# Immunology of the Gut

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# Immunology of the Gut

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In memory of the late Joseph Heremans

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# Introduction

P. J. LACHMANN

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The area that this symposium is going to work over is an extensive one. I was taught at medical school that the absorptive area of the intestine alone was the size of a tennis court, though I cannot vouch for this being true. It is the overlapping fields of gastroenterology in its widest sense—to include even dental caries, in which perhaps not all gastroenterologists regard themselves as practitioners—that have been brought together with immunology in this symposium. Immunological mechanisms can be thought of as protecting the *milieu intérieur* from the *milieu extérieur*, at any rate as far as macromolecules are concerned. The mechanisms for doing this have to apply not only to the normal, sterile tissues of the body, which are protected from most macromolecules except for the insect's sting and the doctor's needle, but also to regions like the gut and to some extent the respiratory tract where the *milieu extérieur* has succeeded in getting inside the *milieu intérieur* and where foreign molecules in the form of both food and microorganisms exist in quantities which, compared with the quantities that immunologists usually deal in, are astronomical.

It is interesting here to draw a distinction between the respiratory tract and the gut. The respiratory tract is protected from antigenic material by a number of mechanical barriers, but once antigenic material gets down to the lung it is almost as antigenic there as when it is introduced parenterally. In the gut, on the other hand, there is no mechanical protection; and in parts of the gut, at any rate, foreign macromolecules are present in large quantities; but in this site they are not antigenic to any substantial extent. Therefore it is not surprising that specialized immunological mechanisms have evolved around areas like the respiratory tract and particularly the gut to deal with these special situations, and these special mechanisms are concerned intimately with the secretory immunoglobulins and especially with IgA. This is the particular immunoglobulin with the characterization of which Joe Heremans was in-

timately involved, and his absence, through his premature death, makes not only this meeting but the whole immunological community very much the poorer. Others are going to discuss IgA—its production, its functions and the cells that make it. The other limb of the immune response, cell-mediated immunity, will not be left out, and I am relieved to see that even complement will have a mention!

The interactions of immunology and clinical medicine have always been very much two-way. It is not just that work on immunology has expanded the field of medicine but also very much that studies of individual patients, the subtle experiments of nature, have made considerable contributions to our understanding of immunology. By no means the worst example of this is the role that studies of multiple myeloma and myeloma proteins have had in increasing our knowledge of immunoglobulin structure, function and genetics, and IgA and IgG, both of which are to be discussed, give good examples of this. It is still a very active field and we shall hear in some detail about the current status of the work on alpha chain disease, that extraordinary situation where partial immunoglobulin molecules are produced and secreted.

The study of immunity deficiency is a second good example of the two-way interaction, and here too work is still very active. We shall hear not only about the effects of primary immune deficiencies, of the sort where the immunodeficient child develops infections; but also about the paradoxical situation, which is now being increasingly appreciated, where minor forms of immune deficiency may become manifest not in increased sensitivity to infection as much as in increased liability to produce allergic diseases due to inappropriate immunological reactivity.

In their turn, studies of immunology have led to great advances both in the understanding and the prevention of disease. Perhaps prophylactic immunization against infection is still the major man-made change in the pattern of morbidity throughout the world, and it is to be hoped that in this area there is still much progress to be made. One could imagine, for example, that a vaccine against dental caries might have almost as much effect on morbidity in the world as a vaccine against cholera, though perhaps not as much as a vaccine against hookworm or malaria (although the latter is not directly relevant to the gut). To develop effective prophylactic immunization one has to understand the processes by which immunological mechanisms bring about immunity. This is a curiously complex topic in which much interest has been taken again in the past few years after a period when there was relatively little work devoted to it. The mechanisms seem to be different for all the major classes of pathogenic organisms. One might say that they are understood badly for bacteria, and worse for worms! We are going to hear about both these topics—about

immunity to the pathogenic variants of the normal gut flora, and about immune mechanisms directed towards nematodes, where there remains the long-standing question of whether the IgE system does have any useful function, and if it does, whether it is in relation to nematodes in the gut.

One major understanding which has come from the study of the immunology of infection is that the same mechanisms which give rise to immunity can also give rise to allergic tissue damage and can themselves contribute to the manifestations of the very infectious diseases against which they also provide protection. This is true both of overt infectious disease (it has, for an example in the gut, been claimed that the diarrhoea of shigella dysentery is largely allergic in nature) and also of diseases that are not obviously infectious at all. For example, brain damage in subacute sclerosing panencephalitis is now recognized as being due to allergic reactions to measles virus infection. The extent to which mechanisms of this kind may be involved in inflammatory bowel disease is a subject of great importance, and this is also to be discussed in the symposium.

Such allergic reactions are not restricted to antigens of infectious organisms, and a consideration of disease caused by allergic manifestations to dietary antigens will be a fitting conclusion to a meeting where I am sure there will be plenty for us all to mark, learn and inwardly digest!

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# The secretory IgA system of the gut

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*Abstract* Most commonly, humoral immunity manifested in the gastrointestinal tract of mammals is due to the presence of secretory IgA antibodies. Antibody specificities have been detected in the secretory IgA of gut secretions to a wide range of naturally occurring viral and bacterial components and to test antigens such as chemically modified proteins. Much of the IgA found in gut secretions is synthesized and secreted locally by the abundant plasma cells of the lamina propria. Development of methods for establishing local protective immunity in the gut requires knowledge of the origins of these plasma cells and of the whereabouts of their precursors when they are susceptible to antigen-driven proliferation and/or maturation.

The Peyer's patches have been shown to contain a population of B lymphocytes especially rich in precursors for IgA plasma cells and in cells which can repopulate gut lamina propria with such IgA plasma cells. The Peyer's patches also appear to 'sample' gut antigens, in that small amounts of antigens are passed intact through their dome epithelial cells.

Recent experiments bearing on the origins, differentiation and maturation, antigen sensitivity, migration and lodging of precursors for gut IgA plasma cells are discussed. We use the following three systems: (1) congenic transfer of cells from different murine lymphoid cell sources or mixtures of these (CB20 → BALB/c or BALB/c → CB20) and the use of allo-antisera to IgA allotypic determinants to assess their potential to impart an adoptive IgA antibody response to the recipient and to repopulate its histocompatible lamina propria with IgA plasma cells; (2) clonal precursor analysis (the method of Klinman) both to enumerate antigen-sensitive cells in different tissues of mice and to evaluate their potential to generate plasma cells making particular isotypes and idiotypes of antibodies; (3) use of pairs of Thiry-Vella loops in rabbits, each member either bearing or lacking a Peyer's patch, and quantitation of antibodies of each isotype and of total secretory IgA to assess the response of each loop with the time after local immunization. The results from all three systems provide strong evidence for the importance of Peyer's patches in supplying cells responsible for local humoral immunity and suggest both a differentiative pathway for IgA precursors and their whereabouts when antigen may cause the expansion of a population of specific cells.

Most commonly, humoral immunity manifest in the gastrointestinal tract of mammals is due to the presence of secretory immunoglobulin A (sIgA) antibodies. Thus, in order to devise effective immunization procedures leading to the establishment of local protective immunity in the gut, one must gain some knowledge of the following: (1) the origins and locations of the cells responsible for the synthesis and secretion of sIgA; (2) the stages at which they and/or their precursors are susceptible to antigen-driven proliferation and their whereabouts when such specific expansion may be possible after both primary and secondary challenge; (3) the interactions these cells must undergo with other cell types or with humoral factors—antigens, mitogens, hormones, etc.—before their maturation to IgA plasma cells; and (4) the migration routes and any tissues of temporary domicile favoured by the cellular progenitors of the gut IgA response and any selective lodging properties that they may develop *en route* to intestinal lamina propria. Observations to be presented by ourselves and by Dr Ahlstedt later in this symposium (Ahlstedt *et al.*, pp. 115–129) suggest that an understanding of these aspects of the development of humoral immunity in the gut may also be germane to the appearance of sIgA antibodies in other secretory (exocrine) tissue. Of course, another process relevant to the occurrence of sIgA antibodies in the gut that is not directly related to the generation of a local IgA response involves the passage of the IgA antibodies across an epithelial cell barrier into the intestinal or glandular lumen, and this will be considered later by Dr Brandtzaeg (Brandtzaeg & Baklien, this volume, pp. 77–108).

Our ability to formulate these particular areas of inquiry pertinent to the development of humoral immunity in the gut follows directly from a series of basic observations made during the past 17 years, many by Professor Joseph Heremans and his colleagues. The Heremans group isolated that isotype of immunoglobulin (Ig) from human serum which we now call IgA and defined some of its characteristic properties, such as its lower isoelectric point and higher sugar content relative to other Igs and its propensity to occur in a number of polymeric forms (Heremans *et al.* 1959). Using immunohistochemical methods we were then able to show the synthesis of the IgA isotype by a class of human or rabbit plasma cells different from those making IgG or IgM (Bernier & Cebra 1965; Cebra *et al.* 1966). This separate population of IgA cells assumed greater significance when considered with the finding by Hanson, Tomasi and colleagues from their two groups that the concentration of IgA in human milk and other exocrine secretions was considerably greater than that of any other isotype of Ig (Hanson, 1960, 1961; Tomasi & Zigelbaum 1963; Tomasi *et al.* 1965). The Tomasi group characterized the human sIgA as some sort of polymer of serum IgA containing 'extra' antigenic sites (Tomasi *et al.* 1965) which were later found to occur on a separate polypeptide now

called secretory component (SC) (South *et al.* 1966). Yet another distinct polypeptide, called J chain, was later found in sIgA, IgM and polymeric serum IgAs (Halpern & Koshland 1970). Shortly after the isolation of human sIgA (Tomasi *et al.* 1965) we were able to purify its homologue from rabbit milk (Cebra & Robbins 1966) and deduce from it the molecular weight and polypeptide chain composition of sIgA: four pairs of heavy ($\alpha$) and light (L) polypeptides + one SC (mol.wt. = 60–70 000) + one J chain (mol.wt. = 15 000) (Cebra & Small 1967; O'Daly & Cebra 1971). In a comprehensive study the Heremans group went on to show that IgA either predominated over all other Ig isotypes in secretions or at least was more concentrated in secretions than in serum from all of many mammalian species examined (Heremans & Vaerman 1971). The rabbit represents a rather extreme case of IgA distribution since the concentration of this isotype, which is the major one in secretions, is about 20-fold higher in milk and 5–10 fold higher in intestinal secretions than in serum (Cebra & Robbins 1966; Robertson & Cebra 1976). An appreciation of the protective role of sIgA in the gut lumen has evolved in parallel with the molecular characterization. Although sIgA antibodies appear neither to react with Fc receptors of any cell type—and therefore do not 'opsonize'—nor to activate complement starting with Cl (Eddie *et al.* 1971), they do appear to be effective simply by complexing with antigen in the gut or at other mucosal surfaces. Thus sIgA antibodies can specifically neutralize toxins, prevent viral attachment to host target cells, and diminish adherence of bacteria to mucosal surfaces and hence the probability of colonization by them (see Ogra *et al.* 1975; Smith *et al.* 1966; Gibbons 1974; Freter 1970).

The local synthesis of much of the sIgA in secretions was inferred from the finding by Tomasi's group of many IgA plasma cells in the interstitium of human salivary glands (Tomasi *et al.* 1965) and by Heremans and his colleagues that human gut mucosa contained up to 200 000 IgA plasma cells per $mm^3$ in the lamina propria (Crabbé *et al.* 1965), or an estimated $7.5 \times 10^{10}$ of such cells in the entire gut (Heremans 1975). The lamina propria of the rabbit intestine contains a similarly large number of IgA plasma cells (Crandall *et al.* 1967). Reflecting the 10–20 fold difference in IgA concentration between secretions and serum, these IgA cells are markedly compartmentalized in lamina propria and exocrine tissue—where they comprise 85% of all plasma cells—away from most IgG and IgM plasma cells which are found in spleen and peripheral lymph nodes in the company of only 2–5% IgA cells (Crandall *et al.* 1967; Cebra *et al.* 1966). In an effort to deduce how this compartmentalization of IgA plasma cells was achieved in the rabbit, we sought a source for cells which could repopulate the gut lamina propria of lethally irradiated animals among a variety of lymphoid tissues. Among the tissues tested were Peyer's

patches, which are situated in the mucosa of the small bowel and are quite distinct from the IgA plasma cell-rich surrounding lamina propria. The histology of mammalian Peyer's patches, especially those of the rabbit, has been thoroughly described (Faulk *et al.* 1971; Waksman *et al.* 1973). Large follicles of B lymphocytes, containing many dividing cells in the deeper and lateral regions of each, are characteristic of this lymphoid tissue. Smaller, thymus-dependent areas rich in T lymphocytes occur between the B cell follicles and closer to the dome epithelium.

Using the allotypic determinants present on the L chains of rabbit Igs as markers of cellular origin, we were able to show that Peyer's patches and appendix were enriched sources of cells which could repopulate the gut lamina propria of lethally irradiated rabbits with IgA plasma cells (Craig & Cebra 1971). Relative to peripheral lymph nodes, blood or spleen, Peyer's patches contained many more immediate precursors of IgA plasma cells as judged by the pokeweed mitogen-stimulated appearance of IgA plasma cells *in vitro* upon microculture of cells from the different sources and by the number of IgA plasma cells generated in spleens of recipients of the various cell populations (Craig & Cebra 1971; Jones *et al.* 1974; Craig & Cebra 1975). A sub-population of Peyer's patch lymphocytes was identified which bore no detectable *endogenous* membrane IgM but did carry as surface markers L chain and Fab ($\alpha$) determinants associated with IgA (Craig & Cebra 1975; Jones & Cebra 1974). We were able to isolate this sub-population of cells, which usually comprised $<10\%$ of Peyer's patch lymphocytes, by fluorescence-activated cell sorting (Jones *et al.* 1974). Almost all of the immediate precursors for IgA plasma cells were found in this sub-population (Jones *et al.* 1974). A similar small minority of B lymphocytes with surface IgA has been detected in mouse Peyer's patches (McWilliams *et al.* 1974; Guy-Grand *et al.* 1974) although the potential of this sub-population has not been evaluated.

An 'antigen sampling' role has also been ascribed to the Peyer's patches, since macromolecules and even bacteria may pass through or by their dome epithelial cells and arrive intact in the midst of B lymphocyte areas (Bockman & Cooper 1973; Carter & Collins 1974). Heremans and his colleagues have made the very important observation that oral administration of either sheep erythrocytes or ferritin to germ-free mice results in the sequential appearance of IgA antibody-forming cells to the former in mesenteric nodes and then spleen and the appearance of ferritin-binding IgA cells after 30 days in gut lamina propria (Crabbé *et al.* 1969; Bazin *et al.* 1970). Gowans and his group and others (Guy-Grand *et al.* 1974; Gowans & Knight 1964; Griscelli *et al.* 1969; McWilliams *et al.* 1975) have shown that a small population of rapidly dividing lymphocytes in rat thoracic duct lymph or in mouse and rat mesenteric

lymph nodes can accumulate in the gut lamina propria. Some of these thoracic duct cells bear surface IgA and some may contain specific cytoplasmic IgA antibodies (Williams & Gowans 1975; Pierce & Gowans 1975). It is tempting to suggest that IgA precursors have their first encounter with antigen in the Peyer's patches. Thus we arrive at a consideration of the areas for inquiry suggested at the start by directing our attention to the antigen-sensitivity and immunological potential of Peyer's patch cells themselves.

## DO PEYER'S PATCHES CONTAIN ANTIGEN-SENSITIVE B LYMPHOCYTES?

Attempts to stimulate the appearance of antibody-forming plasma cells in Peyer's patches by a wide variety of antigens and immunization schedules have consistently failed (see Henry *et al.* 1970; Coppola *et al.* 1972). Similarly, efforts to adoptively transfer an antibody response with patch cells alone have been unrewarded (Katz & Perey 1973; Knudson *et al.* 1975). However, transfers of mixtures of appendix cells with autologous thymocytes into X-irradiated rabbits or of patch cells with spleen cells into syngeneic, cyclophosphamide-suppressed mice led to an increased number of IgM antibody-producing cells over that observed when appendix or patch cells were omitted from the inoculum (Ozer & Waksman 1972; Coppola *et al.* 1972). A more recent example of such 'synergism' with patch cells involved their adoptive transfer to syngeneic, X-irradiated mice along with bone marrow cells and a resulting anti-sheep erythrocyte response including some IgA antibody-producing cells (Knudson *et al.* 1975). Because of the experimental design the tissue source of the antibody-producing cells could not be ascertained. Finally, attempts to elicit an *in vitro* IgM response to sheep erythrocytes with Peyer's patch cells alone have been successful with cells of rabbit origin (Henry *et al.* 1970) and likewise for mouse cells, except that there is some controversy about the need for accessory, adherent peritoneal cells (Kagnoff & Campbell 1973) or not (Jones *et al.* 1976).

We proposed that much of the difficulty in demonstrating the expression of a primary antibody response by patch cells—especially an IgA antibody response—encountered using either an adoptive transfer or an *in vitro* assay was due to the failure to maintain either viable recipients or cultures sufficiently long enough. Thus we have adoptively transferred lymphoid cells into sub-lethally irradiated (600 R) syngeneic mice to assess both their potential to respond to antigen and their time-course of response, and into similarly irradiated congenic recipients differing in Ig allotype from the cell donors to establish the origin of any antibody-producing cells. In our first experiments

TABLE 1

Time-course of PFC response in recipients of Peyer's patch cells

| *Day of assay* | *No. of animals* | *Mean plaques/spleen* | | | |
|---|---|---|---|---|---|
| | | *IgM* | *IgG1* | *IgG2* | *IgA* |
| 7 | 2 | 740 | 0 | 0 | 0 |
| 10 | 3 | 633 | 443 | N.D. | 516 |
| 12 | 4 | 690 | 2120 | 4240 | 1200 |
| 15 | 2 | 310 | 3040 | N.D. | 740 |
| 19 | 3 | 520 | 612 | 814 | 570 |

DBA/2J → DBA/2J.
$10^7$ Peyer's patch cells + $10^7$ thymus cells.

$10^7$ Peyer's patch cells, $10^7$ spleen cells or $3 \times 10^7$ peripheral lymph node cells (excluding cells from mesenteric nodes) from normal DBA/2 mice were transferred along with $10^7$ syngeneic thymus cells into X-irradiated DBA/2 recipients. After 24 hours the mice were challenged with $5 \times 10^8$ sheep erythrocytes given intraperitoneally. The recipients were sacrificed from 7–19 days after challenge and their spleens were assayed for IgM, IgG1, IgG2, and IgA antibody-producing cells (PFC) by the Jerne plaque assay technique. Table 1 shows that by day 7 the Peyer's patch cell recipients expressed only IgM PFC. From day 10 onwards IgA and IgG PFC were also detected. Cells expressing either of these isotypes reached a maximum number around day 12–15 and declined thereafter. Table 2 shows that the IgA PFC detected 12 days after antigenic challenge are only observed in recipients of patch cells plus thymocytes and not in mice receiving mixtures of cells from other sources or thymocytes alone. In order rigorously to ascertain the source of the IgA PFC and to rule out the possibility that the response obtained in sub-lethally irradiated syngeneic recipients is due to host B cell regeneration followed by some undefined interaction with donor cells, we did congenic transfers using CB20 mice (Lieberman *et al.* 1974) as the presumptive B cell donors and BALB/c as the thymocyte

TABLE 2

Comparison of PFC responses in recipients of lymphocytes from different sources

| *Type of transfer* | *Day of assay* | *No. of animals* | *Mean plaques/spleen* | | | |
|---|---|---|---|---|---|---|
| | | | *IgM* | *IgG1* | *IgG2* | *IgA* |
| $10^7$ spleen + $10^7$ thymus | 12 | 3 | 884 | 3730 | 2940 | 6 |
| $3 \times 10^7$ lymph node + $10^7$ thymus | 12 | 3 | 504 | 3190 | 2610 | 5 |
| $10^7$ Peyer's patch + $10^7$ thymus | 12 | 4 | 690 | 2120 | 4240 | 1200 |
| $10^7$ thymus | 12 | 2 | 57 | 0 | 0 | 0 |

DBA/2J → DBA/2J.

TABLE 3

PFC response by recipients of Peyer's patch or peripheral lymphoid cells in a congenic transfer

| *Type of transfer* | *Day of assay* | *No. of animals* | *Mean plaques/spleen* | | | | $\frac{Ig2^b}{IgA} \times 100$ *spleen plasma cells* |
|---|---|---|---|---|---|---|---|
| | | | *IgM* | *IgG1* | *IgG2* | *IgA* | |
| $10^7$ CB20 PP[a] + $10^7$ BALB/cJ thymus | 7 | 2 | 2490 | 0 | 0 | 0 | N.D. |
| $10^7$ CB20 PP + $10^7$ BALB/cJ thymus | 10 | 3 | 630 | 950 | 0 | 610 | 100 |
| $2 \times 10^7$ CB20 LN + $10^7$ BALB/cJ thymus | 10 | 3 | 700 | 106 | 0 | 0 | N.D. |

[a]PP, Peyer's patch cells; LN, lymph node cells.

donors and recipients. These two strains are histocompatible (H-$2^d$) but their Ig molecules can be distinguished by allotypic markers. For instance, allo-antisera can be raised which specifically distinguish the IgA molecules of the two congenic strains which express allelic $C_\alpha$ genes (Ig-$2^a$ and Ig-$2^b$ for BALB/c and CB20 respectively) (Herzenberg 1964). Table 3 shows that cell transfers between such mice resulted in IgA PFC by day 10 only when Peyer's patches were the source of donor B cells. At this time, essentially all of the IgA plasma cells in recipients' spleens also carried the donor IgA allotype marker, as judged by staining with fluorochrome coupled anti-alpha chain and anti-Ig-$2^b$. Thus Peyer's patches of the donor were the source of the antibody-forming IgA plasma cells and do contain antigen-sensitive cells capable of generating IgA plasma cells while spleen and peripheral nodes appear to contain many fewer of such cells. The results also suggest that Peyer's patches share with other lymphoid tissue the potential to generate IgM, IgG1 and, at least in the DBA/2 strain, IgG2 plasma cells.

## ENUMERATION OF FREQUENCY OF ANTIGEN-SENSITIVE PRECURSORS IN PEYER'S PATCHES AND ASSESSMENT OF THEIR POTENTIAL TO EXPRESS VARIOUS Ig ISOTYPES

To enumerate the precursor frequency in different lymphoid populations we have employed the technique of clonal analysis developed by Klinman and his colleagues (Klinman 1969) and evaluated the clonal precursor cells (CPC) sensitive to the phosphorylcholine (PC) determinant, since considerable information is available concerning splenic CPC reactive with this hapten (Gearhart *et al.* 1975). Briefly, mice to be used for scoring CPC were carrier-

TABLE 4

Frequency of anti-phosphorylcholine (PC) precursors

| *Donor* | *Total no. cells analysed* | *Total clones/ $10^5$ B cells* | *No. clones/$10^5$ B cells with T15 idiotype* | *% anti-PC response represented by T15 idiotype* |
|---|---|---|---|---|
| Spleen | $630 \times 10^6$ | $2.28 \pm 1.78$ | $1.77 \pm 1.63$ | $74 \pm 28$ |
| Peyer's patch | $94 \times 10^6$ | $3.13 \pm 1.14$ | $1.23 \pm 0.84$ | $39 \pm 15$ |

primed with *Limulus* haemocyanin 6–8 weeks before cell transfer. They were then lethally irradiated (1300 R) and one day later were injected with limiting doses of the lymphoid cells to be tested. After 16 hours the mice were sacrificed and their spleens were sliced into 1.0 mm cubes. The spleen fragments were individually cultured in Dulbecco's modified Eagle's medium with 10% agammaglobulinaemic horse serum in the wells of microtitre plates along with an optimum concentration of PC conjugated to haemocyanin through a tri-peptide spacer ($5 \times 10^{-7}$ M in PC). At intervals culture fluid was removed from the wells and fresh medium was added. The fluids from the wells were individually assayed for anti-PC antibody, isotype of anti-PC antibody and for the presence of a common idiotype of anti-PC antibody from BALB/c mice (prototype myeloma protein TEPC 15) (Potter & Lieberman 1970) using radioimmunoassays. If the appropriate, limiting dilutions of lymphocytes are delivered to the scoring animal, then the total amount of antibody and its isotype(s) and idiotype in a positive spleen fragment can be considered to be the product of a single clone and to reflect that potential of a single precursor expressible in the CPC assay by its progeny (Klinman 1969; Gearhart *et al.* 1975). Table 4 compares the frequency of anti-PC CPC with and without the potential to express TEPC 15 idiotype in spleen and Peyer's patches. The frequency of CPC in both these tissues, based on B cell content, is similar to that previously observed for spleen (Gearhart *et al.* 1975). The lower frequency of CPC in patch cells that express the TEPC 15 idiotype than in spleen is conspicuous and provides a hint that the two cell sources may be subject to different mixtures of naturally occurring PC-determinants which may selectively expand out different sub-populations of anti-PC precursor cells. Table 5 compares the CPC from the two tissues with respect to isotypes of anti-PC antibodies expressed by their clonal progeny. The CPC of splenic origin predominantly generate clones which make only IgG1, IgM and IgG1 or IgM, IgG1 and IgA anti-PC. The population of CPC from Peyer's patches is conspicuously different in that their resultant clones produce IgM and IgA or only IgA anti-PC with a very high incidence. Fig. 1 graphically illustrates

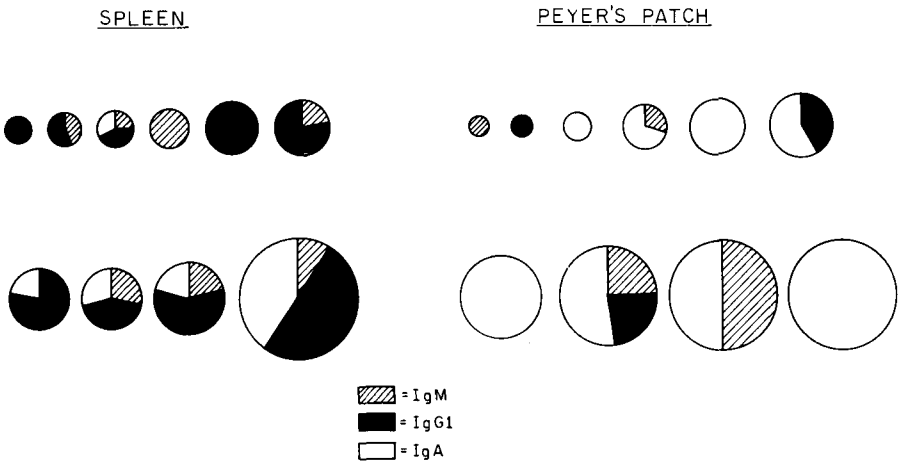


FIG. 1. Anti-phosphorylcholine (PC) response of B lymphocyte-derived clones expressing the TEPC 15 idiotype marker. The area of each circle represents the size of the clone estimated from total output of product antibody. The sectors shown in some circles represent the proportion of total antibody present as a particular isotype (IgA, IgM, IgG1). The clones depicted were chosen to be representative of much larger samples derived from both spleen and Peyer's patch.

this difference in populations of CPC as well as the relative sizes of the clones generated by the two cell sources as judged by their total output of anti-PC antibody (proportional to area of circles). There seems to be no correlation between clone size and isotype(s) expressed. Thus the Peyer's patches are as rich a source of antigen-sensitive CPC as the spleen but their potential to generate clones making IgA antibodies is far greater. The Peyer's patches are known to differ from other lymphoid tissue in some or all of the following characteristics: (1) T lymphocyte areas segregated away from B cell follicles;

TABLE 5

Isotype distribution

| *Heavy chain class* | *% B cell clones with TEPC 15 idiotype* | |
|---|---|---|
| | *Spleen* | *Peyer's patch* |
| $\mu$ | 10 | 6 |
| $\gamma 1$ | 16 | 6 |
| $\alpha$ | 2 | 46 |
| $\mu, \gamma 1$ | 26 | 3 |
| $\gamma 1, \alpha$ | 6 | 8 |
| $\mu, \alpha$ | 4 | 26 |
| $\mu, \gamma 1, \alpha$ | 36 | 8 |

(2) no organized antigen-trapping reticulum; (3) excessive numbers of suppressor T lymphocytes; (4) deficiency in accessory cells; (5) extensive B lymphocyte proliferation without *in situ* maturation to plasma cells (see: Faulk *et al.* 1971; Waksman *et al.* 1973; Bockman & Cooper 1973; Kagnoff & Campbell 1974; Kamin *et al.* 1974). Thus one unique feature of the Peyer's patch may be that it provides an environment that permits B cell division—at least in part antigen-driven—without providing those interactions necessary for maturation to plasma cells. One might suppose therefore that the patch B cell population contains cells that, on the average, are more divisions removed from the pre-B or 'virgin' B cell than those of any other lymphoid tissue. Such cells may be more likely to generate clones which express IgA. Whether, in addition to permitting such B cell divisions, the milieu of the Peyer's patch can also influence the course of differentiation (perhaps gene rearrangement) or development (gene expression) is still conjectural.

However, having demonstrated that Peyer's patches contain antigen-senstitive precursors for IgA plasma cells and that they are enriched sources of precursors for clones which express IgA, we must consider their role in populating the lamina propria of gut mucosa. We have already discussed observations by others that dividing cells from mesenteric nodes and thoracic duct lymph appear to selectively lodge in gut (p. 10). However, such short-term selective accumulation in gut has not been shown for cells of Peyer's patches. Our own studies related above have clearly shown that allogeneic patch cells contained a sub-population of B cells that could fully repopulate intestinal lamina propria with IgA plasma cells. We have now examined such repopulation in mice histocompatible with the donated cells in a system which permitted unequivocal assignment of the B cell source of IgA plasma cells.

## REPOPULATION OF HISTOCOMPATIBLE INTESTINAL MUCOSA WITH IgA PLASMA CELLS FROM CONGENIC DONORS

As before, our lymphoid cell donors were CB20 mice and the recipients were sub-lethally (600 R) X-irradiated BALB/c congenic mice. Each animal received either $10^7$ patch cells or $2 \times 10^7$ peripheral lymph node cells from CB20 mice either with or without $10^7$ BALB/c thymocytes. The addition of exogenous thymus cells appeared to have no effect on the repopulation observed. The recipients were sacrificed at various times after cell transfer. Spleens were rendered into single-cell suspensions for replicate cell monolayer preparation by cyto-centrifugation (Dore & Balfour 1965) and random cryostat sections of the small intestine were made. Each film and section was consecutively stained with fluorescein (F)-labelled anti-$\alpha$ and rhodamine (R)-labelled anti-Ig-

TABLE 6

Relative repopulation of spleen and gut lamina propria by donor-derived IgA plasma cells

| *Cell source*[a] | *Day* | *Exp. no.* | *% Ig-2$^b$/α in spleen*[b] | *Relative α+ area of gut analysed*[c] | *Average % Ig-2$^b$/α in gut*[d] |
|---|---|---|---|---|---|
| PP | 6 | 1 | 0 | – | 0 |
| PP | 6 | 1 | 0 | – | 0 |
| PP | 7 | 2 | 0 | – | N.D. |
| PP | 7 | 2 | 0 | – | N.D. |
| PP | 8 | 3 | 93 | 517 | 42 |
| PP | 10 | 2 | 100 | 498 | 47 |
| PP | 10 | 2 | N.D.[a] | 693 | 49 |
| PP | 10 | 2 | N.D. | 967 | 48 |
| PP | 12 | 1 | 100 | 2391 | 58 |
| PP | 14 | 3 | 90 | 375 | 96 |
| PLN | 10 | 2 | 15 | 662 | 21 |
| PLN | 10 | 2 | N.D. | 486 | 15 |
| PLN | 10 | 2 | N.D. | 498 | 9 |
| PLN | 14 | 3 | 45 | 423 | 36 |

[a] PP, Peyer's patch cells; PLN, peripheral node cells; N.D., not done.
[b] % of Ig-2$^b$ plasma cells of total IgA plasma cells. About 200–400 and 20–100 IgA plasma cells were present per $2 \times 10^5$ nucleated cells in spleens of recipients of PP and PLN cells respectively.
[c] About 20 plasma cells comprise 100 units of area.
[d] Determined from area measurements.

2$^b$ (anti-CB20 IgA allotype). Differential cell counts were made on the monolayers. Fields of the gut sections were randomly chosen on the basis of content of F anti-α-staining cells alone and these were photographed first and then rephotographed under filter conditions permitting visualization of any R anti-Ig-2$^b$ positive cells. Each set of transparencies from a single field was magnified, projected in turn onto the same tracing paper, and the areas of α and Ig-2$^b$ positive cells were recorded. The latter cells were always a sub-set of the former. The IgA and allotype positive areas were measured using a planimeter and the extent of repopulation was expressed as the percentage of the total α-positive area which was Ig-2$^b$ allotype positive. Table 6 indicates that very few, if any, donor-derived IgA cells appear in spleen or gut through days 6–7. However, by day 8–10 there is a sudden appearance of donor IgA plasma cells in lamina propria of patch cell recipients, when they comprise about 40–50% of the total IgA cell population. By day 14 almost all gut IgA cells of patch cell recipients are of donor origin. The peripheral nodes seem considerably less effective at recolonizing recipients with IgA plasma cells and contribute only 10–20% of such cells by day 10 and 36% by day 14. Of course, as donor-derived cells are

repopulating the gut, the host's own cells are diminishing in number. Our finding that almost complete replacement of intestinal IgA cells occurs by 14 days in Peyer's patch cell recipients is consistent with a previous estimate, based on a different measurement, of 4.7 days as the half-life of mouse IgA cells in lamina propria (Mattioli & Tomasi 1973). By 14 days the host cells should have endured three half-lives and should have diminished to about 12% of their original number. Thus comparison of different cell sources for their potential to repopulate by other methods should be more meaningful at early times (days 8–10) when the host compartment is still substantial and hence provides a more accurate internal standard.

Peyer's patch cells can certainly repopulate gut lamina propria with IgA plasma cells. However, it is still not known whether they must temporarily reside in other tissues before gaining their propensity to lodge in lamina propria or whether they pass directly to the gut, there to mature into plasma cells. Another property of these IgA precursors which has not yet been evaluated is whether they can divide after lodging in the lamina propria and before maturation into plasma cells. A truly 'local' secondary response would seem to require such a reserve potential for antigen-stimulated division.

On the basis of some of the principles deduced by ourselves and others we have attempted to devise a model system for evaluating the role of Peyer's patches after the introduction of antigen into the gut lumen (Robertson & Cebra 1976).

## A MODEL SYSTEM FOR THE STUDY OF INDUCTION OF HUMORAL IMMUNITY IN THE GUT

Many investigators have been able to stimulate the appearance of specific IgA antibodies in mammalian secretions (Blackman *et al.* 1974; Montgomery *et al.* 1974; Yardley *et al.* 1976). Particularly, Hanson and his colleagues have been able to correlate the appearance of IgA antibody-secreting cells in human milk with colonization of mothers' guts with a distinctive serotype of *E. coli* (Goldblum *et al.* 1975). Most of these studies suffer from the drawbacks that it is technically difficult to identify the precise tissues where B lymphocytes respond to antigen and to directly implicate Peyer's patches in this response. Thus, sites of earliest response of B cells to antigen could include: (1) Peyer's patches; (2) lamina propria; (3) mesenteric lymph nodes; (4) spleen and other lymphoid tissues reached by antigen via the bloodstream after absorption by the gut.

Using rabbits we have constructed a series of animals having two 20-cm isolated ileal loops (Thiry-Vella), each bearing or lacking a Peyer's patch, by

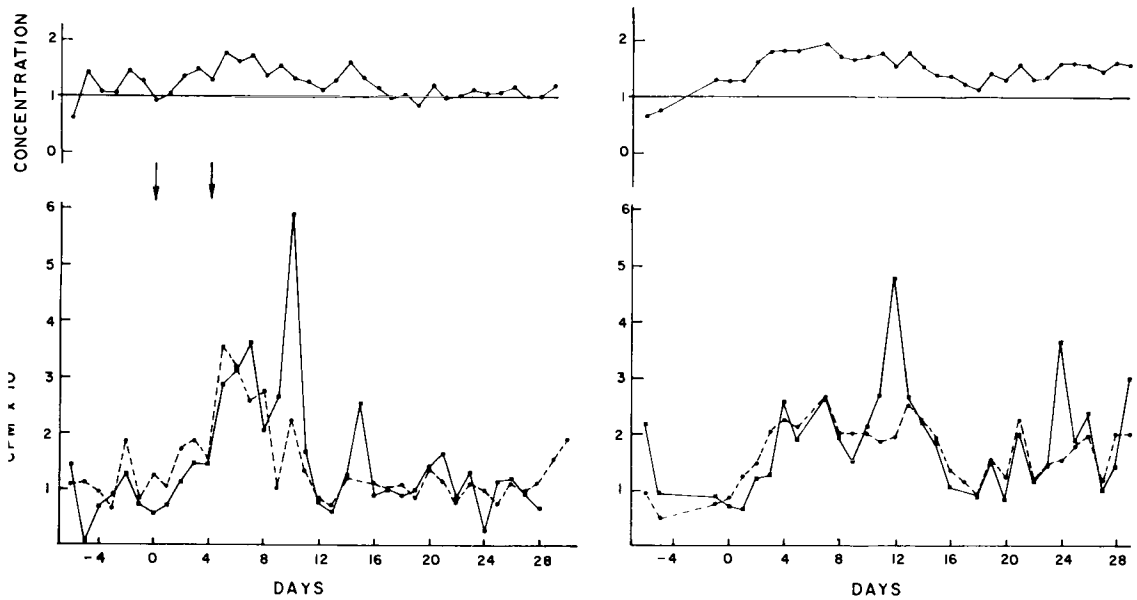


FIG. 2. Analysis of daily loop secretions of rabbit 1 (containing Peyer's patches) which received two doses of antigens, DNP-KLH and *Salmonella typhimurium*, into the loops on day 0 and day 4 (↓). Data for the stimulated loop (SL) are shown on the left and the control loop (CL) on the right. Symbols: -•--•-, specific IgA anti-DNP activity as determined by $^{125}$I anti-α in the radioimmunoassay (RIA) at a 1/600 sample dilution; ——▪——, total anti-DNP activity as determined by $^{125}$I anti-Fab in the RIA at a 1/600 sample dilution. The relative sIgA concentration (•——•) was determined at a 1/3000 sample dilution by $^{125}$I anti-Fab and anti-α BAC in the RIA. The value of '1' for the SL is 1.6 mg sIgA/ml secretions; for the CL, 2.3 mg/ml. The specific anti-DNP activity has been normalized by a factor which reflects the daily fluctuation of total sIgA.

the method used by Yardley and his group (Keren *et al.* 1975). These loops, though isolated, still maintain their mesenteric attachment and thus their neurovascular supply. Seven days after surgery, antigens—2,4-dinitrophenylated keyhole limpet haemocyanin (DNP-KLH) (400 μg) and heat-killed *Salmonella typhimurium* LT-2 Zinder ($4 \times 10^7$)—were introduced into one of the loops (distal) and saline was introduced into the other control loop (proximal). After 12 hours the remaining antigens were flushed out of the loops with air and the introduction of antigen was repeated at the desired times. The secretions of the loops were collected at intervals and the total sIgA, total antibody and total IgA antibody concentrations were measured by radioimmunoassay using appropriate insoluble antigens (Elson & Taylor 1974; Klinman & Taylor 1969). The specific serum response was also monitored. Fig. 2 shows that a specific IgA anti-DNP response could be elicited by two applications of the mixture of antigens into the distal loop on days 0 and 4 (Robertson & Cebra 1976). The

TABLE 7

Absolute concentrations of anti-DNP antibodies and of sIgA in the intestinal loop fluids

| *Animal* | *Loop* | *Peyer's patch*[c] | *Pre-immune levels* | | *Maximum response levels* | |
|---|---|---|---|---|---|---|
| | | | *Total IgA (mg/ml)* | *Anti-DNP (μg/ml)* | *Total IgA (mg/ml)* | *Anti-DNP (μg/ml)* |
| 1 | SL[a] | + | 3.1 | 33 | 4.5 | 138-216 |
| | CL[b] | + | 3.4 | 51 | 6.8 | 147 |
| 2 | SL | + | 1.7 | 16 | 2.2 | 87 |
| | CL | + | 1.1 | 27 | 2.7 | 49-86 |
| 3 | SL | + | 3.1 | 22 | 4.8 | 60 |
| | CL | + | >5 | 27 | >5 | 80 |
| 4 | SL | + | 2.9 | 6 | 4.1 | 25-36 |
| | CL | + | 5 | 15 | >5 | 72 |
| 5 | SL | — | 3.7 | 5 | >5 | 10 |
| | CL | — | 3.2 | 4 | 5.5 | 14 |
| 6 | SL | — | 2.7 | 23 | >5 | 44 |
| | CL | — | 2.8 | 17 | 4.7 | 36 |
| 7 | SL | — | 5.4 | 5 | >5 | 24 |
| | CL | + | >5 | 13-34 | 3.4-6.5 | 34-42 |
| 8 | SL | + | 1.5 | 4 | 5.6 | 45 |
| | CL | — | 0.072 | 7.7 | 0.43-0.96 | 44 |

[a] SL, stimulated loop.
[b] CL, control loop.
[c] +, Peyer's patch present in loop; —, Peyer's patch absent from loop.

time-course of appearance of anti-DNP antibodies was somewhat variable but often showed an early rise (days 4–8) followed by a slight decline and then another modest rise to a rather constant level, which occurred later (days 12–20). Most often, the change in antibody concentration with time followed the same course in secretions of the distal (immunized) and proximal (control) loop. Almost all the anti-DNP present in the secretions was IgA and this was in the form of dimers and higher polymers. Immunohistochemical staining showed plasma cells making anti-DNP in the lamina propria of the loops. Throughout the 20–40 days during which loop secretions could be monitored, no rise in serum anti-DNP titres of any isotype could be detected. The maximum concentration of IgA anti-DNP often rose 5–10 fold over pre-stimulation values to 100–200 μg/ml (Table 7). However, if the loops lacked Peyer's patches, the increase in anti-DNP in secretions was either slight or did not occur at all (Table 7). We believe that this model system implicates the Peyer's patch in the response to antigens impinging on the gut mucosa. The observations are consistent with antigen-stimulated B cell division in the patch follicles followed by departure of these cells via the mesenteric nodes, through the circulation and back to the lamina propria of all sections of the gut. The possibility of temporary residence of these cells in mesenteric nodes and further antigen-driven

proliferation and maturation before further emigration is also consistent with our findings.

We have tried to provide some recent findings pertinent to the stimulation of humoral immunity in the gut and to sharpen the areas of inquiry we posed at the outset as relevant to this process. I am sure that subsequent contributors to this symposium will further elucidate the mechanisms for both the eliciting and the functioning of gut immunity.

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# Discussion

*Soothill:* How secure is your evidence that the Peyer's patch is relevant to the IgA response in the experiments with ileal loops?

*Cebra:* The general finding in about 20 rabbits is that if there is an IgA response, it is manifest in the secretions of both loops, and the time-courses are almost identical. If the loop receiving antigen lacks a patch, there is no response in either loop, or a minimal response in both; but if the loop receiving antigen bears a patch, the response tends to be higher in both loops, though not as high in the control loop as when both loops have a patch. The overall net increase in IgA antibodies when both loops have a patch is 10-fold, sometimes 20- or 25-fold, from about 20 μg/ml of background antibody to 200 μg/ml. If neither loop contains a patch, there is 10–20 μg/ml of antibody before stimulation which may rise to 30 or 35 μg/ml with antigen.

*Porter:* So the segmental immunization reported by Ogra & Karzon (1969) is really determined by where you do the immunization within a loop preparation? I assume they must have been applying antigen high up in the intestine, away from the Peyer's patch region?

*Booth:* No; Ogra used colonic segments.

*Cebra:* It is possible that one can, in the absence of a patch, stimulate a local immune response. One unanswered question is whether the cells that arrive in the lamina propria have a residual potential for antigen-driven proliferation. Such a potential would be necessary for a true secondary response. It has been shown that a small fraction of antigen can pass intact through gut epithelium

in areas lacking a patch (Walker *et al.* 1972), so I would not rule out stimulation in the absence of a patch. However, I think our results show that a patch is essential for effective stimulation.

*Soothill:* Have you shown that this is not simply a result of the particular region of the bowel you are dealing with?

*Cebra:* No. In our choice of sites for stimulation we have favoured loops from the distal ileum, which is richer in patches and is known to play a role in stimulation by certain gut organisms that accumulate proximal to the ileal-caecal valve (Carter & Collins 1974).

*Soothill:* The result could be an effect of organisms present acting as adjuvants?

*Cebra:* Yes. In fact, there could be an enhanced response due to the salmonellae given with the antigen. We are now giving the antigen without salmonella organisms.

*Lehner:* A possible adjuvant effect is important, but it depends on the sequence of adding adjuvant to the antigen; this certainly applies to lipopolysaccharides (Johnson *et al.* 1956; Lagrange & Mackaness 1975). Have you tried to give lipopolysaccharide first and then antigen? This would not increase the humoral antibody response but might cause suppression.

*Cebra:* We have not done this but we are doing various extensions of this experiment, involving the use of LPS and varying the order of addition, and also we are assessing the effect of carrier-priming in a hapten–protein system. You are probably partly right in thinking that LPS is affecting the response. At least three-quarters of the animals show an increase in total IgA in the loop as well as the increase in specific antibody. We think this increase in IgA concentration may have something to do with the bacteria we put in the loop.

*Gowans:* Your evidence that you get a similar antibody response in both loops when you give antigen into only one loop contradicts other evidence in sheep which shows clearly that the highest concentration of specific antibody is found in the challenged loop (Husband & Lascelles 1974). I will be reporting similar results in rats (p. 39). How do you reconcile these differences?

*Cebra:* Pierce & Gowans (1975) have shown that antigen can influence IgA cell lodging. Probably at least two factors are involved in determining the magnitude of the response in different segments of the gut. One is a random lodging of B lymphocyte precursors of IgA plasma cells in the lamina propria after stimulation and emigration of cells from the patch through the mesenteric lymph nodes. The other factor might be a selective accumulation and/or proliferation of cells at sites adjacent to antigens or antigen-bearing material in the gut. Most of our rabbits are immunized two to four times, and immunization is still going on when we see the first responses. Perhaps by using small amounts

(<400 μg) we are not detecting as big a role for local antigen in determining sites of IgA secretion as others have found.

*Gowans:* Did you study the time-course of the response? Is there any time at which the response is highest in the loop with a patch?

*Cebra:* The overall response may be less in the non-immunized loop but it follows very much the same time-course.

*Porter:* I want to take up the question of the class of antibodies. In your studies with *Trichinella* infections in rabbits (Crandall *et al.* 1967) you showed that the first cells in the response were IgM cells. Our work in the pig (Allen & Porter 1973) shows that in the early local immune response of the intestine to bacterial antigens, or in normal bacterial colonization in the developing pig, IgM cells far outnumber IgA cells over the first 4–5 weeks of life. The early antibody response is largely IgM. McNeish has similar findings in relation to *Escherichia coli* infections in infants in which IgM was the chief immunoglobulin (McNeish *et al.* 1975). Of course, IgM is a major secretory immunoglobulin. Your precursor cells in the loop experiments are probably μ-negative in their surface characteristics. If you had looked at your antibody response in the loops in terms of IgM, might you have found different characteristics of the response?

*Cebra:* Perhaps. There is always the possibility of inactivation and degradation of IgM antibodies before assay; if IgM antibodies are more susceptible we might have missed them in assaying the loop secretions. Had we looked at antibody-forming cells in the lamina propria, which we are doing now (which is harder because one cannot disperse lamina propria cells and do plaque-forming cell analysis), we might have seen significant numbers of IgM antibody-forming cells in the gut.

Let me try to reconcile two kinds of data in my paper. I spoke of immediate precursors for IgA plasma cells. These are important in the earliest phase of the IgA response. This early IgA response could even be regarded as a secondary response by patch cells that may be many divisions away from 'virgin' antigen-sensitive cells and may have been primed by naturally occurring antigens. I think that these immediate precursors are surface IgA-positive cells that can generate large clones of IgA plasma cells. The antibody responses seen later (days 10, 12, 15 after stimulation) are probably derived from μ-bearing cells that give rise to the μ plus α clones. Such μ+ cells may also be abundant clonal precursors in the Peyer's patches. In other words, I think antigen-sensitive cells may be at different stages of maturity in the patches. One doesn't know the importance of T cell interactions in influencing the expression of IgA by clonal progeny of μ+ antigen-sensitive cells originating in the patch or in selectively stimulating maturation of the daughters derived from α+ cells. The time-sequence and relative numbers of IgM and IgA

antibody-forming cells may vary, depending on the amount of T cell cooperation.

*Porter:* In your adoptive transfer studies did your μ-negative cells give rise predominantly to IgA producers?

*Cebra:* Yes. Plasma cells derived from μ-negative Peyer's patch cells found in either spleen or gut lamina propria of allogeneic rabbit recipients predominantly were IgA producers. Probably the precursors of these cells are equivalent to those cells in mouse Peyer's patches that generate clones which produce only IgA. However, other precursors found in mouse Peyer's patches can give rise to clones expressing IgM and occasionally IgG1 as well as IgA. It is probable—although not formally shown—that these latter precursors bear IgM on their surface.

*Pierce:* You mentioned a biphasic antibody response in your loops. Was the second phase of that response similar in magnitude to the first, and equal in both loops? Have you an explanation for this observation?

*Cebra:* We often but not always see the biphasic response. If one phase is missing it is usually the earlier one. An explanation may be derived from our clonal precursor analysis: at any given time one has in the patch an assortment of cells having equal proliferative potential; any can give rise to, say, a thousand antibody-secreting cells. Some of these cells in the patch are differentiated to the point where they can provide clones expressing only IgA, and I think these are responsible for the earliest phase of the secretory IgA response. These precursors could be regarded as secondary cells, even though they have not been deliberately primed. Other antigen-sensitive cells which probably bear IgM occur in the Peyer's patches and these may be even more abundant than the precursors which generate clones expressing only IgA. These go through further divisions after antigen stimulation before producing daughters that make IgA. I would postulate that these cells account for the later phase of the secretory IgA response. It also seems possible that the development of maximal expression of a secretory IgA response is dependent on the time-course of the generation of cooperating T cells.

*Gowans:* You have never shown that μ-bearing cells are the precursors of cells which eventually make IgA, have you?

*Cebra:* No, not formally, but we have shown that a high proportion of antigen-sensitive cells from the patch reactive with the phosphorylcholine determinant can generate clones which produce IgM and IgA. Assaying with the DNP rather than the phosphorylcholine determinant, the picture is a little different (P. J. Gearhart & J. J. Cebra, unpublished work 1976). Almost half of the clones derived from the spleen or patch contain some cells which express IgM. However, there are hardly any clonal precursors for DNP in the

unimmunized mouse which generate clones making only IgA. Almost all of the derivative clones expressing IgA also make IgM or IgM plus IgGl. While we have not shown in these assays that μ-bearing cells go on to make IgA, there is a strong suggestion that some of their daughter plasma cells do.

*Pierce:* Have you any evidence of a third or fourth wave or is this response in the loops only biphasic, not multiphasic? Secondly, you gave antigen repeatedly. Do you time these responses in relation to the first dose of antigen or the last? Is it possible that the biphasic response is related to the schedule of multiple doses?

