg/kg) was i.p. injected to male Donryu rats (200g) once a day for 4 days. The total area of gastric erosions and TBA reactants in the gastric mucosa were determined 24 h after the final administration. Polyethylene glycol (PEG)-SOD (150,000 U/kg, i.v.) and/or catalase (90,000 U/kg, s.c.) were injected with every C48/80 injection. Allopurinol (50mg/kg) was orally administered 48 and 24 h before the initiation of C48/80 treatment and with every C48/80 injection. Anti-PMN antibody (10ml/kg) was i.p. injected 18 h before

Table 1 Effects of various agents on PAF-induced gastric injury

Agents	Injury score	TBA reactants(nmol/g wet wt)
Control	2.5±0.2 (n=16)	113.2±5.2 (n=5)
SOD	1.7±0.3*(n=9)	100.9±3.4 (n=5)
Catalase	2.0±0.3 (n=8)	107.3±5.4 (n=5)
SOD and Catalase	1.3±0.3*(n=9)	94.0±3.8*(n=5)
Allopurinol	1.4±0.3 (n=7)	100.2±5.0 (n=5)
Anti-PMN Ab.	0.7±0.2*(n=6)	96.0±4.8*(n=5)

mean±S.E. *p<0.05

Table 2 Effects of various agents on C48/80-induced gastric injury

Agents	Area of erosions(mm^2)	TBA reactants(nmol/mg protein)
Control	73.0±31.4 (n=10)	0.512±0.104 (n=10)
SOD	38.5±25.6* (n=10)	0.405±0.059 (n=10)*
Catalase	46.0±19.6* (n=10)	0.427±0.070 (n=10)*
SOD+Catalase	28.8±29.0**(n=10)	0.398±0.087 (n=10)*
Allopurinol	22.5±17.5* (n= 8)	0.392±0.055 (n= 8)**
Anti-PMN Ab.	11.2±24.4**(n=16)	0.371±0.088 (n=10)**

mean±S.D. *p<0.05, **p<0.01

the initiation of C48/80 treatment and with every C48/80 injection. Gastric mucosal blood flow was measured after C48/80 injection.

3.Results

3.1 CHANGES IN GASTRIC MUCOSAL BLOOD FLOW AND TBA REACTANTS IN THE GASTRIC MUCOSA

Gastric mucosal blood flow was significantly decreased, and hemorrhagic erosions and hyperemia were observed in the glandular stomach and TBA reactants in the gastric mucosa were significantly increased after PAF or C48/80 injection.

3.2. EFFECTS OF VARIOUS AGENTS ON GASTRIC INJURY AND ON TBA REACTANTS IN THE GASTRIC MUCOSA

In PAF-induced gastric injury (Table 1), injury score was significantly inhibited by the treatment with SOD, SOD plus catalase and anti-PMN antibody. The increase in TBA reactants in the gastric mucosa was significantly inhibited by the treatment with SOD plus catalase and anti-PMN antibody.

In C48/80-induced gastric injury (Table 2), both the total area of gastric erosions and the increase in TBA reactants in the gastric mucosa were significantly inhibited by the treatment with SOD, catalase, SOD plus catalase, allopurinol and anti-PMN antibody.

4. Discussion

In PAF or C48/80-induced gastric injury, gastric mucosal blood flow was decreased and TBA reactants in the gastric mucosa were increased. Gastrinc injury and TBA reactants in the gastric mucosa were inhibited in rats treated with SOD and/or catalase, and in PMN-depleted rats. These results suggest that PMN-derived oxygen radicals may play a pathogenetic role in gastric injury induced by PAF and C48/80.

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Expression of procoagulant activity in crude cell suspensions and in cell subpopulations of equine bronchopulmonary lavage cells

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1. Introduction

In previous studies we had seen, that cell free supernatants of equine respiratory secretions contained procoagulant activity (PCA) which was correlated with the severity of chronic pulmonary disease and with the quantity of neutrophils in the secretions (Grünig et al. 1988). The purpose of this study was to investigate which equine lung cell type produces PCA.

2. Materials and Methods

Bronchopulmonary lavage was performed in 10 healthy horses and in 39 horses with chronic pulmonary disease. Cell subpopulations were recovered by discontinous density gradient centrifugation on either Percoll or Metrizamide gradients. PCA was determined in cell free supernatants, in crude cells, and in cell subpopulations.