*Cebra:* The phases of the response do not seem to be related to the timing of antigen doses, since the last antigen administration is usually at the beginning of the response and most of our observations have been made after stimulation on days 0 and 4. It is not clear if there are subsequent waves of secretory IgA responses because the system gets rather 'noisy' later.

*Pepys:* Have you any data on a delta-like heavy chain on the Peyer's patch cells, in view of the work showing that delta is a major heavy chain isotype on such cells in mice (Vitetta *et al.* 1975)?

*Cebra:* No.

*Parrott:* In your adoptive transfer experiments, you always added thymocytes, evidently because there are not enough T cells in Peyer's patches. If you took Peyer's patches from germ-free mice or from mice of other ages, would you need to add the thymocytes?

*Cebra:* For the adoptive transfer of a sheep red blood cell response you can leave out the thymocytes; there are sufficient T cells in the patch. However, if you remove the T cells from the Peyer's patch cell population, there is no appreciable adoptive antibody response transferred with the residual cells. If T cells from a primed animal are added to a Peyer's patch cell inoculum, more IgA PFC cells arise in the spleens of the recipients and these appear sooner than in recipients of patch cells alone. We feel that there is a T cell-dependence of the expression of the IgA plaque-forming cells (PFC) and part of the delay in the appearance of IgA PFC until 10–12 days after cell transfer might be due to the necessity for generating sufficient T cells. We are now investigating the role of suppressor and cooperating T cells in this adoptive transfer system.

*White:* You mentioned the dome epithelium and the implication seemed to be that antigens were entering through this epithelium. Where is the antigen? You said that there is no antigen-retaining reticulum in Peyer's patches. Is the antigen associated with antibody?

*Cebra:* The work I quoted was that of Bockman & Cooper (1973) and of Walker *et al.* (1972). When ferritin or peroxidase has been introduced into the gut lumen the active protein has been found in vesicles in dome epithelial cells,

and presumably must be transported through these cells since the proteins are also found among the patch lymphoid follicles. Very quickly thereafter the active proteins may be found trapped in the reticulum of draining mesenteric lymph nodes. I don't know what the mechanism is by which protein is transported across the dome epithelial cell. Perhaps the mechanism is similar to that found in studies by Rodewald (1975) on transport of maternal immunoglobulin across the absorptive gut epithelium of newborn mice and rats. There appears to be a membrane-bound receptor for immunoglobulin which becomes bound to membrane within vesicles upon endocytosis. After trans-cellular passage the immunoglobulin is ejected on the basal side of the cell by a reversal of endocytosis.

Cells that can take up antigen have been observed scattered through the patch (Faulk *et al.* 1971), so I would not exclude any trapping of antigen there, but there is no organized antigen-trapping reticulum in the patches. I know of no studies concerning the role of antibody in transport of antigen into the patch or in trapping it there.

*Evans:* You mentioned the difficulties of dispersing lamina propria cells for plaquing studies. Methods have been described for doing plaquing on sections (Berenbaum & Stringer 1970). Have you found these to be impractical on intestine?

*Cebra:* We have not been able to make the plaquing method work for gut sections because of non-specific lysis. We want to follow the cells after adoptive transfers to the lamina propria and we have resorted to an immunohistochemical method.

*Parrott:* Your main thesis is that the Peyer's patches populate the whole of the lamina propria. Dr Crabbé did beautiful studies (Crabbé *et al.* 1970) showing that there are more IgA cells in the villi close to the Peyer's patches than in more distant villi, and we (Parrott & Ferguson 1974) have pointed out that there are just a few lymphatic connections directly from Peyer's patches to those near villi where you can show with labelled cells that a few cells go straight through.

*Cebra:* I am familiar with these histological studies (Crabbé *et al.* 1970) and agree that lateral migration of IgA precursors directly from patches into the adjacent lamina propria may be an alternative means of populating the lamina propria. However, I believe that most observations support the lymph/blood circulation route as the major one leading to population of lamina propria.

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*Immunology of the Gut*
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# The natural history of the cells producing IgA in the gut

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*Abstract* The IgA-secreting cells in the lamina propria of the small intestine are derived from large lymphocytes which enter the blood by way of the thoracic duct and then migrate into the gut where they complete their differentiation into plasma cells. Three aspects of this cellular traffic have been examined in rats.

1. The cells in thoracic duct lymph which give rise to IgA-secreting cells in the lamina propria are among those which carry surface IgA. Blast cells lacking surface immunoglobulin migrate mainly into the Peyer's patches and do not contribute to the IgA response.
2. Studies on a secondary antibody response to cholera toxoid, in which the challenge was given into a Thiry-Vella loop, showed that the antibody-containing blast cells in thoracic duct lymph were derived from Peyer's patches. The mesenteric nodes contributed little, if anything, to the cellular response in the lymph.
3. The idea that secretory component is a signal for the emigration of large lymphocytes from the blood into the lamina propria lacks experimental support. Secretory component does not bind to the IgA on the surface of thoracic duct cells. On the other hand, antigen in the gut may play an important part in immobilizing large lymphocytes in the lamina propria once they have migrated.

Immunity against potentially harmful microorganisms and macromolecules which may enter by way of the gut is provided by antibodies which are secreted into the intestinal lumen. The plasma cells which synthesize these antibodies are unusual in two ways: they are situated locally in the lamina propria of the intestine and not in lymphoid tissue; and in man and most animal species the predominant class of immunoglobulin which they synthesize is IgA. There is now evidence that the population of IgA-secreting cells in the gut is maintained by a traffic of large lymphocytes which enter the blood by way of the thoracic duct and then migrate from the blood into the intestinal lamina propria where they complete their differentiation into plasma cells (Gowans & Knight 1964; Guy-Grand *et al.* 1974: Pierce & Gowans 1975). This paper is concerned with

three aspects of the generation and delivery of this remarkable local immunity: (1) the nature of the cells in thoracic duct lymph which migrate into the gut, (2) the origin of these cells, and (3) the factors which may influence their accumulation in the lamina propria.

Although IgA antibodies play an important protective role against enteric pathogens and their products it must be emphasized that both the small and large intestine of normal animals contain large numbers of IgA-secreting plasma cells. The antigens against which these antibodies are directed have not been identified but the density of plasma cells does not appear to be related in a simple way to the bacterial load in different parts of the intestine: the bacterial content of the large gut is considerably higher than that of the small, but this is not reflected in the density of plasma cells in the respective laminae propriae. Possibly, the absorption of sensitizing doses of dietary proteins constitutes a potential hazard against which the secretion of specific IgA antibodies is the normal response. The complex abnormalities sometimes associated with selective IgA deficiencies also suggest that the full range of function undertaken by IgA has probably not yet been uncovered. This paper is only indirectly concerned with the functions of IgA antibodies but it is necessary to point out that the cells in the gut which synthesize them are being continually generated in normal, healthy animals. The present experiments have been carried out on inbred strains of rats, raised under specific pathogen free conditions but later held in a conventional animal room for various periods before study. Unless otherwise stated the rats were not intentionally immunized.

## ORIGIN OF INTESTINAL IgA-SECRETING CELLS FROM THORACIC DUCT LYMPHOCYTES

The thoracic duct collects lymph by way of the intestinal lymphatics from the whole intestinal bed, that is, from the large and small gut and from its associated lymphoid tissue, the mesenteric lymph nodes and the Peyer's patches (Fig. 1). In the absence of intentional immunization, both the small and large lymphocytes in thoracic duct lymph are derived almost exclusively from the intestinal bed. This has been established in rats by comparing the cell output from the thoracic duct with that from the intestinal lymphatics (Mann & Higgins 1950; G. Mayrhofer & J. L. Gowans, unpublished). The large lymphocytes, which make up less than 10% of all the cells in thoracic duct lymph, are formed by cell division in the intestinal lymphoid tissue because very few of them normally recirculate from the blood back into the lymph (Gowans & Knight 1964), and because they virtually all become labelled *in vivo* following a continuous 12-hour infusion of tritiated thymidine (Gowans 1959).

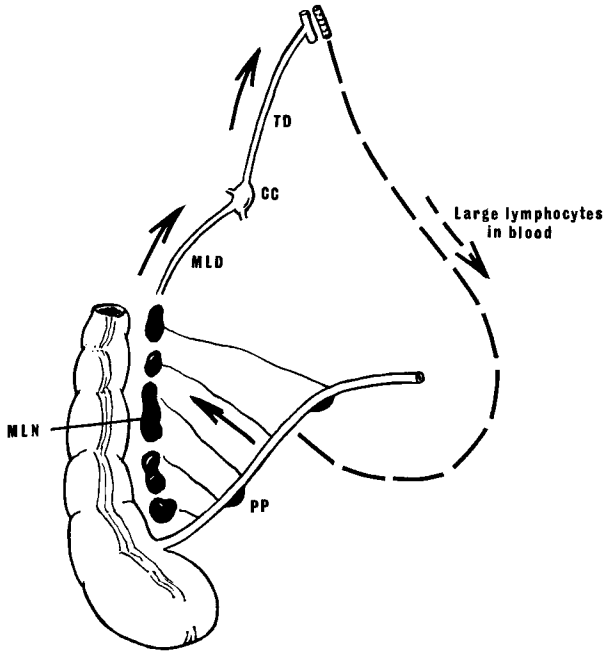


FIG. 1. The migration of large lymphocytes from the blood into the lamina propria of the small intestine and their origin from the lymphatic bed drained by the thoracic duct (TD). CC: Cisterna chyli; MLD: mesenteric (intestinal) lymph duct; MLN: mesenteric lymph nodes; PP: Peyer's patch.

Large lymphocytes in thoracic duct lymph, unlike the small, can be labelled by a brief incubation *in vitro* with radioactive precursors of DNA. The identification of such labelled cells by autoradiography after transfusion into syngeneic rats established that at least some of the plasma cells in the lamina propria of the intestine are derived from DNA-synthesizing lymphocytes which normally enter the blood by way of the thoracic duct. It was therefore proposed that the large lymphocytes were attracted into the lamina propria by the antigens which had originally provoked their formation in the intestinal lymphoid tissue (Gowans & Knight 1964), a view which will be reconsidered later. The subsequent discovery that the predominant class of immunoglobulin synthesized by intestinal plasma cells is IgA (Crabbé *et al.* 1965) led to the obvious conclusion that the large lymphocytes in thoracic duct lymph are the precursors of the IgA-synthesizing cells in the gut and this point has now been established (Guy-Grand *et al.* 1974; Pierce & Gowans 1975). Table 1 shows the results of a transfusion experiment in which large lymphocytes from the thoracic duct

TABLE 1

Proportion of labelled donor thoracic duct lymphocytes which contained internal IgA in gut of recipient rats

| | *% labelled cells with internal IgA in lamina propria* | |
|---|---|---|
| | *1* | *2* |
| Jejunum | 72 (72) | 70 (91) |
| Ileum : upper | 84 (49) | 64 (142) |
| : lower | 77 (74) | 67 (143) |
| Colon : ascending | 50 (48) | 66 (29) |

Donor thoracic duct lymphocytes labelled *in vitro* with tritiated thymidine (1 h, 37°C, 0.5μCi/ml, specific activity 5 Ci/mmole). Total of $2–8 \times 10^8$ cells given i.v. to each of two syngeneic rats which were killed 24 h later. Autoradiographs exposed for 14 days. Total number of labelled cells counted in gut sections in parenthesis; table shows % of these which also contained IgA by immunofluorescence. Technique for observing double label in Williams & Gowans (1975).

were identified in the intestine of syngeneic recipients both by their radioactive label and by their content of cytoplasmic IgA. It can be seen that about 70% of all the labelled cells in the lamina propria of the small intestine, and possibly a somewhat smaller proportion in the large intestine, also contained IgA. Labelled cells not containing IgA may have produced it at a later time or some may have been T blasts.

The fate of large lymphocytes has also been studied by estimating the distribution of radioactivity among the organs of rats after an intravenous transfusion of syngeneic thoracic duct cells previously labelled *in vitro* with a radioactive precursor of DNA (Hall *et al.* 1972). Fig. 2 illustrates a series of experiments of this kind which confirm that the tissue in which the greatest proportion of injected radioactivity accumulates is the small intestine (about 20–40% of the injected activity) and that a much smaller fraction localizes in the large intestine. Fig. 2 makes the additional point that a large fraction of the radioactive label appears in the urine, indicating that many of the transfused cells had died. This may merely reflect the damage sustained by the cells during handling *in vitro* or, possibly, that a proportion of the large lymphocytes are at the end of their lifespan and normally die in the lymph or in the blood.

Studies on the fate of DNA-synthesizing cells teased from lymph nodes are more difficult to interpret since the extent to which they reflect the population which normally migrates into the lymph is not known. However, large lymphocytes from mesenteric lymph nodes are second only to those from the thoracic duct in the proportion which localizes in the lamina propria (Griscelli

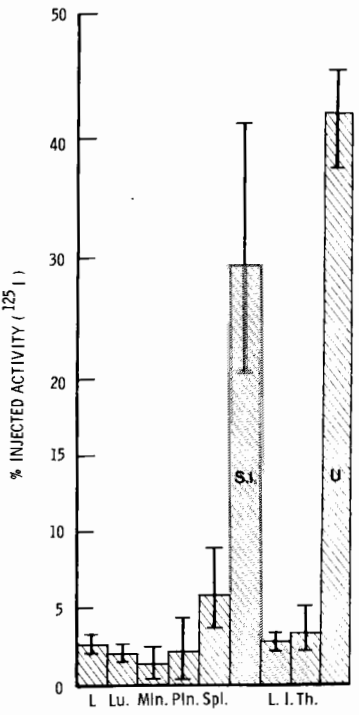


FIG. 2. Proportion of injected radioactivity recovered in whole organs of AO strain rats 24 h after an i.v. injection of syngeneic thoracic duct cells ($0.3–1.6 \times 10^8$) labelled *in vitro* with [$^{125}$I]iododeoxyuridine ($^{125}$IUdR) (1 h, 37°C, 0.1 μCi/ml). Mean and range for 5 recipients. Liver (L), lungs (Lu), mesenteric lymph nodes (Mln), inguinal, axillary, brachial and cervical nodes (Pln), spleen (Spl), small intestine (S.I.), large intestine (L.I.), thyroid (Th) and urine (U).

*et al.* 1969); large lymphocytes from peripheral nodes elsewhere show no predilection for the lamina propria (Griscelli *et al.* 1969). Paradoxically, DNA-synthesizing cells from Peyer's patches are reported not to home to the gut (Guy-Grand *et al.* 1974; McWilliams *et al.* 1975) although, as will be discussed later, Peyer's patches are the major source of the gut-seeking lymphocytes. The nature of the lymphocytes from bronchus-associated lymphoid tissue which populate the lamina propria is not known (Rudzik *et al.* 1975*b*).

The transfusion experiments make it clear that some IgA-secreting cells in the lamina propria are derived from precursors in thoracic duct lymph but they do not precisely identify them. The problem is complicated by the possibility that cells in the lymph not in DNA synthesis may also be precursors and by the heterogeneity of the blast cells themselves. These complications have been partially resolved by studying the distribution of surface and internal IgA

TABLE 2

Homing to the intestine of DNA-synthesizing large lymphocytes from the thoracic duct. Fate of all labelled blasts compared with that of subpopulation of blasts which lacked surface Ig

| *Experiment* | *% injected radioactivity recovered in intestine after transfusion of:* | | | |
|---|---|---|---|---|
| | *Undepleted TDL*[a] | | *Depleted TDL*[b] | |
| | *S.I.* | *L.I.* | *S.I.* | *L.I.* |
| 1 | 32.1 | 3.6 | 42.8 | 4.6 |
| 2 | 40.6 | 4.7 | 32.0 | 3.3 |
| 3 | 43.8 | 5.5 | 37.5 | 4.0 |

[a] Normal thoracic duct lymphocytes (TDL) from inbred AO or PVG/c strain rats.
[b] TDL depleted of all cells carrying surface Ig by rosetting with sheep erythrocytes coated with rabbit $F(ab')_2$ anti-rat Fab and spinning through Isopaque/Ficoll (Parish & Hayward 1975; Mason 1976). About $10^8$ normal or depleted TDL which had been labelled *in vitro* with tritiated thymidine (1h, 37°C, 0.5 μCi/ml, specific activity 5Ci/mmole) given i.v. to syngeneic recipients. Recipients killed at 24 h and whole small (S.I.) or large (L.I.) intestine incinerated on sample oxidizer and radioactivity (c.p.m.) measured by scintillation counting.

among rat thoracic duct lymphocytes. About half the large lymphocytes in thoracic duct lymph and also about half of those which label *in vitro* with tritiated thymidine contain considerable amounts of cytoplasmic IgA. Cells with internal IgA may account for up to 5% of all the lymphocytes in lymph from conventional rats but cells containing immunoglobulin of other classes are very rare. All the lymphocytes which contain cytoplasmic IgA also carry surface IgA which is not acquired by absorption from the lymph; a further 1–5% of all the cells carry surface IgA but lack internal IgA and these are mainly small lymphocytes (Williams & Gowans 1975). Similar values have been obtained for mouse thoracic duct cells by Guy-Grand *et al.* (1974). The cells in lymph carrying surface immunoglobulin of all classes can be efficiently removed by rosetting them with appropriately prepared sheep erythrocytes and then separating the rosettes from the non-rosetting cells by spinning through Isopaque/Ficoll (Parish & Hayward 1974; Mason 1976). Table 2 indicates that about the same proportion of blast cells in both the depleted and undepleted populations migrate into the intestine. However, autoradiography showed that the labelled cells which had originally lacked surface immunoglobulin accumulated predominantly in the T areas of Peyer's patches and to some extent in the villi at their margins; the few which were found in the lamina propria did not contain IgA. T blasts from the auricular nodes of mice (Parrott & Ferguson 1974) and from the thoracic duct of rats (Ford 1975), activated by oxazolone and a graft-versus-host reaction respectively, also migrate into the T areas of Peyer's patches but not into the lamina propria. It is not clear whether T blasts

which have been normally activated by gut antigens show a greater tendency to migrate into Peyer's patches than those generated by other means; nor is their function known.

It can be concluded from these experiments that the blast cells in thoracic duct lymph which home into the lamina propria are those which carry IgA on their surface but it has yet to be established whether the small lymphocytes with surface IgA but not in DNA synthesis are also part of the precursor population.

## THE ORIGIN OF IgA-CONTAINING CELLS IN LYMPH

The presence of IgA-containing cells in thoracic duct lymph can be interpreted in the light of the studies of Morris and his colleagues (Hall & Morris 1963; Hay *et al.* 1972) on the cellular response in efferent lymph which follows regional stimulation with antigen: the cells with internal IgA arise in intestinal lymphoid tissue in response to antigenic stimulation from the gut and are then released into the thoracic duct lymph. The study of a specific response to a defined antigen has made this interpretation more certain and has enabled the origin of the lymph-borne precursors of the intestinal IgA response to be determined. The candidates for the tissue of origin are clearly either the Peyer's patches and/or the mesenteric lymph nodes (Fig. 1).

Pierce & Gowans (1975) showed that when rats which had been primed intraperitoneally with cholera toxoid were given an intraintestinal boost with fluid toxoid, a wave of antitoxin-containing large lymphocytes (ACC) appeared in the thoracic duct lymph. They were first detected about two days after challenge and their numbers increased rapidly during the next 1–2 days to reach a maximum of about 200 000 ACC/h; at the height of the response 1% of all thoracic duct lymphocytes were ACC, of which about 80% contained IgA. In rats which had been primed orally, the intraintestinal boost produced ACC which contained IgA exclusively (Table 3).

A proportionally smaller ACC response in thoracic duct lymph was observed when the boosting dose of toxoid was given into the lumen of a Thiry-Vella loop (Markowitz *et al.* 1964) which had been constructed from about one-quarter of the total length of the small intestine (Fig. 3). On the other hand, no response was obtained after challenging loops from which the Peyer's patches had been removed surgically some time previously (Fig. 3). In other animals the long chain of mesenteric lymph nodes was removed and time allowed for the torn lymphatics to regenerate so that the normal flow of lymph from the intestine into the thoracic duct was re-established. A normal ACC response to challenge with cholera toxoid could be generated from Thiry-Vella loops prepared in such lymphatectomized animals (Fig. 3).

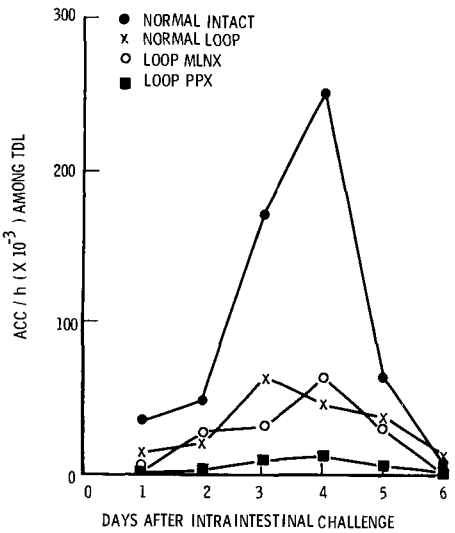


FIG. 3. Output of antitoxin-containing cells (ACC) in thoracic duct lymph after intraintestinal challenge with 1.0 mg of fluid cholera toxoid in rats primed 14 days previously with 0.1 mg of toxoid in CFA intraperitoneally. Challenge into duodenum of 5 normal intact rats (●); and challenge into Thiry-Vella loops of either 8 normal rats (×), of 4 rats from which the mesenteric lymph nodes (MLN) had been removed surgically (○), or of 3 rats in which the loops lacked Peyer's patches (PP) (■).

TABLE 3

Ig class of cholera antitoxin in thoracic duct lymphocytes (TDL) after intraintestinal challenge of primed rats

| *Immunization* | | *No. rats* | *Ig class in ACC* *% ACC with internal:* | | |
|---|---|---|---|---|---|
| *Primary* | *Secondary* | | *IgA* | *IgM* | *IgG2* |
| Intraperitoneal | Oral or intraduodenal | 5 | 82 (76-88) | 0 | 9 (6-14) |
| Oral | | 2 | 100 (99, 100) | 0 | 0.5 (1, 0) |

Rats primed with either 0.1 mg cholera toxoid in CFA i.p. or 25-40 mg toxoid in drinking water over 8-16 days. Challenge with either 5 mg toxoid orally or 1 mg intraduodenally 14 days after priming. Thoracic duct cannulated 3 days after challenge and lymph collected for 24 h. Ig class in TDL by autoradiography after incubation with $^{125}$I-rabbit F(ab')$_2$ anti-rat Ig; internal cholera antitoxin by immunofluorescence. Values are mean and range. (From Pierce & Gowans 1975.)

TABLE 4

Effect of a thoracic duct fistula on the appearance of antitoxin-containing cells (ACC) in gut after intraduodenal challenge with cholera toxoid in primed rats

| *Rat* | *Immunization* | *Density of ACC in lamina propria (mean/mm³ × 10⁻³ ± S.E.)* | | |
|---|---|---|---|---|
| | | *Jejunum* | *Ileum* | *Colon* |
| Normal (6) | Primary only | 0.7 ± 0.1 | 2.0 ± 0.5 | 3.1 ± 1.1 |
| Normal (5-7) | Secondary | 10.3 ± 2.0 | 14.9 ± 2.1 | 5.7 ± 1.3 |
| With TD fistula (6-7) | Secondary | 2.0 ± 0.6 | 2.8 ± 0.6 | 7.2 ± 1.0 |

Rats primed i.p. with 0.1 mg cholera toxoid in complete Freund's adjuvant and challenged intraduodenally with 1 mg of fluid toxoid 14 days later. In one group of rats a thoracic duct fistula was established just before challenge. Observations 19 days after priming. Number of rats in parenthesis. (From Pierce & Gowans 1975.)

These experiments show that the IgA antibody-containing large lymphocytes in thoracic duct lymph were derived from Peyer's patches. We had considered the possibility that although the precursors may have been discharged from Peyer's patches, their induction by antigen might have occurred upstream in the mesenteric nodes where antigen-trapping would probably be more efficient. In the event, the mesenteric nodes apparently contributed little to the response and engagement with antigen must have occurred in the Peyer's patches.

The wave of ACC which appeared in the lymph of primed rats after intraduodenal challenge with cholera toxoid was followed about two days later by the accumulation of IgA-containing ACC in the lamina propria of the small intestine (Pierce & Gowans 1975). This intestinal accumulation of ACC did not occur if a thoracic duct fistula had been established just before challenge: the density of ACC in the jejunum and ileum of rats with thoracic duct fistulae was not significantly different from the density observed in rats which had received primary immunization only (Table 4). Thus, the ACC which accumulated in the lamina propria were derived *exclusively* from lymph-borne cells. This conclusion does not refute the claim that cells from bronchus-associated lymphoid tissue may contribute to IgA production in the lamina propria (Rudzik *et al.* 1975*b*). It does, however, show that an immune response triggered in the intestine itself is delivered entirely by cells derived from the intestinal lymphoid mass.

The first evidence for the origin from Peyer's patches of IgA-secreting cells was provided by Craig & Cebra (1971). They showed that suspensions of cells teased from Peyer's patches gave rise to IgA-containing cells in the intestine and spleen of irradiated allogeneic rabbits. The splenic localization was

probably an artefact of allogeneic transfer (Rudzik *et al.* 1975*a*) but the experiments clearly revealed the potentiality of cells from Peyer's patches in comparison with lymphoid tissue elsewhere. The precursors in Peyer's patches were among the cells with IgA on their surface (Jones & Cebra 1974). In view of all the evidence for the origin of IgA-secreting cells from Peyer's patches it is surprising that blast cells from Peyer's patches do not home into the gut (Guy-Grand *et al.* 1974). If this is not a consequence of cell damage during handling *in vitro* then it must be assumed that the lymph-borne precursors of the response only enter into DNA synthesis after their release from the Peyer's patches into the lymph, although this seems unlikely.

The mechanism of the remarkable class commitment shown by cells in Peyer's patches remains unexplained. It is not known whether Peyer's patches accumulate IgA precursors from the general recirculating pool of B lymphocytes or whether the environment of the Peyer's patches dictates their differentiation, perhaps from B cells initially carrying surface IgM. In any event, Peyer's patches have the capacity to accumulate IgA memory cells from elsewhere, because intraintestinal challenge produced a brisk secondary response of ACC in the lymph in rats which had been primed intraperitoneally, a route which does not engage lymphoid tissue in the intestinal bed (Pierce & Gowans 1975).

## FACTORS AFFECTING THE MIGRATION OF LARGE LYMPHOCYTES INTO THE LAMINA PROPRIA

In discussing the factors which affect the localization of large lymphocytes in the intestine it is necessary first to consider the extent to which this migration shows selectivity. On a weight basis the spleen sequesters almost as great a proportion of an injected dose of labelled large lymphocytes as the small intestine. On the other hand, if one asks—into what organ or tissue do most of the large lymphocytes pass in order to execute their function?—then it is clear that the small intestine is overwhelmingly the dominant organ (Fig. 2). The large intestine attracts only a small fraction of all gut-localizing cells (Fig. 2); its behaviour is also anomalous in that a thoracic duct fistula did not prevent the accumulation of ACC in the colon in rats primed and challenged with cholera toxoid (Table 4). Plasma cells in the large gut may turn over relatively slowly or the large and small intestine may differ in the mechanisms by which plasma cells are generated or localized.

In the original work on the homing of large lymphocytes (Gowans & Knight 1964) it was suggested that antigen might play an important part in determining their localization in the gut. It would seem sensible to accumulate plasma cells in those areas of the intestine where the target for the secreted antibody is

TABLE 5

Antigenic challenge into Thiry-Vella loops prepared in rats primed intraperitoneally with cholera toxoid. Selective accumulation of antitoxin-containing cells (ACC) in challenged loop

| | *Loop challenged:* | | |
|---|---|---|---|
| | *Proximal (4)* | *Distal (6)* | |
| | *ACC/cm* | *ACC/cm* | *IgA-containing cells/cm* |
| Duodenum | 13 (10-16) | 15 ( 7-22) | 2094 (1638-2569) |
| Proximal loop | 69 (62-89) | 15 (10-21) | 822 ( 693- 921) |
| Distal loop | 16 (12-20) | 73 (52-121) | 730 ( 662- 870) |
| Ileum | 16 ( 8-26) | 18 (12-25) | 1122 ( 988-1265) |

Proximal and distal Thiry-Vella loops (Markowitz *et al.* 1964) prepared from equal lengths of mid-third of small gut. Continuity re-established by joining jejunum to lower ileum. Loops made 10 days after priming with 0.1 mg cholera toxoid in complete Freund's adjuvant, i.p. Challenge into one loop with 1 mg fluid toxoid 4 days after surgery. Antitoxin-containing cells (ACC) and IgA-containing cells in gut 6 days after challenge scored by immunofluorescence in measured lengths of gut sections cut at 4 μm thickness. Number of rats in parenthesis.

present. Pierce & Gowans (1975) showed that ACC taken from the thoracic duct after challenging primed animals with cholera toxoid could be identified in large numbers in the intestine of normal recipients, whether or not they had been given antigen at the time of transfusion. On the other hand, it was found that in the primed animal itself, the greatest concentration of ACC in the lamina propria always occurred in that region of the intestine which had been challenged with antigen.

Similar evidence for the influence of antigen on the localization of specific antibody-producing cells in the lamina propria has come from studies on Thiry-Vella loops. Rats were primed intraperitoneally with cholera toxoid and two separate loops were prepared from the small intestine 10 days later. A challenge of toxoid was then given into one loop only and the number of ACC was counted in both loops six days later. Table 5 shows that the greatest number of ACC occurred in the loop which received antigen despite the fact that the loops themselves contained fewer total IgA cells than normal small intestine. Similar findings have been reported for responses in Thiry-Vella loops in sheep (Husband & Lascelles 1974).

The idea that antigen plays any part in the localization of large lymphocytes in the intestine is thought to be discredited by the observation that they will home selectively into neonatal or embryonic intestine which is presumptively antigen-free (Halstead & Hall 1972; Parrott & Ferguson 1974). How then is it possible to reconcile these observations with experiments which show that antigen dictates the area in the gut in which most specific antibody-forming cells

accumulate? The dilemma could be resolved if two independent processes are at work. First, some property intrinsic to the small intestine favours the emigration of large lymphocytes into the lamina propria; and second, there is an antigen-dependent immobilization of the cells once they have emigrated. This implies that if the specific antigen is not present in the lamina propria the cells may leave it, re-enter lymphatics and pass by way of the thoracic duct back into the blood. In conventional animals such a recirculation of large lymphocytes would not be observed (Gowans & Knight 1964) because the antigens which gave rise to their formation are naturally present in the gut. The experiments in which ACC homed into the intestine whether or not cholera toxoid was present in the gut (Pierce & Gowans 1975) are incomplete since localization was only studied at one time-interval (5 hours) after transfusion. The same objection applies to studies on neonatal gut where the accumulation of cells in the lamina propria was only studied up to 8 hours after transfusion (Halstead & Hall 1972). The lymphatic obstruction which has been described in grafts of embryonic gut placed under the capsule of the kidney (Ferguson & Parrott 1972) may not allow cells which have emigrated into the lamina propria to leave it again.

The proposed immobilization of large lymphocytes in the lamina propria by antigen can be readily tested but even if it proves to be correct it would leave unexplained the mechanism of the initial migration from the blood which probably occurs through small venules. In anatomical terms it is reasonable to suppose that the signal for migration is provided by the vascular endothelium and that the first event is probably the adherence of the lymphocytes to it. A number of workers have considered the possibility that secretory component, synthesized by the intestinal epithelial cells, may provide the signal for migration because it might combine with the lymphocytes which carry IgA on their surface. This is not an attractive hypothesis because there is no evidence that secretory component is available either in the lamina propria or, more important, in the vascular endothelium. Further, our colleague H.-J. Gross has shown that secretory component does not, in fact, bind to the surface of rat thoracic duct lymphocytes: $^{125}$I-labelled rabbit secretory component bound avidly to the internal IgA in fixed smears of rat thoracic duct large lymphocytes but it failed to bind to the surface of living large lymphocytes in suspension, possibly implying that the IgA on their surface is monomeric. The hypothesis is further weakened by the observation that migration of large lymphocytes into the gut is unperturbed by pre-treating animals with antibodies directed against either secretory component or IgA (McWilliams *et al.* 1975). For the moment, the molecular basis for the emigration of large lymphocytes into the lamina propria of the small intestine remains unsolved.

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## Discussion

*Ahlstedt:* To take up the question of antigen causing the cells to home to particular areas, in our studies with colostral cells, where we treated pregnant women with *Escherichia coli* O83 bacteria, antibody-producing cells were found in the colostrum for over three weeks, while the bacteria were very difficult to detect in faecal samples (Goldblum *et al.* 1975). It would seem unlikely that the antigen is transported from the gut up to the mammary gland and stays there for three weeks, making the cells there produce antibodies.

*Gowans:* So antibody-forming cells are entering the mammary gland and passing out into the colostrum continuously?

*Ahlstedt:* Yes, maybe, if the cells are not proliferating within the gland. This finding may also be peculiar to the mammary gland, since we cannot detect any secretory antibodies in saliva or urine, or any antibodies in serum. We haven't looked at the gut.

*Brandtzaeg:* I agree with Professor Gowans that secretory component apparently has no role in the homing of IgA cell precursors to the gut. We have looked at circulating human B lymphocytes and we detect no surface affinity for secretory component and no J chain on these cells (Brandtzaeg 1976).

*Booth:* Strober and his colleagues (1976) have described a single case recorded of secretory piece deficiency. There was a normal population of IgA cells in the lamina propria.

*Brandtzaeg:* As will be discussed in my paper (pp. 77–108), I doubt very much that that report shows a complete deficiency of the secretory component.

Professor Gowans referred to Hall's recent study (Hopkins & Hall 1976) and used this as an argument that the IgA cells in the lamina propria do not come from peripheral lymph nodes, but have an intestinal source. However, if your conclusions about the role of antigen in B cell trapping are correct, this mechanism may equally well apply to the immunocyte populations in spleen and peripheral lymph nodes in Hall's study. The intestinal lymphoid

I am not sure that this study proves that the blast cells homing to the gut come exclusively from intestinal sources.

*Gowans:* Hopkins & Hall (1976) have shown that radio-labelled blast cells from the intestinal lymph of sheep home preferentially to the small intestine whereas those from the efferent lymph of peripheral nodes elsewhere migrate mainly to the spleen. You are right to emphasize that the blasts in intestinal lymph were formed in response to normal gut antigens whereas the spleen-seeking blasts were a response to intentional stimulation with a mixed bacterial vaccine. So it could be argued that the latter were not given an opportunity to display gut-homing since the specific antigen was not present in the gut. It would be interesting to know whether these blasts entered the lamina propria and then left it again, but this appears unlikely since Hopkins and Hall say that 'they did not reappear in significant numbers in samples of either lymph or blood'.

*Brandtzaeg:* One should stimulate the peripheral lymph nodes with the gut flora, to do a clear-cut experiment.

*Gowans:* I agree.

*Rosen:* Can one interfere with the homing of IgA-forming cells by treating them with anti-IgA antibody, and is the homing population completely eliminated if one treats the thoracic duct suspension with anti-IgA and complement?

*Cebra:* That experiment using mesenteric lymph node cells has been done by McWilliams *et al.* (1976). Anti-IgA antibody plus complement ablates the small extra increment of cells that are found in mesenteric nodes and that lodge in gut rather than elsewhere. These sensitive cells probably account for at least some of the IgA precursors which populate gut lamina propria.

*Gowans:* This experiment is difficult to interpret because treatment with anti-IgA may opsonize the cells for phagocytosis by the liver and spleen.

*Seligmann:* Have you any information on the membrane-bound immunoglobulins of the large dividing cells in thoracic duct lymph which do not have intracytoplasmic IgA, and particularly have you information on IgD?

*Gowans:* I cannot give you a precise answer to your question but from estimates of the proportion of large lymphocytes with surface IgA (Williams & Gowans 1975) and of the proportion of blasts carrying surface Ig of all classes (Mason 1976*a*) I would guess that very few large lymphocytes in rat thoracic duct lymph possess membrane Ig other than IgA. About half do not bind anti-L chain reagents and are presumably T blasts.

We have no reagent for detecting IgD in rats (assuming it exists) but Mason (1976*b*) has concluded that the existence of B cells carrying IgD *exclusively* in rat thoracic duct lymph is very unlikely: the sum of the proportions of **B cells**

with surface IgM, IgG and IgA is about equal to the proportion of all cells which bind anti-L chain antibodies. On the other hand, estimates of the amount of anti-Ig reagents bound to individual cells certainly leave room for cells carrying more than one class, for example IgM *plus* presumptive IgD.

I should emphasize that everything I have said applies to thoracic duct cells from rats which were not intentionally immunized: that is, to blast cells generated in gut lymphoid tissue in response to gut antigens. I would guess that blast cells generated in peripheral nodes elsewhere might carry predominantly IgM.

*Booth:* In loop experiments, a technical problem is that the loop atrophies after about a month and the segment distal to the anastomosis hypertrophies. What is your technique for counting the cells and how do you express the number—per length of tissue or per villus, or what?

*Gowans:* All the experiments were done within a week or so of preparing the loops and the problems you describe were not encountered.

On the second point, we express the density of antibody-forming cells as the number per centimetre of gut in histological sections cut at 4 μm. The unit length includes the whole thickness of the mucosa down to the muscular coat.

*Parrott:* In the primed rats with loops, have you put a non-cross-reacting antigen into the loops, or traumatized the second loop, to get the cells to go to that loop?

*Gowans:* No, we have not tried mechanical, chemical or other immunological stimuli in the second loop.

*Porter:* You highlighted the difference in the populations of lymphoid cells in the colon and small intestine; there is also a difference among the small intestinal lymphoid cells that was first shown by Crabbé & Heremans (1966). The duodenum, for example, showed a considerably higher lymphoid cell population than the jejunum or ileum. We have made a similar observation in relation to intestinal infection with *E. coli* in mono-contaminated germ-free pigs. The response of the gut shows ten times more cells in the duodenum than the jejunum and ileum, yet the antigen density, or the *E. coli* population, is almost inverse, in that it is higher at the lower end of the intestine. I have argued teleologically that this is a good way for the gut to defend itself, in that it is better to pour the antibody out at the upper end of the intestine than at the lower, but it goes against the antigen-trapping idea, and I favour some intrinsic factor which would tend to bind antibody-forming cells in the upper small intestine rather than to mediate at the site of challenge.

*Pepys:* Why does intraperitoneal antigen prime for subsequent gut challenge? Is it because antigens reach the gut or because non-gut blast cells home into the intestine?

*Gowans:* It is surprising that intraperitoneal priming prepares the gut for a secondary response (Pierce & Gowans 1975). I assume that memory cells created by intraperitoneal priming accumulate in Peyer's patches and are thus available for stimulation by the subsequent intra-intestinal challenge. Dr Pierce tells me that subcutaneous priming is much less effective, which is hard to explain. Antigen injected intraperitoneally is collected by the diaphragmatic lymphatics, a few of which join the thoracic duct in the chest; most, however, drain into the mediastinal lymph nodes. I suppose that it is possible that intraperitoneal antigen in complete Freund's adjuvant, which is extremely corrosive, may stimulate Peyer's patches directly by penetrating them from their peritoneal surface, but this has never been established. We need to know what happens to an antigen in CFA which is injected into the peritoneal cavity.

*Pepys:* We have data that may be relevant. We have been looking at the production of IgE antibody in mice after the intraperitoneal injection of 0.1 μg of ovalbumin on alum, with a priming dose at six weeks of age and a booster four weeks later. The mice make good IgE responses. When we killed them some weeks after the booster, we found IgE-containing lymphoid cells in the Peyer's patches, although antigen had not been given into the gut (W. D. Brighton, B. E. Hewitt & M. B. Pepys, unpublished work).

Secondly, on the question of whether lymphocytes are stimulated by antigen, or divide, in the lamina propria, Marsh (1975) has studied both the morphology and the tritiated thymidine incorporation of lymphocytes in the intestinal epithelium of mice. He found that at any one time about 5% are blasts of which many appear to be T cells, but some look like B cells.

*Gowans:* The simple scheme which I put forward envisages each DNA-synthesizing large lymphocyte in the lymph as giving rise to no more than two IgA-secreting cells in the lamina propria. This may well be an over-simplification. We need to know whether IgA-precursor cells which have not yet started proliferating in either the Peyer's patches or the lymph can enter the lamina propria and proliferate and differentiate locally; and whether large lymphocytes which are already in DNA synthesis in the lymph can undergo several further divisions after migration into the gut. The clustering of IgA plasma cells in some villi is certainly consistent with continued local proliferation, although equally consistent with accumulation due to specific attraction.

*Bienenstock:* You mentioned that it isn't known where some of the respiratory tract lymphoid cells go to. We have shown that lymphoid cells in the follicles of the respiratory tract go to the gut and will repopulate the spleen in a similar fashion to cells from Peyer's patches (Rudzik *et al.* 1975).

As far as intraperitoneal priming is concerned, antigen put into the peritoneal

cavity certainly goes up into the mediastinal area and enters what is loosely described as the area draining the respiratory tract (Gerbrandy & Bienenstock 1976). I suggest that this may be a higher localization of potential IgA precursor cells than if the antigen is given either parenterally or orally. I wonder why, in the experiments done with Dr Pierce, oral priming is worse than intraperitoneal priming?

*Pierce:* I am not certain it is worse, because the conditions are not the same. We cannot prime the gut of rats with either a single intraperitoneal or intraduodenal injection of purified fluid toxoid. We obtain good priming when toxoid is injected intraperitoneally with Freund's complete adjuvant but we have not evaluated this combination given into the gut lumen.

*Cebra:* Complete Freund's adjuvant given intraperitoneally with antigen induces a high proportion of cooperating T cells. What is the relative importance of the priming, on the one hand to give cooperating T cells and on the other to affect the B cell compartment of the Peyer's patches? In experiments mentioned earlier (p. 26), if cooperating T cells were transferred together with Peyer's patch cells we found more IgA-forming PFC cells (plaque-forming cells) in the spleen, and at earlier times. The response that Professor Gowans observes may be only one part of the overall secondary IgA response, the earliest phase. Secondary IgA cells may mature in a few days following very few divisions and end up in the lamina propria. Those B cells which are able to mature so rapidly must be already committed to generating IgA plasma cells and also must surely have available a lot of cooperating T cells to facilitate rapid maturation with few cell divisions. Ordinarily, an adoptive transfer of unprimed cells requires seven or eight cell divisions before significant numbers of IgA plasma cells are seen. The rapidity of your response suggests both cooperation early with pre-existing T cells and that the B cells functioning in your system are already committed to generating IgA plasma cells (i.e., are secondary B cells). One wonders whether they have reached that secondary state by priming or whether natural gut stimulation has put a few cells in this state, which are susceptible to early maturation to IgA plasma cells. There may be a reservoir of other cells that can take part in later stages of the IgA response.

*Pierce:* In the rat intraperitoneal priming of the gut immune response does not require Freund's *complete* adjuvant; it is produced equally well by the incomplete adjuvant. This may diminish concern that the response depends on T cell stimulation primarily. It is *not* reproducible by intraperitoneal priming with an alum-precipitated toxoid, or by toxoid plus Freund's adjuvant given subcutaneously.

Second, we have observed apparent species differences in the responses of

rats and dogs. In dogs we can prime the gut by the subcutaneous route *without* Freund's adjuvant, so the requirement for an oil adjuvant given intraperitoneally may apply to rats but not to the system in general.

*Lachmann:* You have discussed afferent and efferent homing, Professor Gowans, and you have evidence that the cells that come from the Peyer's patches are committed to make IgA, but how do they get there in the first place? They must presumably come from bone marrow; but how do the cells there that are committed to make IgA know to go to Peyer's patches?

*Gowans:* We have no idea. Peyer's patches are very odd structures. They possess no afferent lymphatics so they are not regional lymph nodes in the conventional sense. Presumably, antigen reaches them directly by passage through the overlying intestinal epithelium, or through defects in it. On the other hand, they are similar to lymph nodes in that both B and T lymphocytes recirculate through them and that, in cell transfer experiments, they can mount IgM and IgG adoptive antibody responses, at least to sheep erythrocytes. Their special feature is that, *in situ*, Peyer's patches generate cells that make IgA. We know that the precursor B cell in Peyer's patches is a non-dividing (small) lymphocyte (McWilliams *et al.* 1974) which carries IgA on its surface (Jones & Cebra 1974).

Now we come to Dr Lachmann's questions. If the marrow produces virgin sIgA (surface IgA) lymphocytes, then Peyer's patches may simply concentrate them by some unknown mechanism. On the other hand, all sIgA small lymphocytes may be memory cells in which there has been an antigen-driven switch from sIgM → sIgA and it is the facility to mediate this switch which is a particular property of Peyer's patches. A further possibility is that sIgA cells, whether virgin or memory, may undergo non-clonal expansion in Peyer's patches under the influence of locally active mitogens (? gut derived). All this is pure speculation, as I am sure Dr Lachmann will immediately recognize.

*Cebra:* One point directly pertinent to Dr Lachmann's question has to do with transfer of bone marrow cells into irradiated congenic BALB recipients. We can restore the peripheral lymphoid tissue, and then test the peripheral nodes, spleen and Peyer's patches. The bone marrow cells, which bear almost no surface IgA, are able, when they proliferate in the Peyer's patches, to generate cells among which are cells capable of mounting an IgA response. We believe that one unique feature of the Peyer's patches, aside from a possible selective effect on differentiation or development, is that divisions occur there without maturation. That seems to be the key to getting a cell line advanced towards, if not already committed to, generating IgA plasma cells.

*Gowans:* There is, as yet, no evidence for a B cell expansion of this kind in Peyer's patches, is there?

*Cebra:* There is extensive cell division there; moreover, from the results of the clonal precursor analysis using a variety of antigens, a pattern emerges. Antigens most likely to stimulate patch cells naturally and chronically reveal a population of antigen-sensitive precursors that generate large clones making IgA plus IgGl or IgA plus IgGl and IgM. Clonal precursor analysis with rarer antigens reveals antigen-sensitive cells which generate clones making only IgM or IgM plus IgGl. Some change in potential of patch cells appears to occur which is division-related.

*Lachmann:* Can we go any further towards resolving the question of whether there is a switch to IgA production by precursors that are not already fully committed to producing that isotype before they meet antigen? It makes a difference to explanations of the generation of IgA responses whether or not there are cells which make IgM and, after they meet antigen in the bowel, go on to make something else.

*Cebra:* Our data are consistent with the existence of B lymphocytes that can be precursors for IgA plasma cells and in addition have a broader isotype potential. Antigen-sensitive cells reactive with the DNP determinant generate clones making IgM plus IgA, IgGl plus IgA and IgA plus IgGl and IgM. A 'switch' must occur during the generation of a clone to explain how one cell gives rise to a thousand cells, some of which make one isotype and some another. Gearhart *et al.* (1975) showed that all the antibody made by such a clone to the phosphorylcholine determinant bears the same idiotype marker, which means that the same V genes are being expressed in connection with $C_\alpha$, $C_\mu$, and $C_{\gamma 1}$. A major question is how the patch environment, or antigen stimulation, influences the expression of the V gene together with a particular C gene. At that level, we are ignorant of what happens. Many geneticists concede that a translocation or gene rearrangement might occur, and that V and C genes are not a single gene to begin with. However, they consider it less likely that with successive divisions the V gene can move around. The alternative must be expansion (selective replication) of a particular V gene giving $VC_\alpha$, $VC_\mu$, $VC_{\gamma 1}$, etc. and thereafter gene regulation by conventional mechanisms. It is hard to see how antigen could affect gene rearrangement or gene expression, so I suggest that potentials to express various isotypes are in all the primary cells, that regulating components may be in their milieu, and that the number of divisions which a B cell undergoes influences which isotypes are expressed. Perhaps divisions can be stimulated in many ways—hormonally, or by mitogens; antigen might just induce the last division(s) before final maturation when a particular VC gene will be expressed. We don't know how antigen intervenes but I suggest that is by the facilitation of division rather than by acting on the genetic material itself.

*Lachmann:* So you would predict that any cell would go on to make IgA, if you stimulated it to divide intensively enough?

*Cebra:* It is possible that either it or its progeny might do that.

*Pepys:* Parkhouse and his collaborators have demonstrated in mice that the memory cells of some stable clones committed to IgG antibody formation have receptors of IgM class (Abney *et al.* 1976). The clonal cells were treated *in vitro* so as to 'cap' and 'strip' with various anti-mouse immunoglobulin antisera, before being transferred together with antigen and carrier-primed helper cells into irradiated recipients. Pretreatment with anti-$\mu$ or anti-Fab prevented the production of IgG antibody, while anti-$\gamma$ and anti-putative mouse $\delta$ had no effect.

*Cebra:* If both classes are synthesized, there must be two V genes active in each cell and presumably those are identical.

*Gowans:* The recent work of D. W. Mason (1976*b*) should be quoted in connection with that just cited by Dr Pepys. Mason used the fluorescence activated cell sorter to identify the B lymphocytes which generated a secondary IgG anti-DNP response in rats. He found the precursors exclusively among a small population of cells bearing surface IgG. The IgM-positive cells, which constituted the majority of the primed population, contributed little or nothing to the response. I would guess that sIgG memory cells were derived, during priming, from sIgM precursors but this remains to be proved.

*Lachmann:* There is the work from Max Cooper's laboratory showing that all future antibody-forming cells go through a phase where they have IgM on their membranes, and that anti-$\mu$ antibody given in early life will destroy subsequent antibody production, even of IgG (Kincade *et al.* 1970). There must presumably be a time when the cells switch from transcribing the $\mu$ isotype to, say, the $\gamma$ isotype. If there is enough messenger RNA left they can still be making IgM receptors although inside the cell the switch in the DNA being copied may have taken place and any new messenger will be for the $\gamma$ chain.

*Mayrhofer:* To my knowledge, there has been no study of the class of immunoglobulin on differentiating cells in animals undergoing a primary immune response leading to production of IgA. Oral immunization with soluble proteins would be a difficult system to work with because prolonged feeding of antigen appears necessary in order to obtain a secretory antibody response. If a switch of class occurs during differentiation, it might easily be missed. Studies on the surface immunoglobulin of precursors of IgA plasma cells in normal animals are looking at on-going immune responses to normal gut antigens (Craig & Cebra 1975; Williams & Gowans 1975) and therefore do not necessarily bear on the precursors that respond with primary exposure to

antigen. The cholera toxoid system (Pierce & Gowans 1975) gives a brisk IgA response, but requires parenteral priming, the effect of which may be to generate IgA memory cells. One possible reason for the lack of a brisk response to oral immunization might be the failure to engage sufficient antigen with the lymphoid tissue of the gut. It would seem worthwhile to try a live enterovirus as an antigen, as this might deliver a more powerful challenge of shorter duration. After such an infection, specific antibody-forming cells might be found in Peyer's patches or thoracic duct lymph showing evidence of class switching. Gowans & Williams (unpublished) for instance found one rat during the course of their study in which a significant minority of thoracic duct cells containing IgA bore IgM on their surfaces. This was unusual as, in most rats, IgA-containing cells have surface IgA (Williams & Gowans 1975). It could be that this animal was responding to an infective agent that it had not previously encountered at the time of study.

*Seligmann:* There have been two cases of Ig myeloma with IgG inside the cell and IgM with the same light chain on the cell membrane (Seligmann *et al.* 1973). Unfortunately, the idiotypes have not been studied.

*Cebra:* Operationally we always elicit a primary response in our experiments, in the adoptive transfer system, in the clonal precursor analysis and in the direct gut stimulation. But one question is what a real primary response is, and I am suggesting that environmental antigens impinging on the cells in the Peyer's patch could cause divisions to yield cells which immunologists would call secondary B lymphocytes, even though they had not been stimulated deliberately. Lipopolysaccharide or other mitogens might also stimulate cells in the Peyer's patches to arrive at such a secondary state where a cell might display IgM on its membrane but synthesize and secrete another isotype on maturation.

*Davies:* Dr Cebra, in your experiments in which you transferred Peyer's patch cells and compared them with transferred spleen cells, there were some added thymocytes, which did not seem to be essential. You certainly demonstrated that cells from the Peyer's patches can, under the given circumstances, be found in the lamina propria doing their thing. How long were they found? You implied a week or so. I would be interested in the performance of these cells in comparison with that of transferred bone marrow cells. One wonders whether the kinds of activity that you have demonstrated *can* take place, but perhaps don't?

*Cebra:* Transfer of congenic cells from the Peyer's patch, with or without thymocytes, to irradiated BALB/c mice, and then antigen stimulation, results in an IgA plaque-forming response in the spleen. The transfer also results in a repopulation of the lamina propria by about day 14. Almost all the IgA cells

bear the donor marker. At about day 30 total IgA cells decline in the lamina propria. The mice gradually replace the donor cells with their own, presumably from bone marrow.

We have transferred marrow and after 6–8 weeks can recover cells from the peripheral lymph nodes, spleen or Peyer's patch displaying the usual potential of cells from these sources, but bearing the marrow donor's marker, so you can totally repopulate the lamina propria with marrow-derived cells. This repopulation takes 3–4 weeks.

*Davies:* That shows that bone marrow *can* do this, but does not tell one much about the state of differentiation of the cells in the bone marrow. You have recolonized Peyer's patches with bone marrow cells, although the implication is that bone marrow eventually outgrows Peyer's patch cells. Whatever kind of 'stemness' they have, their capacity for persistence, or proliferation, is relatively limited. Is there anything incompatible with the notion that one is dealing ontogenetically with the development of microenvironments within which the synthesis of a particular antibody will be propitiated, rather than a development of cells which will produce a particular kind of antibody?

*Cebra:* I have no evidence for this, but it is an attractive notion, although how the microenvironment could direct the course of differentiation and maturation is not clear. I prefer a version of 'microenvironment' that includes cells or factors that act on B lymphocytes and, during the course of division, favour the expression of IgA by some siblings in a clone but not other isotypes by progeny of the same clone. That kind of immunoregulation, together with stimuli of division, might be all that the Peyer's patch 'microenvironment' provides to generate more IgA precursors than appear elsewhere.

*Davies:* The idea of microenvironments has precedents in the haemopoietic system. It seems likely that certain kinds of haemopoietic stem cells develop along one of two or perhaps three pathways of development in the spleen, and this gives credence to the microenvironmental notion. One wonders whether it applies to lymphoid cells.

*Cebra:* Initially we thought that patch cells displayed a differentiative pathway of potential to express IgM → IgG1 → IgA while peripheral node cells showed a parallel but different pathway of IgM → IgG1 → IgG2. However, with some antigens we saw a pronounced potential for IgG2 expression by Peyer's patch cells, so a clear-cut separate and distinctive development of B cells in different microenvironments does not seem to occur.

*Davies:* Did you see them making IgG2 in the spleen, however?

*Cebra:* Yes, but they were derived from patch precursor cells on adoptive transfer, even though they expressed IgG2 in the spleen of the recipient.

*Porter:* In relation to the sequential development of Ig-forming cells in the

lamina propria, it is some time after birth in the mammal before the IgA cell dominates, and so the role of the IgA cell in the ontogeny of secretory Ig responses should not be over-emphasized. The pig and cow are at least 20 weeks old before there are more IgA cells than IgM cells in the lamina propria. It is almost a year before one sees the adult pattern of about 80% of the cells in the lamina making IgA.

In relation to the sequential development, IgM as a secretory immunoglobulin must be taken seriously into account. One wonders what the consensus is about Cooper's sequence of IgM to IgG to IgA in the chicken, because although IgG cells appear in the lamina they form a very small proportion of the population at any age, in mammals.

*Brandtzaeg:* I agree that the Ig A system has been too much emphasized, and that we should think more about the development of IgM cells. In IgA-deficient patients, IgM cell precursors, and to a smaller extent IgG cell precursors, home to the gut and to other glandular sites, and the local plasma cell population develops to about the same size as the normal IgA cell population.

*Seligmann:* The question is whether there is a *direct* switch from M to A without going through G. There is some evidence in favour of this hypothesis (Cooper *et al.* 1976).

*Porter:* In the normal sequence of development within the first week of life there are at least ten times more IgM cells than IgA cells in any part of the intestine.

*Brandtzaeg:* My point was that the gland-associated development of IgM-(and IgG-)producing cells is greatly enhanced when there is a maturation defect in the IgA system.

*Porter:* So it appears as if there is *no* competence to switch.

*White:* Kincade & Cooper (1973) made the firm assertion that switches do not occur in peripheral tissues, based on the experimental finding that the switch did not occur in bursectomized birds, and we await confirmation or rejection of that. Of course, the bursectomy additionally eliminated another possible peripheral site of switching, namely the germinal centres. On the other hand, the experiments of Martin & Leslie (1974) favour a direct switch from IgM to IgA. Chickens were bursectomized at hatch and treated with anti-$\mu$ antiserum; there was a depletion of IgA and IgM but not IgG. At least some of the switching was thought to occur in the periphery.

*Lachmann:* Cooper's hypothesis goes further and says that the switch is not antigen-driven and happens before the cells see antigen. It is a coherent story and therefore a good one for testing with data!

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*Immunology of the Gut*
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# Significance of immune mechanisms in relation to enteric infections of the gastrointestinal tract in animals

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*Abstract* The impact of bacterial colonization on the alimentary tract in early life is reflected in gross changes in morphology. Subsequent health, if not survival, may largely be determined by a continuum of local intestinal immune mechanisms and it is essential for antibody development during the neonatal period to compensate adequately for declining passive maternal antibody. Consequent upon the development of the gut microflora the lamina becomes infiltrated with immunocytes in which the dominant immunoglobulins produced are IgM and IgA. Both immunoglobulins are transported across the epithelium by a process involving membrane-bound vesicles.

Germ-free and fistulated pigs and calves were shown to be able to respond to oral immunization with *Escherichia coli* O somatic antigens during the first week of life. Resistance to infection with enteropathogenic *E. coli* was significantly enhanced, along with other parameters of nutrition and performance. However, in the young chick, although the intestinal response to infection with *E. coli* was similar to that in the mammal, no response to *E. coli* O antigens could be determined on oral administration in germ-free or local intestinal applications in fistulated birds.

In the mammalian intestine secretory antibodies participate in the control of pathogenic *E. coli* by blocking adhesion to the mucosal epithelium, interfering with the elaboration of surface antigens, inhibiting toxins, and facilitating rapid elimination from the alimentary tract by agglutination and bacteriostasis. In consequence fewer enteropathogens are excreted into the environment, an important feature in modern intensive systems of animal production.

Animal species have evolved in intimate association with a complex microbial flora, and perhaps in consequence most living forms exhibit a strong dependence upon microbial activities. The alimentary tract provides an ideal environment for microbial colonization and the indigenous flora may play an indispensable role in regulating intestinal physiology and biochemistry and exert various morphogenetic effects on the host. The way in which the host tolerates the

proliferation of certain microorganisms, and yet sets up mechanisms for rejection of others, remains to be determined. Colonization by a pathogen always has a traumatic effect on the morphology of the intestinal mucosa, interfering with the dynamics of epithelial cell renewal to the detriment of the function of the organ in nutrition (Kenworthy 1970). Under such circumstances the competence of intestinal immune mechanisms will govern the opportunity for rejection of the pathogen and subsequent survival of the host.

It is well known that *Escherichia coli* preponderates among those pathogens associated with enteric disease in domestic livestock. The animals most at risk are young ones, particularly those raised in modern intensive systems. We have studied the secretory immune mechanisms of the gut with particular reference to their role in effecting a balance in the host–pathogen relationship. In this context we have investigated oral immunization during the neonatal period, placing emphasis on nutritional performance and environment and not limiting the observations to the clinical manifestations of the problem.

## MATERNAL IMMUNITY IN THE NEONATE

Most species have the ability to respond to a variety of antigenic stimuli during late fetal development. Indeed recent studies have shown that fetal lambs may be orally immunized by introducing antigens into the amniotic fluid (Husband & McDowell 1975). However, it is normal for passively acquired maternal immunoglobulins to compensate for the quantitative immunological deficiency of the neonate. The susceptibility of the neonate to enteric colibacillosis is readily evident in those species with chorioepithelial placentation. In such species, exemplified by pigs and calves, survival in the absence of colostrum will barely extend to 24 hours in a conventional environment. The quantitative weight of maternal immunity is evident from measurement of immunoglobulins which accumulate in the blood serum of the neonate. Thus the piglet in the first few hours of life will absorb colostral immunoglobulins through its gut wall amounting to approximately 3 g in total and the calf exceeds this by some 20-fold; even the chicken will take up some 60 mg from the yolk sac. What is of immediate consequence, however, is the class of antibody involved and its mode of antibacterial action in the intestinal mucosa and within the lumen.

A comparative study of immunoglobulin (Ig) classes in the pig, calf and chicken is of interest in this respect (Fig. 1), providing several different features of alimentary tract physiology and of the mode of operation of maternal immunity. The universal role of IgG is seen to operate in terms of its quantitative abundance in each species. The selective characteristics of the ruminant

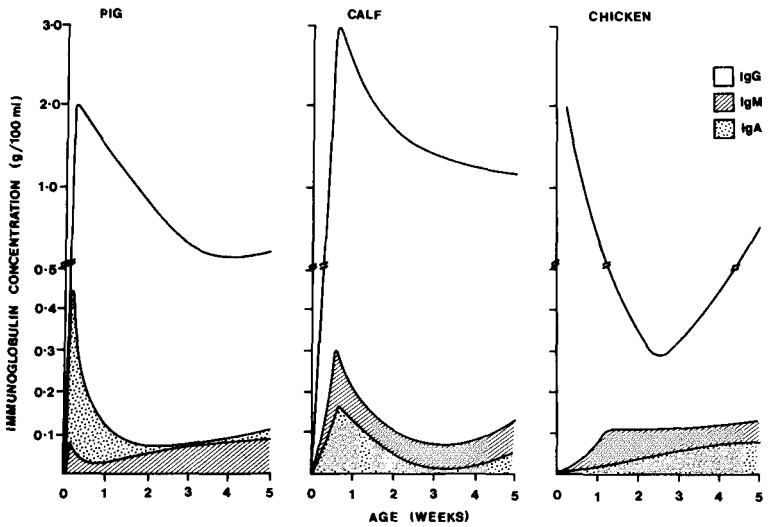


FIG. 1. Comparison of serum immunoglobulin (Ig) profiles of pig, calf, and chicken during the first few weeks of life.

allowing transport of $IgG_1$ into mammary secretions are well known and will not be dealt with here. In both pig and calf, IgM and IgA are transferred and play a significant role in antibacterial immunity. There is no transfer of IgM or IgA via the yolk sac in birds.

In mammals the lactational changes in the immunoglobulin profile of the dam in relation to neonatal requirement are of considerable interest. During the first few days of lactation the total concentration of immunoglobulin falls steeply in the milks of both pigs and cows. It is the rapid fall in IgG during this period which contributes most to the total decline, thus exercising a necessary economy in a maternal immunoglobulin which is transferred almost exclusively from the blood circulation by a transudative process across the mammary acinar epithelium.

In the pig, IgA emerges as the dominant immunoglobulin in the milk (Porter *et al.* 1970) whereas in the cow there is a uniformly low level of immunoglobulin secretion, which is a feature of ruminants in general. Secretory 11S IgA anti-*E. coli* antibodies in porcine colostrum and milk have been shown to operate entirely in the lumen without contributing significantly to antibody in the blood circulation of the neonatal piglet. The physiological patterns of milk antibody secretion, ingestion by the neonate and subsequent passage through the alimentary tract combine to provide a continuous bathing of the intestinal mucosa with maternal IgA antibody. Since the bovine mammary

gland does not continue to secrete high levels of IgA throughout lactation (Butler *et al.* 1972), it is interesting to see how the calf compensates for this deficiency. It exhibits no intestinal selection in absorption of maternal immunoglobulins and temporarily high levels of 11S IgA occur in the blood circulation (Porter 1972). This IgA declines with a half-life of approximately two days, being lost into various external secretions, principally those of the gut. This process continues for a period of approximately 10 days, providing the basis of a short-term barrier to infection.

In this context the recent observations on the appearance of IgA and IgM in the amniotic fluid of embryonating eggs and the intestinal tract of chick embryos are of interest (Rose *et al.* 1974). It is suggested that the maternal IgA and IgM present in oviduct secretions are acquired by the egg as it passes down the oviduct where the white is laid down. These appear in the embryonic gut via swallowed amniotic fluid, giving some analogy to the situation that exists in the newborn mammal.

## DEVELOPMENT OF INTESTINAL SECRETORY IMMUNITY IN THE NEONATE

There are very few lymphoid cells in the intestinal lamina propria of the newborn mammal and lymphoid follicles are poorly defined. In the germ-free state the lymphoid cell population of the lamina propria remains at a negligible level and the lamina becomes infiltrated with lymphocytes and plasma cells in response to the development of a gut microflora. Sequential studies on pigs have shown that during the first days of life, IgM cells outnumber those containing IgA or IgG (Fig. 2) and during this period IgM is the principal immunoglobulin secreted across the crypt epithelium (Allen & Porter 1973*a*,*b*). Immunoelectron microscopical studies suggest that the mechanism of transport of both immunoglobulins is essentially the same. Vesicles containing either IgM or IgA were found in the apical cytoplasm of crypt epithelial cells, suggesting that the immunoglobulin is transported across the epithelium by a process of reverse pinocytosis (Allen *et al.* 1973, 1976).

The relationship between the cellular component of the secretory immune system and the antigenic load in the lumen of the gut may be considered to be one of dynamic equilibrium. Thus the greater the load the higher the level of cellular activity required to counter it. The major antigenic stimulus derives from the enteric flora and it is interesting to note that lipopolysaccharide from Gram-negative organisms appears free in the lumen and is not only a B cell mitogen but also an antigen which promotes preferential synthesis of IgM.

During the early stages of its development an animal is likely to experience challenges by new antigens more frequently than later on. The establishment of

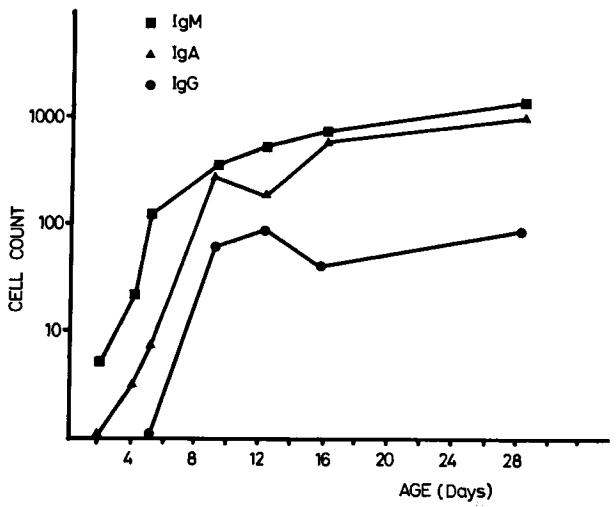


FIG. 2. Sequential development of immunoglobulin-producing cells in the lamina propria of the duodenum in the young pig.

the gut microflora together with a wide range of dietary antigens all contribute to this challenge. As the animal matures, the microflora becomes stabilized and the number of previously unencountered dietary components diminishes to a low level, and the cellular component of the lamina is better able to equilibrate with the luminal environment. Thus it is suggested that local antigenic challenge results in an initial proliferation of IgM-producing cells, to be followed by a possibly greater proliferation of IgA cells. Similarly, at any stage of maturity this development process is likely to occur in response to any previously unexperienced antigen. It is interesting that Crandall *et al.* (1967) showed a relative increase in the numbers of IgM-containing cells in the intestinal mucosa of adult rabbits soon after infection with *Trichinella.* Furthermore, in studies of infants with enteric colibacillosis, IgM antibodies formed an important part of the early response, especially in the youngest children of four or five months of age (McNeish *et al.* 1975).

The pattern of development of immunoglobulin-producing cells in response to antigens from the intestinal lumen can be gauged to advantage in the germ-free animal. In the germ-free pig IgM was demonstrated to play a dominant role in the early phases of the intestinal response to pathogenic *E. coli.* Furthermore, repeated administration of sterile preparations of bacterial antigens resulted in a response similar in terms of cell numbers to that produced by infection with the live organism in a comparable period.

In the young chick, however, no response to *E. coli* O antigens could be

determined on either local application in fistulated birds or oral immunization in germ-free birds. This was surprising since the characteristics of the intestinal response to infection with *E. coli* are very similar to those in the mammal, implicating IgM-and IgA-producing cells in the tissues and antibody associated with these immunoglobulin classes in the secretions (Parry *et al.* 1977).

## ORAL IMMUNIZATION IN PROPHYLACTIC CONTROL OF POST-WEANING ENTERIC INFECTION

The decline and termination of the protective function of maternal immunoglobulin is of signal importance in the complex of predisposing factors in the pathogenesis of post-weaning enteric syndromes. Immune mechanisms only play a significant role in inhibiting microbial proliferation after weaning provided that the intestinal mucosa is alerted to the production and secretion of antibodies long before the organisms achieve pathogenic proportions in the lumen. Thus if intestinal immunity is to have any potential at all, the young animal must be immunized early in life in order for a mucosal blockage to be established against the proliferation of enteropathogens consequent upon weaning. Only a limited number of serotypes are normally associated with diarrhoea syndromes of young farm livestock (Sojka 1971), so suitable prospects for vaccination exist.

The first criterion to establish in support of this rationale was that effective synthesis and secretion of anti-*E. coli* antibodies would occur in response to an antigenic stimulus to the intestinal mucosa of the neonate even in the presence of maternal antibodies. Studies were made in a litter of pigs in which intestinal fistulae were prepared at four days of age; four animals were maintained on the sow and four animals were reared separately and fed on cow's milk. One animal in each group was retained as a control and heat-inactivated *E. coli* were administered to the others. The characteristics of the secretory antibody response were very similar in all six animals, independent of whether the animals were maintained on maternal milk or a reconstituted cow's milk substitute lacking antibody (Fig. 3). Studies in the young calf provided essentially similar data; intestinal secretion of antibodies was registered in colostrum-fed animals in response to the oral administration of bacterial antigens at five days of age. Thus a useful relationship between passive maternal antibody and active intestinal antibody could be established in neonatal life. Since health and performance in young animals could be attributed to the establishment of this continuum, the stage was set to examine the efficacy of the local intestinal immune response in terms of the young animal's resistance to enteric infection.

Imbalance in the host–pathogen relationship will be shown in a range of

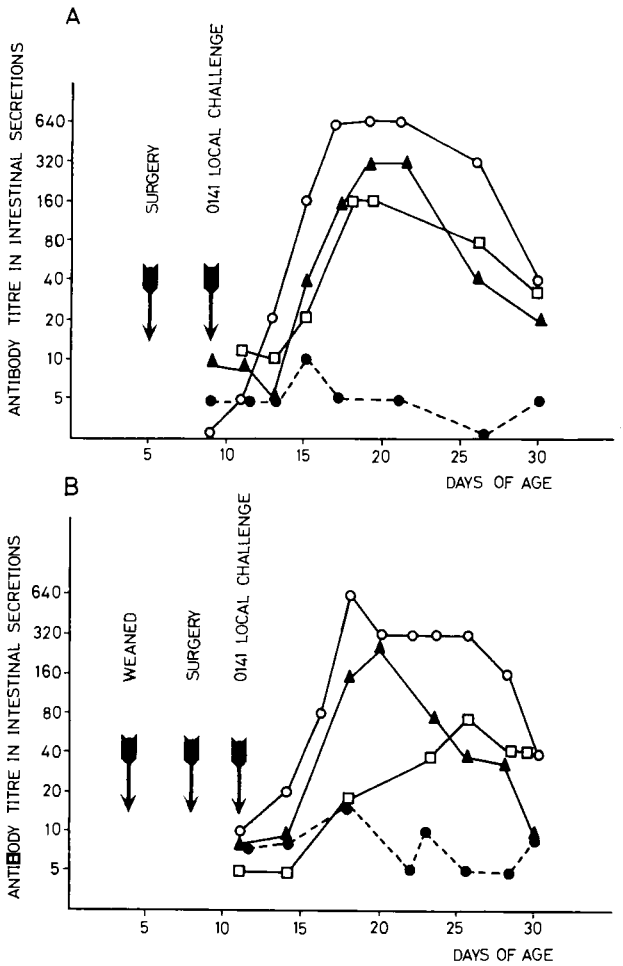


FIG. 3. Local intestinal antibody secretion after administration of heat-inactivated *E. coli* O141 to baby pigs.
(A) Animals maintained on the sow.
(B) Animals weaned at four days of age and raised on reconstituted spray-dried cow's milk. ●, control animals. ○, ▲ and ■, animals locally challenged with heat-inactivated *E. coli* O141.

responses creating a decline in performance, leading finally to overt disease and death. Young farm animals are growing rapidly during this stage of development and we have taken the view that maintenance of intestinal integrity and function against a bacterial challenge should be apparent in terms of animal performance. Thus, whereas the traditional approach to investiga-

tions of *E. coli*-associated enteric syndromes has been towards clinical manifestations of the problem, we have also placed emphasis on the overall nutritional and physiological status of the animal during a vital period of its life.

The requirement for antigens to be administered in repeated doses was met by including them in the diet, thus saving the labour of individual dosing. *E. coli* antigens were added at levels which ensured that on average animals consumed at least 10 times the minimum required dose.

In pilot trials with pigs, orally immunized animals showed a reduction in the natural excretion of pathogenic *E. coli* compared with controls. Significant benefits in weight gain were recorded along with improvements in food conversion, and the incidence of diarrhoea and requirement for medication were reduced (Porter *et al.* 1974*a*). Trials in calves provided much the same data. When calves are reared in units under continuous occupation, health and performance deteriorate in successive batches of animals associated with the bacterial loading of the environment created by previous occupants (Roy *et al.* 1955). It is interesting to note that in such a trial carried out over a period of 12 months involving 16 batches of calves, the overall deterioration in performance shown in control groups was practically abolished by oral immunization.

## IMMUNE MECHANISMS OF HOST RESISTANCE TO ENTEROPATHOGENIC E. COLI AFTER ORAL IMMUNIZATION

Enteropathogenic *E. coli* may represent a substantial component of the intestinal microflora without manifestation of clinical symptoms. The enteric syndrome is usually associated with the establishment of enteropathogens in the anterior regions of the small intestine, a view supported by the observation that the tissues become progressively less responsive to the effects of *E. coli* enterotoxins after the first few feet, comprising the duodenum and upper jejunum. Thus attachment to the intestinal wall is considered to be an important prerequisite of an enteropathogen, enabling it to counter the flushing effects of peristalsis. Furthermore the toxic mechanisms will be optimized by the close proximity of the bacteria with the epithelial cells (Smith & Linggood 1971).

The commonest mechanism of adhesion identified in the Enterobacteriaceae is associated with the presence of filamentous surface antigens. A common K antigen, K88, occurs in porcine enteropathogenic strains and this, unlike other K antigens, is a protein component forming fine filaments on the surface of the bacterium. The antigen is the product of an episomal gene which can be transferred (Ørskov & Ørskov 1966) and elimination of the genetic elements

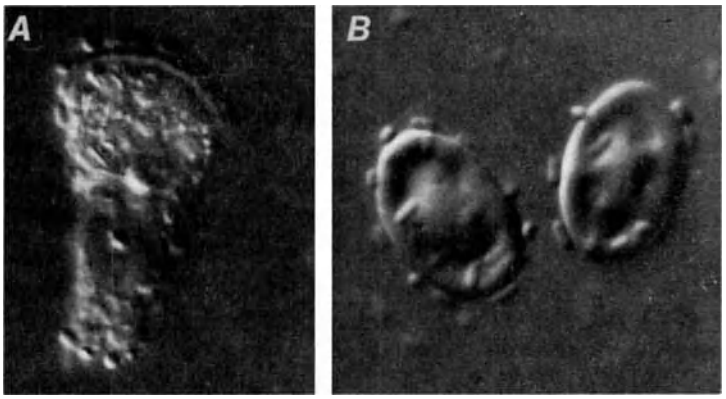


FIG. 4. Adhesion of pathogenic *E. coli* [O8; K87(B), K88ab(L)] to cell membranes shown by interference contrast microscopy.
(A) Intestinal epithelial cell from duodenum of newborn piglet (× 600).
(B) Chicken erythrocyte (× 600).

responsible for its synthesis results in loss of virulence (Smith & Linggood 1971).

Calf and lamb enteropathogenic strains also possess a common K antigen, designated K99, and this too is controlled by a transmissible plasmid (Smith & Linggood 1972). These antigens appear to display specific characteristics of attachment to host intestinal epithelium. Thus K88 determines virulence in the pig and K99 in the calf or lamb and not *vice versa*. This implies the presence of a specific receptor in the epithelial membrane of each species for the bacterial protein determinant. In this context it is of interest that *E. coli* possessing these antigens also agglutinate and adhere to the erythrocytes of certain species (Fig. 4). The erythrocyte and its membrane are a more suitable subject for investigation than the enterocyte and furthermore a simple haemagglutination technique provides a useful method for quantifying the bacterial K antigen.

We have used the chicken erythrocyte to facilitate our investigations of the K88 virulence determinant for the pig; by specific inhibition techniques we have correlated the agglutination of erythrocytes with adhesion to enterocytes. Additionally, both properties can be inhibited by antibodies raised against the purified K antigen. The presence of a possible membrane receptor in enterocytes and erythrocytes is demonstrated indirectly by treating the cells with K88 antigen and subsequently localizing the antigen with fluorescent antibody.

The role of virulence determinants in protective immunization has been examined in relation to porcine neonatal enteritis by Rutter & Jones (1973). Sows were immunized with a crude preparation of K88 antigen and passive protection in the suckling neonate was suitably demonstrated by oral challenge

with enteropathogenic *E. coli*. Parenteral immunization of the sow results in transfer of antibody, mainly by transudative processes, and the antibody class yielded by the immunization protocol used in this study is invariably IgG. An active local antibody response to bacterial adhesion determinants would be expected to enhance the resistance of a young animal to intestinal colonization with enteropathogenic serotypes of *E. coli*, but so far these protein K antigens have not been used successfully as an oral immunogen.

There are, however, indirect means of eliminating the properties of bacterial adhesion. Agglutination may assist in creating bacterial aggregates which may successfully be eliminated by peristalsis, but of greater potential significance is our recent observation that piglets orally immunized with O antigens, and then infected with K88-containing enteropathogens, subsequently excrete only K88-negative *E. coli*. We failed to infect control pigs with cultures prepared from the excreted serotype, whereas the original enteropathogen was demonstrated to be virulent and excreted without modification in the control animals. Furthermore, in an *in vitro* model system in which K88-positive *E. coli* were passaged through media containing antibodies to O antigens, the K88 plasmid was progressively lost so that the level of antigen which was produced in culture declined rapidly over a series of passages. Thus modification of the antigenic composition of enteropathogens occurs in the presence of antibody *in vivo* and *in vitro*. The practical benefits of oral immunoprophylaxis therefore go beyond the immediate characteristics of host resistance, and a very significant advantage to the environment will accrue in terms of a limitation on the excretion of serotypes with virulence determinants, thereby reducing the risk to other animals.

The ability to produce enterotoxin is a prime requirement for enteropathogenicity and this too has been identified with the presence of a transmissible plasmid (Smith & Halls 1968). Enterotoxins of *Vibrio cholerae* and *E. coli* have several similarities and their main function is the creation of a prolonged disturbance of fluid and electrolyte balance. If we take the view that *E. coli* enterotoxins are poorly antigenic, it might be most appropriate to make an indirect approach to the problem via antibacterial mechanisms. Thus, if the bacterium is eliminated, the enterotoxic problem does not arise. In this respect it is significant that Smith & Linggood (1971) had concluded from studies of post-weaning diarrhoea in pigs that the protective function of immunity may be principally associated with bactericidal as opposed to anti-enterotoxic mechanisms.

This concept of indirect protection against enterotoxigenicity was tested in orally immunized pigs in which the main effect of mucosal antibodies was considered to be bacterial agglutination and stasis (Porter *et al.* 1974*b*).

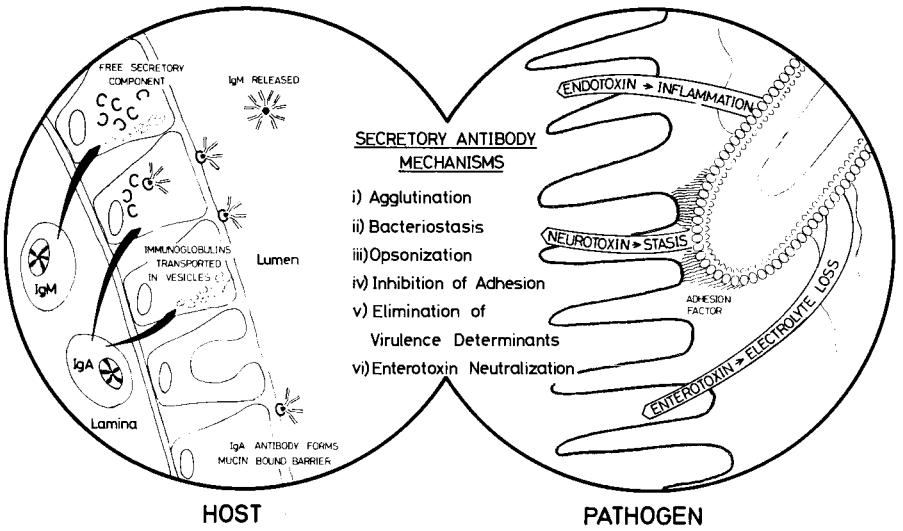


FIG. 5. Schematic representation of host–pathogen interaction in the intestine, showing mechanisms of secretion of antibody (*left*) and virulence characteristics of enteropathogenic *E. coli* (*right*). Defined characteristics of interference with microbial pathogenicity by secretory antibody are listed.

Bacterial proliferation in response to infection during the critical two-week period after weaning was substantially reduced by oral immunization with heat-inactivated antigens, and in consequence so was the severity and duration of diarrhoea. Furthermore, in recent studies orally immunized piglets were used in ligated gut tests to look for an inhibition of the enterotoxigenicity of live pathogens (Balger *et al.* 1975). Such animals were demonstrated to resist approximately 1000 times greater doses of *E. coli* than control animals and the effect was attributed to bacteriostasis. However, perhaps of greater significance are the recent observations of Linggood (1976) using the ligated gut model with cell-free isolates of *E. coli* enterotoxin. In these studies enterotoxin neutralization was observed in orally immunized animals and in addition the activity could be passively transferred with intestinal secretions. Thus enterotoxin inhibition can be mediated directly by mucosal antibodies, without necessarily invoking indirect antibacterial mechanisms.

The variety of ways in which secretory antibodies may mediate in local interference with microbial pathogenicity are summarized in Fig. 5. Most of these mechanisms will be required for solid immune defence.

Finally, in considering the mechanisms of synthesis and secretion of immu-

noglobulins and their presentation as a local antibody barrier on the intestinal epithelium, it is relevant also to consider the distribution of immunoglobulin-producing cells within the lamina propria. If we return to the observation of Smith & Linggood (1971), that in order to cause disease the bacteria needed to proliferate in the upper region of the small intestine, it is significant that immunocytes are more numerous here than anywhere else in the gut. A further link in quantitative terms is apparent when one considers that it is this region of the intestine which is most susceptible to the effects of enterotoxin (Smith & Halls 1967). Thus immune cell function predominates in the most vulnerable area of the gut rather than in the region which carries the greatest microbial population. Probably this characteristic of intestinal function will contribute to the control of the pathogenic component of the intestinal flora not only locally, but generally, by virtue of the release of antibody which will ultimately traverse the length of the alimentary tract in the digesta. In this context, our observation on the effects of low levels of secretory antibody causing loss of virulence determinants in cultures of enteropathogens will be relevant. Thus, in terms of the balance of the host–pathogen relationship, the environmental implications of the excretion of fewer organisms with lower virulence will definitely benefit the host.

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## Discussion

*Pierce:* You suggested that it is necessary to continue to feed the antigen in order to sustain protection, as measured by the presence of coproantibody. In dogs immunized by one of several means that induce a local immune response to cholera toxoid, we obtained protection against live vibrio challenge which far outlasted the presence of preformed antibodies in gut washings (Pierce

1976). Have you considered that it may not be necessary to continually feed the antigen, or to have pre-existing secreted antibody in the gut, to protect the animals?

*Porter:* We have not examined this particular aspect. The mechanisms we have concentrated on would probably require the continuous secretion of antibody; for example, the loss of the virulence determinant, or inhibition of the adhesion determinant, and bacteriostasis, would all be mediated by secreted antibody.

*Pierce:* Perhaps antibody-mediated mechanisms can be effective even in the absence of pre-formed secretory antibody—for example, by a rapid secondary response after re-exposure of a primed animal.

*Porter:* Have you evidence of a rapid secondary response of this type?

*Pierce:* Only in terms of protection against experimental cholera in dogs, which has an incubation period of 12–16 hours. We have shown that significant protection against oral challenge with live *Vibrio cholerae* lasts at least eight months after effective local immunization with cholera toxoid (Pierce 1976).

*Porter:* There is a difference between the level of antibody secretion, and the antibody which would be present in the intestinal epithelium. There will be continuing antibody in the intestinal wall that is not revealed by the crude measurement of antibody in the secretion from the gut loop. However, I do not think that protection will go on for months.

*Lehner:* Dr Pierce's question touched on the problem of whether there is a memory in the secretory antibody (IgA) system, and some of the evidence you showed, Dr Porter, suggests that there wasn't; the antibodies did not appear early, the response was not greater in magnitude, and it didn't last longer. Can you enlighten us about the existence of an IgA memory system?

*Porter:* Professor Parrott has published work on bovine serum albumin (BSA) which indicated tolerance. We were studying a response that will not involve T cell help, since we use the O endotoxin. We use this antigen because it is very stable, is not degraded in mammalian intestine to any extent, and mediates important anti-bacterial mechanisms. We wouldn't have expected that the O antigen would mediate an anti-enterotoxic effect, and Balger's work suggests that it was a bacteriostatic mechanism rather than an anti-enterotoxic one (Balger *et al.* 1975), yet we are getting enterotoxin antigens surviving and producing antibodies that can be transferred between species.

The other important point is the mediation against the adhesion-virulence determinant. We can produce the virulence determinant very easily and can measure it, but we are not yet able to immunize by feeding this protein. In Professor Parrott's work with BSA there was little activity in terms of secreted antibody and yet there was tolerance, after oral feeding of antigen.

*Parrott:* We fed large amounts of protein antigen (BSA) and got partial tolerance (Thomas & Parrott 1974). There was also a small amount of systemic antibody but there were no IgA-secreting cells in the lamina propria; but there was no antibody production after challenge with BSA in Freund's complete adjuvant, so presumably no production of memory.

*Brandtzaeg:* In relation to a possible secondary response, Richard Newcomb repeatedly immunized his own nose with serum albumin from *Alligator mississippiensis*, and found after prolonged immunization an apparent feedback on the secretory IgA response as measured in the secretion (Newcomb & Sutoris 1974). It may be misleading just to look at the secretions, however. For example, if gland-associated secondary immune responses also lead to a gradual switch from production of dimeric IgA to an increased proportion of monomeric IgA and perhaps also IgG, these immunoglobulins will not appear in the secretions. A secondary response may thus be masked because of lack of secretory properties of the antibodies produced. This possibility should be considered in evaluating mucosal secondary immune responses.

*Mayrhofer:* Another factor is that if IgA has a role in preventing antigen absorption by the gut, then any possibility of producing a secondary response by gut challenge with an antigen is reduced for as long as secretory antibody to that antigen persists. This could make it difficult to demonstrate memory in the IgA system of the intact animal. An adoptive transfer of immune lymphocytes into a non-immune animal may be necessary in order to show it.

*André:* We found that a repeat intragastric administration of sheep red blood cells resulted in a spleen response with plaque-forming cells which belonged predominantly to the IgA class. This spleen response was in all points similar to the primary response and in no way suggestive of immunological memory when the animals were given their second immunization after a three-month rest period. In contrast, when the intragastric immunization was resumed two weeks after the priming period, no response was obtained (André *et al.* 1973). It is known that local immunization of the gut impairs its capacity to absorb the corresponding antigen (André *et al.* 1974). But we have also established that intragastric immunization caused an immunological hyporesponsiveness to parenteral challenge and that one is therefore dealing with tolerance and not with failure to absorb antigen from the gut (André *et al.* 1975).

*Porter:* We get different results with bacterial antigens. We get a booster response, not tolerance, by oral immunization. If we orally immunize with the *E. coli* antigens and follow with a single parenteral administration of the same heat-killed antigen, there is a booster response providing an IgM antibody in serum. We haven't looked at the secretions because we wanted to exploit this response in terms of colostral immunity, timing the IgM response to coincide

with formation of colostrum, to get a high IgM antibody titre into the suckling neonate (this is John Chidlow's work). This is distinct from the tolerance observations made by Professor Parrott.

*Lachmann:* Is it significant that you are using on the one hand a T-dependent and on the other a T-independent antigen? The classical demonstration of tolerance following oral immunization is the Sulzberger & Chase phenomenon, where it is to delayed hypersensitivity that feeding hapten renders the animal tolerant (Sulzberger 1929; Chase 1963).

*Porter:* Yes. We are using a T-independent antigen. This is the difference from Dr Parrott's work.

*White:* You said that if you continuously administered the antigens of *E. coli* to pigs you found a response in terms of cell numbers that was like that generated by infection with gut pathogen; thus in the monocontaminated and in the orally immunized germ-free animal the cell responses were similar. In the chicken, whereas we obtained a similar cell response in the monocontaminated bird to that generated in the pig, the oral administration of *E. coli* O antigens produced no cell response. Is that related to adherence of the antigen to the intestinal wall? Does it adhere in the pig and not in the chicken?

*Porter:* I don't think so, but we haven't looked at this question specifically. We have examined the survival of the antigen along the alimentary tract of the chick; we fed iodine-labelled *E. coli* O2 antigen, which is pathogenic for the chick. It survives into the caecum. There is much degradation, since iodine-labelled fragments are absorbed into the blood and excreted in the urine. We wondered whether there is a lack of absorption of the antigen, or perhaps adherence of the antigen, or survival of antigen, in the region of the bursa. Furthermore, the distribution of Peyer's patches along the intestine of the pig gives a greater opportunity for immune stimulation than does the simple presentation of the antigen to the bursa in the chicken.

*White:* You said that antibody does not interfere with the immunization process. Does that depend on the specificity of the antibody?

*Porter:* No. We were using an immunization schedule that reproduced what we envisaged would exist in the sucking pig, in preparation for events at weaning, because the young animal normally then goes through an *E. coli* proliferation lasting two weeks. A survey by Svendsen *et al.* (1974) found 82% morbidity associated with *E. coli* pathogens at weaning. We envisage preparing the young animal and its intestinal immune system to take over from the protection continuously mediated by maternal IgA antibody in its intestine during the suckling period. The sow provides antibody to the gut lumen of the young animal throughout suckling and that presumably could interfere with

any antigenic stimulus, given orally. The experiment I described (p. 60) showed that the maternal antibody did *not* interfere.

*White:* Was it antibody against the K antigen?

*Porter:* No, it was antibody against the O antigen, which was interfering with the production of the adherence factor.

*Booth:* One obvious feature of the gut mucosa is the mucus layer but whenever we look at preparations, by electron microscopy or any other method, we never see it. This applies to all techniques of fixation for electron microscopy. You have made scanning electron micrographs simply showing an impregnated surface of the membrane.

*Porter:* Those electron micrographs illustrate adherence. In an animal that had died because of *E. coli* infection, or is in the worst phases of infection, the anterior small intestine is completely coated with millions of organisms, whereas in an animal excreting the same pathogen and not showing clinical symptoms, the anterior small intestine can be free. So whether the animal shows clinical symptoms is determined by the upper region of the small intestine.

*Booth:* The question is whether the organisms adhere in the small intestine. It has been proved for the mouth.

*Porter:* Yes. The bacterium must gain access to the upper small intestine and adhere where it has close association with the epithelial cell and the capability to multiply and secrete its toxin, because *E. coli* is non-invasive and the effect is mediated by toxin. The interesting point is that the antibodies are mediating not against adhesion but against the ability of the pathogen to produce the adhesion factor.

*Gowans:* Has the ability of virulent bacteria to adhere to the intestinal epithelium any connection with the ability of the organisms to prevent mucus secretion? Mucus does not appear on your list of possible mechanisms, presumably because it is not an immunological mechanism, but one would like to know whether it is important.

*Lachmann:* In fact it is not altogether non-immunological. Eggert (1976) in Professor Coombs' laboratory has evidence of IgA complexes with mucin that mediate antibacterial reactions in saliva.

*Porter:* We have interesting observations in relation to the reaction of the K88 antigen which go some way towards answering this question. If we prepare extracts of this antigen from a virulent organism and treat sections of gut with it, and then with a fluorescent antiglobulin, or do the same with chicken erythrocytes, we see receptor domains for that antigen.

*Booth:* The background of my question was an observation of Tabagchali (1970) using electron microscopy and non-pathogenic *E. coli* in man, showing the bacteria beautifully wrapped up in mucus, which is very puzzling.

*Porter:* We can agglutinate bacteria *in vitro* with anti-O antibody and do not prevent adhesion, so the agglutinate still adheres by available determinants to the gut epithelial cells.

*Bienenstock:* Bloch & Walker studied antigen uptake in the rat ligated everted gut sac. If the rats are pre-immunized or there are immune complexes in the bathing medium, the goblet cells eject mucus (K. J. Bloch & A. Walker, personal communication 1976). It would be interesting to look at the relationship of immune complexes to that.

*Cebra:* You seem to be suggesting, Dr Porter, that IgM could pass into the gut lumen but that IgA could adhere to the mucosal surface of the cells. Perhaps there is an interaction between the Fc region of IgA and the cell surface, or perhaps an Fc–mucin interaction. Have you any observations on the relative adhesion of serum as against secretory IgA to the surface of cells? We had wondered if the passage of IgA across undifferentiated crypt cells might lead to the sequential addition of monosaccharides, leading to a mucin-like oligosaccharide and resulting in a cell surface interaction directly or via a mucin binding. Have you done comparative studies?

*Porter:* No, nothing in that realm, but would you not think that the secretory component mediates this binding effect with mucin?

*Cebra:* I am open-minded on this. But just how different are the two isotypes, IgM and IgA, with respect to mucin binding which in turn may lead to cell adhesion?

*Porter:* We obtained intestinal secretions from Thiry-Vella loops in calves. By the time we put the secretions into the fridge to cool, the mucin had clotted and when we compared the supernatant and the clot, the binding of IgA and IgM was very different. Only IgM went into the supernatant, whereas IgA was bound into the mucin gel.

*Lachmann:* What is the evidence of opsonization by virtue of IgA antibodies?

*Porter:* Rowley made the initial observations on this (Wernet *et al.* 1971) but I believe he has subsequently retracted this thesis.

*Soothill:* Unfortunately Dr Breu was unable to reproduce this phenomenon while working in our laboratory.

*Porter:* There is other work that has shown this. Girard & de Kalbermatten (1970) made similar observations using intestinal antibodies. One should remember also that intestinal antibody function is not solely mediated by IgA; I have emphasized the role of IgM (Fig. 5, p. 65). This may also exert a potent antibacterial effect in gut immunity.

*Lachmann:* IgGl in serum at least is a perfectly good opsonizing and complement-fixing antibody.

*Porter:* There is interesting work by Bellamy (1973) in relation to the appear-

ance of neutrophils in the intestinal lumen, which seems to be antibody-mediated. Bellamy looked in the calf at the transudation of neutrophils into Thiry-Vella loops in response to the local administration of BSA and also *E. coli* antigens. This seemed to be antibody-mediated rather than T cell-mediated, so there may be a basis for opsonization within the gut lumen.

*Lehner:* On the same basis, has anyone shown Fc receptors for IgA?

*Pepys:* There is evidence that IgG and IgA from the plasma bind in the cold to human peripheral blood lymphocytes and monocytes; the immunoglobulins can be removed by washing the cells at 37 °C instead of 4 °C, or by a low pH shock (Kumagai *et al.* 1975; Lobo *et al.* 1975). It has not been established that this binding, particularly of IgA, takes place via receptors for Fc.

*Lehner:* Is there any evidence for the IgA Fc receptor on polymorphs or macrophages by rosetting or by using aggregated IgA and anti-IgA conjugate?

*Pepys:* The work of Kumagai *et al.* (1975) demonstrated the following. A proportion of human peripheral blood lymphocytes and monocytes, prepared by Ficoll-Hypaque separation and washed at 4 °C, can be stained with fluorescent anti-IgA antibodies. If the cells are washed at 37 °C before fluorescent staining, very few stain with anti-IgA. Reincubation at 4 °C of the warm-washed cells with a source of IgA restores the staining with anti-IgA.

*Lachmann:* It has been shown that IgA will not inhibit K cell cytotoxicity (Wislöff *et al.* 1974).

*Rosen:* There is an interesting situation in man where the early feeding to the newborn of poliovirus results in the prompt cessation of poliovirus secretion and in no immunity. This occurs when the mother has a reasonable titre of antibody to poliovirus. The reason is that the human newborn has Fab fragments in the intestine which are washed out at about 48 hours after birth. Fab fragments can even be found in newborn infants with oesophageal atresia. Fab has about 10 times less viral-neutralizing capacity than intact γ-globulin. Nonetheless, these Fab fragments are effective in neutralizing poliovirus.

*Bienenstock:* Chickens are interesting in that they have very few, but large, Peyer's patches; there are only two in the whole gastrointestinal tract, exclusive of the bursa. It might be worth exploring some of the questions we discussed earlier using the chicken, because this species has many IgA cells with relatively few Peyer's patches.

*Porter:* Most characteristics of the secretory immune system in the chicken have an analogy to the mammal; there is an analogue, if not a homologue, to the secretory component, and there is the same sort of response to bacterial infections, yet antigen given orally fails to mediate responses seen in mammals.

*Bienenstock:* One reason may be that the bird doesn't have the Peyer's patches and therefore lacks the lymphoid tissue necessary for the response.

*Parrott:* Perhaps you should sit chickens in a bath of antigen, so that it can get to the bursa—the converse of oral immunization!

*White:* This has been done with HSA. The antigen is cloacally drunk by the chicken; there is not much immediate antibody formation, but there is sensitization to subsequent systemic challenge and you lay down the mechanism for antibody production, the antigen going via the cloaca into the bursa.

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*Immunology of the Gut*
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# Intestinal secretion of IgA and IgM: a hypothetical model

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*Abstract* The secretory component (SC) has recently been found to be associated with IgM in external secretions, although in a less stable complex than secretory IgA. Moreover, SC combines spontaneously *in vitro* with both IgA and IgM. A prerequisite is that the immunoglobulins contain the J chain, which is present only in dimers and polymers. This polypeptide is essential for the formation of an SC-binding site which appears already at the cytoplasmic level in IgA- and IgM-producing immunocytes. Locally formed J-chain-containing immunoglobulins are therefore readily available for complexing with SC present in the membranes of columnar secretory epithelial cells of glandular sites. This complexing initiates pinocytosis and external transport. Immunohistochemically the gland cells are shown to contain SC, IgA and IgM in identical locations, except that SC alone appears in the Golgi zone. Locally formed IgA and IgM antibodies are thus efficiently transferred to the mucosal surface where they exert an immunological exclusion of antigens. Conversely, IgG antibodies, which are not actively drained away from the lamina propria, may rather become engaged in complement activation and cell-mediated cytotoxicity with potentially deleterious effects on the tissue. Secondary to severe inflammatory reactions, secretory epithelium may show decreased production of SC; the selective external transport of SC-stabilized secretory IgA and IgM is thus jeopardized, and a vicious circle may be set up in the mucosa.

Because of its quantitative dominance and unique association with an epithelial secretory component (SC), IgA is considered as the most important immune factor in human exocrine secretions (for reviews, see Tomasi & Grey 1972; Hanson & Brandtzaeg 1973; Heremans 1974). Nevertheless, some individuals appear completely healthy despite a selective lack of IgA, at least in countries with good hygiene. It has been speculated that this may depend on a compensatory local immune mechanism expressed by enhanced local synthesis and secretion of IgM, known to occur in IgA deficiency (Heremans & Crabbé 1967);

Brandtzaeg *et al.* 1968; Eidelman & Davis 1968; Brandtzaeg 1971*a*). The observation that locally produced IgM may exhibit anti-virus activity in IgA-deficient individuals (Ogra *et al.* 1974) supports a possible contribution of this immunoglobulin to mucosal defence mechanisms. On the basis of our quantifications of immunoglobulins in pure glandular secretions we proposed several years ago that IgM should be considered as a secretory immunoglobulin with epithelial transport properties similar to secretory IgA (Brandtzaeg 1968). Indeed, subsequent studies of reptiles and amphibians suggested that IgM probably served the function of protecting mucosal surfaces before the evolution of IgA (Portis & Coe 1975). Recent immunochemical studies of IgM present in human secretions have moreover demonstrated that the 19S pentamers contain SC, which is retained in 60–70% of the molecules after purification (Brandtzaeg 1975*c*). Compared with secretory IgA, the quaternary structure of secretory IgM is less stabilized, since SC shows reactive I determinants after incorporation and depends on an excess of free component for permanent association with the remaining 30–40% of IgM in the secretions.

## A MODEL FOR THE GLANDULAR TRANSPORT OF IgA AND IgM

It was speculated in several early studies that SC may facilitate the entry of extracellular IgA into glandular epithelial cells (Tomasi *et al.* 1965; South *et al.* 1966; O'Daly *et al.* 1971). SC was therefore originally called 'transport piece' (South *et al.* 1966). This suggestion was prompted by the fact that SC was complexed with secretory IgA, but there was no obvious explanation for its postulated transport function.

We proposed originally that a common glandular transport mechanism operates for IgA and IgM independently of SC, perhaps involving unique characteristics ('transfer sites') in the Fc portions of these two immunoglobulin classes and a corresponding epithelial receptor of unknown nature (Brandtzaeg 1968; Brandtzaeg *et al.* 1970). This view was mainly influenced by our early failure to demonstrate a regular association between SC and secretory IgM (Brandtzaeg *et al.* 1968; Brandtzaeg 1971*a*). An epithelial receptor specific for IgG1 has recently been demonstrated in bovine mammary glands, which preferentially transmits this immunoglobulin unchanged from serum during the colostrum-forming period (Kemler *et al.* 1975).