3. Results and Conclusions

All supernatants and unpurified cell suspensions contained PCA. Macrophages were enriched in subpopulations of low density. Neutrophils could not be purified by density centrifugation using either gradient medium although they were increased in subpopulations of high density (#'s 5, 6). This unexpected density shift requires further investigation.

As an example, the results of PCA determinations obtained from metrizamide gradients are discussed in this manuscript (fig. 1). PCA content was highest in cell-subpopulations # 1 and # 6 which contained dead cells (viability 64 %). PCA of subpopulation #6 might have been derived from activated cells or from cell membranes or "cell ghosts" which could not be quantitated. Therefore, this subpopulation was excluded from the

statistical analysis of correlation between PCA and cell content of subpopulations. PCA was positively correlated with macrophage content ($p<0.001$) and negatively correlated with neutrophil ($p<0.02$) and with lymphocyte ($p<0.001$) content. Similar correlations were found with subpopulations recovered from Percoll gradients. Therefore, PCA of lung cells most likely originates from macrophages.

4. References

Grünig, G., Hermann, M., Winder, C., Von Fellenberg, R. (1988) "Procoagulant activity in respiratory tract secretions from horses with chronic pulmonary diseases" Am.J.Vet.Res. 49, 705-79.

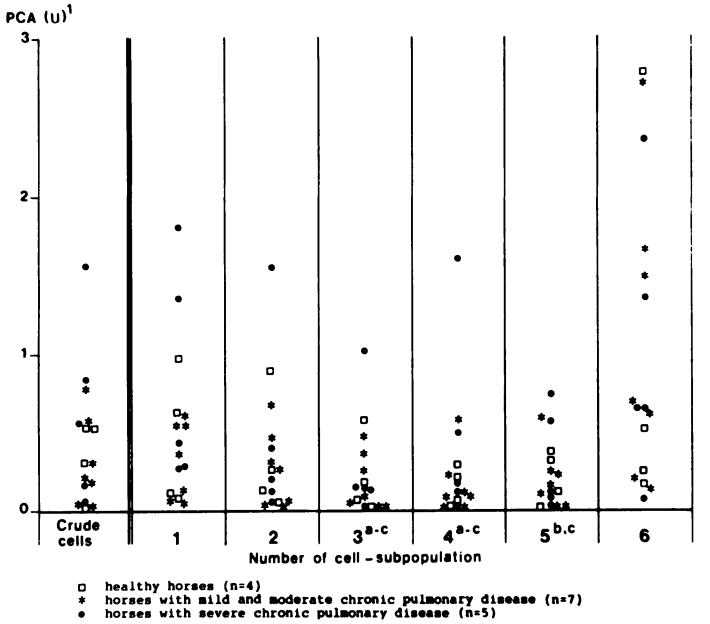

Figure 1: Procoagulant activities induced by crude cells or by cell-subpopulations recovered from discontinous Metrizamide gradients.

1) Clotting times were determined and converted to U according to a standard curve prepared with commercial thromboplastin. PCA was normalized for 3 x 10^4 cells in the assay.
a-c) PCA content significantly ($p<0.05$) different from crude cells[a], from subpopulation # 1,[b] or from subpopulation #6.[c]

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Primary *in vitro* immunisation of rabbit Peyer's patch B-cells with keyhole limpet haemocyanin

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1. INTRODUCTION

The major protective barrier of the gut against ingested pathogens is the gut-associated immune system. Immune response to foreign substances or organisms is modulated by several interactive cellular components of the gut-associated lymphoid tissue (GALT) by direct cell contact and by soluable mediators (lymphokines). If the bulk of the immune system resides in the GALT, then the intestinal lymphocyte populations and mechanisms of cell-mediated immunity become extremely important in carrying out specialized effector functions. Peyer's patch, which appears crucial for antigen recognition, is one such lymphoid compartment. Hence, the purpose of this study is to determine if KLH would be immunogenic in vitro for rabbit Peyer's patch cells. The present study describes primary in vitro immunization, examines the in vitro antibody response by the Enzyme-Linked Immunosorbent Assay (ELISA), and explores the kinetics of the immune response of the Peyer's patch cells.