Subsequent observations, however, have made it increasingly likely that the epithelial membrane receptor specific for IgA and IgM is identical with SC. We have therefore recently proposed a common glandular transport model for these two immunoglobulins including five critical steps as depicted in Fig. 1: (1) J chains are produced and incorporated into IgA dimers or larger polymers

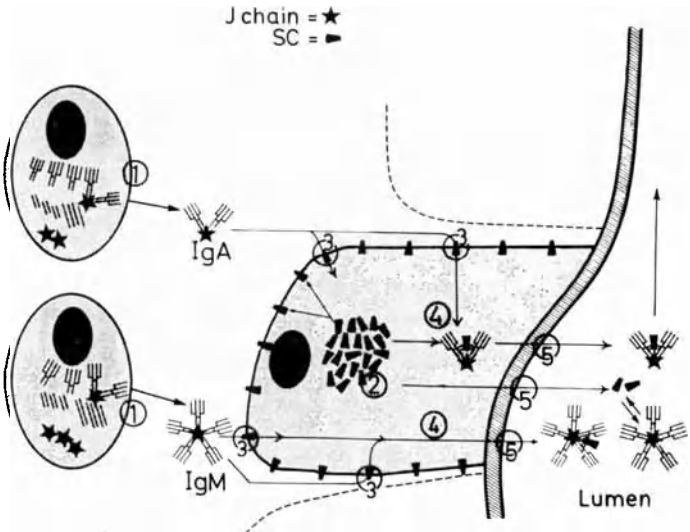


FIG. 1. Schematic representation of gland-associated synthesis and selective external transport of dimeric IgA and pentameric IgM. It is proposed that SC acts as a specific receptor for these two immunoglobulins, and that Ig–SC complexes are formed and become mobilized in the plasma membrane of secretory epithelial cells. The completed secretory immunoglobulins finally reach the gland lumen via the cytoplasm outside the Golgi region. While conjugation of IgA with SC is efficient and gives rise to stable complexes, this is so for only 60–70% of the IgM; the rest of the secreted IgM contains SC in an association that is unstable and depends on excess of free SC in the fluid. The five schematic steps are discussed in the text. (Modified from Brandtzaeg 1974*d*.)

and into IgM pentamers in gland-associated immunocytes; (2) SC is produced by secretory epithelial cells, concentrated in the Golgi zone, and made available for the general secretory process as well as for plasma-membrane incorporation; (3) structural characteristics induced by the J chains in polymeric IgA and pentameric IgM constitute a specific SC-binding site; the polymers will therefore after release from the immunocytes readily become bound to epithelial cells by non-covalent interactions, which apparently depend on the I-determinant-bearing part of the membrane-associated SC (Brandtzaeg 1975*a*); (4) the Ig–SC complexes are then taken up by the epithelial cell by adsorptive pinocytosis and subjected to stabilizing conjugation by disulphide exchange

(enzymically catalysed?); such exchange reactions depend on the immunoglobulin class as well as on an excess of free SC (Brandtzaeg 1974*a*); (5) finally, the completed secretory polymers are extruded into the gland lumen along general secretory pathways (Kagnoff *et al.* 1973). The possibility also exists that some of the Ig–SC complexes floating in the cell membrane may reach the gland lumen without entering the cytoplasm.

In man the covalent conjugation of IgA with SC is efficient and gives rise to polymers which are very stable in the exocrine fluids. SC is thus disulphide-bonded in 75–80% of human secretory 11S IgA (Brandtzaeg 1974*a*), and only about 10% of the IgA dimers separated from human colostrum lack SC (Mestecky *et al.* 1970). As discussed in the introduction, a permanent association between SC and secretory IgM is more dependent on an excess of free SC (Brandtzaeg 1975*c*). The ratio of free to bound SC varies in different secretions (Brandtzaeg 1973*b*) and also depends on the fluid flow rate (Brandtzaeg 1971*d*). In normal human saliva and colostrum the amount of free SC may approach that of the bound component (Brandtzaeg 1973*b*). In pig colostrum, on the other hand, there are only traces of free SC and 60% of the dimeric IgA lacks SC after purification (Bourne 1974). A stabilization of Ig–SC complexes, as regularly seen for human secretory IgA, may hence be explained by surplus SC production and unique possibilities for disulphide-exchange reactions. The fact that there is no other known situation where a receptor becomes permanently attached to the transported molecule (Lamm 1976) is therefore no valid argument against a receptor function of SC. The phylogenetic selection of the secretory IgA system may indeed have its basis in the unique stability conferred on dimeric IgA by covalently bound SC (Lindh 1974), since the less stabilized secretory IgM apparently has a shorter functional survival time, at least in the gastrointestinal secretions (Haneberg 1974 *a,b*).

The proposed model for selective epithelial immunoglobulin reception and transmission is compatible with the fluid mosaic structure suggested for cell membranes (Singer & Nicolson 1972). However, it must be stressed from the outset that our transport model is based mainly on test tube experiments with purified proteins and on the immunofluorescence of dead tissue. Nevertheless, it should be possible in the near future to test several of the proposed steps by kinetic studies on living cells.

## IMMUNOCHEMICAL AND PHYSICOCHEMICAL OBSERVATIONS SUPPORTING THE MODEL

### *1. Quantitation and characterization of human immunoglobulins*

The concentration ratio of IgG:IgA in pure glandular secretions, such as

TABLE 1

Average immunoglobulin concentrations (mg/100 ml) in serum and some exocrine secretions

| *Sample* | *Immunoglobulin class* | | | *Ratio* | |
|---|---|---|---|---|---|
| | *IgG* | *IgA* | *IgM* | *IgG:IgA* | *IgG:IgM* |
| Serum[a] | 1230 | 328 | 132 | 3.8 | 9.3 |
| Colostrum[a] | 10 | 1234 | 61 | 0.008 | 0.16 |
| Parotid secretion[a] | 0.036 | 3.95 | 0.043 | 0.009 | 0.84 |
| Whole saliva[a] | 1.44 | 19.40 | 0.20 | 0.07 | 6.86 |
| Duodenal secretion[b] | 10.4 | 31.3 | 20.7 | 0.33 | 0.50 |
| Jejunal secretion[c] | 34.0 | 27.6 | N.D.[d] | 1.23 | N.D. |
| Colonic secretion[c] | 86.0 | 82.7 | N.D. | 1.04 | N.D. |

[a] From Brandtzaeg *et al.* (1970).
[b] From Girard & de Kalbermatten (1970).
[c] From Bull *et al.* (1971).
[d] N.D., not determined.

colostrum and parotid saliva, is 400–500 times lower than the same ratio in normal serum (Table 1). This suggests that the exocrine glands actively or selectively transmit IgA. The same reasoning applies to the glandular transfer of IgM, although the reduction of the IgG:IgM ratio is less marked (10–60 times) and varies between different secretions (Table 1). Fluids collected from surfaces of mucous membranes contain relatively more IgG, indicating that the external transmission of this immunoglobulin mainly depends on extra-glandular passive diffusion. Such 'leakage' is enhanced by inflammatory processes (Brandtzaeg *et al.* 1970). Comparisons of the parotid transmission of immunoglobulins in patients with G myeloma or macroglobulinaemia have further attested to the selectivity inherent in the glandular transport of IgA and IgM (Brandtzaeg 1971*a*). Moreover, parotid IgA and IgM show similar secretory dynamics on gustatory stimulation of the gland (Brandtzaeg 1971*d*).

Since secretory IgM is chiefly a 19S pentamer (Brandtzaeg 1971*a*, 1975*c*), its immunoglobulin moiety may be derived from serum. Conversely, the IgA pattern in pure glandular secretions is quite different from that in serum, comprising a major J-chain-containing 11S dimer fraction (Tomasi *et al.* 1965; Halpern & Koshland 1970), two minor (heavier than 11S) polymer populations, and only 10–13% monomeric 7S IgA (Brandtzaeg *et al.* 1970). About 80–90% of human serum IgA normally consists of monomers devoid of J chain (see Vaerman 1973); most secretory IgA must therefore originate in gland-associated

immunocytes which produce both IgA and J chain (see later section, p. 87).

The predominance of IgA in external secretions could hence depend on preferential local synthesis alone rather than on selective external transmission. Nevertheless, glandular transfer selectivity for IgA and IgM is firmly substantiated by immunohistochemical observations (see later, p. 89). Since pure glandular fluids contain about ten times more monomeric IgA than IgG (Brandtzaeg *et al.* 1970), transfer selectivity might seem to be more easily explained by an Fc characteristic of IgA and IgM (Brandtzaeg 1968) than by a J-chain-induced SC-binding site (Fig. 1, p. 79). However, a relative enrichment of 7S IgA compared with IgG may well be accounted for by a combination of passive external transmission and degradation. Intercellular diffusion through the epithelium probably includes monomeric IgA derived both from serum and from the abundant local IgA-producing immunocytes, which release a mixture of monomers and dimers (see later, p. 87). As discussed above for IgM, dimers of IgA are probably unstable before covalent conjugation with SC, and may in part be converted to monomers by intraepithelial and intraluminal degradation. Some breakdown of SC-conjugated dimers is also likely, and may explain why a fraction of the monomeric IgA present in colostrum is apparently associated with SC (Mestecky *et al.* 1970).

A preferential glandular transfer of IgA dimers compared with monomers is supported by analyses of monoclonal IgA components appearing in the saliva of patients with multiple myeloma (Coelho *et al.* 1974), and intravenously injected monomeric IgA does not seem to be selectively secreted (for review, see Heremans 1974; Lamm 1976). One possible exception was reported after the infusion of large quantities of plasma into two hypogammaglobulinaemic patients (South *et al.* 1966). However, it could not be excluded that the traces of IgA transmitted to the saliva were polymers.

A varying fraction (10–20%) of IgA in normal serum is composed of dimers and larger polymers (see Vaerman 1973). This fraction is small in view of the abundant polymer-producing IgA cells found in bone marrow (Rádl *et al.* 1974) and especially in the digestive tract (Brandtzaeg 1973*a*, 1974*c*). The fraction of polymeric IgA in lymph from the thoracic duct is not significantly raised (Tomasi & Grey 1972), perhaps because locally produced IgA polymers are rapidly transmitted to the gut lumen. Moreover, Rádl *et al.* (1975) showed that most IgA polymers present in normal human serum lack the J chain. Since there is a dynamic equilibrium between proteins in serum and interstitial fluid, this observation supports the idea that an efficient clearing mechanism selective for J-chain-containing IgA operates in glandular regions. Only about 20% of serum IgM (molecular weight about 1 000 000) is distributed extravascularly (Waldmann & Strober 1969); a J-chain-dependent clearing mechan-

ism would therefore *a priori* be less efficient for IgM than for serum-derived dimeric IgA (molecular weight about 320 000). It should also be considered that the two molecular species are competing for the same epithelial receptor if this indeed is SC. The proposed transport model could easily explain the lack of external transmission of intravenously injected secretory 11S IgA (Stiehm *et al.* 1966; Butler *et al.* 1967) since the SC-binding site of these polymers is occupied.

*2. In vitro combination of SC with IgA and IgM*

The discovery of SC as a regular subunit of human secretory IgA (Tomasi *et al.* 1965) stimulated a series of attempts to show specific affinity between SC and IgA. Tomasi & Bienenstock (1968) first reported that reduced and alkylated SC added to whole serum combined fairly specifically with IgA. Hanson *et al.* (1969) mixed native free SC with purified IgA and obtained a low yield of poorly stabilized complexes. We likewise found that the affinity of native free SC was relatively specific for serum IgA, although there was some evidence of SC–IgM association (Brandtzaeg 1971*c*). Thompson (1970) had previously reported that small amounts of SC–IgM complexes might occur in sera of IgA-deficient individuals, and also found that IgM in the duodenal juice of one such patient to some extent was associated with SC.

The first indication that a particular conformation is necessary for an efficient binding of free SC came from studies of rabbit secretory IgA; when the polymer had been separated from its bound SC it became highly active in recombination with free SC (Lawton *et al.* 1970*b*; O'Daly & Cebra 1971). Mach (1970) and Rádl *et al.* (1971) independently demonstrated the importance of the IgA-dimer conformation by studying the combination between human myeloma proteins and free SC isolated from colostrum. Complexing with IgM was also noted in these studies, but only polymers larger than 19S pentamers seemed to be active (Rádl *et al.* 1971). In a subsequent study we were unable to confirm the latter finding (Brandtzaeg 1974*a*). By contrast, we showed that SC is able to combine with 19S IgM as readily as with dimeric IgA (Fig. 2*a*,*c*), and that specific complex formation depends on non-covalent interactions (Brandtzaeg 1974 *a*,*b*). This would be a prerequisite for a receptor function of membrane-associated SC. Our observations have been verified by Weicker & Underdown (1975).

When Mach published his results in 1970 the J chain had just been detected in polymeric IgA and IgM (for review, see Inman & Mestecky 1974; Koshland 1975); in an addendum to his paper he therefore postulated a role for this polypeptide in the SC-binding process. We obtained the first evidence to

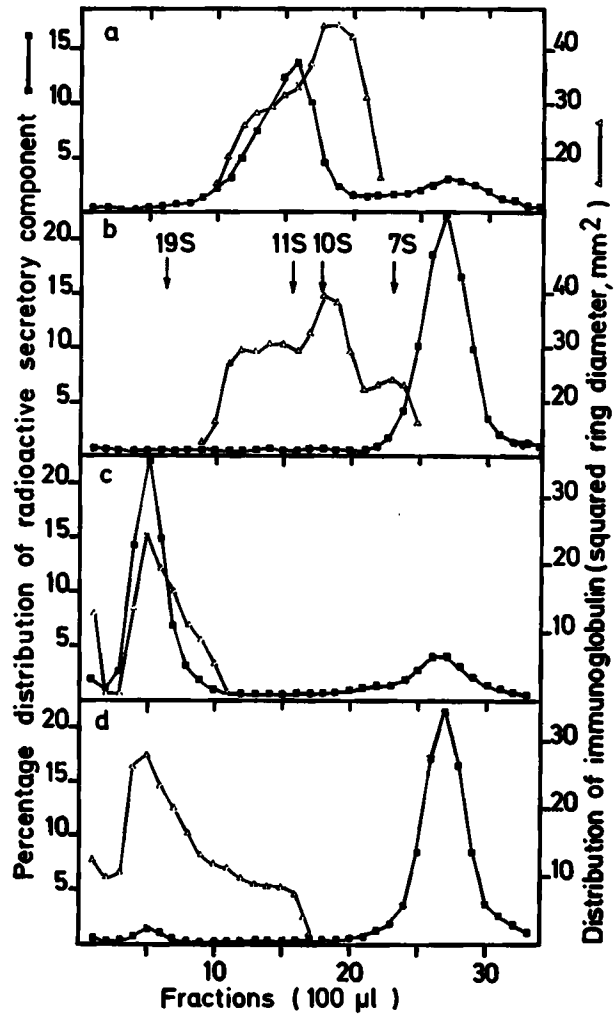


FIG. 2. Ultracentrifugation analyses (42 000 r.p.m., 20 h, 10–35% linear sucrose gradients) of immunoglobulin polymers after incubation with radioactive SC (2.5 µg). The immunoglobulin distribution was determined by testing each fraction in single radial immunodiffusion; SC distribution was shown by scintillation counting. Bottom of gradients is to the left. The positions of four marker proteins are indicated (vertical arrows) as reference for S values. Samples: (*a*) 100 µg of J-chain-containing polyclonal IgA polymers (mainly 10S dimers); (*b*) 100 µg of J-chain-deficient monoclonal IgA polymers; (*c*) 50 µg of J-chain-containing monoclonal IgM 19S pentamers; and (*d*) 110 µg of J-chain-deficient monoclonal IgM polymers.

support such a function of the J chain when we found that an IgM polymer lacking it failed to bind SC *in vitro* (Eskeland & Brandtzaeg 1974). In a subsequent study we quantified immunochemically the reductive release of J chain in twenty-four IgA preparations (Brandtzaeg 1976*b*). Five polymeric fractions that contained only 0.2–0.8 mg J chain per 100 mg protein showed an SC-binding capacity of 6–12% compared with 69–82% for polymers containing more than 4.0 mg J chain per 100 mg (Fig. 2). Monomeric IgA without contaminating J-chain-positive polymers did not bind SC. The report of Jerry *et al.* (1972) has caused some confusion in this respect. They found that in the presence of a large excess of SC obtained from reduced secretory IgA, covalent complexing took place with monomeric IgA of the subclass $\alpha_2$ and genetic variant $Am_2$ (+). These findings must be clearly distinguished from our results which are based on non-covalent interactions with small amounts of native free SC.

We have thus demonstrated that the J chain is mandatory for a spontaneous association of SC with IgA and IgM, but the mechanism of its function in this binding process is still unknown. One possibility is that the SC-binding site is located in the Fc region of the Ig polymers, the conformation of which may depend on J-chain incorporation. Direct non-covalent interactions between SC and the J chains may be another explanation, although the purified polypeptide only marginally blocks the binding of SC to dimeric IgA and 19S IgM (Brandtzaeg 1975*d*). Antibody to J chain, on the other hand, efficiently blocks the SC-binding site of these polymers (Brandtzaeg 1975*d*). Such experiments are difficult to interpret, but the results are compatible with the hypothesis that polymer-incorporated J chains have acquired a configuration conducive to specific interactions with SC.

Contrary to the present view (see Koshland 1975), our recent studies of human J chain have indicated that it occurs as a dimer in IgA and as a trimer or two dimers in IgM (Brandtzaeg 1975*b*). IgM should hence show stronger SC affinity than IgA if this property is determined by the J chain. Indeed, the binding of SC to IgM is less inhibited by high salt concentrations (Brandtzaeg 1974*a*), and on a molar basis pentameric IgM has five to thirty times better affinity for SC than dimeric or trimeric IgA in competitive tests (P. Brandtzaeg, unpublished data). It is tempting to speculate that these observations reflect the 'bonus effect' of a higher molar J-chain content in IgM. Thus, if our glandular transport model is correct, the relative amount of bound J chain may have biological consequences by enhancing the epithelial reception and transmission of IgM, whose local synthesis normally is inferior to that of dimeric IgA (see below, p. 86).

## IMMUNOHISTOCHEMICAL OBSERVATIONS SUPPORTING THE MODEL

### *1. Characterization of intestinal immunocyte populations*

The reliability of immunohistochemical observations depends on the quality of the fluorochrome conjugates as well as on the tissue-processing technique (for review, see Brandtzaeg & Baklien 1976). Our aim is complete retention of diffusible immunoglobulins in one piece of the biopsy specimen (see Fig. 11 *a-c*, p. 100) and extensive removal of these components from another with preservation of immunocytes and facilitated characterization of their cytoplasmic content (see Fig. 11*e-h*, p. 100). The former piece is fixed directly in cold alcohol, whereas the latter is first extracted by washing in isotonic buffered saline (Brandtzaeg 1974*d*).

TABLE 2

Immunoglobulin-containing cells in normal adult intestinal mucosa

| | *Cell numbers expressed as mean, observed range, and average % contribution* | | |
|---|---|---|---|
| | *IgA cells* | *IgM cells* | *IgG cells* |
| Jejunum | 104(60-163)<br>81% | 21(8-36)<br>17% | 3.2(1-8)<br>2.6% |
| Ileum | 37(21-61)<br>83% | 5(1.5-10)<br>11% | 2.5(0.3-6)<br>5% |
| Large bowel | 129(28-237)<br>90% | 8(1-27)<br>6% | 6(2-14)<br>4.2% |

Table modified from Brandtzaeg & Baklien (1976). Data based on a 'mucosal tissue unit' constituting a 6-μm-thick and 500-μm-wide block of tissue including the mucosa at full height from the muscularis mucosae.

A marked preponderance of IgA-producing cells is found at all levels of the human intestinal tract (Table 2). We have calculated that almost $10^{10}$ such immunocytes normally occur per metre of small bowel. The share of IgM-producing cells varies between 6% and 17%, being highest proximally, and IgG cells are normally even fewer. The immunocytes are not homogeneously distributed throughout the various mucosal layers. In the normal large bowel about 60% occur in the luminal 200-μm zone, with decreasing numbers towards the muscularis mucosae. In the small bowel about 70% occur in the 200-μm zone around the base of the villi. Marked alterations are seen associated with bowel disease. Thus, in coeliac disease an increase takes place for all immunocyte classes, in relative terms most prominent for IgG and IgM cells.

In ulcerative colitis and Crohn's disease the IgG cell response is very dramatic (for review, see Brandtzaeg & Baklien 1976).

Gland-associated IgA immunocytes were initially thought to produce monomers which subsequently polymerized by complexing with SC (Fig. 10*a,b*, p. 97). Random combination of the polymer subunits was supported by the apparent occurrence in human colostrum of hybrid 11S IgA isoagglutinins containing both κ and λ light chains (Costea *et al.* 1968). But molecules hybrid with regard to allotypic markers could not be detected in rabbit colostral 11S IgA (Lawton & Mage 1969). Also, studies of suspended rabbit appendix immunocytes indicated a direct release of dimeric IgA (Cohen & Kern 1969). Our finding of J chains with partially buried antigenic determinants in human intestinal IgA cells is likewise indicative of dimer production (Brandtzaeg 1976*a*). Moreover, SC-affinity tests on tissue sections (Brandtzaeg 1973*a*) show that the J-chain-containing IgA and IgM immunocytes (Fig. 3) can bind SC to their cytoplasm *in vitro* (Brandtzaeg 1974*c*). This non-covalent affinity characteristic (Brandtzaeg 1974*a*) demonstrates that the polymer subunits to a substantial extent are 'correctly' aligned already at the cytoplasmic level. Thus, when these immunoglobulins are released into the extracellular fluid, they are readily available for spontaneous complexing with membrane-associated SC of columnar epithelial cells.

The intestinal IgA immunocytes are heterogeneous with regard to cytoplasmic SC affinity (Brandtzaeg 1973*a*) and J-chain content (Fig. 3*a-c*). This indicates that the cells produce varying proportions of monomers and polymers. According to our glandular transport model most of the monomers should be drained away by the vessels and thus contribute to the pool of serum IgA.

Very little conclusive information is available about the origin of the intestinal immunocytes, but they apparently develop from blast cells seeded into the mucosa (for review, see Brandtzaeg & Baklien 1976; Lamm 1976). Most of these blasts probably express J-chain synthesis, since this seems to be turned on early in the immune response (Brandtzaeg & Berdal 1975; Brandtzaeg 1976*a*). We have therefore proposed that circulating J-chain-containing blasts regardless of immunoglobulin class may contribute to the predominant gland-associated population of dimer-producing IgA immunocytes (Fig. 4). In individuals with selective IgA deficiency there is a block in the final B cell differentiation; according to the maturation scheme (Fig. 4), IgM- and IgG-producing cells should then accumulate adjacent to the glands, which agrees with immunohistochemical observations (Brandtzaeg *et al.* 1968; Savilahti 1973).

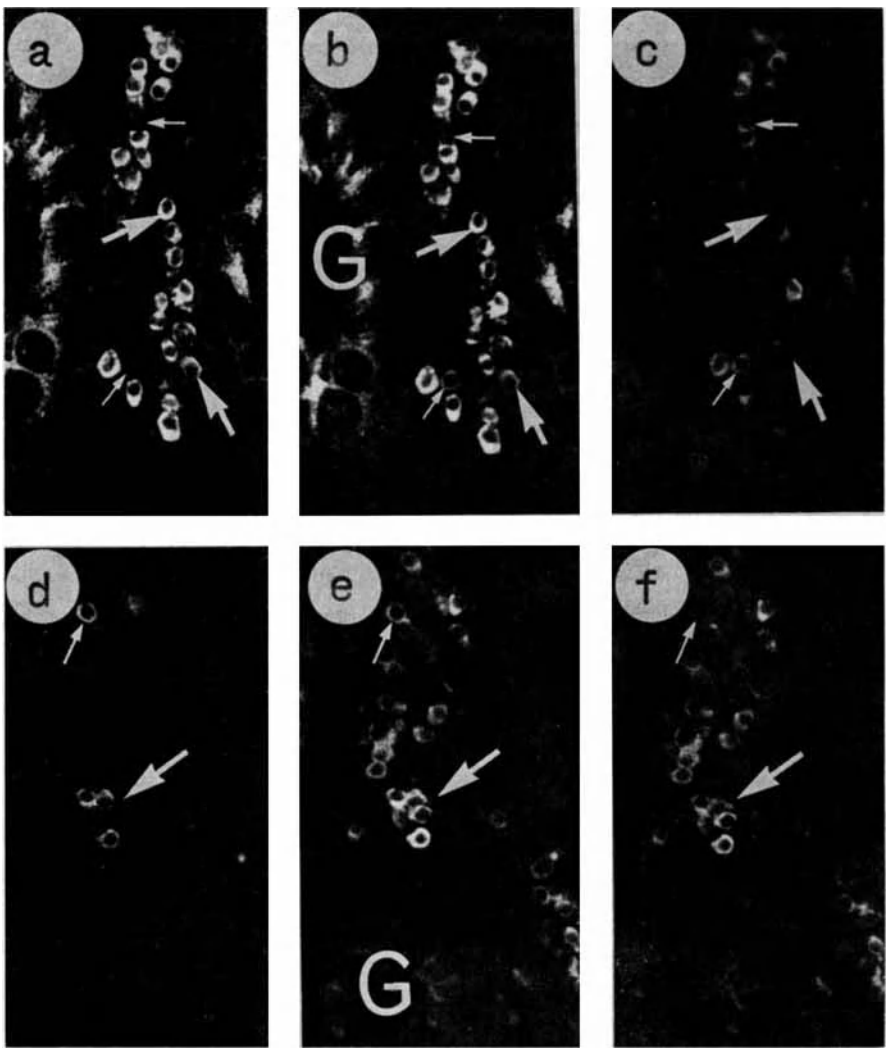


FIG. 3. Immunohistochemical demonstration of J chain in human intestinal IgA and IgM immunocytes. Sections of a saline-extracted specimen of normal rectal mucosa were preincubated for 60 min in 6M-urea, pH 3.2, to expose concealed J-chain determinants.
(*a-c*) Double tracing of 'green' IgA and 'red' J chain shown by selective filtration of green (*a*) and red (*c*) fluorescence and double exposure (*b*) in the same field. Most IgA immunocytes are J-chain-positive, although of varying intensity; a few are negative (*large arrows*). Two J-chain-positive cells are of another immunoglobulin class (*small arrows*).
(*d-f*) Double tracing of 'green' IgM and 'red' J chain shown by selective filtration of green (*d*) and red (*f*) fluorescence and double exposure (*e*) in the same field. Three IgM immunocytes are J-chain-positive (*large arrow*), whereas one is negative (*small arrow*). Some J chain is revealed in the gland cells (*G*). Magnification: × 340.

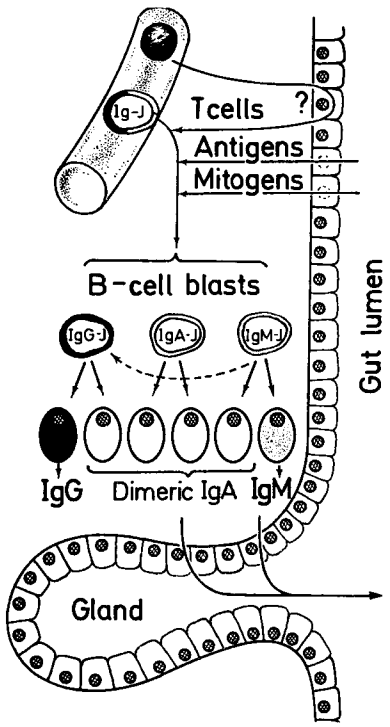


FIG. 4. Hypothetical scheme for the maintenance of a normal gland-associated immunocyte population. Circulating J-chain-positive B cell blasts of IgG, IgA, or IgM class become continuously seeded into the lamina propria, where further clonal differentiation is induced by a 'second signal' mediated by helper T cells or directly by antigens or mitogens. During this differentiation most cells end up as IgA dimer producers due to switching of class expression in the precursors. The direction of differentiation may be from IgM to IgG to IgA or directly from IgM to IgA. If an insufficient amount of J chain is synthesized by mature IgA cells, a mixture of dimeric and monomeric IgA will be produced (not indicated in figure). Monomeric IgA will, like IgG, not be transported externally by gland cells. (From Brandtzaeg & Baklien 1976.)

### 2. *Interstitial and epithelial distribution of immunoglobulins and SC*

In sections of directly fixed mucosal specimens the connective tissue ground substance exhibits diffuse fluorescence for IgG, particularly intense in the basement membrane zones along epithelia and vessel walls (Figs. 5*a* and 6*a*). The epithelium is virtually devoid of cytoplasmic IgG, but there may be an irregular faint fluorescence related to interstices in the glands and especially in the epithelium facing the gut lumen, indicating intercellular diffusion. Some IgG is also occasionally seen in goblet cells (Fig. 6*a*). An influence of the biopsy procedure on the epithelial IgG distribution cannot be excluded.

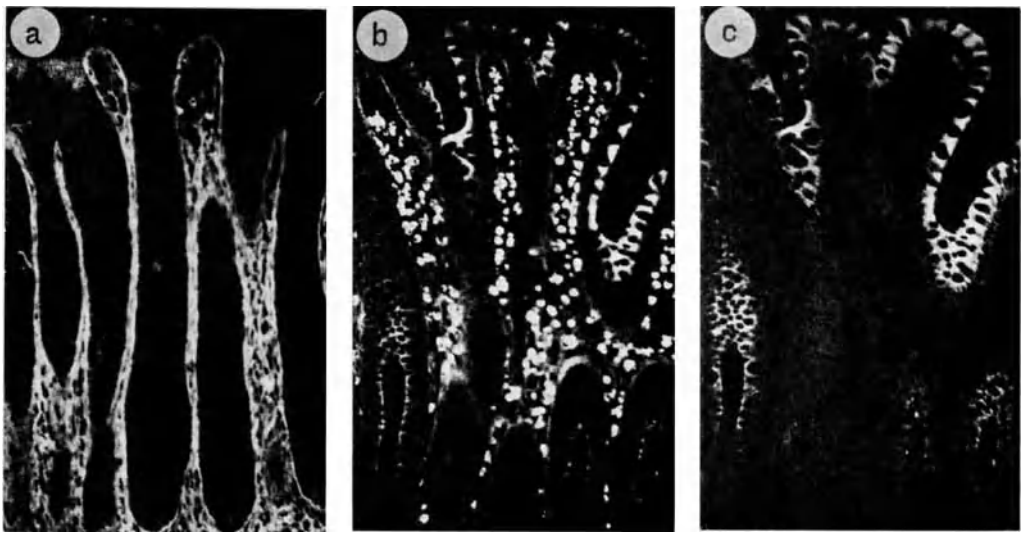


FIG. 5. Immunohistochemical localization of immunoglobulins and SC in normal rectal mucosa fixed directly in alcohol to retain diffusible protein components. Comparable fields shown after single tracing of 'red' IgG in one section (*a*) and double tracing of 'red' IgA (*b*) and 'green' SC (*c*) in the adjacent section. Note that IgG predominates in connective tissue ground substance and basement membranes but is hardly detectable in epithelium. IgA is located in numerous immunocytes, but only small amounts are seen extracellularly in this specimen. In the epithelium IgA is especially prominent apically in the columnar cells of the crypts, whereas little is present in surface epithelium (at the top). SC shows an overall distribution in epithelium similar to that of IgA. Magnification: × 100.

In most normal specimens the lamina propria contains much less extracellular IgA than IgG, despite extensive local synthesis of the former (Fig. 5*b*). This is compatible with an efficient external transport of the locally produced dimeric IgA. However, in connection with an intensified mucosal immune response, the specific background staining is very bright and obscures many of the IgA immunocytes (Figs. 6*c* and 11*c*). The crypt epithelium shows staining for IgA in the cytoplasm and in relation to interstices. The intracellular fluorescence is bright in the apical part of the columnar cells near the crypt openings, especially in the large bowel. Much less IgA is present in the surface epithelium (Fig. 5*b*) and hardly any occurs in the epithelium covering the villi of the small intestine (Fig. 6*c*).

The ground substance shows relatively little IgM staining, except for basement membrane zones and vessel walls. The epithelial distribution mimics that of IgA, but the fluorescence intensity is normally weaker (Fig. 6*b*). IgM is most readily demonstrated in colonic and rectal glands (Fig. 7*b*), as noted before

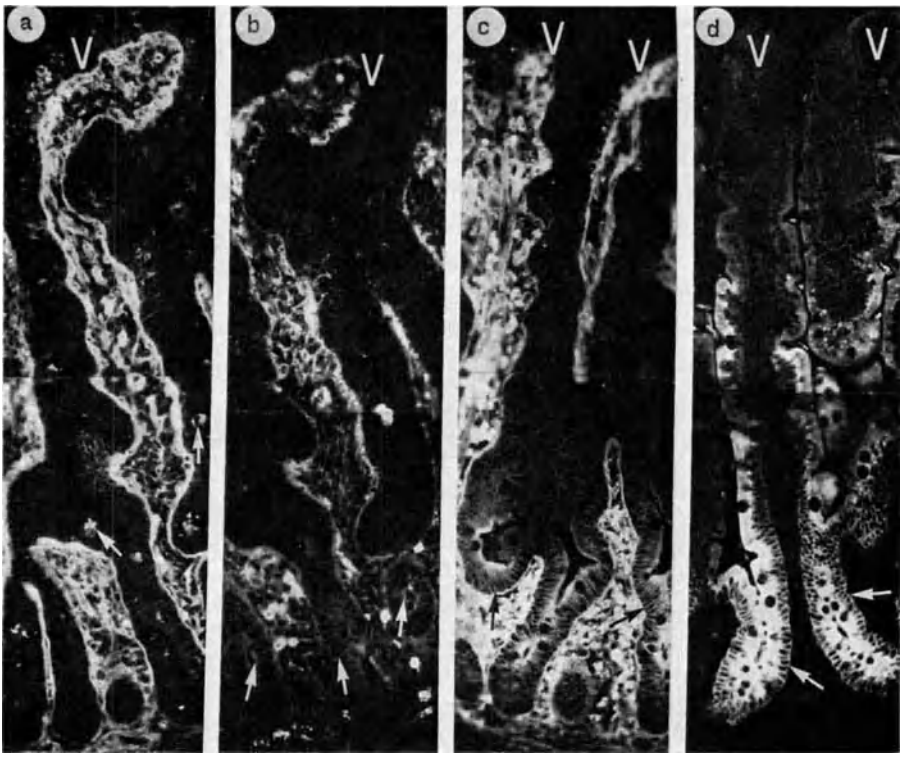


FIG. 6. Immunohistochemical localization of immunoglobulins and SC in morphologically normal duodenal mucosa from a patient with *Giardia lamblia* infestation. The specimen was fixed directly in alcohol to retain diffusible proteins. Single tracing with rhodamine conjugates in four serial sections: (*a*) IgG; (*b*) IgM; (*c*) IgA; and (*d*) SC. Lamina propria is rich in extracellular IgG, but also contains unusually large amounts of extracellular IgA, probably because of extensive local production by numerous IgA immunocytes which are difficult to discern against the bright background. Smaller amounts of extracellular IgM are present and some IgM immunocytes can be seen. Columnar crypt cells contain cytoplasmic SC and there is also distinct staining related to their basal and lateral borders (*arrows* in *d*). The SC concentration is gradually diminished in epithelium covering the villi (*V*). Mucinous content of goblet cells is unstained. Epithelial distribution of IgA parallels that of SC with distinct staining related to lateral borders of crypt cells (*arrows* in *c*) as well as apically in their cytoplasm. Similar but much weaker epithelial staining is seen for IgM (*arrows* in *b*). Cytoplasmic IgG fluorescence is absent from columnar crypt cells, but some striated staining is seen related to epithelial interstices. Some goblet cells contain IgG (*arrows* in *a*). Magnification: × 50.

in normal (Chen 1971) and especially in IgA-deficient individuals with enhanced local synthesis of IgM (Heremans & Crabbé 1967; Savilahti 1973). Paired staining with contrasting colours reveals no difference in the epithelial distribution of IgA and IgM in colonic glands (Brandtzaeg 1975*c*).

There is reason to believe that most of the 'intercellular' staining for IgA and IgM in crypt epithelium may be interpreted as cell-membrane fluorescence. Firstly, if passive diffusion from the lamina propria was the only explanation, the concentration of IgG between the epithelial cells should greatly exceed that of IgA and especially that of IgM. A possible molecular filtration in the basement membrane should moreover favour IgG. Secondly, all epithelial IgG can be removed by washing the tissue specimens, whereas IgA and IgM may still be found related to the borders of epithelial cells (Fig. 11*e-h*, p. 100). This indicates that IgA and IgM are adsorbed to the cell membranes, as proposed before (Brandtzaeg 1974*e*). The presence of IgA in crypt cell membranes has likewise been suggested at the ultrastructural level (Brown *et al.* 1975).

Many conflicting reports have appeared about the cellular origin of SC. Rossen *et al.* (1968) proposed that it is a plasma cell product, and Tourville *et al.* (1969) claimed that the goblet cell is the major intestinal source of SC. Other workers have refuted these findings (Munster 1972; Søltoft & Söeberg 1972; Poger & Lamm 1974). Our studies (Brandtzaeg 1973*b*, 1974*d*) have demonstrated SC in the columnar crypt cells of the large bowel and generally also in the surface lining cells (Fig. 5*c*). In the small bowel it is present in the columnar cells of the glands, decreasing in concentration in the epithelium covering the villi and rarely reaching their tips (Fig. 6*d*). Regardless of the tissue-processing technique, we have been unable to demonstrate SC associated with the mucinous content of goblet cells. At the ultrastructural level SC has been detected on the lateral membranes of both goblet and columnar crypt cells (Brown *et al.* 1975). Poger & Lamm (1974) failed to demonstrate SC corresponding to cell membranes by immunofluorescence, but we see specific staining related to the intercellular and basal borders of glandular cells, especially with highly sensitive rhodamine conjugates (Figs. 6*d* and 7*d*). This

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FIG. 7. Immunohistochemical localization of immunoglobulins and SC in a directly alcohol-fixed autopsy specimen from the colon of a 32-week-old boy with intractable diarrhoea and marasmus. There was a maturation defect in his B cell system until the age of 5 months, when marked synthesis of IgM and IgG began. From that age immunocytes appeared in his bowel mucosa with a predominance of IgM-producing cells. Single tracing with rhodamine conjugates is shown in serial sections: (*a*) IgG; (*b*) IgM (corresponding fields); (*c*) IgA; and (*d*) SC (corresponding fields). Lamina propria contains large amounts of extracellular IgG and IgM, whereas IgA is localized to immunocytes. In the crypt epithelium IgM is located in the cytoplasm of columnar cells and along their lateral borders (*arrows* in *b*). Some striated staining is seen for IgG. IgA is present only in crypt cells situated adjacent to small groups of IgA immunocytes. SC is ubiquitously present in the cytoplasm of columnar epithelial cells and along their lateral and basal borders (*small arrows* in *d*). *Large arrows* point to similar locations in (*c*) and (*d*). Magnification: × 90.

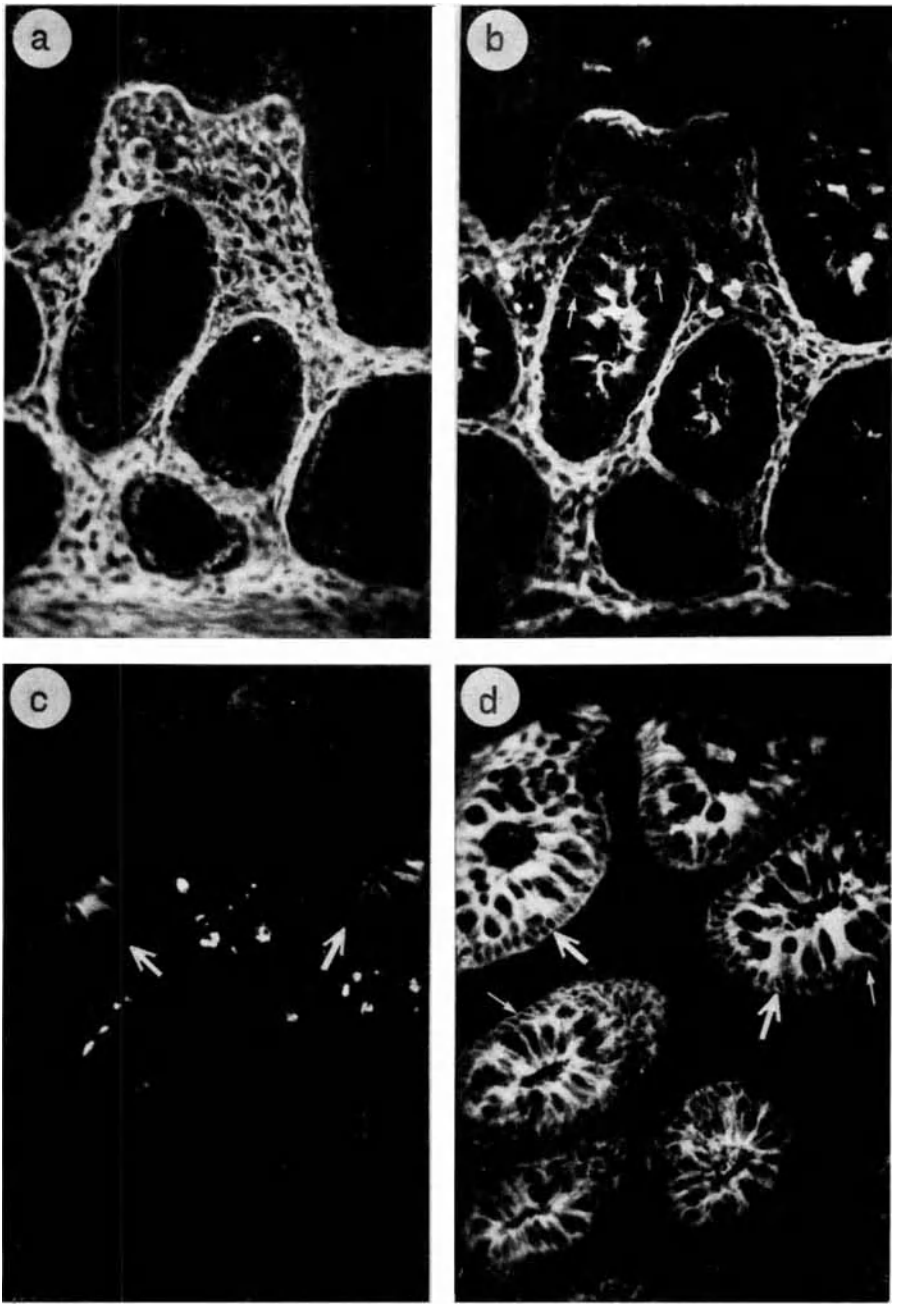


FIG. 7

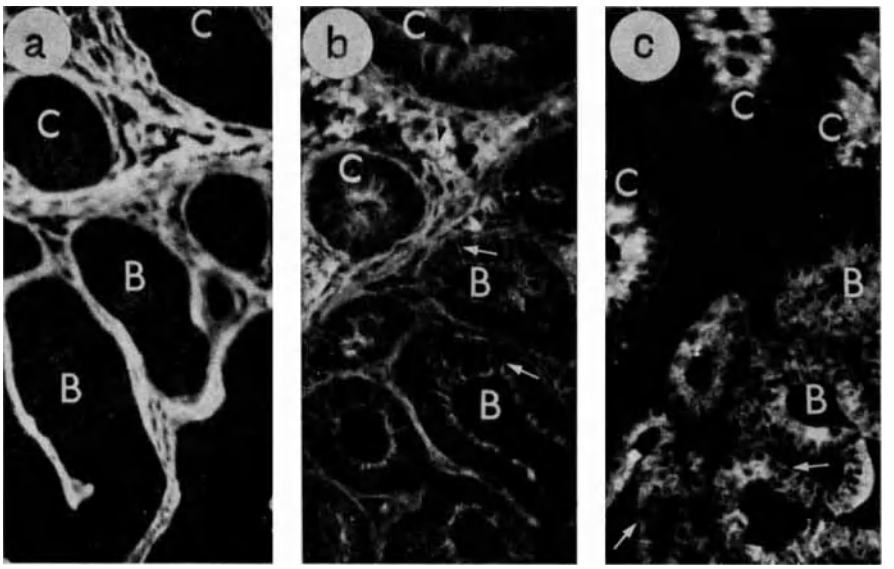


FIG. 8. Immunohistochemical localization of immunoglobulins and SC in a directly alcohol-fixed specimen of duodenal mucosa from an adult with coeliac disease. The bottom of two or three crypts (*C*) and some Brunner's glands (*B*) are shown. Single tracing with rhodamine conjugates in serial sections: (*a*) IgG; (*b*) IgA; and (*c*) SC. Note differences between crypts and tubules of Brunner's glands with regard to IgA and SC distribution. Distinct localization of IgA and SC is seen related to the borders of tubule cells (*arrows* in *b* and *c*), whereas IgA in apical cytoplasm and accumulations of SC in Golgi zone are typical for columnar crypt cells. Stroma of Brunner's glands contains IgA, but there are no IgA immunocytes, in contrast to the mucosal lamina propria which is crowded by such cells (*arrowheads* in *b*). Note abundance of IgG in connective tissue, but absence of IgG staining in epithelium (*a*). Magnification: × 200.

feature may well represent membrane-associated SC. Indeed, Huang *et al.* (1976) recently succeeded in establishing long-term cultures of colon carcinoma cells bearing SC which regenerated after removal by trypsinization. These findings lend strong support to the proposed receptor function of SC.

The congruent distribution of SC, IgA and IgM in epithelium showing immunoglobulin transport further suggests that SC is involved in this process (Figs. 5*b*,*c* and 6*b*-*d*). When the local synthesis of IgA is limited to a few immunocytes, the immunohistochemical appearance indicates that IgA passes directly into the adjacent SC-containing epithelial cells (Fig. 7*c*). O'Daly *et al.* (1971) made similar observations in rabbits, using allotypic markers to trace the local immunoglobulin products after B cell transfer experiments. Conversely, when there is a pronounced IgA synthesis in the gut the immunoglobulin may apparently diffuse over a considerable distance before it is taken up by the

epithelial cells. This is exemplified in Fig. 8, which shows unusually prominent IgA transport by a group of SC-producing Brunner's glands devoid of IgA immunocytes. However, the interstitial fluid surrounding the tubules contains appreciable amounts of IgA, probably derived from the mucosal lamina propria which in this patient with coeliac disease is very rich in IgA-producing cells.

Compared with the crypt epithelium, the staining for SC and IgA is intense in relation to the borders of the epithelial cells in these Brunner's glands (Fig. 8*b*,*c*). This may indicate that relatively more SC–IgA complexes accumulate in the gland cell membranes and perhaps reach the lumen without entering the cytoplasm, as has previously been suggested for respiratory and salivary glands (Brandtzaeg 1974*e*). That the striated staining does not represent intercellular IgA is indicated by the lack of IgG in the epithelial interstices of the tubules (Fig. 8*a*).

Although the epithelial distributions of SC and IgA are in general very similar, the detailed staining patterns of crypts revealed by double tracing are not completely congruent (Brandtzaeg 1974*d*,*e*). A common feature is the fluorescence apparently related to the cell membranes, as discussed above. Intracellularly, both SC and IgA are present in the apical portion of the cytoplasm, but SC alone is distinctly concentrated in a granular pattern corresponding to the Golgi zone (Fig. 9). Ultrastructural studies of rabbit mammary glands lend support to these observations, since SC but no IgA could be found in the Golgi elements of the epithelial cells, whereas both components were present in more apically located vesicles (Kraehenbuhl *et al.* 1975). Moreover, the antigenic properties of SC present in human colonic crypt cells indicate that it exists in a free form in the Golgi zone and in a bound form apically in the cytoplasm (Brandtzaeg 1974*e*; Poger & Lamm 1974).

## OTHER PROPOSED MODELS FOR THE GLANDULAR TRANSPORT OF IgA

There is now general agreement that secretory IgA represents the synthetic product of two cell types. Since free SC is concentrated in the Golgi zone of serous secretory epithelial cells (Fig. 9), it seems unquestionable that it is produced by these cells. The synthesis of SC may be regarded as independent of IgA and IgM because it appears to be normal in hypogammaglobulinaemic individuals (see Brandtzaeg & Baklien 1976) and occurs in the fetus before the initiation of immunoglobulin production (Ogra *et al.* 1972). However, there may be as yet undefined interrelations between SC and immunocytes. Thus, Hassall's corpuscles synthesize SC (Tomasi & Yurchak 1972) and the maturation of IgA-producing cells is clearly thymus-dependent (for review, see Lamm

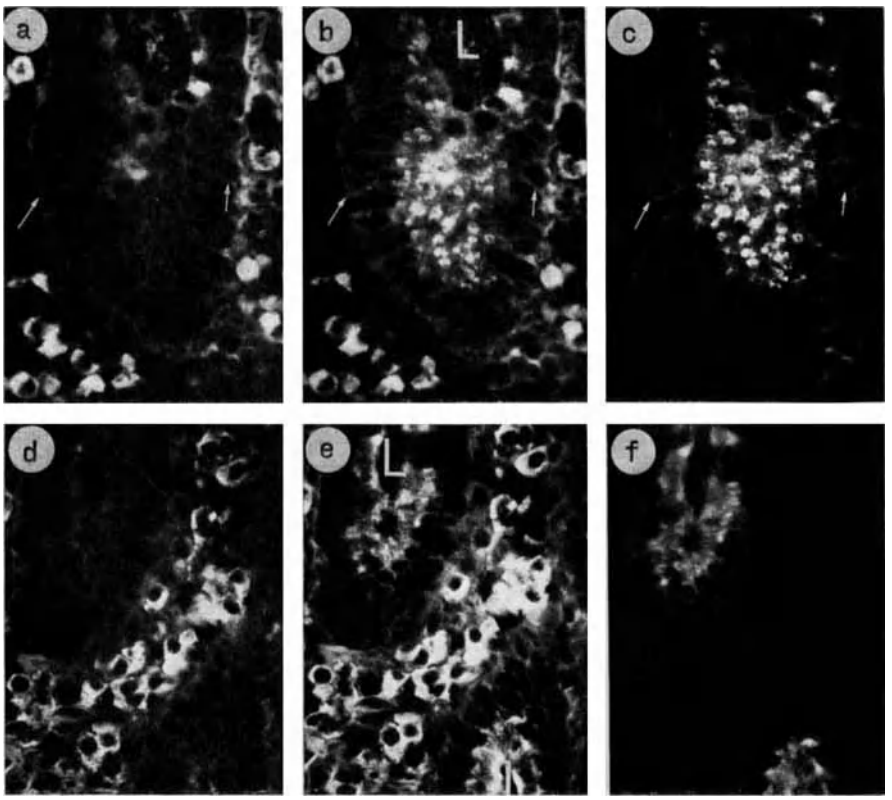


FIG. 9. Double tracing of IgA and SC in directly alcohol-fixed intestinal specimens: (*a-c*) Oblique section through crypt in colon mucosa from a patient with ulcerative colitis. (*d-e*) Section through two hypertrophied crypts in duodenal mucosa from an adult with coeliac disease. (*a*) Selective red filtration for IgA; (*c*) selective green filtration for SC; and (*b*) double exposure in same field. Many columnar cells in the colon crypt have been cut through the Golgi zone where granular accumulations of SC (*c*) and absence of IgA (*a*) are distinctly shown. Both IgA and SC occur in the apical part of the cytoplasm close to the lumen (*L*) of the gland, and also related to lateral and basal borders of columnar cells (*arrows*). Mucinous content of goblet cells is unstained. (*d*) Selective green filtration for IgA; (*f*) selective red filtration for SC; and (*e*) double exposure in same field. The duodenal specimen contains numerous IgA immunocytes and considerable amounts of extracellular IgA in lamina propria. Epithelial distribution of SC and IgA is similar to that seen in the colon crypt, but SC is not so distinctly concentrated in the Golgi zone. Magnification: × 510.

1976). Lawton *et al.* (1970*a*) obtained some evidence from tissue culture experiments with rabbit mammary glands of an influence of IgA on the synthesis of SC. Moreover, it is claimed that some unidentified cells in lymphoid organs such as bone marrow, spleen and lymph nodes are able to produce SC (Lai A Fat *et al.* 1974).

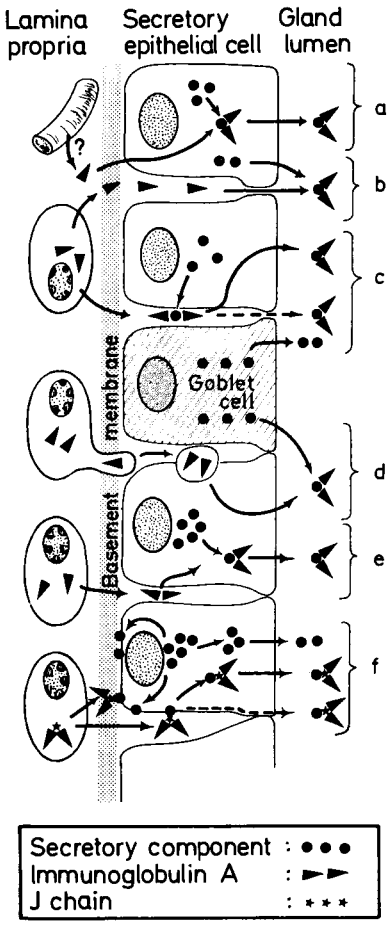


FIG. 10. Schematic representation of glandular transport of secretory IgA as proposed by various research groups. The six models depicted are discussed in the text.

In the first model proposed for the glandular secretion of IgA (Tomasi *et al.* 1965; South *et al.* 1966) it was envisioned that the SC-producing epithelial cell mediated the immunoglobulin transport, and that the union of two IgA monomers took place by intracellular complexing with SC (Fig. 10*a*). The IgA monomers were thought to be produced by local plasma cells, but a contribution from serum was not excluded. It was not clear whether the epithelial transport was active, or whether it depended on high concentrations of locally formed IgA. This model was recently supported by Shiner & Ballard (1973) using commercial immunofluorescent antibodies to 7S IgA, 11S IgA and

SC. However, their results are questionable since the reagents obviously did not discriminate between monomeric and dimeric IgA, nor between free and bound SC.

Heremans & Crabbé (1967) challenged the view that IgA follows an intracellular route through the epithelium. They felt that the apical IgA fluorescence seen in intestinal glands should be ascribed to adsorbed mucus rather than to cytoplasmic IgA. On the other hand, they stressed the localization of IgA in epithelial interstices and proposed that most intestinal IgA diffuses between the epithelial cells into the gut lumen where complexing with SC takes place (Fig. 10*b*).

A direct passage of IgA into the lumen would be restricted by the apical tight junctions between the epithelial cells. Tomasi and co-workers (Tourville *et al.* 1969; Tourville & Tomasi 1969; Franklin *et al.* 1973) have therefore maintained the view that IgA combines with SC in the epithelial interstices; most of the complexes then enter the glandular cell and are subsequently extruded into the lumen (Fig. 10*c*). This model raises many questions. Why should the basement membrane selectively allow IgA (and IgM) to diffuse into the epithelial interstices? Why is SC usually undetectable in sera of hypogammaglobulinaemic individuals (Brandtzaeg 1971*b*) if it is regularly secreted into the intercellular spaces? Why do the secretory IgA molecules go into the epithelial cell? These authors (Tourville *et al.* 1969; Tourville & Tomasi 1969) have moreover claimed that the mucous-type glandular cell is especially active in the production of SC, but it is not clear how this fits into their transport model.

Allen *et al.* (1973) tried to follow the intestinal secretion of IgA in pigs at the ultrastructural level. They suggested that pseudopodia from plasma cells adjacent to the glands are sloughed off as vesicles which cross the basement membrane into the epithelial interstices and thereafter enter the columnar cells (Fig. 10*d*). In this way IgA could be protected during the entire external transfer. After release into the gut lumen IgA combines with SC, which also according to the latter authors is derived mainly from goblet cells. The ultrastructural localization of IgA and SC in human intestinal epithelium does not agree with these findings (Brown *et al.* 1975), and the proposed model cannot explain the transport of IgA by glands lying at a considerable distance from the IgA-producing cells (Fig. 8, p. 94).

The recent immunohistochemical study of Poger & Lamm (1974) agrees to some extent with our findings. Firstly, the serous secretory epithelial cell is identified as the major source of SC. Secondly, when IgA is transported through this cell, free SC is present in the Golgi zone, whereas the bound component occurs in the apical part of the cytoplasm. Since no indication of

membrane-associated SC was obtained, their observations were interpreted (Lamm 1976) to suggest that the assembly of secretory IgA takes place inside the epithelial cell after the fusion of pinocytotic and SC-containing Golgi vesicles (Fig. 10*e*).

If the epithelial uptake of IgA occurred as a 'fluid' or 'bulk' pinocytosis, the latter model would not easily explain the selectivity in the transport. One possibility mentioned by Lamm (1976) is that SC protects IgA against degradation by intracellular enzymes, whereas IgG and other proteins included in the pinocytotic vesicle are degraded. Even fragments of IgG should retain some antigenicity, however, and the complete lack of cytoplasmic IgG staining in glandular epithelia (see previous section, p. 89) speaks against an entry of this protein into intact secretory cells. Thus, a selection of IgA most likely takes place at the epithelial cell membrane by means of a receptor. According to our transport model this receptor is specific for J-chain-containing IgA and IgM (Fig. 1, p. 79). Receptor–substrate complexes formed on the cell surface are either taken up by adsorptive pinocytosis or may float in the membrane and reach the gland lumen without entering the cytoplasm (broken arrow in Fig. 10*f*). Which route is preferred may depend on the cellular distribution of SC, which apparently varies among different glands (Fig. 8). Although there is no formal proof demonstrating the identity of the epithelial receptor with SC, circumstantial evidence is accumulating to support our view. Heremans (1974) in his recent review also favoured a similar model for the transport of IgA.

Some authors argue against a participation of SC in glandular immunoglobulin transport (Weicker & Underdown 1975) by referring to the lack of *in vivo* (Newcomb & Ishizaka 1970) and *in vitro* association between SC and IgE (P. Brandtzaeg, unpublished data). This argument is not valid, however, since the previous assumption of selectivity in the secretion of IgE comparable to that of IgA does not hold true (Nakajima *et al.* 1975); a relative enrichment of IgE in some exocrine fluids compared with serum apparently depends on local synthesis combined with passive diffusion through epithelial interstices. The function of SC has further been confused by the recent suggestion that it may act as a γ-glutamyltransferase; a possible involvement of this enzyme activity in the glandular transport or function of secretory IgA has been discussed (Binkley & Wiesemann 1975). However, we have distinctly separated the γ-glutamyltransferase activity of colostrum from SC and secretory IgA (P. Brandtzaeg & A. Winsnes, unpublished data). In our opinion, therefore, the only established activity of SC is to complex spontaneously with J-chain-containing IgA and IgM by specific non-covalent interactions. This property is certainly compatible with its proposed receptor function.

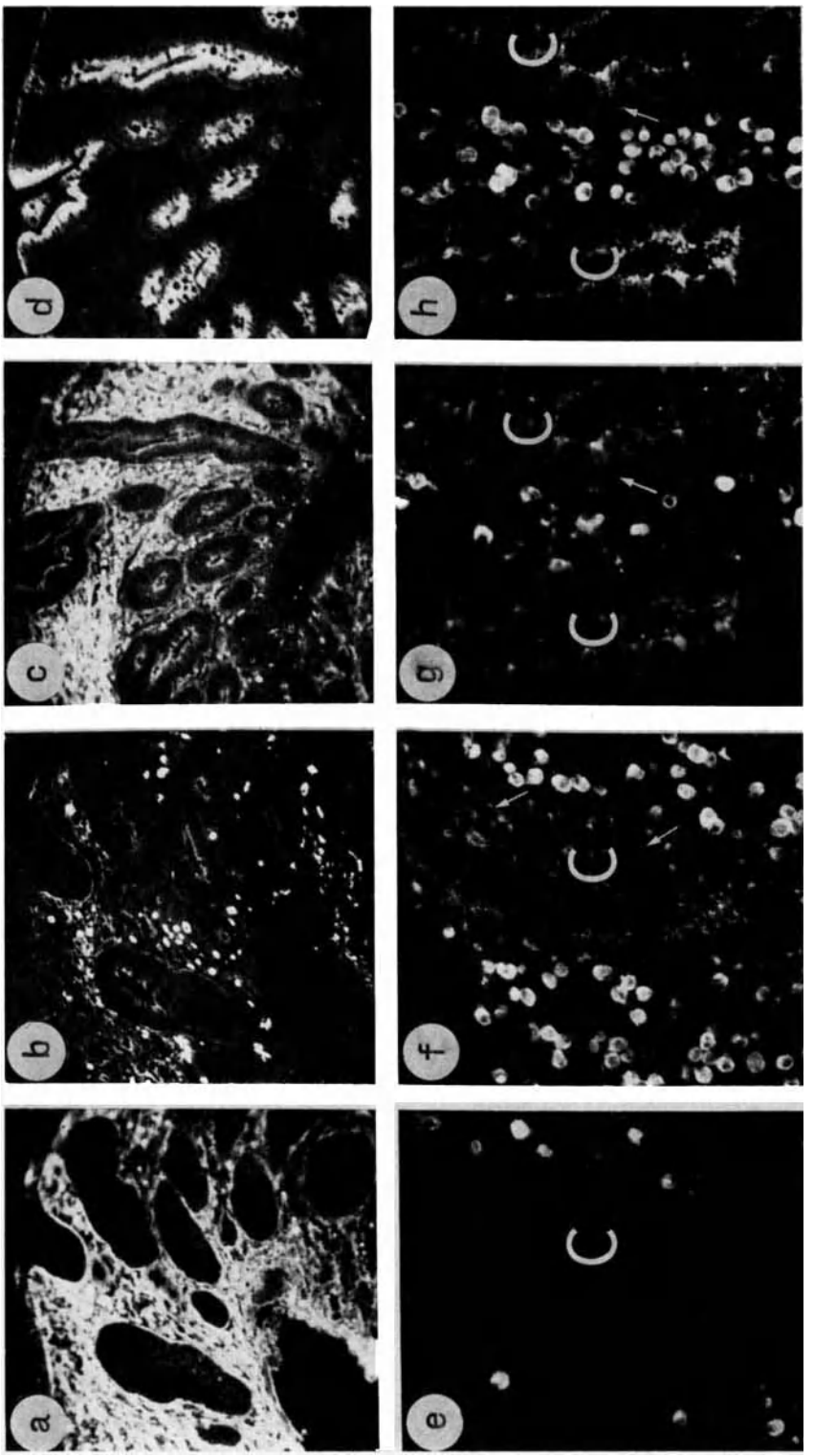


FIG. 11

## GLANDULAR IMMUNOGLOBULIN TRANSPORT IN DISEASE

A defect in glandular immunoglobulin transport has not been convincingly shown to be a primary cause of intestinal disease. The SC pattern of duodenal and jejunal mucosa from patients with coeliac disease (Fig. 11*d*) mimics that of the normal colon, and signs of intracellular IgA and IgM transport are seen along the entire hypertrophied crypts (Fig. 11*b,c*). We do not agree with Shiner & Ballard (1973) who claimed that there is a marked 'backflow' of secretory IgA into the lamina propria in this disease. In our hands the usual epithelial distribution of SC and IgA is found (Fig. 9*d-f*). The same holds true for normal-appearing glandular epithelium in ulcerative colitis and Crohn's disease, where SC may be present to the very brink of the ulceration (Fig. 12*a-c*). However, pathological epithelium may contain reduced amounts of SC, and there seems to be a parallel decrease (Fig. 12*d,e*) and sometimes complete lack (Fig. 12*f,g*) of intracellular IgA and IgM transport. Green & Fox (1975) and Das *et al.* (1975) have likewise reported altered SC distribution in Crohn's disease and idiopathic proctitis. These findings must be ascribed to localized secondary events, and no significant overall reduction of SC synthesis is shown when mucosal specimens from various intestinal diseases are cultured *in vitro* (McClelland *et al.* 1976).

A few reports have recently appeared indicating deficient SC synthesis as the primary cause of disease. A 52-year-old male (Krakauer *et al.* 1975) and a 15-year-old boy (Strober *et al.* 1976) with severe diarrhoea were found to have normal levels of serum immunoglobulins including IgA, but secretory IgA and SC were virtually undetectable in saliva and intestinal fluid. Moreover, the intestinal mucosa of the latter patient showed only negligible IgA production

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FIG. 11. Immunohistochemical localization of immunoglobulins and SC in directly fixed (*a-d*) and saline-extracted (*e-h*) specimens of jejunal mucosa from an adult with coeliac disease. Single tracing with rhodamine conjugates in serial sections: (*a*) IgG; (*b*) IgM; (*c*) IgA; and (*d*) SC. The extracellular concentration of IgG is high as usual, but in coeliac disease with intensified local immune responses large amounts of extracellular IgA are also present in lamina propria and obscure the visualization of numerous IgA immunocytes. Extracellular IgM is less abundant, and most IgM cells can be discerned. Hypertrophied crypts produce SC along their entire length, and also cells lining the surface show some SC staining. The epithelial distribution of IgA parallels that of SC. Small amounts of cytoplasmic IgM can likewise be seen in the crypt epithelium, whereas faint IgG staining seems to be restricted to epithelial interstices. Double tracing of: (*e*) 'red' IgG and (*f*) 'green' IgA (same field); and of: (*g*) 'red' IgM and (*h*) 'green' IgA (same field). Extracellular immunoglobulins have been removed from lamina propria by the extraction procedure, and immunocytes of all classes are clearly revealed against the dark background. IgG has also been completely removed from lateral and basal borders of crypt cells (*C*) ,whereas some IgA and IgM seems to be retained along the epithelial cell membranes (*arrows*). Magnification: (*a-d*) × 80; (*e-h*) × 190.

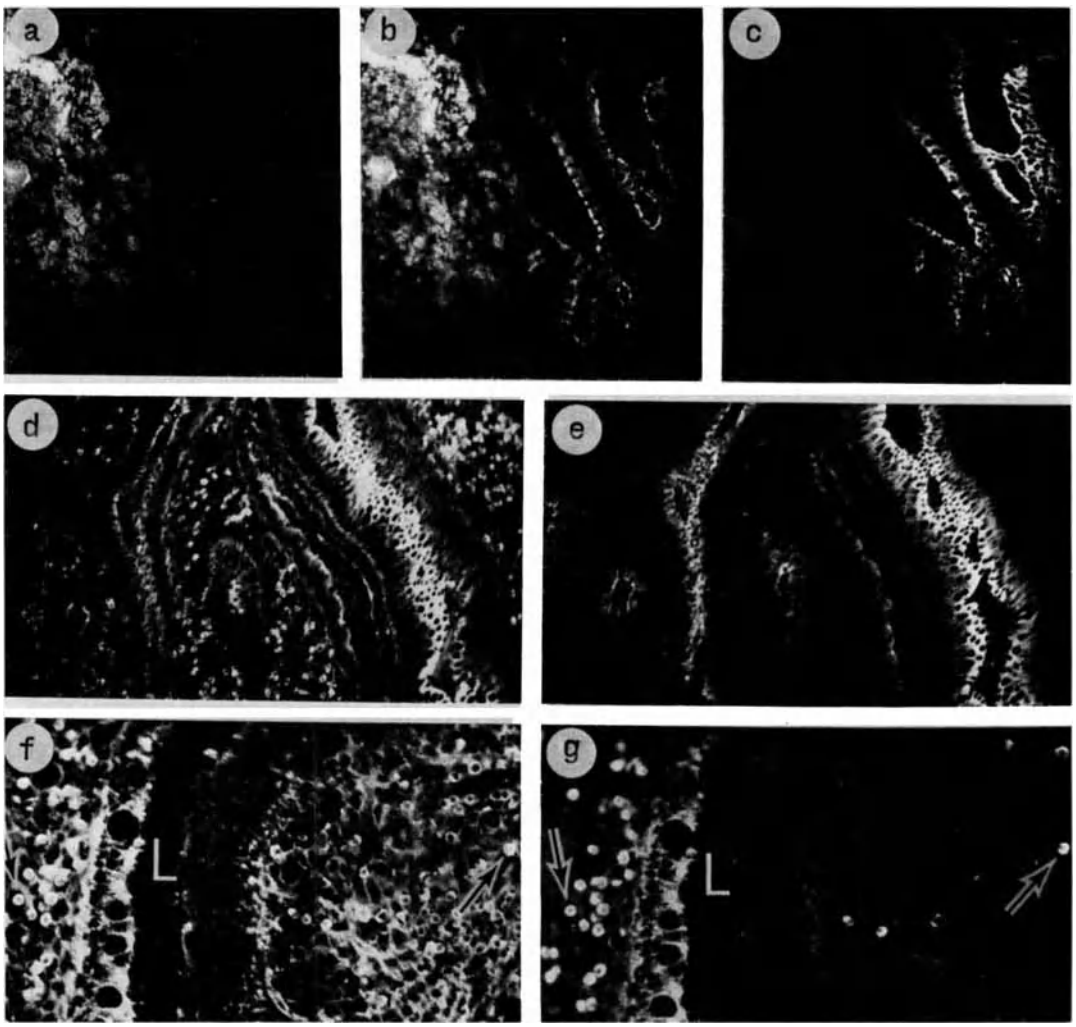


FIG. 12. Immunohistochemical localization of epithelial IgA and SC in inflammatory bowel disease. (*a-c*) Double tracing of 'green' lactoferrin and 'red' SC in rectal mucosa of patient with ulcerative colitis shown by selective filtration of green (*a*) and red (*c*) fluorescence and double exposure (*b*) in same field. Note that there is a normal distribution of SC almost to the brink of the ulceration, which appears as an accumulation of lactoferrin-containing neutrophilic granulocytes. (*d, e*) Tracing of IgA (*d*) and SC (*e*) in comparable fields from neighbouring sections of large bowel mucosa of a patient with Crohn's disease of colon. The pathological epithelium shows highly varying content of SC, and there are parallel variations in the epithelial distribution of IgA. (*f, g*) Double tracing of 'green' IgA and 'red' IgG in saline-extracted specimen from the colon of a patient with ulcerative colitis shown by double exposure (*f*) and selective filtration of green fluorescence (*g*) in same field. Examples of identical positions in the two pictures are indicated by arrows. Note that the crypt epithelium to the left of the lumen (*L*) apparently shows a normal distribution of IgA in columnar cells, whereas there is no intracellular immunoglobulin in the pathological epithelium facing the dense IgG-cell infiltrate to the right. Magnification: (*a-e*) × 100; (*f, g*) × 240.

in tissue culture. A common feature was a 20-fold increase of the IgM concentration in jejunal aspirates, indicating normal epithelial transport combined with enhanced local synthesis. A maturation defect of B cell blasts being seeded into the mucosa might explain the fact that intestinal IgA production apparently was replaced by IgM. According to our proposed glandular transport model, however, IgM should not be transmitted to the gut lumen without the participation of SC. Lack of SC synthesis in the two patients was not directly shown by immunohistochemistry or tissue culture techniques, neither was any attempt made to demonstrate SC bound to IgM in the intestinal fluid. Critical definition of these important points in such rare patients would be invaluable for the understanding of mucosal immunity.

A primary defect in SC-synthesizing capacity has been proposed as a possible cause of the sudden-infant-death syndrome (Ogra *et al.* 1975). Decreased amounts of SC were found in mucosal tissue extracts and sections from most of eight patients studied. The authors speculated that this deficiency might have jeopardized the respiratory mucosal defence. However, it was not excluded that a defect in SC production could be secondary to a recent virus infection of the epithelium. Williams *et al.* (1976) detected SC in submandibular gland extracts from all of ten such patients. A primary SC deficiency therefore seems unlikely as a general aetiological factor in this syndrome.

## BIOLOGICAL IMPLICATIONS OF SELECTIVE GLANDULAR IMMUNOGLOBULIN TRANSPORT

It may be conceived that a normal immunological homeostasis is maintained in the intestinal mucosa through a critical balance between the various immunoglobulin classes. Polymeric IgA and IgM act as a first line of defence by immunological antigen exclusion at the mucosal surface. Antigens by-passing this exclusion mechanism may meet corresponding antibodies of all the three major immunoglobulin classes in the lamina propria. IgM and IgG are able to activate complement and IgG may participate in antibody-dependent cell-mediated cytotoxicity, but adverse reactions are normally most likely moderated within the mucosa by blocking antibody activities of IgA, which lacks phlogistic properties.

The initial phase of an intestinal immune response is characterized by increased synthesis and external transport of IgM and IgA. When noxious influences are counterbalanced by such a response, only moderate and reversible alterations of the local immunological homeostasis take place. An example is probably seen in coeliac disease; a pronounced mucosal IgM and IgA response develops but also a disproportionately increased local formation of

IgG, which indeed may be responsible for a considerable fraction of the gluten antibodies (Brandtzaeg & Baklien 1976). Since IgG is not actively transferred to the body exterior, antibodies of this class are of little value in the immunological exclusion of antigens and may rather have adverse effects within the mucosa.

Persistent and excessive stimulation of the intestinal B cell system leads to a pronounced local overproduction of IgG, as seen in ulcerative colitis and Crohn's disease (see Brandtzaeg & Baklien 1976), and the immunological homeostasis is severely altered. Antigens gain increased access to the lamina propria through epithelial breaks and because of defective external transport of SC-stabilized secretory IgA and IgM by injured epithelium. The IgG-cell response will be maintained and intensified by a continuous exposure of the interior of the body to a massive antigen load.

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## Discussion

*Cebra:* You suggested that secretory component may be a membrane component of the epithelial cells and be found on their basal borders. Have you any idea about the selectivity of secretory component as a receptor explaining differences in the concentrations of exported Ig? If you compare IgM with IgA1 and IgA2, are the changes in proportions outside in gut lumen explained by a difference in specificity?

*Brandtzaeg:* So far there is no evidence for any difference between IgA1 and IgA2 dimers in SC binding properties. There is one study indicating binding of SC to monomeric IgA2 of the genetic variant $Am_2$ (+), but that experiment was done with a large excess of SC obtained from colostral IgA by reduction (Jerry *et al.* 1972). The situation was therefore not comparable to the specific, non-covalent interaction I have discussed.

*Cebra:* Have you looked at the relative proportions of IgA2 and IgA1 cells in the lamina propria? There is a difference in the proportion of the two

isotypes in the secretions and in the circulation, and one wonders what the basis for the difference is.

*Brandtzaeg:* This information is based on one publication (Grey *et al.* 1968) which has not been confirmed, and there are as yet no published cellular studies.

*André:* You gave evidence of some IgG cells synthesizing J chains. Do these cells bind secretory component?

*Brandtzaeg:* The IgG producers containing J chain do not bind SC, neither do J-chain-positive IgD cells. The SC-binding site depends on the incorporation of J chain into IgA or IgM polymers. Apparently J chain is unable to combine with IgG and IgD.

*Gowans:* How firm is the evidence that there is a J-chain specific binding site for secretory component?

*Brandtzaeg:* The evidence is two-fold. Firstly, you need the presence of J chain in the IgM or IgA polymers in order to obtain SC binding. Secondly, you can block the binding site by means of antibody to J chain. The latter experiment is difficult to interpret, however, because of the possibility of non-specific steric hindrance.

*Lachmann:* When you say you need the J chain, have you taken *polymeric* forms of IgM and IgA that are free of J chain and shown they have not bound secretory piece?

*Brandtzaeg:* Yes; that was shown in Fig. 2. SC likewise binds only to the cytoplasm of J-chain-producing IgA and IgM cells when tested on tissue sections.

*Cebra:* According to Hanly *et al.* (1973), there may well be a requirement for J chain but there seems also to be a second requirement, for the hinge region of the Fc fragment. They can prepare Fc dimer from IgA2 molecules which fails to bind SC. One can infer that perhaps one requires the J chain but also a part of the heavy chain around the hinge.

*Brandtzaeg:* I agree completely, because J chain isolated from polymeric IgA does not block the binding reaction to any great extent. Also cells containing native free J chain (we call it 'free' when it is in an IgG or IgD plasma cell) do not bind secretory component, so free J chain apparently has very low affinity for the secretory component. The Fc portion of Ig polymers is thus essential for the SC-binding site. Why it is important is not known. Is it because of the conformation of the heavy chains or because of the configuration of the bound J chains? Our results favour the latter possibility.

*Vaerman:* It has also been proved by Mach (1970) that dimeric IgA which was re-formed from reduced IgA and from which J chain had been removed could not bind.

*Gowans:* Where precisely do you think the membrane-bound secretory

component is located? You illustrated a striated pattern which you suggested might indicate a distribution along the sides of the epithelial cells. However, another picture showed basement membrane staining. Do you think the receptors are arranged on the basement membrane, or on the epithelial cells?

*Brandtzaeg:* I think they are on the plasma membrane, and this is supported by the work of Huang *et al.* (1976).

*Gowans:* Has this ever been demonstrated on suspensions of intestinal epithelial cells? The epithelium can be readily dissociated into a cell suspension.

*Brandtzaeg:* We have not been able to produce such cell suspensions with the capacity to make SC, which would be required for proving the point.

*Cebra:* Some beautiful studies were made by Kraehenbuhl *et al.* (1973) using microperoxidase-labelled Fab anti-secretory component. He used normal sections and looked at the epithelial cells, and found a staining of the whole cell membrane going round into the intercellular space before the tight junction. This distribution of SC is very much like the one that Rodewald (1975) finds for Fc receptors on the absorptive epithelium in the neonatal rat.

*Brandtzaeg:* As I mentioned (p. 95), Kraehenbuhl *et al.* (1975) also showed that SC but not IgA is present in the Golgi complex in rabbit mammary gland cells. The same sort of findings as you mention have been made by Brown *et al.* (1975) showing a plasma membrane localization of IgA plus SC, in human gut sections examined electron microscopically.

*Gowans:* Can you really tell that it is on the surface in sections?

*Cebra:* This technique uses thick sections which are 'marinated' in the Fab-microperoxidase and then cut into ultra-thin sections.

*Brandtzaeg:* I agree that the final proof of a model such as I have been suggesting must be obtained on cells in suspension. The first step in this direction is the studies reported by Huang *et al.* (1976). I hope that they will do further experiments on cultivated colon cancer cells.

*Evans:* With the Sainte-Marie technique you could lose up to 50% of antigenic activity of IgG, especially if it is at an extracellular site. Scott (1976) found that with jejunal biopsies which had been washed for up to two days in phosphate-buffered saline and then sectioned as frozen sections, IgG was often still present in the stroma.

*Brandtzaeg:* It depends on the technique and on the type of tissue. The dimensions of the tissue piece in one direction must not exceed 2–3 mm if one is to get rid of the diffusible proteins. But our slides show a satisfactory absence of background staining for IgG.

*Evans:* This might be denaturation. Have you tried it with frozen sections?

*Brandtzaeg:* Why is it not denatured in the plasma cells, then?

*Evans:* It may be much easier to denature material at an extracellular site.

*Brandtzaeg:* I have doubts about frozen sections in relation to the specificity of extracellular staining.

*Evans:* The specificity control I used was the same as one usually uses in such studies, namely absorption and blocking studies.

*Brandtzaeg:* I cannot answer your question but I doubt that one will have pronounced denaturation at 4 °C in our washing process, and the alcohol procedure is the same as for the directly fixed material. The fact that diffuse background staining is present, despite a 48-hour washing of tissue pieces that were too big, speaks against extracellular denaturation of Ig.

*Vaerman:* I was struck by the lack of IgG staining between the epithelial cells. Andersen *et al.* (1963) claimed that the gut was an important site of IgG catabolism. This suggests that some IgG reaches the gut lumen. I am surprised then to find no IgG staining, especially in view of the high concentration of IgG in the extracellular fluid. This implies that if there is some IgG there, it is very quickly removed from the spaces between the epithelial cells by an unknown mechanism.

*Brandtzaeg:* Our directly fixed material indicates that there is some intercellular staining of IgG in the crypts, and especially in all types of surface epithelium, like the tips of the villi and the surface of the colon. It depends on the type of epithelium: in the Brunner's glands and salivary glands the epithelium seems to be very tightly packed, because there we hardly see any IgG staining, while it is easily seen in columnar surface epithelia. Normally IgG is not present in the cytoplasm of epithelial cells.

*Ferguson:* You touched on the technical problems of fluorescence staining related to extracellular fluid. Do you think that when there is extravascular staining with anti-immunoglobulin and anti-complement conjugates (on the basement membrane or elsewhere), this can be taken to imply the presence of immune complexes? I am thinking in the context of mucosal appearances in coeliac disease, Crohn's disease and ulcerative colitis.

*Brandtzaeg:* We find extravascular immunoglobulins of all classes and also extravascular complement factors (Baklien & Brandtzaeg 1974), but whether they are present in complexes, I do not know. One can't tell from tissue sections.

*Porter:* Allen and I showed that IgA was transported in vesicles (Allen *et al.* 1973). This particular vesiculation takes place outside the epithelial cell. We showed the vesicles within the intercellular channels. Our model includes the pushing of a pseudopodium from the plasma cell into the intercellular space. Would you feel that in the transport of this vesicle, secretory component is necessary?

*Brandtzaeg:* Let me first ask you whether you still feel that there are vesiculations from the plasma cell going into the interstices.

*Porter:* We have seen these inside and outside the epithelial cell, within the intercellular channel.

*Brandtzaeg:* How then is IgA transported considerable distances from plasma cells to epithelium, if it depends on such vesicles?

*Porter:* I agree that free IgA may be transported by transudation on to the secretory component. My question is whether you feel that the secretory component would be a necessary receptor in terms of transporting that vesicle.

*Brandtzaeg:* I have no idea about these vesicles because I am not working at the ultrastructural level, but in the study of Brown *et al.* (1975) such vesicles were not found.

*Lachmann:* Can one conclude that there seems to be some difference of opinion on whether secretory piece, either bound on cell membranes or free in solution, acts as a receptor for IgA? We have been told that secretory piece occurs on the membranes of epithelial cells and picks up material, presumably either directly from a plasma cell, or from solution. But on the other hand there seems to be no good evidence that secretory piece is an important receptor for localizing IgA-forming cells in the lamina propria. If the secretory piece is not the receptor responsible, is the IgA itself the identifying molecule? There would seem to be no convincing evidence for this either.

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*Immunology of the Gut*
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# Antibodies in human serum and milk induced by enterobacteria and food proteins

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*Abstract* Ingestion of *Escherichia coli* O83 bacteria by adults resulted in a transient irregular colonization leading to a serum antibody response in only four out of 14 cases examined. In all of three pregnant women, however, IgA antibodies against *E. coli* O83 antigen were released from colostral cells after similar bacterial ingestion although no serum antibody response was noted. The findings indicate a link between the antigenic exposure of the gut and secretory antibodies of the IgA class, presumably locally formed in the mammary gland.

Antibodies of the secretory IgA class registered in colostrum may, at least partly, reflect the antigenic exposure of the gut. These antibodies are probably important in protecting against *E. coli* infections in the neonate, as suggested by the findings of antibodies in human milk against O and K antigens of non-enteropathogenic as well as enteropathogenic serotypes of *E. coli*. Furthermore, in milk of women from low socio-economic groups in Pakistan, neutralizing antibodies were present against enterotoxins of *E. coli* bacteria and occasionally against *Vibrio cholerae* enterotoxins.

In addition, secretory IgA antibodies against food proteins were detected in human milk. This suggests that intestinal exposure to such antigens could stimulate a local immune response in the gut resulting in triggered lymphoid cells homing to the mammary gland. These human milk secretory IgA antibodies against bovine milk proteins may help to prevent cow's milk allergy in infants on mixed feeding, since these infants tend to have a lower serum antibody response to cow's milk proteins than infants fed mostly artificially. Furthermore, children suffering from cow's milk protein intolerance and gluten enteropathy may have higher serum levels of antibody to cow's milk protein antigens than normal children, possibly reflecting increased permeability of the intestinal mucosa for various antigens.

Intestinal exposure to antigens has been found to stimulate the immunological system in man as well as in animals (see Crabbé *et al.* 1970; Lodinová *et al.*

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1973; Sagie *et al.* 1974). The resulting immune response, mostly registered as an increase in antibody titre, may be local as well as systemic.

Immunity resulting from intestinal exposure to microbial antigens appears to be important in the body's defence against enteric infections. A protective role of acquired local immunity has been demonstrated in cholera where antibacterial as well as anti-enterotoxin antibodies have been found to be effective (Fubara & Freter 1973; Holmgren *et al.* 1975). Protection mediated by local antibodies against other enteric organisms such as *Escherichia coli*, *Salmonella* and *Shigella* has also been indicated (reviewed by Gerrard 1974 and Hanson *et al.* 1976*a*). Local immunity in the form of secretory IgA antibodies not only provides protection against mucosal adhesion of bacteria or toxins, but may also protect the mucosa from contact with potential allergens such as food proteins (Walker *et al.* 1972, 1974*b*, 1975; Soothill 1974, 1976). Furthermore, the local antibodies can serve a regulatory role with regard to their continued synthesis by abrogating further antigenic contact with the lymphoid cells in the gut mucosa (Fubara & Freter 1973; Walker *et al.* 1972, 1974*b*). Recent observations suggest a close relation between antigenic stimulation in the gut and the appearance of secretory IgA antibodies in the mammary secretion (Allardyce *et al.* 1974; Goldblum *et al.* 1975; Montgomery *et al.* 1974). This paper surveys studies of the relationship between the systemic serum antibody response and the local responses in the gut and the mammary gland against enteric microbial antigens and food proteins.

## SERUM ANTIBODY RESPONSE AFTER INTESTINAL EXPOSURE TO E. COLI

Colonization of the gut in infants with *Escherichia coli* O83 bacteria gives rise to a specific serum antibody response if the *E. coli* O83 establishes itself as a resident strain dominating the aerobic faecal flora (Lodinová *et al.* 1973). In contrast, we found that if healthy adults were exposed to these bacteria by the ingestion of two doses, resulting in *E. coli* O83 as one of many transient strains in the gut, serum antibody responses were detectable in only four out of 11 individuals (Jodal *et al.* 1976) (Fig. 1).

Serum antibody responses presumably stimulated from the gut in healthy children as well as in adults may vary with the bacterial antigens involved. Thus, the anti-*E. coli* O6 serum antibody levels are lower than those against *E. coli* O2, O4 and O75 (Table 1) (Ahlstedt & Jodal 1976; Jodal *et al.* 1976). Yet the virulence of these bacteria of various types may be similar, as suggested by their relative frequency in urinary tract infections and in faecal flora (Grüneberg *et al.* 1968; K. Lincoln & G. Lidin-Janson, personal communication). Thus a tolerogenic effect of the O6 antigen, as previously observed in

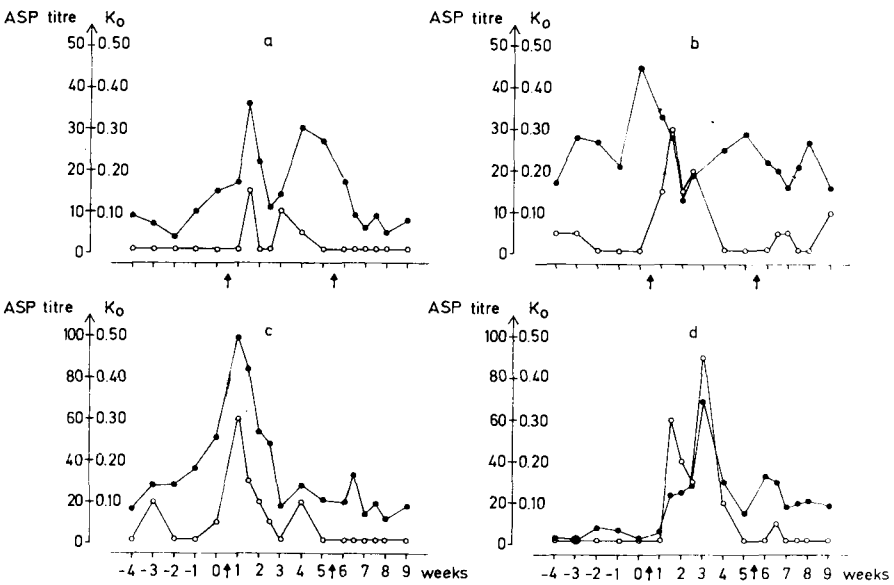


FIG. 1. The antibody titre (ASP) (—• —) and the antibody avidity (Ko) (— ○ —) to the *E. coli* O83 antigen in four healthy adults (*a–d*) after ingestion of the bacteria. Two ingestion times are indicated (↑).

TABLE 1

Mean serum antibody titre against *E. coli* O antigens, determined with the ASP technique

| | *Antibody titre against* | | | | |
|---|---|---|---|---|---|
| | *O2* | *O4* | *O6* | *O75* | *O83* |
| Children | 54[a] | 47[a] | 35 | 46 | not done |
| Adults | 40[b] | 40[b] | 26 | 40[2] | 22 |

[a] Differs significantly from O6 ($P < 0.05$).
[b] Differs significantly from O6 and O83 ($P < 0.01$).

parenterally immunized mice (Ahlstedt *et al.* 1973), might possibly explain the lower anti-O6 titres observed.

## MILK ANTIBODY RESPONSE AFTER INTESTINAL EXPOSURE TO E. COLI

Microbial antigens induce a local response in the gut demonstrable as IgA coproantibodies (Crabbé *et al.* 1970; Holmgren *et al.* 1975; Lodinová *et al.* 1973). Such a response is difficult to study, because of the problems of

obtaining representative material and the risk of enzymic degradation, even though laborious experimental systems have recently been successfully applied (e.g. Svennerholm & Holmgren 1976; Pierce & Sack 1976; Walker *et al.* 1974*a*). We have studied the link between the antigenic exposure of the gut and antibody release from the mammary gland. The results indicate a close relationship between the intestinal antigenic stimulation and the antibodies in the mammary secretion (Goldblum *et al.* 1975). This prompted us to study in greater detail lymphoid cells and antibodies in the human milk as a reflection of the maternal gut immunity.

Human milk contains considerable numbers of lymphoid cells, up to 8% of which secrete IgA antibodies to *E. coli* O antigens as detected with the plaque haemolysis-in-gel technique (Ahlstedt *et al.* 1975). This high frequency of cells producing antibodies against only one group of antigens present in the gut suggests that they may represent a rather selective lymphoid cell population. The triggering of this cell population may occur within the intestinal tract. Thus three pregnant women ingested bacteria of the harmless *E. coli* O83 strain used in about 200 neonates by Lodinová *et al.* (1973). One or two ingestions by the women of $10^9$ bacteria resulted within a few days in strikingly high numbers of cells in their milk which formed antibodies against the O83 antigen (Fig. 2). Actually, between 0.1 and 1% of the milk cells formed anti-O83 antibodies, again indicating that the cells recorded belong to a selective cell population forming antibodies against enterobacterial antigens present in the gastrointestinal tract. Since we did not see any corresponding serum antibody response it is not likely that the local antibody response was due to the transport of antigen from the gut to the mammary gland. Therefore we favour the hypothesis that the observed IgA-producing cells were antigenically triggered in the Peyer's patches and then homed to the mammary gland. This is in accordance with the findings of Craig & Cebra (1971) that cells from the Peyer's patches can repopulate the intestinal mucosa of irradiated animals with IgA-producing lymphoid cells. It is also in agreement with the recent demonstration by Pierce & Sack (1976) that lymphoid cells containing antibodies against an antigen used for peroral immunization can be found in the thoracic duct lymph.

Human milk contains high levels of antibodies against many enterobacterial antigens. Thus Gindrat *et al.* (1972) found antibodies against numerous *E. coli* O antigens. Using the enzyme-linked immunosorbent assay (ELISA), we found such antibodies to be predominantly of the secretory IgA type, although IgG and IgM were also demonstrated (Ahlstedt *et al.* 1975).

Analysis of antibodies in milk from well-nourished healthy Swedish mothers and undernourished Pakistani mothers showed quite similar antibody levels to

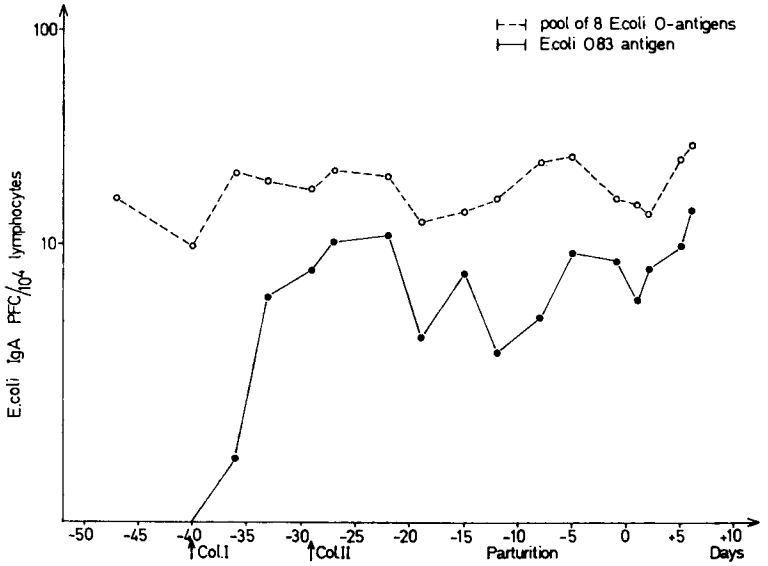


FIG. 2a

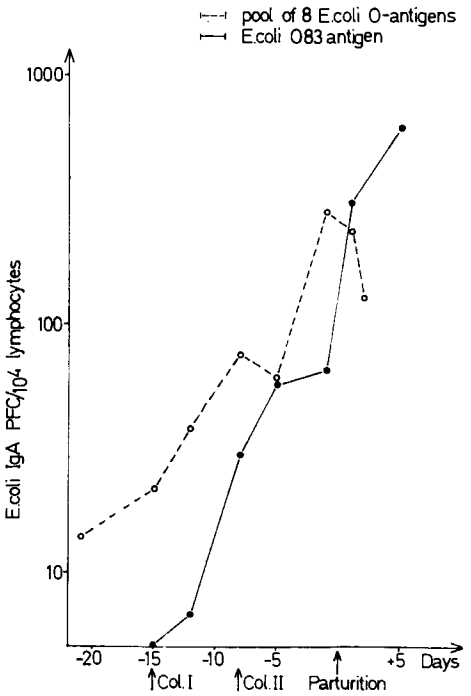


FIG. 2b

For legend, see p. 120

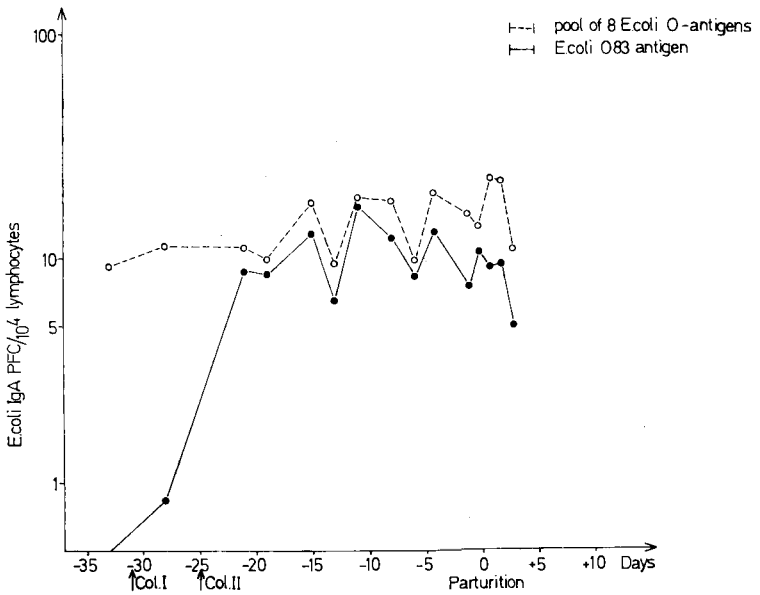


FIG. 2c

FIG. 2. Antibody-forming cells registered as plaques in colostrum samples from three women after ingestion of *E. coli* O83 bacteria. —•—, antibodies against the O83 antigen. - - o - -, antibodies against a pool of O antigens from the eight most frequent strains occurring in urinary tract infections. (*a*), (*b*) and (*c*) show the responses of the different women. (From Goldblum *et al.* (1975), by permission of the Editor of *Nature*.)

*E. coli* O antigens. This was noted regardless of whether a pool of the Swedish or Pakistani *E. coli* O antigens was used in the assays (Carlsson *et al.* 1976). Thus the finding that individuals suffering from protein calorie malnutrition are deficient in their secretory immune response (Sirisinha *et al.* 1974) could not be confirmed by studying the mammary gland secretion. There were indications, however, that the milk volumes were smaller from the undernourished Pakistani mothers than from the healthy Swedish mothers, resulting in a smaller output of the antibodies. In the Pakistani milk samples we also consistently found antibodies against the O antigens of enteropathogenic *E. coli* bacteria while such antibodies were less common in milk from Swedish mothers (Table 2).

Antibodies against the important virulence antigens of the capsule (K) of the *E. coli* bacteria are commonly present in the milk (Table 3 and Hanson *et al.* 1976*a*; Carlsson *et al.*, in manuscript). Of particular interest is the almost

TABLE 2

Milk antibody levels to enteropathogenic *E. coli* in Pakistani and Swedish mothers, measured with the enzyme-linked immunoabsorbent assay (ELISA)

| *Determined with antiserum against* | *Pakistani mothers (n = 13)* | | *Swedish mothers (n = 20)* | | *P* |
|---|---|---|---|---|---|
| | $\bar{x}$ | *Range* | $\bar{x}$ | *Range* | |
| IgA | 19.1 | 5.6–70.6 | 5.7 | 2.9–13.7 | $< 0.01$ |
| SC[a] | 27.1 | 0 –79.8 | 3.3 | 0 –22.3 | $< 0.01$ |
| IgG | 0.8 | 0 – 4.2 | 0.2 | 0 – 1.8 | N.S. |
| IgM | 2.9 | 0 –17.5 | 0 | 0 | N.S. |

[a] SC, secretory component.

TABLE 3

Antibodies to *E. coli K* antigens: ratio of antibody levels in milk/serum

| | $\bar{x}$ | *Range* | *n* |
|---|---|---|---|
| K1 | 3.36 | 0.48– 8.9 | 13 |
| K3 | 4.70 | 0.98–15.3 | 13 |
| K6 | 2.87 | 1.18– 6.7 | 12 |
| K13 | 4.27 | 1.38–14.3 | 10 |
| K52 | 2.68 | 0.97– 6.73 | 13 |

consistent presence of anti-K1 antibodies (Table 3) in spite of the previously shown poor immunogenicity of the K1 antigen (Kaijser *et al.* 1973). Since this K1 antigen has been found in 84% of the *E. coli* strains causing neonatal meningitis (Robbins *et al.* 1974), the presence of such antibodies may be of particular significance during the neonatal period.

Another antibody activity in human milk of potential biological importance is directed against the enterotoxins of *E. coli* and *Vibrio cholerae*. Striking differences were noticed between the milk specimens of Pakistani and Swedish women with regard to neutralizing activity against *E. coli* enterotoxin. This was tested with the adrenal cell morphology assay (Donta *et al.* 1974) using a few (2–5) minimal effective doses of enterotoxins and the milk samples diluted 1:50 to avoid non-specific cell reactions. Most of the Pakistani milk samples neutralized the enterotoxin of two different *E. coli* strains, whereas only a single Swedish milk sample had a partial neutralizing effect (Table 4). It seems likely that this difference reflects a more frequent intestinal exposure to enterotoxigenic *E. coli* of the Pakistani than of the Swedish women, resulting in the production of milk antibodies. Interestingly, neutralizing activity against *V.*

TABLE 4

Presence of neutralizing antibodies to *V. cholerae* and *E. coli* enterotoxins in human milk

| *Origin of milk* | *Neutralization of toxin from:* | | |
|---|---|---|---|
| | *V. cholerae*[a] | *E. coli I*[a] | *E. coli II*[a] |
| Pakistan | 1/18 | 18/18 | 12/18 |
| Sweden | 0/16 | 1/16 | 1/16 |

*Method:* adrenal cell assay (Donta *et al.* 1974).
[a] 2-5 minimal effective doses of the enterotoxins were used.

*cholerae* enterotoxin was seen much less frequently (Table 4), indicating both that the anti-*E. coli* enterotoxin activity was specific rather than due to immunological cross-reactivity of cholera toxin antibodies, and that enterotoxigenic *E. coli* are much more common than *V. cholerae* as a 'normal' antigenic stimulus for the Pakistani population (Holmgren *et al.* 1976).

## SERUM AND MILK ANTIBODY RESPONSE TO FOOD PROTEINS

The appearance of serum antibodies to food proteins such as cow's milk proteins has long been recognized. Thus Lippard *et al.* (1936) showed antibodies to bovine milk proteins in almost 100% of infants under one year of age who had been fed cow's milk. Investigating sera from healthy children with the ELISA (S. P. Fällström *et al.*, unpublished), we registered antibodies of the IgG and IgA classes to cow's milk proteins in most of the children. Antibodies of the IgE class were very rarely found (Fig. 3 and Table 5) while low levels of IgM antibodies were consistently present. Such findings are in accordance with previous observations (for review see Gerrard 1974). In children with acute gastroenteritis we noted the same levels of antibody to cow's milk proteins as in the healthy controls (Table 5).

Serum antibodies to cow's milk proteins have been shown by many techniques in patients with cow's milk protein intolerance (reviewed by Hanson & Johansson 1970). This has not been useful diagnostically, however, since discrimination from other patients and normals has been poor. Using the ELISA we also found rather variable patterns in infants with cow's milk protein intolerance and gastrointestinal symptoms (Table 5), even though they all had their diagnosis verified by provocation tests twice repeated. Increased levels of IgG, IgA and sometimes of IgE anti-cow's milk protein antibodies were noted in some individuals. However, no consistent antibody increase was found. Children with coeliac disease showed similar patterns to those of

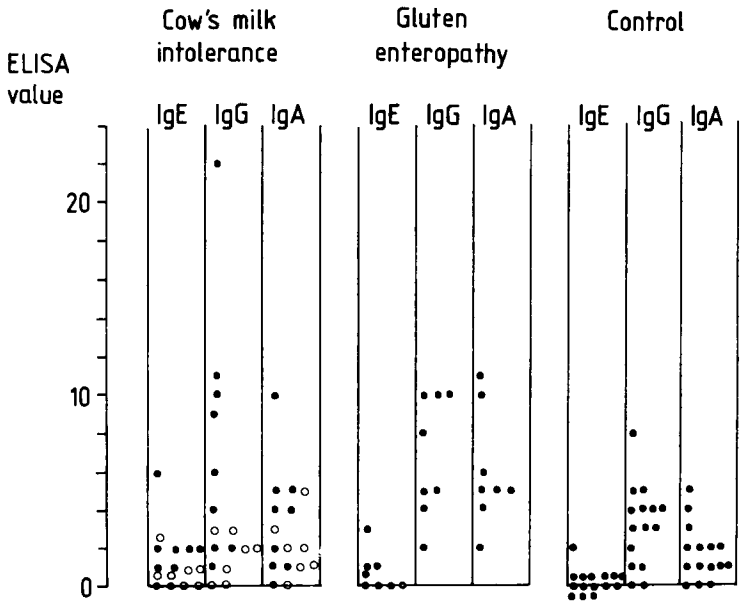


FIG. 3. Antibody levels in serum determined with the enzyme-linked immunoabsorbent assay (ELISA) to cow's milk protein antigens in children with cow's milk protein intolerance or gluten enteropathy and in healthy children. •, chronic disease; o, acute disease.