2. MATERIALS AND METHODS

2.1 Primary Immunization Assay. Rabbit Peyer's patch cell suspension (from 6-8 weeks, male, NZW, Hazleton, Denver, PA), with a final cell density of $3x10^6$ cells/ml in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY), were set in round-bottom, 96-well, microtiter culture plates (Costar, Cambridge, MA) using our established primary in vitro microculture method previously described(1). Wells were immunized with various KLH concentrations in a range from 0.5 ng/ml to 150 ng/ml in triplicate cultures. Microculture supernatants were serially assayed from separate microculture plates (for day 7, 9, 12 and 14) for antibody production.

2.2 Assay for Antibody Production. Anti-rabbit immunoglobulin activity was estimated by ELISA. Round-bottom, soft polyvinyl, 96-well microtiter plates were coated with KLH proteins in a 0.01 M carbonate buffer (pH 9.8), positive and negative control included, blocked with phosphate-buffered saline (pH 7.4) containing 0.1% bovine serum albumin. (PBS-BSA) washed 5 times, supernatants (0.1 ml) were transferred to microtiter plates and incubated overnight at 4°C. The next day plates were washed 5 times, followed by IgG fraction IgM-specific, peroxidase conjugated anti-rabbit immunoglobulin (Cappel, Melvern) 1:3000 in (PBS-BSA) was added, then substrate (2, 2^1-azino-di-(3-ethyl benzthiazoline) sulfate), Solution A, and hydrogen peroxide, Solution B (Kirkeguard and Perry, Gaithersburg, MD) addition. "Developed" ELISA plates were read at wave length 405 nm by a Dynatech (Dynatech, Alexandria, VA)MR580

ELISA reader. The data collected from the reader was directed to a VMS/VAX computer for storage and descriptive statistical analysis of each assay.

3. RESULTS

To determine if a KLH protein would be immunogenic in vitro and produced antibodies toward itself, rabbit Peyer's patch cells in microculture as described above, were immunized with KLH, produced antibody was examined by ELISA. The results of ELISA assays obtained for an experiment are shown in the figure below; it shows a typical antibody response of Peyer's patch cells immunized in vitro with KLH protein and towards KLH, increasing culture time in days and the antibody response as optical density units of x-axis, y-axis, respectively. Each point along the curve represents the results of triplicate microcultures and the immunizing doses are shown. The graph compares the immunizing doses on different culture days. It can be seen that maximum response was at day 12, at a dose of 5 ng/ml, and dose responses are parallel. These results are similar to the rabbit spleen cell response to in vitro immunization with KLH.

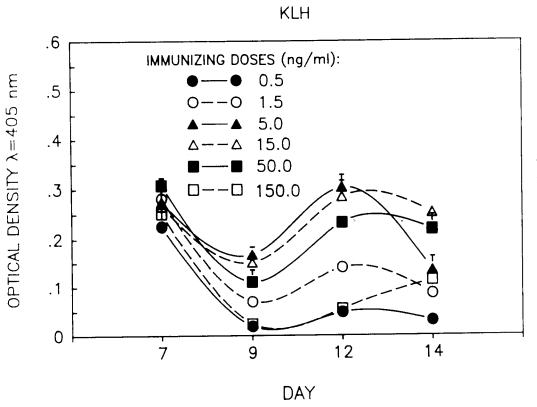

Figure 1. Peyer's patch cell IgM antibody response to KLH

4. DISCUSSION

The present study demonstrates that a rabbit Peyer's patch cell suspension can be successfully, and reproducibly, immunized in vitro with KLH, and can be subsequently assayed for antibody production by ELISA. It is accepted that the lymphoid organ responsible for initiating the immune response to enterically deposited antigens is the Peyer's patch. In conclusion, the success of this model system suggests that the Peyer's patch, a more appropriate gut-associated lymphoid tissue (GALT), may be a useful test system for potential enteric vaccine constructs.

5. REFERENCE

1. Axelrod, D.A. (1985) Primary and secondary in vitro immune response of the rabbit Peyer's patch and spleen to RDEC-1 pili, Clinical Immunology and Immunopathology 36, 371-377.

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