TABLE 5

Antibodies to cow's milk proteins determined with the enzyme-linked immunoabsorbent assay (ELISA) (median and range)

| *Protein* | *Ig* | *Cow's milk protein intolerance* | | *Gluten enteropathy* | *Gastroenteritis* | *Control* |
|---|---|---|---|---|---|---|
| | | *Chronic* | *Acute* | | | |
| Cow's milk proteins | E | 1 (0–6) | 0 (0–2) | 0 (0–3) | 0 (0) | 0 (0–2) |
| | G | 6 (0–22) | 2 (0–5) | 6.5 (2–10) | 4 (1–5) | 3 (0–8) |
| | A | 4 (0–10) | 2 (0–5) | 5 (2–11) | 3 (0–7) | 1 (0–6) |
| α-Casein | E | 0 (0–2) | 0 (0–1) | 0.5 (0–2) | 0 (0–1) | 0 (0–1) |
| | G | 4 (0–10) | 3 (1–4) | 4 (1–10) | 4 (3–5) | 3 (0–11) |
| | A | 1 (0–7) | 1 (0–2) | 4 (1–8) | 1 (1–5) | 1 (0–4) |
| β-Casein | E | 0 (0) | 0 (0–1) | 0 (0) | 0 (0–1) | 0 (0–1) |
| | G | 1.5 (0–5) | 3 (0–4) | 0.5 (0–3) | 1 (1–5) | 1.5 (0–5) |
| | A | 1 (0–3) | 0 (0–1) | 0 (0–2) | 3 (1–5) | 1 (0–2) |
| β-Lacto-globulin | E | 0 (0) | 0 (0) | 0 (0) | 0 (0–1) | 0 (0) |
| | G | 5 (0–6) | 4 (1–5) | 3.5 (1–5) | 2 (1–3) | 4 (0–5) |
| | A | 0 (0–2) | 0 (0–1) | 2.5 (1–5) | 1 (0–2) | 2 (0–5) |

children with cow's milk protein intolerance (S. P. Fällström *et al.*, unpublished), which presumably means that both groups of patients have a defective gut mucosa and perhaps an insufficient local immune response, permitting the penetration of various antigenic molecules. According to our hypothesis that antigenic exposure in the gut is important for antibody formation in the mammary gland, human milk should contain antibodies not only against microbial antigens but also against food material which may come into contact with the intestinal lymphoid tissue. Therefore we analysed human milk for the presence of antibodies to bovine milk proteins with the ELISA. In all 20 samples investigated we found considerable amounts of secretory IgA antibodies against bovine $\beta$-lactoglobulin and against $\alpha$- and $\beta$-casein (Fig. 4) (S. P. Fällström *et al.*, unpublished). These studies illustrate the intimate relationship between food constituents and the gut mucosa, where resorption of native proteins may result in stimulation of immunocompetent cells within the Peyer's patches and in more central lymphoid tissues, thus inducing local as well as systemic immune responses (Lippard *et al.* 1936; Sagie *et al.* 1974; Soothill 1976; Walker 1976).

## PROTECTIVE EFFECT OF MILK ANTIBODIES

A protective role of the secretory IgA antibodies from human milk detectable as coproantibodies with retained antibody activity in the breast-fed infant (Kenny *et al.* 1967; Gindrat *et al.* 1972) has been indicated against neonatal sepsis/meningitis (Winberg & Wessner 1971) and infantile intestinal infections caused by enteropathogenic species of *E. coli* and by *Shigella* (Mata & Urrutia 1971). Obviously it is a practical arrangement for the mother to provide the baby with secretory IgA antibodies against the enterobacteria she herself has been and is exposed to and which may colonize and eventually infect the neonate. Such protection would be of particular significance in developing areas of the world with undernutrition, because this condition seems linked with a defective first line of defence (Walker 1976).

Secretory IgA antibodies in the gastrointestinal tract probably protect primarily by preventing adhesion of the pathogens or their toxic products to the mucosa (Fubara & Freter 1973; Holmgren *et al.* 1976). Such a function of milk secretory IgA antibodies transferred from the mother to the infant could possibly be one of the determining factors behind the reported lower content of *E. coli* in the intestinal flora of the breast-fed than of the artificially fed infant (Bullen & Willis 1971). On the other hand, the anti-*E. coli* antibodies in milk directed against the O or the K antigens of *E. coli* do not appear to prevent bacterial colonization of the gut of the infant with the respective *E. coli* strains

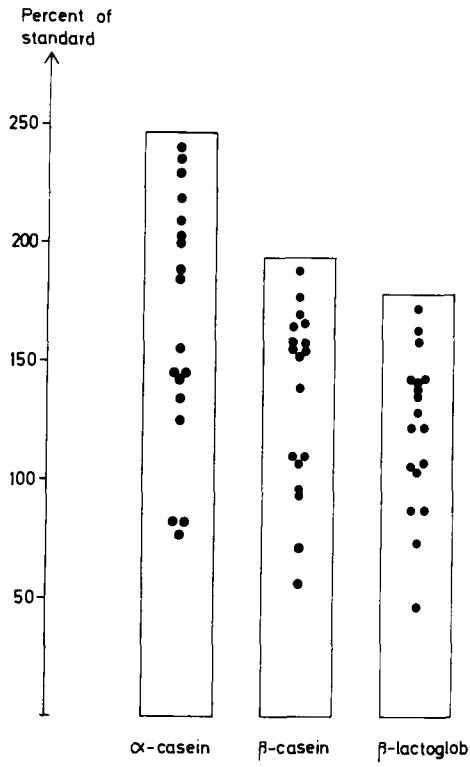


FIG. 4. Levels of antibody to cow's milk protein antigens in human milk from healthy mothers determined with the enzyme-linked immunoabsorbent assay (ELISA).

(Gothefors *et al.* 1976; B. Carlsson *et al.*, unpublished). It is possible that the antibodies play a role by selecting mutants of relatively low virulence, as suggested by Gothefors *et al.* (1975) for babies fed with human milk.

That antibodies may induce continuous changes in an exposed bacterial flora has been indicated at the local level for *V. cholerae* in the mouse gut (Sack & Miller 1969), for *V. fetus verseralis* in the bovine vagina (Corbeil *et al.* 1975) and for *E. coli* in the human urinary tract (Olling *et al.* 1973; Hanson *et al.* 1976*b*,c). Such changes can be detected as variations in bacterial surface characteristics (Sack & Miller 1969; Hanson *et al.* 1976*a*,*b*; Olling *et al.* 1973; Lindberg *et al.* 1975*b*) and possibly by the extent of the symptoms induced by the infection (Lindberg 1975*a*; Verrier-Jones *et al.* 1975).

Since it is difficult to measure local secretory IgA antibody production in the gut directly it may be practical to use milk secretory IgA antibodies as a reflection of the antigenic exposure of the gut. Determination of milk secretory

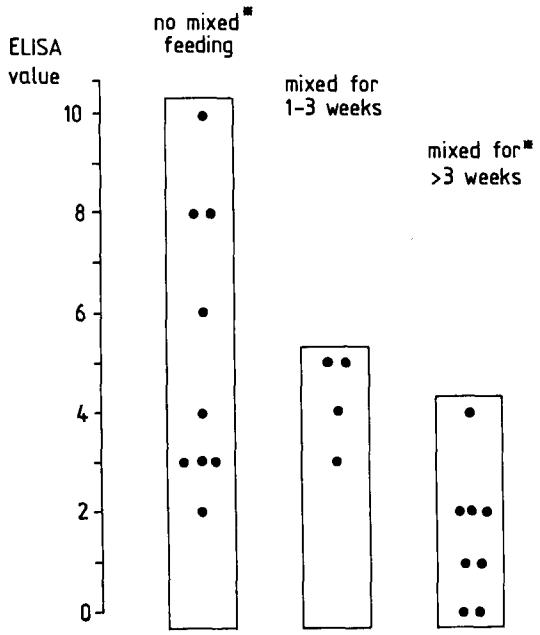


FIG. 5. Influence of breast-milk feeding on the serum IgG antibody response to cow's milk proteins in infants, as determined with the enzyme-linked immunoabsorbent assay (ELISA). *, difference significant at $P > 0.01$.

IgA antibodies may give a picture of the epidemiological situation by showing which microbial antigens have stimulated and are stimulating the antibody response in the gut.

The presence in human milk of antibodies against food antigens such as cow's milk proteins may also be important. These milk secretory IgA antibodies may prevent food allergies by hindering the absorption of the intact food proteins. In accordance with this hypothesis, a lower frequency of gastrointestinal allergies has been observed in children who are fed both breast milk and artificial nutrients as compared to those fed only artificial nutrients (Soothill 1974, 1976). The lower levels of antibodies to cow's milk proteins registered in sera from healthy children given mixed feeding, compared to sera from children given only artificial feeding, fits nicely with the proposed function of secretory IgA antibodies in human milk of preventing the absorption of intact immunogenic/allergenic food constituents (S. P. Fällström *et al.*, in manuscript) (Fig. 5).

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## Discussion

*Porter:* Your thesis was very nicely demonstrated by the work of Professor Bohl on pigs (Bohl *et al.* 1972). He has been interested in the transmissible gastroenteritis (TGE) virus. He found that sows that had suffered an enteric infection with TGE virus generated IgA antibodies in the colostrum and milk which subsequently gave solid protection of the neonate against deliberate

infection with that virus, whereas sows that had been parenterally immunized with an attenuated virus developed IgG antibody which did not give solid protection in the neonate.

The IgA antibody demonstrated by Bohl was quite unassociated with serum antibody in the sow, so it was clear that there must have been a traffic of cells from the gut to the mammary gland to produce there locally the IgA which protected the neonate. In that respect, this work gives credence to your overall philosophy on this protective function.

*Brandtzaeg:* Haneberg (1974) has shown that it is probably only during the first month that colostral IgA antibodies survive passage through the gut of the infant. Do you have any ideas about this, Dr Ahlstedt?

*Ahlstedt:* Most studies dealing with antibodies in human colostrum and milk have been done on samples taken within a month after parturition. The protective capacity of the antibodies in this milk has been indicated but is not proved. Further, the protective effect of the antibodies in relation to that of other factors in the milk has to be established. If the antibodies are not of protective value for the infant after one month of age, why are they present in the milk? To protect the breast? But why then the close relation between antigenic exposure of the gut and antibodies in the milk?

*Pierce:* Survival of IgA through the gut may depend in part upon inhibition of gastric acid secretion. This inhibition occurs during the first few months of life in suckling children but not in children fed cow's milk (Maffei & Nobrega 1975), which suggests that either the suckling process or a component of the mother's milk is responsible. Inhibition of acid secretion would protect IgA from acid pepsin degradation.

*Brandtzaeg:* This shows up in the pH of the faeces of breast-fed children.

*Pierce:* The pH of the faeces reflects events that take place in the small intestine and colon, particularly lactose degradation. Faecal pH is not necessarily a direct reflection of what happened in the stomach.

*Porter:* On this point about the survival of IgA in the gut, in relation to whether gastric enzymes develop or not, the pig is a good model for the human infant!

We studied the passage of anti-*E. coli* IgA antibodies along the intestine in piglets fed sow's milk (Porter *et al.* 1970). The way the IgA separates from the milk clot in the stomach and passes into the small intestine and the speed at which this happens assists in the antibody activity in the gut. Within five minutes of feeding the milk the IgA appears in the duodenum, and it passes through the small intestine and appears in the ileum within one hour, so that the whole processes takes about ninety minutes. The piglet feeds about once every ninety minutes, so one has a fast passage of IgA and a continuous coating of maternal antibody. Going back to Bohl's observations, it is par-

ticularly interesting that where he took milk from sows immunized by the gut route with TGE virus and subsequently infected the neonate with the virus, he got total protection, with no pathological effects in the gut. When he fed milk from sows in which IgG antibody was present he found a massive degeneration in the mucosa of the neonate but the first yard of the intestine appeared to be protected, as though the IgG survived that far and thereafter protection was absent. It looks as if, in this species at least, IgA has physiological features that support the local maternal defence of the mucosa. I would suspect it is probably similar in the human.

*André:* We have some preliminary results that are relevant. We immunized pregnant guinea pigs by the oral route with sheep red cells on the day of parturition. IgA against sheep red cells was found in the milk ten days later. The newborn guinea pigs were fed with this milk. One or two months later they were injected with sheep red cells and compared to controls. Their response was markedly decreased (C. André, unpublished work 1975).

*Lachmann:* If you immunize into the mammary gland do you get a rise in IgA antibody in the gut?

*André:* We haven't tried this.

*Pierce:* Dr Ahlstedt, have you examined the morphology of the cells in milk which presumably contain or secrete IgA? Do they look like plasma cells or immunoblasts?

*Ahlstedt:* We have tried to study the morphology of the cells, but it is difficult to obtain good samples to examine. However, we do know that we have antibody specificity in the plaques, which indicates that the cells are of B cell type, probably plasma cells, and not epithelium cells.

*Gowans:* How do the cells reach the colostrum? What is the route they take from the capillaries to the acini?

*Pepys:* The mammary gland during lactation is not a nicely organized exocrine gland. It is an apocrine gland with shedding of cell fragments and whole cells, including lymphocytes, polymorphs and epithelial cells.

*Evans:* There are not many polymorphs in the interstitium of the lactating mammary gland, surely?

*Soothill:* We have certainly found both polymorphs and macrophages in human milk. How they get there, we don't know.

*Cebra:* I wonder if there is any contradiction between Dr Gowans' finding that secretory component does not bind to IgA immunoblasts and your being able to facilitate plaque formation by plasma cells in the milk with anti-SC. Is this just a matter of the stage in maturation of the IgA-secreting cells? Presumably the plasma cells have to obtain secretory component from outside sources, since they don't make it?

*Ahlstedt:* I don't know the stage in maturation since we haven't been able to do morphological studies, as I said. On the formation of *secretory* IgA plaques, we know that there is antigenic specificity in the plaque, indicating that the centrum cell is a plasma cell *making* IgA and not an epithelium cell *releasing* secretory IgA. However, we cannot exclude that the antibody coming out of the plasma cell takes up secretory component formed by an epithelium cell nearby and becomes a secretory IgA antibody, absorbing on to the red cell coated with antigen and picking up the developing anti-secretory component antiserum. I think we have epithelium cells present, since we have not found a good way of getting rid of them.

When culturing the milk cells we have found that the binding between the IgA molecule and the secretory component is very strong, confirming the findings of Dr Brandtzaeg. Further, the cultured cells produce dimeric secre-

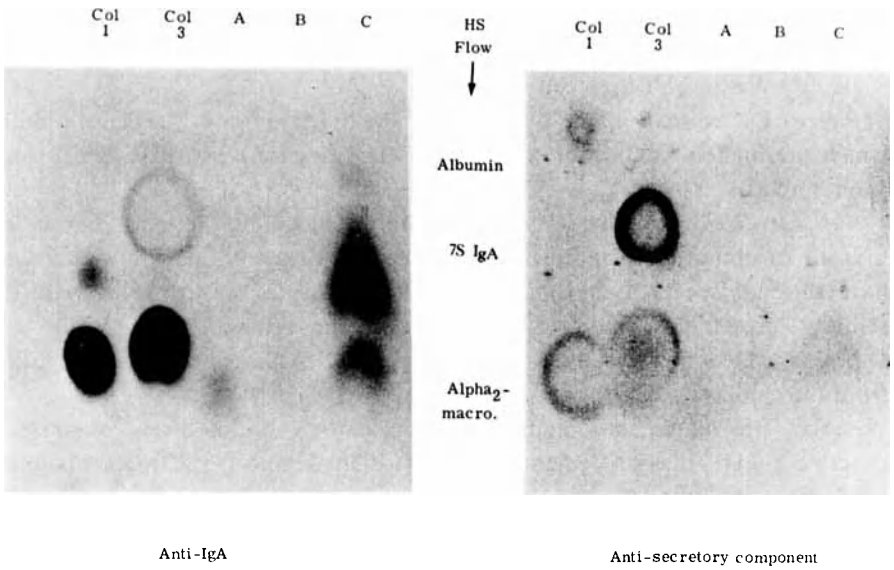


FIG. 1. (Ahlstedt). Thin layer immuno-gel filtration, using Sephadex G-200 superfine, of the supernatants A, B and C from 5-day cultures of IgA-producing cells from colostrum obtained two days *post partum*. Cols. 1 and 3 are colostrum obtained one and three days *post partum* from another woman. Comparison of these patterns shows that all five samples contain protein of the size of secretory IgA (heavily stained on the left and large rings on the right in Cols. 1 and 3). Although the colostrum samples contain fragments of IgA as well as secretory component filtering more slowly than secretory IgA, there are no indications that the secretory component was released from the IgA in the culture supernatants during the filtration. The filtration positions of some human serum proteins are indicated (centre). Cultures A and B contained $10^6$ cells/ml and C, $3.9 \times 10^6$ cells/ml.

tory IgA, very little free secretory component and monomeric IgA. When a sample from a culture was run using the immuno-gel filtration method of Hanson *et al.* (1971) we found very little separation of the formed secretory IgA during filtration (Fig. 1). The secretory component of small molecular size demonstrable in Col. 1 in the figure is not seen in preparation C, although IgA in a similar filtration region was demonstrated in both Col. 1 and in preparation C.

*Lachmann:* You showed us that you do not get asymmetric overlapping plaques when you develop with antiserum to SC but that you get perfectly round plaques. Presumably this is because there is secretory piece everywhere. Might you not have expected that if you need cooperation between two cells making different components to obtain a plaque the plaques would be asymmetrical?

*Ahlstedt:* If we have two cells, one making IgA antibodies, which then attach to the antigen-coated red cells and pick up the secretory component formed by the other cell, perhaps the plaque would be somewhat askew, but probably not very much.

*Lachmann:* I would have expected that in some circumstances you would get haemolysis on one side only.

*Ahlstedt:* The diffusion rate of the secretory component is rather rapid and the distances are small between cells of varying types, giving a high probability for an even distribution of the secretory component.

*Bienenstock:* The problem of whether dimeric IgA has a selective transport advantage was briefly mentioned by Dr Brandtzaeg. The question of whether it really has, and whether the antibodies synthesized in the bowel, presumably circulating, can be selectively transported out into the secretions, is one on which I know of no direct evidence in man. The problem is that man has monomeric IgA, primarily in serum, and also dimeric IgA, primarily in secretions. Many animal species have dimeric IgA in the serum, and many people have shown transport advantages for IgA in the serum going out into the secretions. One of the continuing problems is why in man one has primarily monomeric IgA in the serum, and what that is due to, and whether dimeric IgA has a selective transport advantage from the serum into the gastrointestinal and other glandular secretions.

*Brandtzaeg:* According to Heremans' (1974) calculations, the level of dimeric IgA in human serum is about the same as the level in dog serum.

*Vaerman:* That study has been criticized by Rádl *et al.* (1975). It was previously said that there is about 10% polymeric IgA in normal human serum, but they now find that it is as low as perhaps 1%. They used three criteria to say that there were very few true polymers in serum, but rather some aggregates. These aggregates were not J-chain-containing, they did not bind secretory

component, and thirdly they lacked a 'configurational polymeric' antigenic determinant. So we may have to revise our estimates.

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*Immunology of the Gut*
Ciba Foundation


# Immunological responses to bacterial plaque in the mouth

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*Abstract* A heavy load of bacteria, referred to as dental plaque, accumulates at the junction between the teeth and gum. Bacterial plaque may be considered to have three functional components: (*a*) cariogenic organisms, (*b*) organisms inducing gingival inflammation and periodontal disease, and (*c*) adjuvant and tolerizing agents, such as lipopolysaccharides, dextrans and levans. Sequential investigation of plaque accumulation in man has shown a correlation between gingival inflammation and both lymphocyte transformation and macrophage migration inhibition. An adjuvant effect of *in vivo* plaque accumulation was manifested by the enhancement of T lymphocytes in the mixed leucocyte culture reaction and of B lymphocytes, as shown by the increased response to lipopolysaccharide. It may be significant that a substantial component of bacterial plaque consists of dextrans and levans, produced by certain streptococci and actinomyces, and lipopolysaccharides from Gram-negative bacteria. These bacterial products are B cell mitogens which may have an adjuvant or tolerizing effect on immune responses. The relationships between immunogenicity, mitogenicity, adjuvanticity and tolerogenicity of lipopolysaccharides, levan and dextran have not been clearly defined. However, important variables of the polyglycans are the molecular weight, type of branching, negative charge, epitope density, degradability, dosage and the sequence between mitogen and antigen. Dental plaque in man is a focus of B cell mitogens and T cell antigens which may modulate the immune responses in such a way as to induce a protective response in the development of caries and a damaging response in periodontal disease.

A heavy load of bacteria, referred to as dental plaque, may accumulate at the junction between the teeth and gum, containing $2.5 \times 10^7$ aerobic and $4.6 \times 10^7$ anaerobic organisms per mg of plaque (Gibbons *et al.* 1964). Bacterial plaque accumulates under the influence of a fine consistency, largely roughage-free diet, with a high content of refined sugars, prevalent in countries with Western dietary practices. The development of daily oral hygiene measures has attempt-

ed to limit the accumulation of dental bacterial plaque. If oral hygiene is deliberately not practiced, up to 50 mg of removable bacterial plaque will accumulate on the teeth and influence the immune responses to oral bacteria (Lehner *et al.* 1974*a*).

Dental plaque is deposited on the teeth, adjacent to the crevicular epithelium which is supplied by blood vessels possibly of the post-capillary venule type (Egelberg 1966) and through which passes a constant traffic of neutrophils, lymphocytes and monocytes (Attström & Egelberg 1970; Skapski & Lehner 1976). A conservative estimate of the total surface area of crevicular epithelium round 28 teeth is of the order of 760 $mm^2$ and this can increase up to about 7600 $mm^2$ in periodontal disease (Arne 1963). It is therefore evident that dental plaque, consisting of bacteria and their products, of which lipopolysaccharides (LPS; Mergenhagen *et al.* 1961), dextrans and levans (Wood 1967) are most important, is found adjacent to an extensive epithelial surface. This can be penetrated by bacterial products, as was shown by autoradiography after the application of tritiated LPS to the gingival sulci of dogs (Schwartz *et al.* 1972).

Bacterial plaque may be considered to have three functional components: (*a*) cariogenic organisms, of which *Streptococcus mutans*, *Lactobacilli* and *Actinomyces* are most important; (*b*) organisms inducing gingivitis and periodontitis, such as *Veillonella*, *Fusobacteria*, *Bacteroides* and *Actinomyces*; and (*c*) adjuvant and tolerizing agents, the most potent being lipopolysaccharides (Johnson *et al.* 1956), dextrans (Battisto & Pappas 1973; Howard *et al.* 1975) and levans (Miranda *et al.* 1972). The interaction of bacteria with the adjuvants and tolerizing agents in plaque may induce immune responses which could inhibit or enhance the development of caries and periodontal disease.

In this paper the results of studies on cell-mediated immune (CMI) responses to oral bacteria and their products will be discussed, with particular reference to the modulating effect of *in vivo* bacterial plaque on the *in vitro* CMI functions and their relationship to the disease indices of caries and gingival inflammation in man.

## LYMPHOPROLIFERATIVE RESPONSES TO PLAQUE ORGANISMS

Peripheral blood lymphocytes in man are sensitized to dental plaque antigens and there is a significant correlation between the degree of lymphocyte stimulation and the periodontal index (Ivanyi & Lehner 1971; Horton *et al.* 1972*a*). A corresponding sensitization of lymphocytes to the following plaque bacteria has been shown: *Veillonella alcalescens*, *Actinomyces viscosus*, *Bacteroides melaninogenicus* and *Fusobacterium fusiforme* (Ivanyi & Lehner 1970). It appears that plaque bacteria can be chemically altered into mitogens,

for when alkali-treated organisms were used to stimulate lymphocytes there was no differentiation between the proliferative response of lymphocytes from controls and patients with periodontal disease and some of the altered bacterial antigens induced greater stimulation than phytohaemagglutinin (Kiger *et al.* 1974).

The cells responding to plaque antigens are both T and B lymphocytes (Mackler *et al.* 1974) and this may be accounted for by the finding that protein antigens of *Veillonella* stimulate T lymphocytes, whereas the lipopolysaccharide from *Veillonella* elicits a response only in B lymphocytes (Ivanyi & Lehner 1974).

## LYMPHOPROLIFERATIVE RESPONSES INDUCED BY B CELL MITOGENS

Lipopolysaccharides, dextrans and levans are mitogens which activate a broad spectrum of B lymphocytes (Gery *et al.* 1972; Greaves & Janossy 1972; Andersson *et al.* 1972; Coutinho & Möller 1973). These polymers are found in large amounts in dental plaque; lipopolysaccharides from Gram-negative bacteria, dextran synthesized by *Strep. mutans* and *sanguis* and levan formed by *Strep. salivarius* and *Actinomyces viscosus*. They induce a low but significant lymphoproliferative response in B lymphocytes in man (Ivanyi & Lehner 1974) and do not seem to be inhibited by the serum inhibitory factor in severe periodontitis. Levan induced the highest stimulation of lymphocytes, with a mean of 4.2 ($\pm$0.7), followed by LPS (2.6 $\pm$ 0.5) and dextran (2.3 $\pm$ 0.3). Lymphocyte stimulation induced by levan and LPS was significantly increased in gingival and periodontal disease, as compared with controls ($P < 0.05$–$0.01$). However, the optimal doses were highest for levan (500 $\mu$g per ml of culture), followed by dextran (50 $\mu$g) and LPS (10 $\mu$g).

## MODULATING FACTORS IN SERUM

Lymphocyte activity can be modulated *in vitro* by serum factors, by stimulating or inhibitory antibodies, or their immune complexes. Activation of lymphocytes by *Veillonella* seems to depend not only on the presence of sensitized lymphocytes but also on stimulating factors present in sera from patients with gingivitis or mild and moderate periodontitis (Ivanyi *et al.* 1973). A depression of lymphocyte transformation, in the presence of autologous serum from patients with severe periodontitis, has been ascribed to a serum inhibitory factor (Ivanyi *et al.* 1973). The response of these lymphocytes, however, can be restored by substituting autologous for homologous serum from patients with gingivitis or moderate periodontitis. These results can be interpreted by assuming that

blocking antibodies on the surface of lymphocytes might be counteracted by deblocking antibodies, as is found in tumours (Hellström & Hellström 1970).

## SOLUBLE MEDIATORS

Sensitized lymphocytes release macrophage migration inhibition factor (MIF; Ivanyi *et al.* 1972) which might serve to localize macrophages at the site of antigenic activation of lymphocytes. Furthermore, an osteoclast-activating factor is produced and this can cause bone resorption, as measured by the release of $^{45}Ca$ from fetal rat bone (Horton *et al.* 1972*b*).

## CYTOTOXIC MECHANISMS

A cytotoxic assay has been described in which specific activation of sensitized lymphocytes causes non-specific cytotoxicity of target cells *in vitro* (Ivanyi *et al.* 1972). This mechanism is antibody-independent and probably different from lymphotoxin, which is released by lymphocytes and damages fibroblasts (Horton *et al.* 1973). Activated lymphocytes can thus damage fibroblasts and other cells and can cause destruction of the supporting bone. Antibody-dependent cytotoxicity by K cells has not yet been described in periodontal disease.

## THE ROLE OF MACROPHAGES

Macrophages can be localized to the site of microbial attack by chemotaxis and by the release of MIF and chemotactic factor from lymphocytes, and they may take part in several functions. The cells are essential for the induction of an immune response and for lymphocyte differentiation and proliferation *in vitro* (Sjöberg *et al.* 1972). Removal of macrophages may impair T lymphocyte stimulation by *Veillonella* antigens and B lymphocyte stimulation by lipopolysaccharides, levan and dextran (Ivanyi & Lehner 1974).

Macrophages may play an important part in adjuvanticity, because bacterial adjuvants induce the formation of macrophage granulomata (Suter & White 1954), macrophage chemotactic factors (Wilkinson *et al.* 1973), sequestration of lymphocytes in lymphoid tissues, which appears to be macrophage dependent (Frost & Lance 1973), and the increased antibody formation resulting from transferring macrophages, containing adjuvant and antigen, to syngeneic mice (Allison 1973). Tolerance to T-dependent antigens induced by B cell mitogens might be mediated by macrophages (Ivanyi 1976), for the suppressive effect of LPS or levan on T cell proliferation induced by *Veillonella* or PPD is mediated by the interactions of LPS and levan with macrophages.

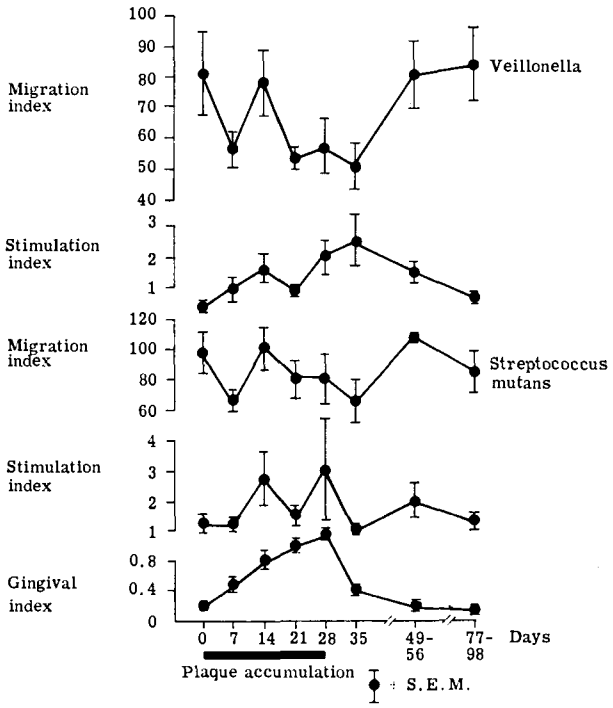


FIG. 1. Sequential cell-mediated immunity to *Streptococcus mutans* and *Veillonella alcalescens* induced by accumulation of bacterial plaque.

Dental plaque or endotoxin may stimulate macrophages directly to synthesize and release lysosomal hydrolases (Page *et al.* 1973). Activation of guinea-pig macrophages by endotoxin, however, requires B lymphocytes (Wilton *et al.* 1975). There is now evidence that endotoxin exerts a direct effect on macrophages, stimulating them to synthesize collagenase, though soluble mediators from B lymphocytes may enhance this response (Mergenhagen *et al.* 1976). The acid hydrolases and collagenase may be responsible for much of the tissue damage in periodontal disease.

## SEQUENTIAL IMMUNE RESPONSES IN EXPERIMENTAL GINGIVITIS IN MAN

Sequential changes in CMI responses were examined in young healthy subjects who abstained from oral hygiene for 28 days, to find out whether the cellular responses were closely related to the clinical changes, or whether they were a consequence of long-standing bacterial stimulation (Lehner *et al.* 1974*a*). Accumulation of dental bacterial plaque and the associated gingival inflammation were correlated with an increase in lymphocyte transformation and release of MIF (Fig. 1). These were induced by sonicates of autologous bacterial

plaque, a number of Gram-negative organisms, *A. viscosus*, and some unrelated antigens. Both cellular responses were of limited duration and had returned to base-line values 28 days after plaque was removed. The close temporal relationship between CMI and gingivitis suggests the following sequence: accumulation of a heavy antigenic load induces CMI responses which lead to the gingival inflammation and later periodontal destruction. This is substantiated by the evidence from the use of immunosuppressive and immunopotentiating drugs (see below, p. 146).

## IMMUNOPOTENTIATION BY DENTAL PLAQUE IN VIVO

The possibility that dental plaque may exert an adjuvant effect *in vivo* has been raised on the basis that an increase in DNA synthesis and release of MIF by lymphocytes can be induced *in vitro* not only by related antigens but also by such unrelated stimulants as the mixed leucocyte culture reaction (MLC) and purified protein derivative of tuberculin (PPD) (Fig. 2; Lehner *et al.* 1974*a*,*b*). Although purified cell preparations were not examined, both T and B lymphocyte functions were enhanced in the subjects who had accumulated plaque for 28 days, as tested by the MLC reaction for T lymphocytes (Johnston & Wilson 1970) and lipopolysaccharide activation for B lymphocytes (Ivanyi & Lehner 1974). There was no detectable increase in antibody titres to the plaque bacteria but an increase in serum immunoglobulins was found. This is consistent with the view that LPS, dextran and levan act as polyclonal B cell activators (Coutinho & Möller 1973). It should, however, be noted that whereas immunogenicity and tolerogenicity to levan are dependent on macrophages and epitope density, this is not so with mitogenicity (Desaymard & Ivanyi 1976).

In view of these findings, it has been suggested that dental plaque may act as an endogenous adjuvant acting on both T and B lymphocytes. Among a multitude of antigens in dental plaque, lipopolysaccharides (Johnson *et al.* 1956) from Gram-negative bacteria, and dextran (Battisto & Pappas 1973) synthesized by *Strep. mutans* and *Strep. sanguis* have adjuvant properties. Dextran given to mice enhances the responses of lymphoid cells both to T-dependent (Concanavalin A) and T-independent mitogens (LPS; Alevy & Battisto 1976*a*). This is comparable to the enhanced responses of lymphocytes in man in the T-dependent (MLC) and T-independent (LPS) reactions induced by bacterial plaque accumulation *in vivo* (Lehner *et al.* 1974*b*). It is suggested that dextrans and other B cell mitogens found in dental plaque have a comparable function in man to that of dextran injected into mice. The proliferative response of human lymphocytes by *Veillonella* has been also potentiated *in vitro* by levan and LPS (Ivanyi 1976). The enhanced responses are mediated

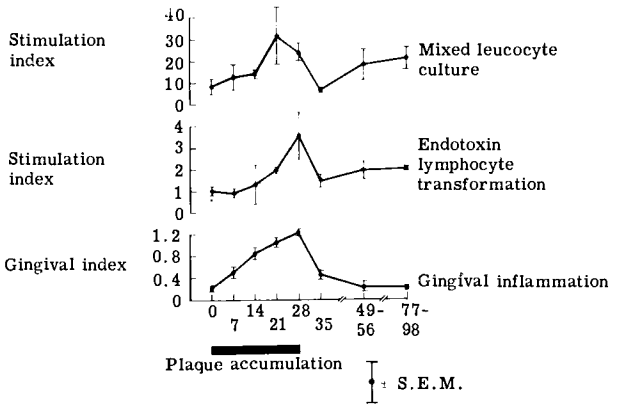


FIG. 2. Sequential responses of T and B lymphocytes in the mixed leucocyte culture reaction and to endotoxin induced by accumulation of bacterial plaque.

through soluble factors elaborated by T lymphocytes and some affect T cells and others, B cells; corresponding receptors are found on thymus cells and bone marrow cells, respectively (Alevy & Battisto 1976*b*). A similar but non-specific factor derived from T cells has been described in relation to LPS (Waldmann & Munro 1975).

Considerable quantities of LPS, dextran and levan are found in dental plaque and they may be responsible for the predominant B lymphocyte proliferation, resulting in plasma cells, in gingivitis. The relationship between mitogenicity and adjuvanticity has not been clarified, as LPS and dextran induce proliferation of B cells (Andersson *et al.* 1972; Coutinho & Möller 1973; Ivanyi & Lehner 1974) and yet the adjuvant effect may be T-cell dependent (Allison & Davies 1971; Battisto & Pappas 1974).

The mechanism of action of adjuvants is complex but four points need to be emphasized in relation to dental plaque. (*a*) The persistence of dental plaque along the gingival margin enables LPS, dextran and levan to be released continuously over an extensive epithelial surface. (*b*) Dental plaque or LPS are potent agents causing release of lysosomal hydrolases from macrophages and these may be involved in the action of adjuvants (Page *et al.* 1973; Spitznagel & Allison 1970). (*c*) In order for dextran to potentiate immune responses (Diamantstein & Blitstein-Willinger 1975) and to activate the alternative pathway of complement (Hadding *et al.* 1973), it must be negatively charged. Indeed, sulphated macromolecules are found in dental plaque, some of which are sulphated glycoproteins (Baumhammers & Stallard 1966; Rolla *et al.* 1975). Negatively charged polysaccharides can be formed by *Strep. mutans* and

*Strep. sanguis* which incorporate labelled phosphate into soluble and insoluble high molecular weight polysaccharides (Melvaer *et al.* 1974). In addition to the negative charge of dextrans which may be necessary for adjuvanticity, the α1–3, α1–6 and α1–4 linkages may influence their adjuvanticity, especially as the α1–3 linked dextran is resistant to degradation and is slowly metabolized. (*d*) Recruitment and proliferation of immunocompetent cells both in the gingival mononuclear cell infiltration and the draining lymph nodes may follow exposure to high local concentrations of antigens.

## FUNCTIONAL RELATIONSHIP BETWEEN IMMUNOPOTENTIATION BY PLAQUE AND THE EFFECT ON CARIES AND GINGIVITIS

Increasing the load of oral microorganisms and their products by allowing plaque to accumulate has led to an enhanced lymphoproliferative response stimulated by *Veillonella*, which can be considered as a representative organism in the development of gingivitis, and by *Strep. mutans*, which is a principal cariogenic organism. Bacterial plaque was allowed to accumulate on the teeth of young healthy subjects for 28 days by abstention from any oral cleansing.

The proliferative response of lymphocytes to *Strep. mutans* and *Veillonella* was determined and the stimulation indices (SI) were plotted against each subject's 'decayed, missing and filled' caries index (DMF) and gingival index (GI) before and during plaque accumulation (Lehner *et al.* 1976*a*; Figs. 3 and 4). All but two of the SI before plaque accumulation were less than 2 and all the GI were less than 0.4, so that these could not be correlated. However, with plaque accumulation the SI of lymphocytes induced by the oral organisms increased and showed a negative correlation with the DMF and a positive correlation with the GI (Figs. 1 and 2). Significant negative correlations between the SI ($\log_{10}$) and the DMF index was found with *Strep. mutans* ($P < 0.01$) and *Veillonella* ($P < 0.05$). In the analysis of SI against GI the positive correlations with *Veillonella* also reached a significant level ($P < 0.02$).

It seems that the immunopotentiating effect of bacterial plaque may not only act as a polyclonal mitogen, but that the increase in the mitogenic response of lymphocytes may be governed by previous sensitization to the bacterial antigens. Thus, polyclonal activators might recall the immunological memory for a variety of antigens and these bear a functional relationship to the relevant diseases. A positive correlation of the SI of lymphocytes with the gingival index of inflammation is consistent with other features associating CMI with a damaging effect on the gingiva. The negative correlation between the response of lymphocytes to *Strep. mutans* and the DMF suggests a protective relationship between CMI and caries; it is similar to that between serum antibodies and

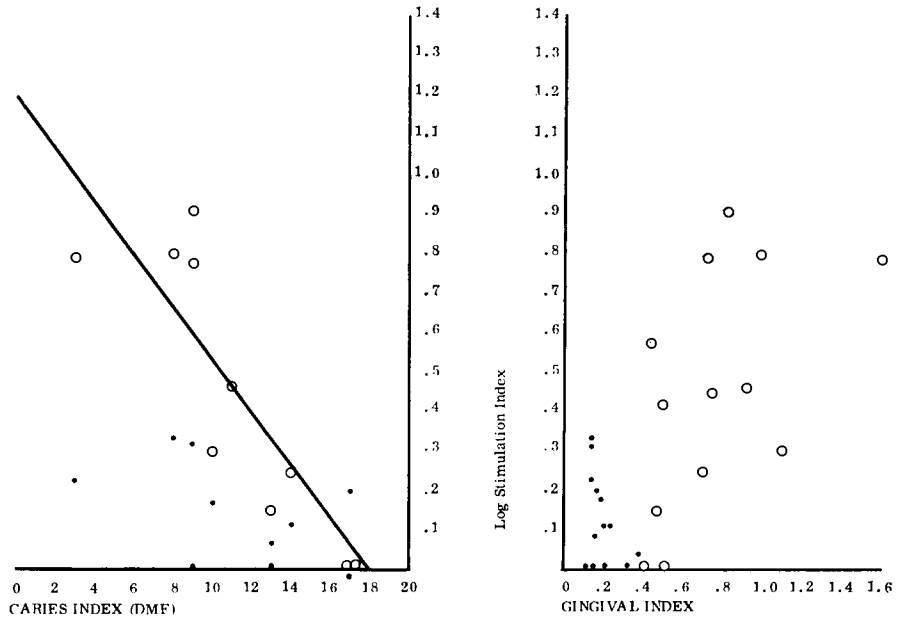


FIG. 3. Relationship between the stimulation indices of lymphocytes to *Streptococcus mutans* and the caries and gingival indices before (•) and during ( o ) plaque accumulation.

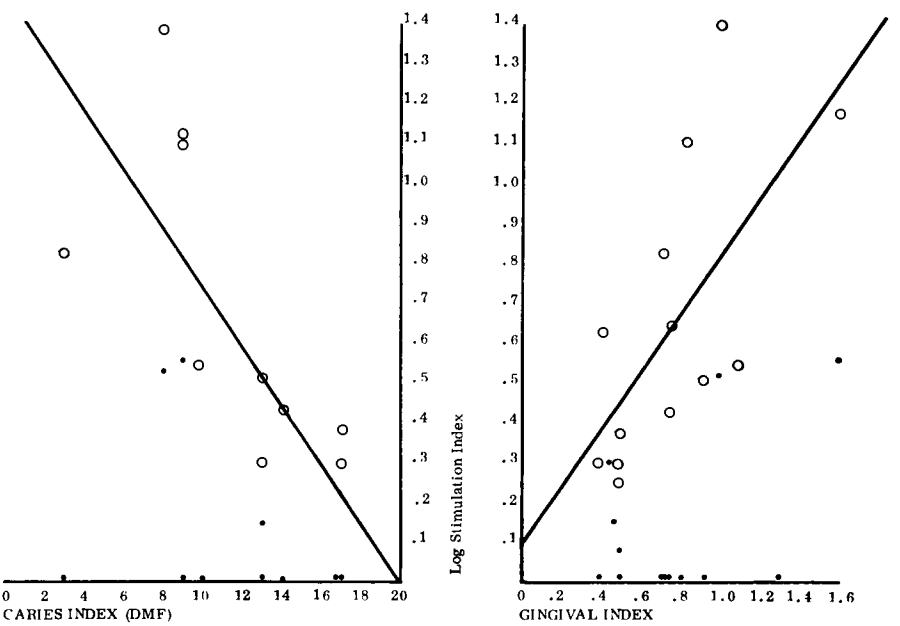


FIG. 4. Relationship between the stimulation indices of lymphocytes to *Veillonella alcalescens* and the caries and gingival indices before (•) and during ( o ) plaque accumulation.

DMF (Challacombe *et al.* 1973; Challacombe 1974; Challacombe & Lehner 1976), so that the lower the caries index, the higher are the serum antibody titres and responses of lymphocytes. Furthermore, a significant increase in lymphocyte proliferation and serum antibodies to *Strep. mutans* is found in monkeys immunized against dental caries (Lehner *et al.* 1976*b*). The CMI to cariogenic bacteria is recalled only under the immunopotentiating conditions of bacterial plaque and this might be a measure of the protective potential of the subject against dental caries. That the immunological recall may have some specificity is suggested by a lack of relationship between the proliferative response to PPD or PHA and the DMF and GI.

## THE EFFECT OF BACTERIAL PLAQUE ON IMMUNOLOGICAL MEMORY

To test the hypothesis that dental bacterial plaque *in vivo* may both potentiate and recall previous sensitization to microorganisms, the plaque accumulation experiment was repeated in five subjects 210 days after plaque was removed and good oral hygiene reinstituted (Lehner *et al.* 1976*a*). Significant lymphocyte transformation was found earlier, it was greater in magnitude and it lasted longer in the second as compared with the first plaque accumulation experiment (Fig. 5). These immunological features are usually ascribed to secondary antibody responses, or to enhancement of an existing immune state. It suggests that bacterial plaque *in vivo* can induce a recall of immunological memory for plaque antigens on the cellular level. It is not clear at present whether this is a measure of T or B lymphocyte memory, but both might be involved, since pooled dental plaque and *Veillonella* specifically can stimulate T and B lymphocytes to undergo transformation into blast cells (Mackler *et al.* 1974; Ivanyi & Lehner 1974), though *Strep. mutans* seems to stimulate only T lymphocytes (unpublished obervations). It must be assumed that however good the oral hygiene might be, a small amount of dental plaque is always present from the time of eruption of the teeth—that is, from about four months of age. Indeed, there is now evidence that sensitization of fetal lymphocytes to plaque antigens might take place *in utero* in women with periodontal disease (Horton *et al.* 1976). Cord blood lymphocytes from these infants are stimulated by plaque antigens, and this has been interpreted to mean that plaque bacteria enter the vascular gingiva of the mother, cause a transient bacteraemia and then may cross the placenta to sensitize fetal lymphocytes.

## IMMUNOSUPPRESSION INDUCED BY B CELL MITOGENS

Although LPS is best known for its adjuvant properties, it may also suppress

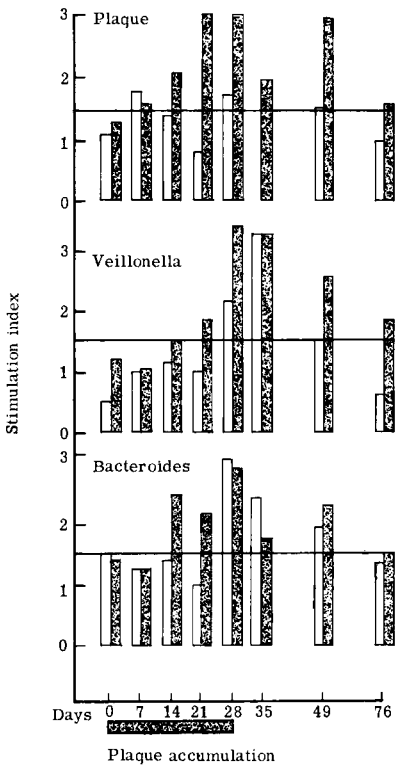


FIG. 5. Mean sequential lymphocyte transformation in five subjects with first (□) and second (■) episodes of experimental gingivitis.

immune responses if given before the antigen. LPS given to mice before sheep red cells causes a depression in food-pad swelling, but when given after the antigen it enhances the foot-pad reaction (Lagrange & Mackaness 1975). The *in vitro* findings with human lymphocytes are comparable with the *in vivo* effects of LPS on the T-cell mediated hypersensitivity in mice, for exposure of human lymphocytes to LPS or levan 24 hours before *Veillonella* results in suppression of *Veillonella*-induced T cell proliferation and, conversely, if the sequence of antigen and mitogen is reversed, T cell proliferation is enhanced (Ivanyi 1976).

High zone B cell tolerance can be induced in mice by polysaccharides; 1–10 mg of a branched native levan, with a molecular weight of $20 \times 10^6$, rapidly induces direct B cell tolerance, and if the levan is depolymerized to a molecular weight of 10 000 it still retains its tolerogenicity but not its immunogenicity

(Miranda *et al.* 1972). Low zone tolerance is induced by immunization with 10–100 $\mu$g of levan (Howard & Courtenay 1974). Dental plaque contains 1% dry weight of levan (McDougall 1964), it may account for about 5% of soluble hexose of the plaque matrix and *Strep. mutans* produces β-(2–1) linked soluble levan (Baird *et al.* 1973). High and low zone tolerance can also be induced with a predominantly α1–6 linked near-linear dextran and depolymerization reduces both immunogenicity and tolerogenicity, both of which are lost with a molecular weight of 20 000 (Howard *et al.* 1975). However, a more branched α1–3 linked dextran is a poor tolerogen and 10 mg of this dextran induces only minimal tolerance, although 1 mg is a potent immunogen (Howard & Courtenay 1975). Dental plaque contains about 8.5% of soluble dextran, having predominantly α1–6 linkages (Wood 1967) and about 1.4% of insoluble dextrans with predominantly α1–3 linkages (Hotz *et al.* 1972). *Strep. mutans* is capable of synthesizing a continuous series of dextrans with a variable proportion of α1–6 and α1–3 linkages (Guggenheim 1970; Lewicki *et al.* 1971; Baird *et al.* 1973) and these may have a complex effect on tolerance, though this has not been yet demonstrated against unrelated antigens. The net result between factors favouring enhancement and tolerance may then depend on the proportion of α1–3 and α1–6 linked dextrans, the negative charge these may carry, epitope density and on the sequence of adding mitogen and antigen.

## THE EFFECT OF IMMUNOSUPPRESSIVE AND IMMUNOPOTENTIATING DRUGS ON GINGIVAL INFLAMMATION

The influence of CMI responses on gingival inflammation has been studied by using immunosuppressive and immunopotentiating drugs. Patients having long-term immunosuppressive treatment show decreased gingival inflammation (Schuller *et al.* 1973) and this is correlated with negative lymphoproliferative responses to oral microorganisms (L. Ivanyi & T. Lehner, unpublished). In contrast the immunopotentiating drug Levamisole, given to 50 subjects, induced a significant enhancement in the *in vitro* lymphoproliferative response stimulated by *Veillonella* ($P < 0.01$) and other antigens (Ivanyi & Lehner 1976); this was correlated with a significant increase in the gingival index of inflammation ($P < 0.001$). The inverse effects of immunosuppressive and immunopotentiating drugs on the *in vivo* gingival inflammation and the *in vitro* lymphoproliferative response strengthens the view that gingival inflammation is modulated by the cellular immune responses to plaque antigens and mitogens.

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## Discussion

*Lachmann:* Is the message that if we don't clean our teeth, we shall be protected against caries, but we shall get gum disease?

*Lehner:* No, one can't expect such protection, because eventually the large number of bacteria and the amount of acid produced would overcome the immunological defence mechanism.

*Davies:* Is the rate of accumulation of plaque dependent on diet? And is plaque accumulation only to be found in the human species?

*Lehner:* The answer to the second question is that it can be found in any animal, if you give it the appropriate diet. The Western, civilized diet—a soft, fibre-free diet—is conducive to the accumulation of plaque, and I suspect that in primitive times this was not the case. In fact, we know that in the developing countries the prevalence of caries is smaller than in the UK. In order for caries to be produced four factors may be involved: (*a*) a sugar-rich diet to be converted to acid; (*b*) acidogenic organisms; (*c*) the tooth itself; and (*d*) antibodies that may modulate caries formation.

*Beeson:* Did you always have the plaque removed mechanically in your subjects or did some of them merely begin brushing their teeth again?

*Lehner:* It was always removed mechanically by professional oral hygienists, so as to make quite certain that it was done thoroughly. It is an experiment which is highly reproducible in the sense that the inflammation appears with bacterial plaque accumulation, and disappears with removal of plaque.

*Beeson:* You don't think that the mechanical effect of brushing the gums might have something to do with it?

*Lehner:* I think you are probably right, in one sense. Back in the 1930's when focal sepsis was a popular subject, it was shown that trauma to the gum may cause a transient bacteraemia (Okell & Elliott 1935). If that was the case, as it almost certainly must have been, one should find immunization, instead of depression, during the bacteraemia caused by the removal of plaque.

*Brandtzaeg:* For the last experiments you mentioned that the caries index was recorded before plaque accumulation. Was the gingival index measured before or afterwards?

*Lehner:* The caries index, as you know, does not change within 28 days, so that would be constant. The gingival inflammation was measured before, during and after plaque accumulation, and maximum stimulation indices are given during the 28 days, mainly between 14 and 28 days. The gingival index was also the maximum index recorded.

*Brandtzaeg:* You implied that the influence on the immune system takes place in the mouth, by penetration of plaque components through the crevicular epithelium, but can you exclude that people with dirty mouths swallow a lot of mitogens and antigens, and that the immunological stimulation thus takes place in the gut?

*Lehner:* This is very much a possibility. You are right that the organisms, and therefore the polysacccharides, might well be swallowed and therefore the immune responses could take place through the gut. On the other hand, there is an extensive epithelial surface in the mouth, and all the relevant immunological cells are present at the site of plaque accumulation, in the gingival crevice epithelium, so that I wonder if we need to invoke another site for absorption.

*Brandtzaeg:* The surface will be small compared with the epithelial surface of the gut, however.

*Lehner:* Yes, except that because of the persistence of plaque and the inflammatory response it induces in the adjacent epithelium, the local permeability may be greatly increased.

*White:* Dr Brandtzaeg started to ask about the mechanism of polyclonal stimulation; how do you think the bacteria enter the blood? Okell & Elliott (1935) said that one had wobbly teeth and one wobbled them and the bacteria could be recovered by blood culture. Is that the mechanism?

*Lehner:* I think the situation is much more complex. First of all, even if you chew energetically on hard meat, you will probably produce a transient bacteraemia, and therefore you do not need to have loose teeth to force bacteria into the blood stream. Secondly, in an interesting study Horton *et al.* (1976) showed that cord blood lymphocytes are sensitized to maternal dental bacterial plaque antigens only from mothers with gingivitis associated with bacterial plaque. This suggested that fetal lymphocytes may get sensitized to dental bacterial plaque *in utero*. Furthermore, polyclonal stimulants, such as lipopolysaccharides, can penetrate the gingival epithelium in the rabbit and thereby presumably sensitize the animal (Schwartz *et al.* 1972).

*White:* I don't know what you would take as evidence of polyclonal stimulation in man, but to me this possibly arises in malaria, in trypanosomiasis, and in infectious mononucleosis. All these conditions, interestingly enough, are associated with the development of heterophile antibody to sheep red cells.

I wonder whether, if your patients stopped brushing their teeth, their titre of antibody in a Paul-Bunnell test would go up?

*Lehner:* I use the term polyclonal B cell mitogen in the sense used by Coutinho & Möller (1973), meaning that antibodies are formed not only to the immunizing antigen but also to unrelated antigens. In man antibodies do not increase under the influence of bacterial plaque accumulation but there is an increase in the T and B lymphocyte response, assessed by the mixed lymphocyte (MLC) reaction and lymphoproliferative response to lipopolysaccharides (Lehner *et al.* 1974).

*Ferguson:* When techniques such as lymphocyte transformation are used in routine clinical immunology laboratories, the reference range for normal values is very wide. From what you have said it is clear that some of this variation may be related to dental hygiene. Can I ask if these dental students had exceptionally clean mouths?

*Lehner:* I wish I could say that they had! In fact, if they did not have clean teeth, we would clean them up before the experiment so that the gingival inflammatory index would be minimal.

*Ferguson:* In the general population, what proportion of young people in their twenties will have substantial gingival inflammation?

*Lehner:* It is difficult to say, but you find some gingival inflammation from the age of eruption of the teeth. There is no norm for normality here, because plaque is accumulated all the time. It is a question of the amount of bacterial plaque and presumably the amount of lipopolysaccharides and dextrans which accumulate.

*Rosen:* How did you separate the B cells? This is a matter of some importance. You cite the experiments of Coutinho & Möller (1973) and those are valid for B cells of mice. Most laboratories working with human lymphocytes cannot show that endotoxin, laevan or dextran are mitogenic for human B lymphocytes. You therefore require very fastidious separation of human B cells to prove that there is no T cell contamination.

*Lehner:* I accept this. In the literature, most people have made the point that lipopolysaccharides and dextran are poor mitogens in man, but there is a significant stimulation, at a low level (Oppenheim & Perry 1965). Our results were also at a low level of stimulation, with an index of two to three, but this is *not* the case in patients with gingivitis and periodontitis, when stimulation indices may reach higher levels (Ivanyi & Lehner 1974).

*Rosen:* Was this done on purified human B cells?

*Lehner:* No, it was not (Ivanyi & Lehner 1974). The responses of unfractionated cells were compared with those of purified human T cells, by eliminating the B cells with anti-immunoglobulin-coated beads. This resulted

in a T cell population which was $> 96\%$ pure. The latter yielded a highly significant decrease in lymphocyte transformation to the B cell mitogens, but not to the T cell antigen, so that the former response was by B cells. But you are right that we ought to show that this is so on purified human B cells.

*Bienenstock:* What is the response to dextranase itself, or any other products of *Strep. mutans* that may be significant in the production of plaque?

*Lehner:* I am not aware that *Strep. mutans* produces dextranase, but it has been used experimentally in attempts to remove dental plaque and prevent caries, though the results have not been encouraging.

*Bienenstock:* Is there any other enzyme produced by microorganisms which is thought to be instrumental in the production of plaque?

*Lehner:* Dextran sucrase, also called glucosyl transferase, plays an important part in the development of plaque; it converts sucrose into dextran. There is also a fructosyl transferase which converts sucrose into levan. We have studied the antibodies and the lymphoproliferative response to these enzymes and found a negative correlation between haemagglutinating antibodies and dental caries (Challacombe *et al.* 1972). It is not clear at present if antibodies to dextran sucrase would prevent the formation of dextran, which is said to glue bacteria to the tooth surface.

*Gowans:* I am not sure what the increased stimulation indices for dextran and LPS imply. Do they imply a higher proportion of B cells in general, or a higher proportion of B cells specifically sensitized to these two products?

*Lehner:* If we use a specific antigen such as *Veillonella* an increased stimulation index implies a larger number of sensitized lymphocytes, just as, in subjects exposed to mycobacteria, PPD *in vitro* induces higher stimulation indices, indicating that a larger number of lymphocytes have been sensitized. This is not the case with the B cell mitogens such as LPS or dextran, which may induce polyclonal stimulation of antibodies and potentiate both T and B lymphocyte responses. Alevy & Battisto (1976) have shown that dextran given to mice enhances the responses of lymphoid cells both to T cell (Concanavalin A) and B cell (LPS) mitogens; these responses are mediated through soluble factors elaborated by T lymphocytes and some affect T cells and others, B cells.

*Gowans:* So if you removed the B lymphocytes, you would not get the response?

*Lehner:* That is correct. I should also quote here earlier work by Allison & Davies (1971) who found that thymectomized mice show a decreased adjuvanticity by Freund's adjuvant and lipopolysaccharides, as tested by antibody formation to bovine serum albumin.

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*Immunology of the Gut*
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# Sites of synthesis and localization of IgE in rats infested with *Nippostrongylus brasiliensis*

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*Abstract* The tissue and cellular localization of IgE has been studied in normal rats and rats infested with the enteric parasite, *Nippostrongylus brasiliensis*. The results of the study do not support the suggestion that IgE is a secretory immunoglobulin with a physiology analogous to that of IgA. The lamina propria of the small intestine and the colonic and pulmonary mucosal surfaces contain numerous anti-IgE-binding cells, but these have been shown to be mast cells and not plasma cells. The major sites of IgE synthesis were the regional lymph nodes of the small intestine and the lungs, which contained large numbers of IgE-secreting plasma cells. Smaller numbers of IgE-secreting plasma cells were also found in peripheral lymph nodes, some of which were distant from tissues known to have direct contact with either larvae or adult worms. Peyer's patches, the intrapulmonary lymphoid tissue and the spleen contained few, if any, IgE-secreting plasma cells. The significance of the IgE which was readily demonstrated in germinal centres of Peyer's patches and several lymph nodes is not known. In contrast to infested animals, the lymphoid organs of normal rats rarely contained any IgE-containing cells. Thoracic duct lymph from infested animals contained only few IgE-containing large lymphocytes, similar in number to cells containing IgM or IgG but only 1/50 as many as those containing IgA. An unexpected observation was that mast cells in mucosal organs appear to contain intracellular IgE, differing in this respect from connective tissue mast cells. Mast cells lying between epithelial cells, the 'globule leucocytes', also appear to contain intracellular IgE and it is suggested that such cells may be responsible for the presence of IgE in exocrine secretions. This study highlights the need for careful identification of cells appearing to contain IgE and suggests reasons for the widely differing reports of the numbers of IgE-secreting plasma cells in human intestinal biopsies.

Antibodies of immunoglobulin class IgE have been shown to sensitize mast cells in man and a variety of other species for release of histamine and the subsequent manifestations of immediate-type hypersensitivity (type 1 hypersensitivity of Coombs & Gell 1963). Antibodies of this sort are variously referred to as homocytotropic, reaginic or anaphylactic antibodies. A protective role

for such antibodies or for the hypersensitivity phenomenon to which they predispose has not been clearly demonstrated, while the pathological effects are the cause of considerable morbidity and mortality in medical practice.

In man, some 10% of the population are more or less severely afflicted by some symptom attributable to IgE/mast cell mediated hypersensitivity (Aaronson 1972). One is therefore provoked to ask why, in the face of a significant mortality produced by this mechanism, the trait persists at such a high level in the population. The answer must be that either now, or in man's relatively recent past, the capacity to mount strong IgE-mediated immune responses has held a selective advantage. The maintenance of this potentially harmful trait might be an example of a balanced polymorphism and it is therefore important to discover the circumstances where IgE antibodies are protective.

One possibility is that IgE and immediate-type hypersensitivity have importance in the host's immune response to parasites. During infestation with a variety of metazoan parasites, idiosyncratic factors affecting IgE responses appear to be largely over-ridden and most members of the population respond by producing large amounts of IgE. Populations of humans in areas where parasites are endemic have generally elevated levels of serum IgE (Bennich & Johansson 1971; Ogilvie & Jones 1973) and eradication of the parasites leads to a decline in circulating IgE (Grove *et al.* 1974). Similar observations in animals (Ogilvie 1967; Wilson & Bloch 1968; Jarrett & Bazin 1974) have also suggested that IgE antibodies may be important elements in the immune response to parasites but there has been no conclusive evidence that either proves or disproves this important possibility.

While systemic anaphylaxis may cause serious symptoms and death following injection of antigen into sensitized individuals, most manifestations of immediate-type hypersensitivity occur at mucosal surfaces of the body and are localized mainly at the site of contact with antigen. Sensitization to environmental and food antigens also occurs mainly at mucous membranes, although circulating homocytotropic antibody subsequently sensitizes mast cells throughout the body. It is therefore of interest to know where IgE antibodies are synthesized and whether they have a particular localization in mucous membranes and secretions. IgE has been detected in a variety of secretions (Ishizaka & Newcomb 1970; Hobday *et al.* 1971; Deuschl & Johansson 1974; Nakajima *et al.* 1975), with some suggestion that active secretion increases its concentration relative to that which would be found in a simple exudate of serum proteins. Ishizaka *et al.* (1969) and Tada & Ishizaka (1970) have investigated sites of IgE synthesis by immunofluorescence techniques and have concluded that IgE is mainly synthesized in the lamina propria and the regional lymph nodes of mucosal organs. An analogy has therefore been drawn between IgE

and IgA, where the latter is undoubtedly a class of immunoglobulin specialized both chemically and by site of synthesis for incorporation into exocrine secretions. However, the case that IgE is a secretory immunoglobulin is by no means as strong as that for IgA. The recent availability of monoclonal rat IgE from myeloma serum (Bazin *et al.* 1974) stimulated an investigation of the sites of IgE synthesis in rats, using fluorescent antibody monospecific for rat IgE. The results of studies in normal rats and in rats infested with the nematode parasite *Nippostrongylus brasiliensis* do not support the contention that IgE is a secretory immunoglobulin with a physiology similar to that of IgA.

## METHODS

Fourteen-week-old female Wistar *rats* were infested by subcutaneous injection on the dorsum of the neck with 2000 third-stage larvae of *N. brasiliensis*. The larvae were obtained from the Wellcome Laboratories, Beckenham, Kent and were produced by the method of Keeling (1960). Infestation of animals was confirmed by faecal egg counts, and faeces were virtually free of eggs by 12 days after injection of larvae. Some rats were given secondary and tertiary infestations by injection of 2500–3000 third-stage larvae 5 weeks and 30 weeks respectively after the primary infestation.

Purified monoclonal *rat IgE* was supplied by Dr H. Bazin (Brussels). *Rabbit anti-rat IgE* was raised against myeloma IR2 IgE and purified by Dr A. F. Williams (Oxford). The antibody was rendered monospecific by absorption with a Sepharose 4B column to which were coupled the proteins of normal rat serum and lymph together with purified $IgG_2$ and anti-idiotype antibody was removed by adsorption to and elution from a myeloma IR162 IgE-Sepharose 4B column. The purified antibody was conjugated with fluorescein isothiocyanate (FITC) and before use was absorbed with thymus and lymph node cells from specific pathogen-free rats. *Goat anti-rabbit Ig* (GAR) was obtained as a conjugate with FITC from Cappel Laboratories (Downingtown, Pa., USA). A 1:50 dilution of this serum was absorbed with mesenteric lymph node cells from a *Nippostrongylus*-infested rat before use. *Rabbit anti-rat IgA* was the same reagent as was used by Williams & Gowans (1975).

*IgE* was detected in tissue sections by incubation with *FITC-anti-IgE* followed by washing and further incubation with FITC-GAR to improve the fluorescence. Omission of the middle layer or replacement with normal rabbit IgG abolished fluorescence, as did absorption of the anti-IgE with a 2.5-fold excess of purified rat IgE. *IgA* was demonstrated by a direct method, essentially as described by Williams & Gowans (1975).

Where sections were for study of IgE-containing cells only, *tissue specimens*

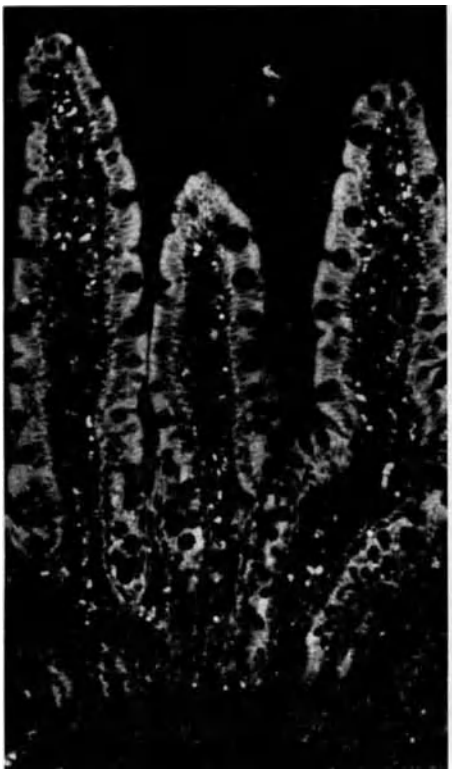
were fixed in cold ethanol and embedded in paraffin (modified from Brandtzaeg 1974). When identification of mast cells was also necessary, the tissues were fixed in cold Carnoy's fluid instead of ethanol. Non-specific eosinophil fluorescence was modified with Lendrum's stain (Johnston & Bienenstock 1974) and mast cells were demonstrated by their affinity for Alcian blue (Enerbäck 1966*a*, *b*). Tissue fixed in Carnoy's fluid could be stained with Alcian blue before incubation with fluorescent reagent without interfering with the fluorescence of FITC conjugates. Sections photographed for fluorescent cells could then be counterstained with Safranin and the same areas re-examined for mast cells by conventional microscopy.

*Homocytotropic antibody responses* against *N. brasiliensis* were measured by passive cutaneous anaphylaxis (PCA) in Wistar R rats by the method of Ovary (1964), using a saline extract of adult worms as the challenging antigen.

## CELLS BINDING ANTI-IgE IN MUCOSAL ORGANS

The mucosal organ most extensively studied for secretory antibody production has been the small intestine. Food and bacterial antigens provide a stimulus for the population of the intestinal lamina propria with IgA-secreting plasma cells in newborn animals and in germ-free animals after exposure to a conventional environment (Crabbé *et al.* 1968, 1969, 1970). The small intestines of *normal rats* were therefore examined for plasma cells secreting either IgE or IgA. In sections stained with the indirect method for IgE, rare weakly fluorescent cells were seen in the lamina propria (Fig. 1). Although the nature of these cells has not been pursued, the intensity of fluorescence was much less than that of IgE-secreting plasma cells. In contrast, when sections from the same tissue blocks were stained by the direct method for IgA, large numbers of IgA-secreting plasma cells were present throughout the length of the small intestine (Fig. 2). Therefore, local synthesis of IgE does not occur to a significant extent in the normal small intestine. The lamina propria of the colon and of the bronchi in normal rats also lacked IgE-secreting plasma cells.

Two *hyperimmune rats* were killed 10 and 20 days after secondary infestation with *N. brasiliensis* (PCA titres 1:2048 and 1:128 respectively). The lamina propria of the small intestine in these animals contained large numbers of brightly fluorescent cells that appeared to contain IgE (Fig. 3). Although the worm burden is carried mainly in the proximal jejunum, the fluorescent cells were relatively uniformly distributed along the length of the small intestine. Many of the cells had the appearance of typical plasma cells, but several differences were noted from the IgA-secreting plasma cells in the lamina propria. IgA-secreting plasma cells mainly occupied the bases of villi, in an



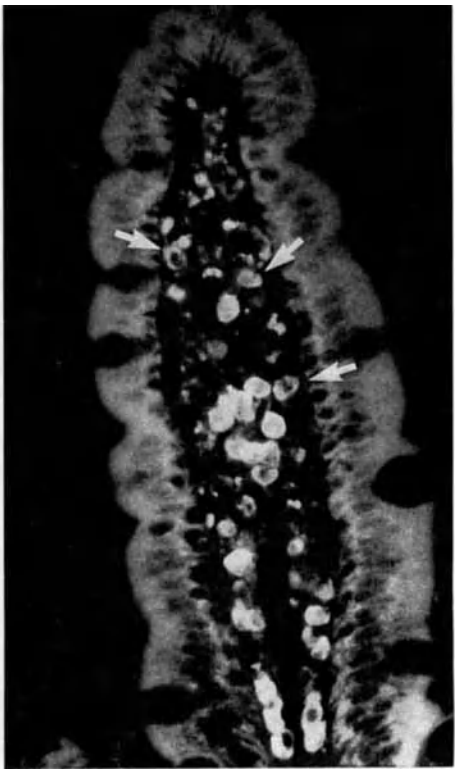


FIG. 1 FIG. 2

FIG. 1. Section of normal rat ileum, stained with FITC-anti-IgE and FITC-GAR. All fluorescence in the lamina propria comes from eosinophils. This, and the autofluorescence of the epithelium, are of quite different colour to the specific green fluorescence of FITC. No specifically fluorescent cells are present. Cf. Figs. 2 and 3. × 340.

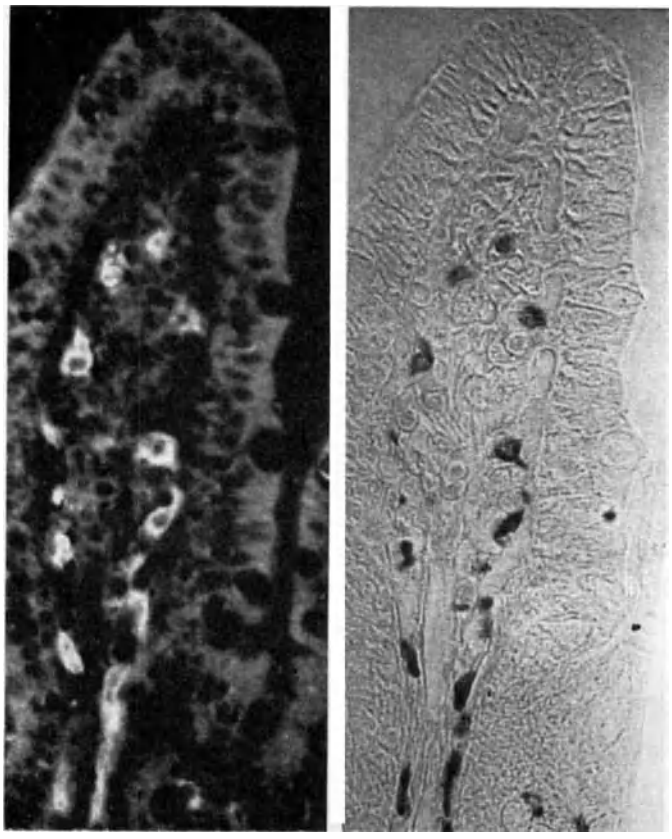
FIG. 2. Section cut from the same block as Fig. 1 and stained with FITC-anti-IgA. Villus containing numerous IgA-secreting plasma cells. Note the axial position of the cells. Fluorescent objects in the tip of the villus and arrowed cells are eosinophils. × 830.

axial position, whereas the anti-IgE-binding cells were usually subepithelial and extended to the villus tips. Especially at the bases of villi and in the crypts, anti-IgE-binding cells were quite frequently found between cells of the epithelium. Although the cells appeared to contain IgE, their morphology and distribution was similar to that of mast cells in rats infested with *N. brasiliensis* (Miller & Jarrett 1971).

Mast cells could not be demonstrated in ethanol-fixed tissue by staining with either toluidine blue or Alcian blue, as was found by Enerbäck (1966*a*), and

FIG. 3. Section of ileum from a rat 10 days after the second infestation with *N. brasiliensis*, stained with FITC-anti-IgE/FITC-GAR. Note the large number of specifically fluorescent cells in the lamina propria, occupying mainly subepithelial positions. Several cells *(arrowed)* lie between epithelial cells. × 840.

only weak staining was obtained with acridine orange (Jagatic & Weiskopf 1966). A rat was killed 10 days after a tertiary infestation (PCA titre 1:16 384) and its tissues were fixed with cold Carnoy's fluid. This fixative preserved the affinity of mucosal mast cells for Alcian blue (Enerbäck 1966*a*) and did not interfere with detection of immunoglobulins. As in the other hyperimmune animals, the lamina propria of the small intestine contained large numbers of anti-IgE-binding cells. Defined fields were photographed for fluorescence and then re-examined for the presence of Alcian blue-positive mast cells. Comparison of Figs. 4*a* and 4*b* shows that all fluorescent cells are also stained by Alcian blue. Few, if any, fluorescent cells in the lamina propria were not mast cells. Therefore, although there is greatly increased synthesis of IgE in rats



*a* *b*

FIG. 4. Section of ileum from a rat 10 days after the third infestation with *N. brasiliensis*. The tissue was fixed with cold Carnoy's fluid and has been stained sequentially with Alcian blue and FITC-anti-IgE/FITC-GAR.

(*a*) Photographed for fluorescence. All cells are specifically stained.

(*b*) The same field as (*a*) after counterstaining with Safranin. All fluorescent cells are mast cells stained with Alcian blue. × 980.

infested with *N. brasiliensis*, synthesis of IgE antibodies does not occur locally in the lamina propria of the small intestine.

The colon is not infested by the parasite, although it may be exposed to antigen. The lamina propria in infested animals contained considerable numbers of fluorescent mast cells (Fig. 5), but no IgE-secreting plasma cells. Fluorescent mast cells (the so-called 'globule leucocytes') were also present between epithelial cells of the colonic glands.

The lungs of infested animals contained large numbers of fluorescent mast cells (Fig. 6). These had the same staining properties as intestinal mast cells

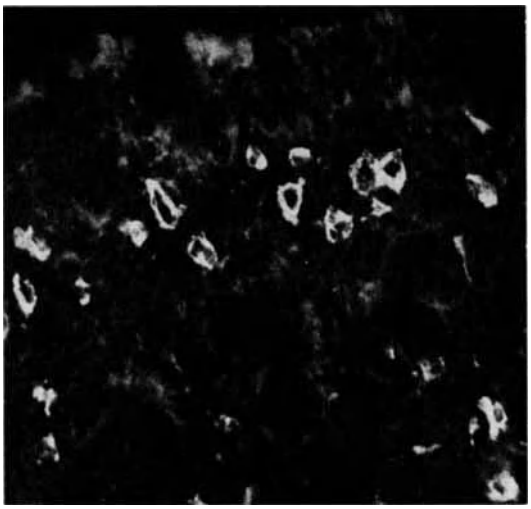


FIG. 5. Section of transverse colon from a rat 10 days after the second infestation, showing cells with specific fluorescence in the superficial layer of the lamina propria and between colonic glands. Fixed and stained as in Fig. 4. The fluorescent cells are mast cells. × 870.

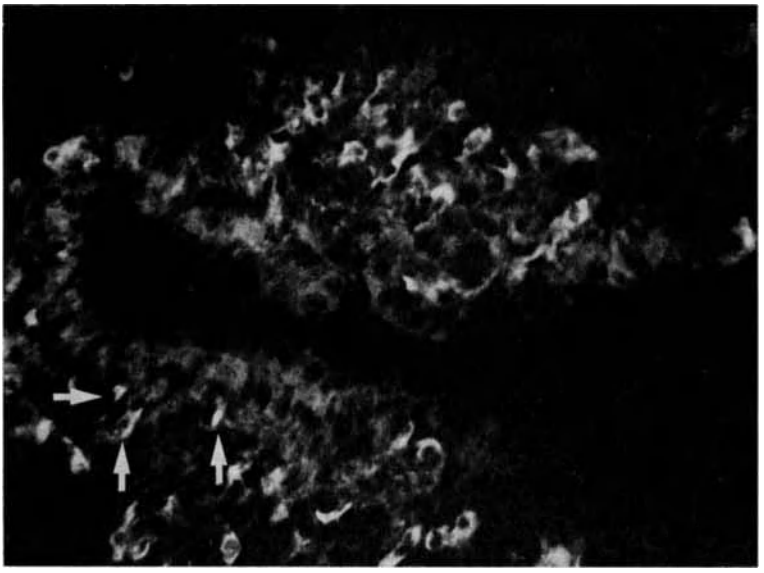


FIG. 6. Section of lung from a rat 10 days after the third infestation, showing a terminal bronchiole surrounded by specifically fluorescent cells. Three fluorescent cells lie between cells of the respiratory epithelium *(arrowed)*. Fixed and stained as in Fig. 4. The fluorescent cells are mast cells. × 830

and also appeared to contain IgE. In the bronchi, fluorescent mast cells were present in the lamina propria and between cells of the respiratory epithelium from the major bronchi down to the terminal bronchioles. No IgE-secreting plasma cells were seen either in the bronchial lamina propria or in the intra-pulmonary lymphoid nodules. Granulomata produced in the lungs by the passage of larval worms (Taliaferro & Sarles 1939) contained large numbers of fluorescent mast cells. These foci of mast cells may explain the IgE-containing 'germinal centres' described by Ishizaka *et al.* (1969) in the parenchyma of the lungs of monkeys infested with a pulmonary parasite.

## IgE AND MUCOSAL MAST CELLS

The fluorescence of the mucosal mast cells does not appear to be limited to the cell perimeter, but to emanate also from the cytoplasm of the cells (Fig. 7). This contrasts with the fluorescence of mature mast cells in connective tissues such as the capsules of lymph nodes, where the appearance is that of binding of the FITC conjugate to the cell membrane (Fig. 8). From these appearances of the fluorescent cells, it seems possible that mucosal mast cells may actually *contain* IgE, in addition to surface-bound IgE, while connective tissue mast cells may only have surface IgE. This suggestion is not wholly novel, as mast cells in dogs infested with *Ascaris* also have the appearance of cytoplasmic fluorescence when stained to demonstrate IgE (Halliwell 1973). Further studies are in progress to assess this possibility by more definitive criteria.

## SITE OF IgE SYNTHESIS

Adult worms in the small intestine are thought to be the main source of immunizing antigen during infestation with *N. brasiliensis* (Ogilvie & Jones 1971). The most likely site of IgE synthesis is therefore the gut-associated lymphoid tissue. Normal *Peyer's patches* bound no anti-IgE. The Peyer's patches of hyperimmune rats did not contain mature IgE-secreting plasma cells but did bind anti-IgE. The mucosal poles of the germinal centres had bright fluorescence in a coarse dendritic pattern, while the serosal pole was unstained (Fig. 9). The mantle of small lymphocytes was stained in a fine reticular pattern, but the interfollicular T-dependent areas (Parrott & Ferguson 1974) and the sub-epithelial corona were unstained. The significance of the IgE in the germinal centres is not known, and although Peyer's patches may be important in the generation of the IgE response to the parasite, they do not appear to be sites of IgE secretion.

The *mesenteric lymph nodes* were major sites of IgE secretion in infested rats.

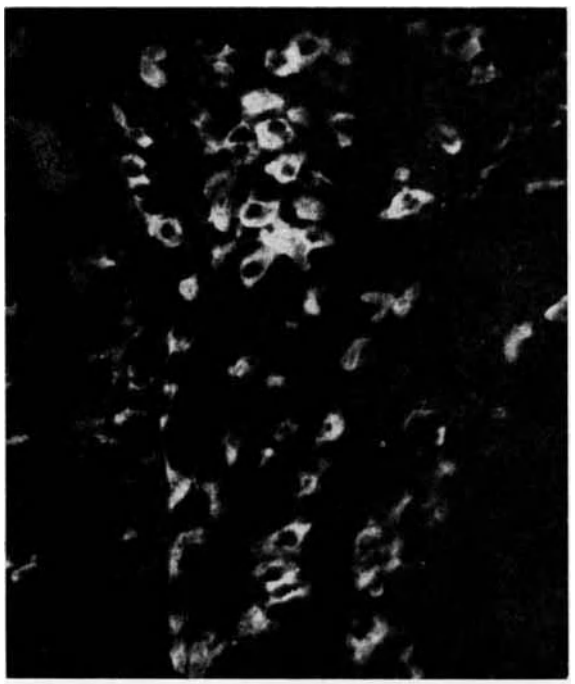


FIG. 7

FIG. 8

FIG. 7. Section from the same block as Fig. 4, stained in the same way. Many fluorescent cells are present in the lamina propria of the villus. The same cells were all also stained by Alcian blue. Note the similarity to plasma cells and the appearance suggesting that the cytoplasm of the cells is stained. × 840.

FIG. 8. Section of the capsule of an axillary lymph node from a rat 10 days after the second infestation stained with FITC-anti-IgE/FITC-GAR. Mature mast cells in the connective tissue stain in a pattern suggesting that only the cell membrane is stained. × 730.

Very few cells bound anti-IgE in normal lymph nodes, but in those from hyperimmune rats the medullary cords contained large numbers of brightly fluorescent cells. These had no affinity for Alcian blue and by exclusion are plasma cells (Fig. 10). Anti-IgE-binding cells were rare in the medullary sinuses and where present were found to be mast cells. This suggests that the

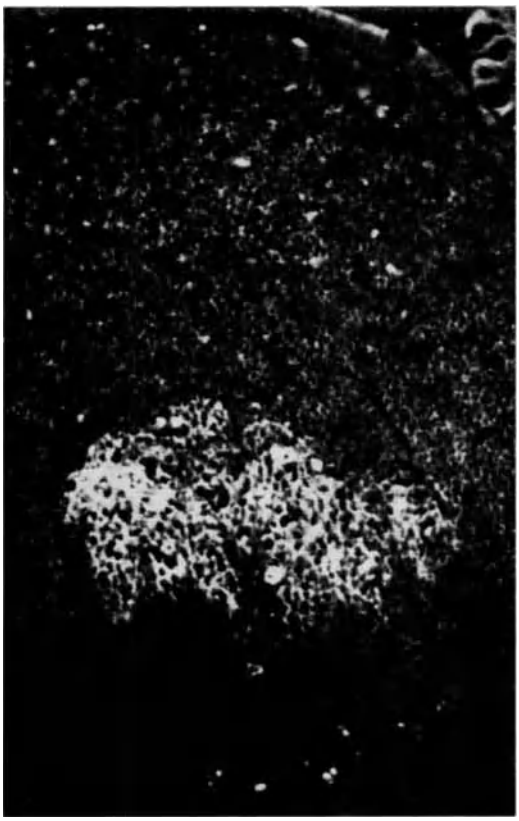


FIG. 9. A Peyer's patch from a rat 10 days after the second infestation, stained with FITC-anti-IgE/FITC-GAR. The mucosal pole of the germinal centre is brightly stained in a coarse dendritic pattern, while the serosal pole is unstained. The mantle of small lymphocytes is also stained, in a fine reticular pattern. × 330.

IgE-secreting cells in the medullary cords are a sedentary population that does not migrate into the efferent lymph. Many germinal centres of the lymph node had brightly fluorescent caps of a coarse dendritic pattern, but the medullary pole of the germinal centre was unstained. The small lymphocytes of the follicles had a fine reticular pattern of fluorescence while the interfollicular recirculatory areas and the paracortex were unstained.

The *lung* is an organ of passage and maturation of the larval parasite. However, the intrapulmonary lymphoid tissue associated with the large bronchi did not contain IgE-secreting plasma cells in either normal or hyperimmune rats. In further studies (on PVG/c rats), the mediastinal lymph nodes of hyperimmune rats were studied, as these hypertrophy during infestation.

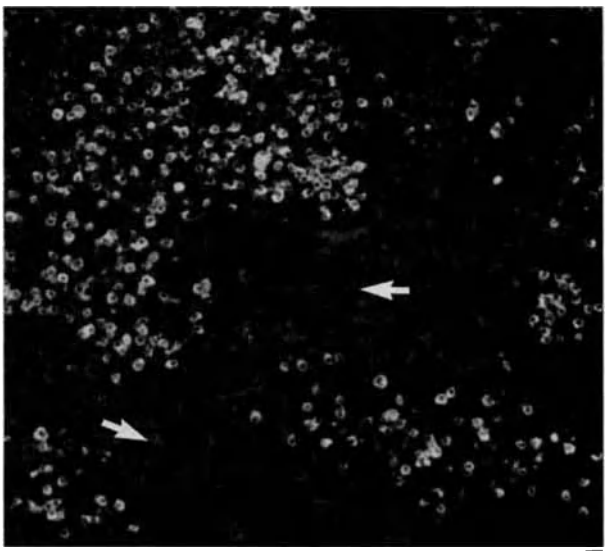


FIG. 10. Section of the medulla of the mesenteric lymph node of a rat 10 days after the third infestation, fixed and stained as in Fig. 4. The medullary cords contain large numbers of IgE-secreting plasma cells. Two large medullary sinuses *(arrowed)* do not contain any fluorescent cells. × 340.

Sections of these lymph nodes from a rat 10 days after a secondary infestation (PCA titre 1:2048) contained large numbers of IgE-secreting plasma cells in the medullary cords. The density of fluorescent cells and the appearance of the follicles was similar to the findings in the mesenteric lymph nodes of hyperimmune animals. It therefore appears that, as with the small intestine, IgE synthesis occurs in the draining lymph nodes of a mucosal organ and not locally in the lamina propria of the organ or in specialized lymphoid tissues closely associated with the mucosa.

The *spleen* was found to contain very few anti-IgE-binding cells in either normal or hyperimmune rats. *Peripheral lymph nodes* of normal rats contained no anti-IgE-binding cells. The axillary, para-aortic and popliteal lymph nodes of hyperimmune rats all contained fluorescent germinal centres and small numbers of IgE-secreting plasma cells in the medullary cords. The axillary lymph node drains the site of injection of larvae, but the other lymph nodes do not drain areas directly exposed to either larvae or adult worms. Synthesis and localization of IgE in these lymph nodes may result from a systemic antigenaemia during infestation, or from anomalous migration of larvae to extra-intestinal sites (Weinstein 1955).

FIG. 11. A fluorescent large lymphocyte in a smear of thoracic duct lymph from a rat 7 days after the second infestation with *N. brasiliensis*. The smear was stained with FITC-anti-IgE and FITC-GAR. Most of the cells were unstained. × 1500.

## IgE-CONTAINING CELLS IN THORACIC DUCT LYMPH

Plasma cells in the lamina propria of the gut are known to arise from precursors in thoracic duct lymph (TDL) that are large lymphocytes (Gowans & Knight 1964), and at least some of which contain internal IgA (Williams & Gowans 1975). At the point of cannulation in the abdomen, the thoracic duct drains the entire intestinal lymphatic bed and its associated lymphoid tissue. Approximately 40% of large lymphocytes in normal rat TDL contain internal IgA, and such cells constitute about 2–4% of all thoracic duct lymphocytes. Cells containing either IgM, IgG2a or IgG2b are very much rarer in normal animals, amounting to approximately 0.1% for IgM and 0.2% for these combined IgG subclasses. Plasma cells containing IgM, IgG2a or IgG2b are very rare in the intestinal lamina propria of normal rats (unpublished results). It was therefore of some interest to know whether TDL contained IgE-synthesizing large lymphocytes during infestation with *N. brasiliensis*. Smears of washed thoracic duct lymphocytes obtained from a PVG/c rat cannulated seven days after a secondary infestation were fixed in ethanol and stained to detect either IgE- or IgA-containing cells. 4.6% of all cells contained IgA at this time, while 0.1% of cells contained IgE. The IgE-containing cells had the morphology of large lymphocytes (Fig. 11). This observation, together with the absence of IgE-containing blast cells in the medullary sinuses of the mesen-

teric lymph node, shows that the traffic of IgE-containing cells in TDL is small compared to that of IgA-containing cells, even during a period of intense IgE synthesis. In this respect, IgE is similar to other classes of immunoglobulin that are not specialized for secretion, and the absence of IgE-secreting plasma cells from the lamina propria of the gut is also consistent with this generalization.

## DISCUSSION

Two main conclusions can be drawn from this work. Firstly, IgE is not synthesized locally in the lamina propria of those mucosal organs of infested rats through which absorption of parasite antigens might be expected to occur. IgE antibodies made during infestation are synthesized by plasma cells in the regional lymph nodes of these organs. The second main conclusion is that although IgE-secreting plasma cells are absent from the lamina propria of the infested gut, large numbers of cells are present that appear to contain IgE. These cells have been shown by their histochemical properties to be mast cells.

Normal rats had few anti-IgE-binding cells in any of the mucosal organs examined. Even after challenging with *N. brasiliensis*, which stimulates synthesis of large amounts both of specific IgE (Ogilvie 1967; Wilson & Bloch 1968) and total IgE (Jarrett & Bazin 1974), IgE-secreting plasma cells were not found in the lamina propria of the small intestine although the main antigenic stimulus is thought to arise from adult worms in this organ (Ogilvie & Jones 1971). Similarly, although the lung has direct contact with the migratory larval stage of the infestation, no IgE-secreting plasma cells were detected in the lamina propria of the bronchi. Therefore, neither food and environmental antigens nor parasite antigens stimulate local IgE synthesis in the intestine or the lung. This finding contrasts with the local synthesis of IgA that occurs in the intestine following initial exposure to food and bacterial antigens (Crabbé *et al.* 1968, 1969, 1970; Tomasi & Grey 1972) or oral immunization with a specific antigen (Pierce and Gowans 1975).

The thoracic duct lymph of a rat undergoing a secondary response to infestation with *N. brasiliensis* contained only rare IgE-containing large lymphocytes. These were only 1/50th as numerous as cells containing IgA and were in about the same proportion to the total cells as are those containing IgM and IgG in normal TDL. Therefore, during a period of greatly accelerated IgE synthesis, IgE-containing cells were present in TDL only in numbers similar to those containing non-secretory classes of immunoglobulin in normal rats. At this time the mesenteric lymph node contained large numbers of IgE-secreting plasma cells and it is noteworthy that these were present in the medullary cords and absent from the medullary sinuses. These findings are in

accordance with the absence of IgE-secreting plasma cells from the lamina propria of the intestine.

Synthesis of IgE occurred mainly in the mesenteric and mediastinal lymph nodes of the infested rats. This is in agreement with work of Ishizaka & Ishizaka (1975) and Ishizaka *et al.* (1976), where use of different methods led to the conclusion that these lymph nodes were major sites of IgE synthesis in *Nippostrongylus*-infested rats. These workers also found the spleen to contribute very little to the production of IgE in response to the parasite. However, it does appear that the spleen can be an important source of IgE when antigen and appropriate adjuvant are administered by a parenteral route (Ishizaka & Ishizaka 1975). In the present study, it was found that peripheral lymph nodes contained IgE-secreting plasma cells and IgE-containing germinal centres. Thus, although IgE may be synthesized predominantly in lymphoid tissue draining the gut and the lung when antigen is presented via these organs and in this way bears some resemblance to IgA, it is different from IgA in that its synthesis can be stimulated in the spleen and peripheral lymph nodes by appropriate immunization. Parenteral immunization usually elicits the appearance of very few IgA-containing specific antibody-forming cells (Crabbé *et al.* 1969) and weak serum IgA responses (Ogra *et al.* 1968; Newcomb *et al.* 1969).

In summary, IgE is not synthesized locally in the lamina propria of mucosal organs by plasma cells and no precursor large lymphocytes containing IgE are found in TDL. IgE does not share with IgA the property of binding secretory component (Newcomb & Ishizaka 1970) and therefore probably does not appear in secretions by a similar mechanism to that used by IgA. In addition, while IgE is synthesized mainly in the lymph nodes draining the gut and lungs during infestation with *N. brasiliensis*, it is also synthesized to a lesser extent in peripheral lymph nodes. Other workers have shown that parenteral immunization can cause the spleen to be a major source of IgE antibody. These considerations do not support the hypothesis that IgE is a secretory immunoglobulin with a physiology similar to that of IgA.

The observations already discussed raise several points for discussion in relation to other published work. One intriguing possibility raised by the present study is that intestinal mast cells, including 'globule leucocytes', may contain IgE in their cytoplasm in addition to any IgE that might be bound to the surface membrane. The mast cells of the lung appeared similar to those of the intestine. Some other work has also suggested that mast cells may contain immunoglobulin. Dobson (1966) found that 'globule leucocytes' in sheep could be stained with a conjugate of a polyvalent anti-sheep globulin serum, although he interpreted this finding to mean that these cells contained Russell

bodies and were therefore plasma cells. Halliwell (1973) observed that mast cells in dogs infested with *Ascaris* appeared to contain cytoplasmic IgE and that this pattern of fluorescence could be imparted to mast cells of non-infested dogs by passive transfer of serum with a high titre of anti-*Ascaris* homocytotropic antibody.

The presence of fluorescent mast cells in sections of intestine and lung stained with anti-IgE may explain the differences in the numbers of presumptive IgE-secreting plasma cells that have been reported in human and monkey material. Ishizaka *et al.* (1969) and Tada & Ishizaka (1970) found considerable numbers of IgE-containing cells in the lamina propria of the intestine and lungs of both humans and monkeys. However, other studies by Hobbs *et al.* (1969), Savilahti (1972), Skinner & Whitehead (1974) and Brown *et al.* (1975) have found few or no IgE-containing cells in intestinal biopsies from humans. Although Tada & Ishizaka (1970) considered the possibility that the IgE-containing cells seen by them might be mast cells, the ethanol fixation method used by them would not be expected to preserve intestinal mast cells for staining with toluidine blue (Enerbäck 1966*a*). The present study suggests that anti-IgE-binding cells around peptic ulcers (Brown *et al.* 1975) may be mast cells, as these are common at sites of chronic inflammation. The IgE content of mast cells appears to be related to serum IgE levels (Halliwell 1973) and the reason for the absence of IgE-containing cells in some studies may be that the tissues were obtained from individuals with normal IgE levels. This possibility is supported by the results of recent studies on intestinal diseases with probable allergic aetiology, where serum IgE levels could be expected to be elevated. Large numbers of IgE-containing cells were present in the affected intestinal lamina propria (Shiner *et al.* 1975; Kilby *et al.* 1975; Heatley *et al.* 1975) and the possibility that these might be mast cells was not excluded although mast cells were common in biopsies from patients with cow's milk allergy (Shiner *et al.* 1975).

Although IgE does not appear to be locally synthesized, it is reported to be present in a number of exocrine secretions (Ishizaka & Newcomb 1970; Hobday *et al.* 1971; Bennich & Johansson 1971; Deuschl & Johansson 1974; Nakajima *et al.* 1975), and to be apparently locally synthesized in nasal polypi (Donovan *et al.* 1970). IgE appears to be present in secretions in higher concentrations relative to serum levels than either albumin or non-secretory immunoglobulin, suggesting a secretory mechanism, although this does not involve secretory component (Newcomb & Ishizaka 1970). It is noteworthy that IgE is found in the secretions of organs prone to immediate-type hypersensitivity phenomena, while it is absent from the secretions of the salivary glands (Nakajima *et al.* 1975), which are not usually the sites of allergic reac-

tions. The finding of mast cells that appear to contain IgE in the lamina propria of mucosal organs may provide an explanation for the high concentration of IgE in nasal polyp fluid, as mast cells are common in these lesions. The presence of IgE-containing mast cells (globule leucocytes) apparently passing between epithelial cells of the gut and the lung may provide the mechanism by which IgE finds its way into secretions. Its release could be due to shedding of these cells or to their degranulation by contact with specific antigens.

The mucosal mast cells have several properties that set them apart from connective tissue mast cells and blood basophils. The mucosal mast cells of the intestine and the lungs had a different pattern of fluorescence from mature connective tissue mast cells when stained with anti-IgE. In the latter cells, the fluorescence appeared to be associated with the surface membrane rather than the cytoplasm. Mucosal mast cells also differ from connective tissue mast cells in their histochemical properties (Enerbäck 1966*a*, *b*) and in their response to compound 48/80 (Mota *et al.* 1956; Enerbäck 1966*c*). It is possible that these two sorts of mast cells also differ in their lineage and physiology and that predictions about the behaviour of mucosal mast cells cannot be made on the basis of studies on more accessible cells such as those of the peritoneal cavity.

Accumulation of large numbers of mast cells in the intestines of rats infested with *N. brasiliensis* and synthesis of specific homocytotropic antibody are coincident events which demand a consideration of the role of immediate-type hypersensitivity in immunity to this parasite. A number of arguments can be marshalled against the essential participation of this mechanism, at least in worm expulsion after primary infestation (Ogilvie & Jones 1973), but some immunological (Mulligan *et al.* 1965; Urquhart *et al.* 1965; Barth *et al.* 1966) and pharmacological (Murray *et al.* 1971*a*,*b*; Rothwell *et al.* 1971) studies suggest that IgE and mast cells participate in the events leading to worm damage and expulsion. There is general agreement that worms are damaged by antibodies in the normal course of infestation (Ogilvie & Hockley 1968), that this is necessary though not sufficient to cause expulsion (Jones & Ogilvie 1971), and that worm expulsion can be hastened by passive immunization with specific antisera (Mulligan *et al.* 1965; Barth *et al.* 1966; Jones *et al.* 1970). It appears that the antisera need not necessarily contain homocytotropic antibody (Jones *et al.* 1970).

Despite the apparent independence of IgE antibodies of the passive immune response, it is not yet clear whether mast cell degranulation and histamine release are necessary to allow access of non-secretory classes of immunoglobulin to the worms in the gut lumen—the 'leak–lesion' hypothesis of Urquhart *et al.* (1965). It is known that the number of mucosal mast cells in the intestine falls during the early stages of infestation (Miller & Jarrett 1971; Keller 1971) and

there is some evidence that this is due to release of a mast cell degranulating factor by the worms (Miller & Jarrett 1971; Keller 1971). In the experiments in which passive antibody was administered, it is possible that the role of IgE was replaced by the worm degranulating factor. Specific antibody was present throughout the infestation whereas normally antibodies would not be synthesized until later, and would include specific homocytotropic antibody. The 'leak–lesion' may have operated despite the absence from the transferred serum of specific homocytotropic antibody.

Direct functional studies on the participation of mast cells in worm expulsion have not been made. Mast cell numbers in histological sections at various times during infestation do not give any indication of the rates of turnover of cells or of their pharmacological mediators. The evidence from such studies is not persuasive in either supporting or refuting the role of mast cells. In studies of the effects of passive antibody, the conditions in the intestine at the time of normal worm expulsion may not be reproduced because mast cells are present in normal intestine in relatively small numbers compared to the numbers during the late phases of infestation. This may explain the relatively weak effects of passive immunization. In experiments utilizing active immunization with immune lymphoid cells, the possibility has not been satisfactorily excluded that the transferred cells may contain B cells committed to IgE synthesis on the one hand or, on the other hand, cells capable of differentiating into mucosal mast cells or exerting inductive effects on mast cell differentiation.

One is therefore still able to entertain the possibility that IgE may have importance in immunity to parasites. The evidence discussed above relates entirely to immune mechanisms expelling worms from a primary infestation. Relatively little work has been done on the immunity to subsequent infestation and an important question in the present context is whether IgE and mast cells are involved in the destruction of migrating larvae (Taliaferro & Sarles 1939), possibly by intensifying the local inflammatory response. The present work raises the possibility that IgE itself may be important as an effector molecule, in addition to its function in sensitizing mast cells. If mucosal mast cells are indeed found to contain IgE, then specific degranulation of mast cells by worm antigens may release high local concentrations of IgE antibody, in addition to amines. The unexpected possibility that mast cells may concentrate IgE makes it important to re-examine the role of homocytotropic antibodies in immunity to parasites.

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# Discussion

*Ogilvie:* Rats vary enormously in their capacity to synthesize IgE in response

to this parasite, and in some animals one can never detect any anti-parasite IgE antibodies in their circulation. It has always been argued that this may not mean anything because IgE may be produced locally in the gut wall. Can you shed any light on this point?

*Mayrhofer:* I don't think I can. I have also had rats that have responded normally to worm challenge, with expulsion of the parasites in the usual time, but in which the serum has been negative when tested for specific IgE antibodies against *Nippostrongylus* by passive cutaneous anaphylaxis (PCA) titration. I have not looked at the mesenteric lymph nodes of such animals to see whether IgE is being produced. In the context of the results I have presented, local production of IgE antibody in the gut mucosa is unlikely. However, an important factor could be the local concentration of IgE in association with mast cells in the lamina propria, and serum IgE antibody levels may be poor indicators of this.

*Ogilvie:* Do you know how IgE gets from the mesenteric node to the tissue mast cells?

*Mayrhofer:* No. There is certainly IgE in thoracic duct lymph of infested rats, as judged by PCA assay, but I am not sure how it compares with the serum concentration.

*Soothill:* We adopted a different approach in looking at local IgE production. We (Donovan *et al.* 1969) measured the concentration of IgE in nasal polyp fluid, which provides a readily available sample of tissue fluid. This fluid is not a secretion, of course. We found high concentrations of IgE in nasal polyp fluid, far higher than could be accounted for by the concentration in serum. The levels paralleled the degree of allergy in the patient. Do you think the mast cell in some way concentrates circulating IgE and then releases it into the fluid? Could our effect have been an effect of mast cell binding?

*Mayrhofer:* These tissues contain large numbers of mast cells, so it is possible. The suggestion from our fluorescence studies is that mast cells can concentrate IgE. It is interesting that not all exocrine secretions contain IgE, while IgA appears to be secreted into all that have been examined. Nakajima *et al.* (1975) found that nasal washings from atopic individuals contained IgE but that parotid gland secretions from the same individuals did not have detectable concentrations. The secretions reported to contain IgE by these and other workers (e.g. nasal, bronchial and intestinal secretions) come from organs where allergic manifestations are more or less common. On the other hand, the salivary glands are not usually affected by immediate-type hypersensitivity. It is therefore possible that the IgE in secretions arises from mast cells as a result of contact with allergen.

*Davies:* I wonder why you appear to exclude the possibility that at least some of the IgE is synthesized by mast cells?

*Mayrhofer:* I don't exclude that possibility, although it seems unlikely. The mast cell IgE could either be acquired passively from the circulation or synthesized by the cells. I have thought of trying to distinguish these possibilities by using the phenomenon of allelic exclusion. The rat has two light chain allotypes. If the IgE is made by the mast cells, then only a proportion of the total IgE-containing population in an $F_1$ hybrid possessing both allotypic markers should stain with a labelled antibody directed against one of the allotypic antigens. If passively acquired, the IgE in the mast cells would be a mixture of allotypes and all IgE-containing cells would also stain for allotype.

*Davies:* You could perhaps do it by passive immunization studies with an appropriately labelled antibody.

*Mayrhofer:* Yes. The inference from Halliwell's work is that mast cells take up IgE into the cytoplasm (Halliwell 1973). When serum was transfused from dogs with a high reaginic antibody titre against *Ascaris* into dogs with a low titre, the mast cells in the recipients became fluorescent when stained with fluorescein-conjugated anti-IgE.

*Gowans:* Is the implication of Dr Davies' comment that mast cells are derived from lymphocytes?

*Davies:* No; perhaps cells other than plasma cells can produce antibody.

*Brandtzaeg:* Feltkamp-Vroom *et al.* (1975) recently described IgE staining of human mast cells and showed that the intensity was not related to the serum content of IgE but to the atopic status of the patients.

*Mayrhofer:* That would correspond with Halliwell's observations in dogs. He found that the IgE content of mast cells correlated well with the skin reactivity of the dogs to *Ascaris* extract, but not so well with the serum IgE level.

*Brandtzaeg:* Why do you use the indirect technique for IgE and the direct method to show IgA?

*Mayrhofer:* The fluorescence obtained when FITC-anti-IgE was used alone was rather weak, especially when I was looking for IgE-containing cells in the lamina propria. The absence of brightly fluorescent plasma cells from the lamina propria of normal animals was worrying at first. I added the second fluorescent layer to increase the brightness of positive cells.

*Brandtzaeg:* You have some background, especially some non-specific staining of the epithelium.

*Mayrhofer:* I would contest that; with the conditions of illumination that I use, the yellowish autofluorescence of the epithelium is quite different in colour from the specific fluorescence of fluorescein. It is present even in unstained material.

*Brandtzaeg:* Is there a problem with eosinophilic granulocytes?

*Mayrhofer:* Yes, and for that reason I have routinely used Lendrum's stain (Johnston & Bienenstock 1974) to modify the fluorescence of the eosinophils so that it is readily distinguished from that of specifically stained cells.

*Brandtzaeg:* Have you checked on peroxidase in these fluorescent cells?

*Mayrhofer:* Yes. Neither the IgE-containing plasma cells in the mesenteric lymph node nor the fluorescent mast cells in the lamina propria contain endogenous peroxidase, when tested in paraffin-embedded tissues. Eosinophils, and macrophages in the lymph node medulla, were positive for peroxidase under the same conditions.

*White:* I would like to take up the question of the membrane of the mast cell and its affinity for Ig. One would expect, from all that we know about heterocytotropic hypersensitivity, that the mast cell membrane would have an affinity for a lot of immunoglobulins and that there would be a rapid dynamic equilibrium for IgG antibodies and a slower one for IgE. When we were trying to isolate the two immunoglobulins of the guinea pig, $\gamma_1$ and $\gamma_2$, we observed a difference relative to their attachment to mast cells. The $\gamma_1$ (but not the $\gamma_2$) would go onto rat or mouse mast cells in connective tissues exactly as you show, and give this curious pattern of scalloped fluorescence. So the non-specific fluorescence to which you referred is an interesting phenomenon. In other words, if you take a frozen section of tissue and expose it to immunoglobulin, it would go onto the surface of the mast cell because it has an affinity for it. This is something which in the case of the guinea-pig antibody (and one could narrow it down to a determinant group on the H2 domain of the Fc piece of guinea pig $\gamma_1$) is productive of a brilliant fluorescent reaction. Human IgG has this ability too. This appears to be class-specific for human immunoglobulins. So when you do a double-layer staining reaction in which you put rabbit antibody on (rabbit anti-rat IgE, as it were), I think you would be laying the ground for this kind of result.

*Mayrhofer:* I don't believe that the fluorescence of the mast cells is non-specific. One of the specificity controls that I used was to substitute purified normal rabbit IgG for the specific rabbit anti-rat IgE as the middle layer of the sandwich. When this was done, there was no fluorescence after the addition of the top layer of fluorescent goat anti-rabbit Ig.

*Cebra:* Ishizaka's group (J. S. Urban, T. Ishizaka & K. Ishizaka, unpublished work 1976) have recently shown that after *Nippostrongylus* infection in rats there is a sharp increase in the number of circulating IgE-containing cells. Have you looked at peripheral blood cells and, if so, have you stained these with Alcian blue? I wonder whether Ishizaka's group may not have been detecting basophils in the circulation.

Secondly, what is known about the specificity of the abundant IgE produced after the infection? Kishimoto & Ishizaka (1973) suggested that a distinctive set of 'helper' cells seems to be stimulated by worm cuticle carriers. Thereafter, no matter what determinant is placed on this cuticle, a certain amount of IgE is produced that is reactive with that hapten. I wonder whether such a worm infection might not simply stimulate any kind of responding B cells to express IgE?

*Mayrhofer:* I have not looked for IgE-containing cells in rat blood, but I do know that basophils are notably absent in this species. In a preliminary study, I have not seen a basophilia in rats during infestation with *Nippostrongylus*.

In answer to your second question, one of the outstanding problems is how much of the antibody formed during the parasite infestation is specific. A potentiation of homocytotropic antibody responses to unrelated antigens during helminth infestations has been clearly demonstrated (Orr & Blair 1969; Jarrett & Stewart 1972). However, because of the antigenic complexity of parasites, it is difficult to measure the fraction of the total IgE response to infestation that is antibody directed at parasite antigens and the fraction that is directed against an unknown number of normal environmental antigens. The question has an important clinical aspect. There are epidemiological studies that suggest that allergic diseases are almost absent in areas of the world where parasite infestation is universal (Bennich & Johansson 1971; Godfrey 1975). It has been suggested that the high levels of IgE in parasitized humans may be the result of potentiated reagin responses to environmental antigens. Dr Ogilvie (Ogilvie & Jones 1973) has suggested that the potentiated response may protect the individual from serious allergic reactions to parasite antigens by competing with parasite-specific IgE antibodies for binding sites on mast cells. On the other hand, one could explain the epidemiological evidence by assuming that most of the IgE in infested individuals is directed against parasite antigens and that this antibody protects against allergic responses to environmental antigens by the same mechanism. On the latter hypothesis, man's natural state is to be parasitized and allergy-free and it is hygiene that has caused a proportion of the population to become troubled by allergy. Resolution of this problem may provide clues for the prophylaxis of allergy.

*Evans:* You made some statements about the sensitivity of the mast cell in the rat to alcohol fixation which I didn't understand. If the mast cell were disrupted by fixation, presumably you wouldn't see the IgE?

*Mayrhofer:* I don't think that ethanol fixation disrupts the mast cells, but that the staining properties of the granules are altered. This may involve the interaction of the basic proteins and the acidic mucopolysaccharide, heparin,

in the mast cell granules. Certain fixatives may preserve the acidic groups of heparin so that they are available for subsequent binding with dyes such as Alcian blue.

*Evans:* Can I ask you about the technique used for staining with toluidine blue? Some histochemists when they stain with toluidine blue avoid subsequent exposure of the specimen to alcohol.

*Mayrhofer:* It depends on the conditions of staining. I have found that connective tissue and peritoneal mast cells hold toluidine blue and their metachromasia even after reprocessing through alcohol if they are initially stained in an alcoholic solution of the dye (50% ethanol, pH 3.5).

*Evans:* What technique did you use for the Alcian blue staining?

*Mayrhofer:* I have followed the method used by Enerbäck (1966). Staining is done in 0.7N-HCl. At the pH of this solution, the only dye-binding groups remaining ionized are said to be the sulphates of sulphated mucopolysaccharides. In the gut, the only structures stained are the mast cells and the contents of goblet cells. After counterstaining with Safranin in 0.125N-HCl, Alcian blue is displaced from mucus and the mast cells alone are stained blue.

*Evans:* I believe that in the guinea pig Kurloff cells may be stained with Alcian blue in certain circumstances. Can one stain rat plasma cells with this dye?

*Mayrhofer:* I have stained mesenteric lymph node sections containing IgE-secreting plasma cells with the same method. Plasma cells in the medullary cords, and in particular those secreting IgE, do not stain with Alcian blue. It has been argued in the past that mucosal mast cells and 'globule leucocytes' are Russell body-containing plasma cells (Dobson 1966; Whur & Johnston 1967). To support this view one would have to argue that Russell body cells have histochemical properties different from plasma cells from which they are believed to arise.

*Evans:* The Kurloff cell inclusion looks rather like a Russell body (Marshall *et al.* 1971).

*Mayrhofer:* I think the most convincing distinction is that the mucosal mast cells have been shown to contain histamine and 5-hydroxytryptamine, and are therefore in this key respect related to mast cells elsewhere (Murray *et al.* 1968; Miller & Walshaw 1972).

*Lehner:* If IgE binds onto the membrane of mast cells, they must have Fc receptors for IgE. Have you looked for these?

*Mayrhofer:* No. To look with this sort of approach requires an *in vitro* system, and at present this means adopting either the blood basophil or the peritoneal mast cell as one's model. However, I am not convinced that they are the same as the mucosal mast cell, for reasons I mentioned in my paper.

*Lachmann:* You were suggesting that in the mucosal mast cells the IgE was intracytoplasmic. Wouldn't you have expected that staining to be granular, or at least outlined by the granules? Your micrographs show a diffuse staining.

*Mayrhofer:* Yes. I wouldn't like to say whether the IgE is in the granules or in the soluble cytoplasm. The granules are very small in these cells, and often closely packed.

*Ferguson:* You mentioned that mast cells are distributed throughout the length of the small intestine. My colleague Mr T. T. MacDonald (unpublished work 1975) has examined the changes in the numbers of intestinal mast cells during *Nippostrongylus brasiliensis* infection of rats. He has found expansion of the mast cell population not only along the entire small intestine but also in segments of small intestine which have been transplanted under the kidney capsules as fetal tissue (by the technique of Ferguson & Parrott 1972) and therefore completely isolated from antigenic stimulation via the lumen. It seems to me likely that the mast cells of the intestinal mucosa are derived from lymph and that their precursors follow the same migration pathways as immunoblasts.

*Bienenstock:* The Ishizakas found that if they put rat thymus into long-term tissue culture they obtained fairly pure colonies and the expansion of mast cells (Ishizaka *et al.* 1976). It has been suggested in the past that lymphocytes and mast cells are related. Miller (1971) said that the mastoblasts in animals infected with *Nippostrongylus* are indistinguishable from immunoblasts in these animals. We have shown T cell markers on rabbit basophils, which suggests that these two cell types may be related (Day *et al.* 1975). Burnet (1965) showed that the thymuses of NZB mice had huge infiltrations with what looked like colony expansion of mast cells. So the possibility is there.

*Gowans:* The origin of tissue mast cells, including those in the intestine of animals with intestinal parasites, is unknown and it should be emphasized that the evidence for an origin from lymphocytes is entirely circumstantial. This includes the Ishizaka studies which are a follow-up of the earlier work of Ginsburg & Sachs (1963).

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*Immunology of the Gut*
Ciba Foundation


# The immunological consequences of nematode infection

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*Abstract* Nematode infections in the gut induce a strong immune response which is rapidly detected parenterally. The response is thymus-dependent and long-lasting and involves both antibodies and cell-mediated reactions. The immunological response to unrelated antigens, tumours and other infectious organisms is altered in animals infected with nematodes. Both antibodies and sensitized lymphocytes participate in the immune response which affects the nematodes themselves, and characteristically the lymphocyte-dependent step cannot act in lactating animals and is neither induced nor able to act in young animals. Present evidence suggests that, despite their well-known association with helminth infections, parasite rejection from the gut does not require the participation of IgE antibodies, mast cells or eosinophils.

Homing of lymphoblasts from the mesenteric lymph node or thoracic duct lymph to the small intestine is increased in rats and mice infected with *Nippostrongylus brasiliensis* or *Trichinella spiralis* and the increase is antigenically non-specific. In mice infected with *T. spiralis* this increase is represented mainly by thymus-derived lymphoblasts.

In the UK nematode infections of the gut are studied for two reasons, either because of their importance in reducing animal productivity or because they induce various pathological or immunological changes in the gut which are of interest for reasons unconnected with parasitism. Here we briefly discuss the pathological changes induced in the host before describing the immunological changes induced by nematodes. Their presence in the gut influences the host's response to other antigens and affects the homing of lymphoblasts from the mesenteric lymph node or thoracic duct lymph to the intestine. Worm expulsion is brought about by a complex interaction between various components of the immune response. This fails in young and lactating animals which in consequence do not expel their worms efficiently.

These aspects of nematode parasitism are illustrated mainly from studies

with two nematodes of the small intestine, *Nippostrongylus brasiliensis* in rats and *Trichinella spiralis* in mice, rats or guinea pigs, but some reference is made to studies with other nematodes.

## PATHOLOGICAL CHANGES INDUCED BY NEMATODES

It is well known that heavy infections of nematodes in the gut prevent weight gain by the host and frequently cause diarrhoea (Symons & Fairbairn 1962; Castro & Olson 1967). The changes induced by these parasites in the small intestine have been studied by Symons using the *N. brasiliensis*/rat model and Castro and colleagues with *T. spiralis* in guinea pigs. The pathological changes seen in the parasitized small intestine are not specific to nematode infections but appear to be similar to the changes found in bacterial infections (Sprinz 1962) or in malabsorption conditions such as coeliac disease (Symons & Fairbairn 1962; Castro *et al.* 1967). These changes include hyperplasia of the villi, which shorten and become irregularly shaped and oedematous (Symons 1957; Castro *et al.* 1967). Epithelial cells change in appearance (Castro *et al.* 1967; Symons *et al.* 1971) and their turnover rate increases (Symons 1965). Brush border enzymes such as maltase, leucine aminopeptidase and alkaline phosphatase are reduced (Symons & Fairbairn 1962, 1963). Digestion and absorption of sugars and protein is impaired at the sites where the parasites are found (Symons 1960; Symons *et al.* 1971; Castro *et al.* 1967) but there may be no overall fall in digestion and absorption if the infection is localized in the upper part of the gastrointestinal tract, as the rest of the gut may compensate by increased digestion and absorption (Symons 1960, 1961). This is not possible, however, when the infection occurs in the large bowel, for example in cattle infected with *Oesophagostomum radiatum*, which in consequence are severely affected (Bremner 1969).

These deleterious effects of nematode infections are enhanced by loss of appetite and plasma protein leakage into the gut (Mulligan 1972). The studies of Symons and his colleagues (largely with *Trichostrongylus colubriformis* in guinea pigs and sheep) have shown that, because loss of appetite is exacerbated by leakage of plasma protein into the gut at the parasitized site, protein catabolism exceeds protein synthesis. Protein synthesis in the liver is elevated by the increased synthesis of plasma proteins which occurs (Symons & Jones 1974) and is depressed elsewhere, especially in skeletal muscle and wool follicles (Symons & Jones 1971, 1972, 1975). Consequently, productivity measured, for example, by increases in muscle weight or wool growth, is severely affected, even by light infections of gut nematodes and especially in growing animals (Symons & Jones 1974, 1975).

Apparently all nematodes of the gut (with the exception of *Trichuris* species found in the large bowel, see p. 188) induce high levels of IgE in their hosts. The anaphylactic reactions induced by interaction of specific IgE with worm allergen at the infected site may well account at least in part for the efflux of plasma protein into the gut lumen (Barth *et al.* 1966). Light and electron microscopy have shown that junctional complexes between cells of the villi may break down in the parasitized gut, permitting abnormal leakage of proteins into the gut lumen (Murray *et al.* 1971). There are few studies of the pathology of these infections in immunologically incompetent hosts. Ferguson & Jarrett (1975) have suggested that the distortion of villi in the intestine of rats infected with *N. brasiliensis* is caused by a thymus-dependent immune reaction. Whether any of the other changes associated with these infections are immunologically induced is not known.

Infections with nematodes may also cause increased microbial growth in the gut (Cypess *et al.* 1974*c*; Rutter & Beer 1975). It is possible that the pathological changes found in the nematode-infected gut may be caused by this increase in bacteria at the site of infection rather than by the nematodes themselves. Rutter & Beer (1975) studied conventional and gnotobiotic pigs infected with *Trichuris suis*. The muco-haemorrhagic diarrhoea associated with this infection developed only in conventional pigs. Therefore the diarrhoea may be caused by the spirochaetes and vibrio-like organisms which were observed only in the conventional pigs, in which increased numbers of bacteria were found during the clinical phase of the disease (Rutter & Beer 1975).

## EFFECT OF NEMATODES ON THE IMMUNE SYSTEM OF THE HOST

The presence of nematode parasites in the gut alters the ability of the host to respond immunologically to a variety of antigens (including other infections), mostly given parenterally. The changes reported to date are summarized in Table 1 and show that immunity may be enhanced or depressed. The remarkable effect of *N. brasiliensis* on IgE levels is thought to result from the production of special helper T cells which enhance IgE, or a lack of suppressor T cells (Jarrett & Ferguson 1974; Kojima & Ovary 1975). Otherwise, these results have been explained by suggesting that the nematodes have non-specific effects on the immune system, such as antigenic competition and enhanced macrophage activity (Cypess *et al.* 1974*a*; Lubiniecki & Cypess 1975). Recent studies of cell homing in infected animals (discussed later, p. 189) have shown changes which might explain some of these effects. The effects of *Syphacia* (Table 1) are noteworthy in that they were caused by naturally

TABLE 1

Influence of nematode infections on the ability of the host to respond to other antigens

| *Parasite and host* | *Effect on response* | *Reference* |
|---|---|---|
| | (*a*) *Increased* | |
| *Trichinella spiralis* in mice and rats | Delayed hypersensitivity reaction to BCG | Cypess *et al.* 1974*b* |
| | Resistance to infection with *Listeria* | Cypess *et al.* 1974*a* |
| | Resistance to infection with trypanosomes | Meerovitch & Ackerman 1974 |
| *Nippostrongylus brasiliensis* in rats and mice | IgE production | Orr & Blair 1969; Jarrett & Bazin 1974 |
| | Resistance to tumour growth | Keller *et al.* 1971 |
| | (*b*) *Decreased* | |
| *T. spiralis* in mice | Antibody response to (1) sheep red blood cells (2) Japanese B encephalitis virus | Faubert & Tanner 1971; Lubiniecki & Cypess 1975 |
| | Skin graft survival prolonged | Svet-Moldavsky *et al.* 1970 |
| *N. brasiliensis* in rats | Enhanced tumour growth | Keller *et al.* 1971 |
| | Enhanced *Plasmodium berghei* infection | Golenser *et al.* 1976 |
| *Syphacia oblevata* in rats | Incidence of adjuvant arthritis reduced | Pearson & Taylor 1975 |
| | Antibody response to ovalbumin | Pearson & Taylor 1975 |
| *Nematospiroides dubius* in mice (*Heligmosomoides polygyrus*) | Antibody response to sheep red blood cells[a] | Shimp *et al.* 1975 |

[a] Sheep red blood cells given orally. In all other reports, antigen given parenterally.

occurring infections which might arise in rodents in all conventional laboratory animal houses.

Nematodes induce a mixed immunological response, evoking antibodies in several immunoglobulin classes, a strong cell-mediated response and an inflammatory reaction which includes may cell types—plasma cells, mononuclear cells, mast cells/basophils and eosinophils (Taliaferro & Sarles 1939). In rats infected with *N. brasiliensis*, IgE, IgG, and IgM antibodies against parasite antigens have been detected but no IgA antibodies have been reported (Ogilvie & Jones 1973). There is indirect evidence that antibodies begin to affect these worms on day 8 of the infection in rats and by day 10 the effects are severe (see review, Ogilvie & Jones 1973). However, antibodies have not been detected in the sera of rats before day 17, which is after the parasites have been expelled.

Antibodies directed against *T. spiralis* antigens are found in the IgG1, IgG2, IgA and IgM in sera from mice about 11–15 days after the infection (Crandall & Crandall 1972). IgE antibodies were never detected in some strains of mice and in others were found from the second week after the infection (Rivera-Ortiz & Nussenzweig 1976). Cells which will cause accelerated rejection of these parasites from syngeneic recipients are found in the thoracic duct lymph or mesenteric lymph nodes of infected rats by day 7–8 of a *N. brasiliensis* infection (Ogilvie *et al.* 1976) and by day 8 in the mesenteric lymph nodes of mice infected with *T. spiralis* (Wakelin & Lloyd 1976).

## THE IMMUNOLOGICAL MECHANISM WHICH EXPELS THE WORMS

The mixed immunological response induced by nematodes may make them especially useful in the study of immune reactions in the gut, but it has complicated the analysis of the immune responses which control these helminths. Besides *N. brasiliensis* and *T. spiralis*, detailed studies of the immune mechanism which affects nematodes have been made with *Trichuris muris*, which is found in the large intestine of mice (Wakelin 1975; Wakelin & Selby 1976), and *Trichostrongylus colubriformis* in guinea pigs (Dineen & Wagland 1966; Rothwell *et al.* 1974). In all cases, a strong, long-lasting and thymus-dependent response is induced (Ogilvie & Jones 1973) and in the case of the first three parasites it is clear that their expulsion requires the sequential action first of antibodies and then of sensitized cells from the mesenteric lymph node (Ogilvie & Jones 1973; Ogilvie & Love 1974; Wakelin 1975; Love *et al.* 1976; Wakelin & Lloyd 1976; Wakelin & Selby 1976). There is a variety of evidence which supports the idea that a two-step mechanism is involved. Recipients given either antiserum or cells are partly protected against infection but a level of resistance approaching that induced by an active infection can be conferred on recipients only by giving them both antiserum and cells from infected donors. In the *N. brasiliensis*/rat model, antibodies (IgG1, Jones *et al.* 1970) affect the worms to make them susceptible to the expulsive action of cells but antibodies alone cause either no rejection or a slow rejection of the worms.

The evidence for this is as follows. Irradiated recipients of antiserum expel their worms slowly if at all, and lactating and young (7–9 weeks) rats do not expel their worms, yet their antibody response (both IgE and IgG1) is normal or increased. That is, serum from young or lactating animals will passively protect immunologically competent recipients from infection and their own worms are damaged by antibodies, so that when transferred into mature non-lactating rats they are susceptible to the cellular step (Ogilvie & Love 1974). The cellular step is brought about by cells which require the presence of the

thymus for their induction and have no surface immunoglobulin (Keller & Keist 1972; Ogilvie *et al.* 1976). Therefore, it would seem reasonable to surmise that the effector cells are T cells. The cellular step is induced but cannot act in lactating animals and is not induced in young animals. Furthermore, sensitized cells obtained from mature infected donors do not cause worm expulsion when given to lactating or young recipients harbouring worms already affected by the antibody step (Ogilvie & Love 1974; Love & Ogilvie 1975). Exactly how these cells bring about worm expulsion is not known. They expel worms from rats given 750 rads within five days and these animals have few mast cells or eosinophils at the site of infection (Ogilvie *et al.* 1977). It has been postulated that the release of prostaglandin E is the final effector of immunity to *N. brasiliensis* (Dineen *et al.* 1974) but no explanation has been offered as to how T cells might induce release of prostaglandins. Progress in this area requires a much more detailed understanding of cell changes at the site of infection, information which may come from current studies of cell traffic in parasitized animals (see later, p. 189 *et seq.*).

## THE EFFECT OF IgE ANTIBODIES ON NEMATODES

A prolonged, thymus-dependent IgE response is characteristic of nematode as of most helminth infections. Much of this is not directed against parasite antigens, but IgE levels in general are elevated, for reasons discussed earlier. It is, however, clearly established that IgE antibodies are not directly concerned in the expulsion of nematodes. They are often found in animals unable to expel their worms (for example, in lactating hosts) and sometimes are not detectable in hosts which are strongly immune. The best illustration of this is that IgE antibodies have never been found in mice infected with *Trichuris muris* (D. Wakelin, personal communication), nor in man infected with *Trichuris trichiura* (Rosenberg *et al.* 1971). The immune mechanism which expels *T. muris* (Wakelin 1975; Wakelin & Selby 1976) is probably identical with that which expels *N. brasiliensis* and, as outlined above, this requires IgG antibodies and sensitized T cells.

It is nevertheless possible that the anaphylactic reactions induced by IgE antibodies might have an enhancing effect on the immune mechanisms which actually affect the worms. Barth *et al.* (1966) showed that an ovalbumin-induced anaphylactic reaction may enhance the action of antibodies on *N. brasiliensis*. Also, the 'self-cure' reaction described in certain nematode infections appears to be an anaphylactic reaction, although whether an IgE–worm allergen interaction is the primary effector in this situation or dramatically enhances other mechanisms has not been formally demonstrated. 'Self-cure' occurs when

animals already infected with a particular nematode ingest a large second infection. Within hours, all the parasites are expelled (reviewed in Ogilvie & Jones 1973). This reaction is not predictable, it occurs only in certain strains of hosts (Cypess & Zidian 1975), and hosts in which it occurs may not necessarily resist further infections.

In the *T. colubriformis*/guinea pig infection there is good evidence that 5-hydroxytryptamine (serotonin) acts directly on the worm to cause expulsion (Rothwell *et al.* 1974). The curious feature of this reaction is that it can be adoptively transferred to recipients only with lymphocytes and not with serum (Dineen & Wagland 1966). If IgE-allergen causes release of 5-hydroxytryptamine from basophils in this infection, the essential requirement for lymphocytes can be explained by suggesting that they are necessary for inducing basophil infiltration into the sites of infection.

## INFLUENCE OF PARASITES ON THE TRAFFIC OF IMMUNOBLASTS TO THE SMALL INTESTINE

The main objective in following the traffic of immunoblasts to the small intestine in nematode infection is to study the expulsion mechanism but, as suggested earlier, it may be that a greater gain will be the resolution of those pathological changes which are a direct effect of the parasite itself and those which are a result of the host's response to the parasites. As already stated, the sources of cells which are most effective in transferring immunity to gut parasites are the mesenteric lymph node and thoracic duct lymph. These cell populations contain, particularly after infection, large numbers of activated blast cells which readily take up isotopically labelled DNA precursors or analogues of DNA precursors. As is well known, such cells have a propensity to extravasate into the mucosal layer of the small intestine (Gowans & Knight 1964; Hall *et al.* 1972; Parrott & Ferguson 1974). Immunoblasts could thus, in theory, make close if not direct contact with parasites in the gut, although contact between cells and the nematodes discussed here has never been reported.

In August rats infected with either *T. spiralis* or *N. brasiliensis* larger numbers of [$^{125}$I]iododeoxyuridine-labelled thoracic duct lymphoblasts accumulate in the small intestine than in uninfected controls (Love & Ogilvie 1977) and in NIH mice infected with *T. spiralis*, similar findings were obtained with mesenteric lymph node blasts (Rose *et al.* 1976*a*). The increase occurred early in mice, within 2–4 days of infection, and was due to increased retention of labelled cells within the whole animal and not redistribution from other sites (Rose *et al.* 1976*a*). It was not related to increase in gut size in infected animals and

at the time of nematode expulsion, which occurs about nine days after infection, there was no difference in cell traffic between infected and uninfected mice. In rats the increase in blast cell traffic was somewhat later, not until 9–12 days, it was coincident with gross inflammation, but again was unrelated to increase in gut size. There was a clear indication of increased localization within segments of intestine with the highest worm counts in both rats and mice but none of the increases were antigenically specific. In rats and mice infected with these nematodes, blast cells activated by completely unrelated stimuli were as capable of homing in increased amounts as were cells from donors infected with the same parasite. In normal uninfected mice both T and B blasts home to the gut mucosa (Guy-Grand *et al.* 1974; Parrott *et al.* 1975; Sprent 1976). However, in mice infected with *T. spiralis* the early increased migration of blast cells is almost entirely composed of mesenteric lymph node T immunoblasts. The number of B blasts from mesenteric node suspensions migrating to the gut was the same in infected mice as in normal controls (Rose *et al.* 1976*a*).

Villous atrophy is one pathological change in the infected gut which preliminary observations justify linking with the increased arrival of mesenteric lymph blasts to the small intestine (M. L. Rose & D. M. V. Parrott, unpublished data). By day 4 in mice infected with *T. spiralis* there is a shortening of villi and an increase in intraepithelial lymphocytes in the first segment of the intestine where worm counts and labelled cell accumulation are highest, whilst changes in lower segments remain minimal. There was, however, no indication from autoradiographs of sections of gut that the injected labelled cells were in any way attracted to the worms, although some labelled cells were in the epithelial layer as well as in the lamina propria. At this time too, as preliminary studies show (D. M. V. Parrott, unpublished), there is little polymorph infiltration or other cellular change indicative of inflammation.

Many of the results of cell migration to the infected gut are reminiscent of other situations in which activated T blasts have been shown to cross blood vessels into local areas of inflammation. Newly formed thoracic duct T blasts which appear during an infection with *Listeria monocytogenes* will assemble very readily in an inflamed peritoneal cavity but in an entirely non-specific way (McGregor & Logie 1974). Similarly the induction of peritonitis may cause cells to be diverted from the intestine in nematode infection (Love & Ogilvie 1977). T blasts from lymph nodes draining the skin after the application of a contact sensitizer such as oxazolone will accumulate in sites inflamed by an irritant such as turpentine or an unrelated contact sensitizer as easily as into the site to which the priming chemical has been applied (Asherson *et al.* 1973; Parrott *et al.* 1975). Four days after *T. spiralis* infection large numbers of

peripheral T blasts appear in the small intestine, providing the skin is not inflamed at the same time (Rose *et al.* 1976*b*). These cells do not accumulate in the normal gut (Parrott & Ferguson 1974; Parrott *et al.* 1975).

Even at this early stage these studies of cell traffic in parasitized animals have yielded results which enable us to suggest possible explanations for some of the phenomena described earlier in this paper. For example, the pathological changes usually ascribed to the presence of nematodes might be exacerbated as a result of activation of T blasts by unrelated co-existing infections such as *Listeria monocytogenes*. Non-specific trapping of circulating lymphocytes such as that occurring in the mesenteric lymph nodes of *T. spiralis*-infected mice (Rose *et al.* 1976*b*) could reduce the chances of lymphocyte–antigen interaction in peripheral sites, which might explain alterations in the responsiveness of nematode-infected animals to unrelated antigens. Decreased immune responsiveness might occur because effector T blasts are diverted to the gut in infected animals rather than to the site of antigen location. The absence of specificity in the increased flow of blast cell traffic could well explain the enhanced worm expulsion which occurs in appropriately timed dual infections (Bruce & Wakelin 1974).

Blast cell diversion might also explain the failure of lactating animals to expel nematodes. In rats there are the same number of blast cells in the intestine of lactating as of normal mothers although the gut of lactating animals is $2\frac{1}{2}$ times increased (Love & Ogilvie 1977). In lactating mice there is an increase in blast cell migration to the gut but there is as much if not more migration to the mammary glands (M. L. Rose & D. M. V. Parrott, unpublished data).

It is obvious that all these suggestions require the support of many more experimental data before they can be accepted as anything other than speculation. It is clear, however, that the study of nematode infections of the gut can yield ideas which may be valuable in understanding the role which immune reactions may have in pathological conditions of the gut and the effect of these conditions on the immunological competence of the animal concerned.

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## Discussion

*Bienenstock:* Guy-Grand's work (Guy-Grand *et al.* 1974) and that of Sprent (1976) suggests that T blasts have preferential localization in and homing to the gut epithelium, as opposed to the B blasts which do not reach the epithelium, in this infection.

*Parrott:* I have found some labelled T cells in the epithelial layer, but they don't occur so very often. Most of them are still in the lamina propria, when one uses [$^{125}$I]iododeoxyuridine labelling. If I had used [$^{3}$H]thymidine and looked at longer times after infection, I might have found more T cells in the epithelial layer. This procedure has proved successful in other experiments (Parrott *et al.* 1975).

*Bienenstock:* The obvious question is whether these T cells have anything to do with what is going on in the lumen.

*Parrott:* In nematode infections the T blasts don't appear to be interested in the worms in the gut. There is no inflammatory response near the nematodes, and we don't see any localization or clustering of T cells around the worm.

*Gowans:* When you refer to the homing of T cells, which kind of T cell are you talking about? Are you referring to T-blasts which can be labelled *in vitro* with [$^{125}$I]iododeoxyuridine or [$^{3}$H]thymidine, or to small, non-dividing T cells which can be labelled *in vitro* with radioactive precursors of RNA?

*Parrott:* In the studies referred to here (Rose *et al.* 1976) we labelled the blasts; one can of course also label ordinary T cells and trace them by autoradiography but I haven't seen any small T lymphocyte labelled by [$^{3}$H]-adenosine or [$^{3}$H]uridine in the lamina propria, with the exception of the area immediately around the Peyer's patches (Parrott & Ferguson 1974).

*Lehner:* Dr Ogilvie, you suggested that in the first 10 days of infection antibodies are produced which act on the nematodes, and then lymphocytes take over. Is this an *in vivo* sequence of the immune response, or are you implying that *in vitro* the lymphocytes could affect the worms?

*Ogilvie:* Unfortunately it is difficult to study the effect of antibodies or lymphocytes on this parasite *in vitro*. Fully sensitized and competent lymphocytes are found in rats as early as seven days after infection. If you transfer lymphocytes from a 7-day infected rat to another rat infected simultaneously with antibody-damaged worms they will expel the worms from the recipient within two or three days. But in the rat from which the cells came, lymphocytes apparently cannot act on the worms until the antibody step affects the worms, which does not happen until day 10. I dont' know why it is necessary for worms to be damaged by antibodies before cells can have their effect.

*Lehner:* Is the antibody acting on its own, or is it opsonizing the worms for phagocytosis?

*Ogilvie:* I think the antibody interferes with the parasites' feeding and they become rather unhappy worms! One can mimic all the effects of antibody by simply taking worms out of an infected rat before antibodies affect them and putting them into culture without antibodies. Twenty-four to 48 hours later we find all the changes which we normally associate with antibody action in the cultured worms, including sensitivity to lymphocyte action when cultured worms are put into rats again (Love *et al.* 1975). It seems that when worms are made less fit, either by the action of antibodies or by being kept *in vitro*, they become susceptible to lymphocyte attack.

*Lehner:* When the lymphocytes come along, are the worms killed?

*Ogilvie:* The worms are never killed. If you collect them as they come out of the intestine and put them back they behave like antibody-damaged worms (Love *et al.* 1975).

*Lehner:* Is the lymphocyte response antibody-independent or antibody-dependent?

*Ogilvie:* I think it is antibody-independent. To test the effect of the second step we take antibody-damaged worms from donors (or damage them in culture, as just described) and put them into an irradiated rat. Into that animal we put thoracic duct cells that have been run down a column of plastic beads coated with anti-rat globulin. The cells which are not retained on the column cause rejection of these worms within five days. I do not think antibodies are involved in the rejection of worms by lymphocytes in this experimental situation (Ogilvie *et al.* 1977).

*Evans:* Do you get the same result with lymphocytes that have been cultured?

*Ogilvie:* This experiment has not been done.

*Mayrhofer:* I find it difficult to imagine what causes the expulsion of the worms. We know that antibody damages them and that perhaps lymphocytes do also, but what makes them move down the gastrointestinal tract? Is it gut motility? I ask this because it is well known that the motility and tone of smooth muscle is reduced under the hormonal influences of pregnancy. Could the effect of lactation be similar, acting at a non-immunological level in a sequence of events with an earlier immunologically activated trigger?

*Ogilvie:* The work of Dineen *et al.* (1974) suggests that the final effect is due to prostaglandin E. When prostaglandins were introduced directly into the gut of these animals the parasites were expelled very quickly and, conversely, infected rats given inhibitors of prostaglandins were unable to expel their worms. So the final effect may be due to prostaglandin, released presumably from macrophages or T cells, or eosinophils.

*Schmidt:* Is there any evidence that macrophages play a role in your system?

*Ogilvie:* There is no evidence for their involvement, but if Dineen *et al.* (1964) are correct and the final effect is due to prostaglandins, the macrophage may be a source of prostaglandins.

*Mayrhofer:* It is difficult to argue from such experiments unless one knows the final step in worm expulsion. Failure to expel worms may be attributed to an early event, such as failure of mast cell accumulation or degranulation, whereas the defect may lie at the end of the chain of events.

On a different point, the temporal correlation between mast cell accumulation in the lamina propria and the onset of worm expulsion (or the failure to find such a correlation) has been held to support (or refute) the role of mast cells in immunity to *Nippostrongylus*. However, mast cell numbers as such may not be important. For instance, in the lactating rat, the presence of large numbers of mast cells despite failure to expel worms does not necessarily indicate normal mast cell function. Accumulation may be normal but degranulation or cell turnover may be defective. On the other hand, it is not necessarily correct to argue that mast cells are not involved in worm expulsion from those experiments where mast cells were not found to accumulate until after the expulsive phase. The low numbers during expulsion may in fact reflect intense mast cell activity and rapid cell turnover.

*Ogilvie:* I agree that the reported difference in time of appearance of mast cells in the gut is by itself probably not important; I also accept your comments that the mast cells in lactating rats may not be able to function exactly as they do in non-lactating rats. However, in our more recent experiments we get rapid expulsion of parasites from irradiated rats that have no mast cells in the lamina propria of their intestines (Ogilvie *et al.* 1977).

*White:* What could be the stimulus for the mast cells? Is it a worm-specific stimulus?

*Ogilvie:* I don't think anybody knows the answer to this interesting question.

*White:* How do you account for the finding that a heterologous anaphylactic reaction expels worms?

*Ogilvie:* I suspect that the experiments to which you refer (Barth *et al.* 1966) may have no relevance to the normal sequence of events in this infection. An important reason for my scepticism about the significance of this work is that lactating rats which produce anti-parasite antibodies and in which a violent, parasite-specific anaphylactic reaction can be induced in the intestine (Connan 1973) nevertheless do not expel their worms. This makes me think that the results of Barth *et al.* (1966) (which showed that an anaphylactic reaction induced by a non-parasite antigen enhanced expulsion of worms from rats

passively immunized with antiserum) may be no guide to the mechanism which normally operates.

*Booth:* You mentioned the enlargement of the gut in the lactating rat. This enlargement is almost all accounted for by villous hyperplasia. I don't know any evidence that when there is hyperplasia of the intestine there is hyperplasia of the lamina propria tissue. But is there likely to be any hyperplasia, for example, of plasma cell or lymphocyte populations?

*Ogilvie:* We have not assessed these cell populations in lactating animals. It would be interesting to do this.

*Booth:* The other question is nutrition. When there is loss of protein from the gut, it is our impression from human and animal studies that it never produces a depletion of anything other than *serum* proteins. This situation is completely comparable to nephrotic syndrome with protein in the urine. The rest of the body protein is normal. This would fit in with your concept that whatever disease occurs in these rats results in lack of appetite and lack of intake, and therefore in a kwashiorkor-like picture in the animal. If that is the case, what is the effect on the immune response?

*Ogilvie:* In acute infections, there wouldn't be time for any interference in nutrition to influence the outcome, but in malnourished animals immunity is profoundly affected and this is almost certainly one reason why nematode infections are so important in endemically infected human populations or animals at pasture.

*Ferguson:* In the case of parasites which do not attach to the surface epithelium, I wonder if villi of a certain length are necessary to allow the parasites to remain in one area of the intestine. In *Nippostrongylus* infection, a couple of days before the parasite is expelled the villi are damaged by an immune reaction giving a flat or convoluted surface (Symons 1965; Ferguson & Jarrett 1975). I have always thought that this would be simply an unavoidable side-effect of the immune response, although it is clinically important in reducing the surface area available for absorption—in fact in some animals the appearances are similar to those found in the flat mucosa of coeliac disease. However, it could be that by destroying the villi a cell-mediated immune reaction can stop the worms from having something to twist around. In lactating animals the villi are longer than normal (Craft 1970) and perhaps in such animals the villi do not totally disappear during parasite infection.

Some other species of worm actually penetrate the gut. Is it known how these are expelled? Do they simply fall off the mucosa to be removed by peristalsis also?

*Ogilvie: Trichuris* spp. nematodes penetrate the gut mucosa and immunity to *T. muris* in the mouse has been studied in detail by Wakelin (1975). This work

suggests that the immune mechanism operating in this model is similar to that postulated for *N. brasiliensis*, in that both antibodies and lymphocytes but not IgE antibodies are required.

*Parrott:* In the histological sections I have looked at, the villi although shortened aren't absolutely flat as in coeliac disease. Perhaps the parasites haven't such a good handhold, but they still have something to wind round.

*Ferguson:* I have never seen a patient with untreated coeliac disease and a flat mucosa who also has a parasite infection. However, these infections are uncommon in Britain. Paediatricians in other parts of the world might have epidemiological information on this.

*Booth:* I can't remember having seen parasitic infection in coeliac disease. In tropical sprue we find it, but the morphological change is quite different from that in coeliac disease. If you consider *Strongyloides* infection in man, there are a host of unanswered problems. Firstly, you get an acute reaction with eosinophilia, a type I response and malabsorption, just as you have described. The second type is the endemic one in populations which appear to be immune but where biopsies reveal worms inside the mucosa, nicely wrapped up, and no immune response at all. In between fall patients who may have chronic infections with *Strongyloides* for 10 years and suddenly develop a hyperinfection and die from it.

*Ogilvie:* I would suggest that this is because their cell-mediated immunity is being depressed at that time.

*Pierce:* What is the distribution and class of antibody found on these worms? This might help to elucidate its mechanism of action.

*Ogilvie:* Nematodes have a surface of modified collagen with no cellular structure. This passively absorbs immunoglobulins of a variety of classes. The beginning and end of the gut is also lined with this collagen and has adsorbed immunoglobulin. Nobody knows what antibodies are there.

*Pierce:* These worms have no demonstrated mechanism of attachment, but does that rule out some more or less specific adherence mechanism which might be analogous with the mucosal adherence factors on certain bacteria? And might not mucosal antibody interfere with such an adherence mechanism? I find it difficult to imagine that anything can hang onto the mucosa unless it has some specific adherence mechanism.

*Ogilvie: Nippostrongylus* does not have any obvious adherence mechanism and the worms are found twined between the villi. Even worms such as hookworms which do attach to the mucosa change their position in the gut.

*Lehner:* I think Dr Ferguson might inadvertently have described yet another lymphokine (p. 198)! If this were so, could you reproduce your results using not

the whole cell, the Fc-negative lymphocyte you described, but a soluble mediator prepared from it?

*Ogilvie:* I have tried that kind of experiment, with no success so far.

*Porter:* One of the natural defence mechanisms of the gut is the peristaltic flow. Is there any evidence of stasis preceding infection?

*Ogilvie:* There has been much discussion about this, but the only good experimental study has only just been completed. Castro *et al.* (1976) have shown that the passage of ingesta through the intestine of rats is increased in rats infected with *Trichinella spiralis.*

*Porter:* In pigs with *E. coli* infections, when the animals were weaned onto cow's milk there was substantial stasis of the intestine which would contribute to colonization.

*Schmidt:* Why doesn't the lymphocyte defence mechanism function in lactating rats?

*Ogilvie:* Presumably lymphocyte function is impeded by the direct or indirect action of hormones, possibly resulting in increased cortisol levels which affect the cells. Suppression of cell-mediated reactions at or near parturition has long been known to occur. For example, it has been known for many years by the farming community that if you want to get your cow past the tuberculin test you take her when she is in the early stages of lactation. The farmers knew long before the immunologists that cell-mediated immune reactions are severely depressed early in lactation!

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*Immunology of the Gut*
Ciba Foundation


# Role of the eosinophil

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*Abstract* The gut wall is one of the conspicuous sites of eosinophil accumulation, presumably because of local chemotactic stimuli. It is reasonable to assume that one chemotactic factor is released by the mast cell, which is often found in proximity to the eosinophil. The association of eosinophils and eosinophilia with allergic disorders has long been recognized, and recent work has shown that increased eosinophil production is mediated by the lymphocyte. That process shares characteristics with other immunological actions. An increased rate of eosinophil tissue accumulation and destruction may be the factor which initiates the mechanism for increased production. None of many hypotheses about the 'function' of the eosinophil is substantiated; nevertheless it seems likely that this member of the immunological apparatus, which tends to be distributed in the front line (mucosal and cutaneous tissues), fulfils some normal protective or homeostatic function. Aside from that assumed normal function, there is growing clinical evidence that eosinophils can at times cause host injury, for example in such states as eosinophilic gastroenteritis and endomyocarditis.

Inasmuch as the eosinophil appears to be a component of the immune system, and one of its conspicuous sites of localization is the wall of the stomach and intestine, it seems appropriate that consideration be given to this cell in a symposium on Immunology of the Gut. While eosinophil function cannot yet be described with confidence, much progress has been made within the past decade, and some patterns of its behaviour are beginning to emerge.

The eosinophil is formed in the marrow and circulates briefly in the blood before being deposited in tissues throughout the body. The relative numbers in marrow, circulation and tissues, as estimated from studies in rodents, are of the order of 300–1–300 (Rytömaa 1960; Hudson 1968). It can thus be seen that the number in the circulation at any one time furnishes an inadequate guide to the interplay of processes that accelerate or depress the rate of production in the marrow, or of the local forces that tend to extract the cells from

the circulation. The inaccuracy of blood levels is further compromised by diurnal fluctuations, together with the error involved in the customary clinical method of quantifying these cells by differential count of 100 leucocytes in stained smears of peripheral blood.

In many clinical reports emphasis is laid on changes in the number of circulating cells within a few hours of a given event; this could only be a result of redistribution of preformed cells, and should not be interpreted as a sign of accelerated or diminished bone marrow production. Extensive experience with laboratory rodents indicates that about two days must elapse before an acceleration in the rate of marrow production can perceptibly raise the number of eosinophils in circulation (Spry 1971).

We can say with confidence that this cell differs markedly from the polymorphonuclear neutrophil in its behaviour, despite some resemblances in morphology, phagocytic capacity and enzyme content. Two examples are evident in their responses to adrenal glucocorticoid administration and to acute infection. In both circumstances, blood neutrophils usually increase, whereas eosinophils tend to diminish. Bass showed that these eosinopenic responses are independent, since the eosinopenia of acute infection is not prevented by adrenalectomy (1975*a*,*b*).

## CLINICAL ASSOCIATIONS

Clinicians have long recognized eosinophilia as characteristic of certain allergic states such as drug reactions, asthma and eczema, and have equated the finding of an eosinophil marker—the Charcot-Leyden crystal—with intense local accumulations of eosinophils in the tissues affected. The appearance of eosinophils in response to the application of suspected allergens, in the Rebuck skin window test, has been useful in the diagnosis of allergy to drugs or pollens (Fowler & Lowell 1966).

As to the behaviour of the eosinophil in disorders other than allergies, the literature now amounts to tens of thousands of articles. I have studied a great many of these over the past decade, trying to discern some unifying theme. As is well known, eosinophilia accompanies most infestations by metazoan parasites, some skin diseases, some neoplastic diseases, and a number of poorly understood pulmonary infiltrations. There is no obvious common factor in this peculiar group of associations, beyond such vague suggestions as 'reaction to foreign protein or altered host tissue'. We should note too some rather surprising exceptions. Bacterial and viral infections, certain diffuse skin diseases such as psoriasis, lymphomas other than Hodgkin's disease, serum sickness, necrotizing vasculitis, many granulomatous diseases, infectious mononucleosis,

chronic urticaria, rheumatic fever and graft rejection are all disorders which might be expected to be accompanied by eosinophilia, yet are not.

*The eosinophil in diseases of the gut*

Food allergy may be associated with blood and tissue eosinophilia. The best studies of this subject have been carried out in infants with milk allergy, where the occurrence of increased numbers of eosinophils in blood, as well as local infiltration of gut mucosa in response to milk feeding, has been well substantiated (Waldmann *et al.* 1967; Shiner *et al.* 1975).

Carcinoma of the oesophagus, stomach, pancreas, liver and colon has been associated with blood eosinophilia, as is of course true of cancer arising in other tissues (Isaacson & Rapoport 1946; Banerjee & Narang 1967). Eosinophilia may accompany chronic relapsing pancreatitis (Mullin *et al.* 1968), and has been noted during the course of chronic ulcerative colitis (Wright & Truelove 1966).

The entity of *eosinophilic gastroenteritis* deserves special attention here. This designation applies to a group of syndromes characterized by eosinophilic infiltration of one or more layers of the wall of the stomach or small intestine, together with substantial increases in the number of circulating eosinophils (Klein *et al.* 1970; Greenberger & Gryboski 1973). The syndromes seem to divide into three types, depending on the part of the gut wall affected. If the principal accumulation of eosinophils is in the serosa, the clinical manifestation may be eosinophilic ascites. Infiltration of the muscular coat may cause visible thickening or even local tumour formation, leading to partial obstruction, most often in the region of the gastroduodenal junction. Infiltration predominant in the mucosa may cause protein-losing enteropathy, anaemia, and malabsorption. Leinbach & Rubin (1970) made an intensive study of a young man with this disease. Over a period of months they examined scores of samples of gastric and small intestinal mucosa, which revealed a peculiar patchiness of the eosinophilic infiltrations. Where the accumulations were greatest and nearest the surface, there was loss of overlying mucosal villi. Eosinophils and Charcot-Leyden crystals were found in this patient's faeces throughout the period of observation. In most cases of eosinophilic gastroenteritis where suitable tests have been made, the blood level of IgE has been found to be elevated. Affected patients often have histories of food allergy, asthma and eczema.

## EOSINOPHIL CHEMOTAXIS

Much study has been devoted to eosinophil chemotaxis, using the Boyden

chamber technique, or modifications of it. It is obvious that many materials, some derived from lymphocytes and complement fractions, some from basophils and mast cells, and some from normal or diseased organs, can, under a variety of circumstances, exert strong chemotactic attraction for eosinophils. During the 1960's much interest was shown in the demonstration that certain kinds of immune complex attracted eosinophils, and were ingested by them (Litt 1964). This evidence has been reviewed by Parish (1974) and Kay (1974). One of the first defined substances to be assigned the property of eosinophil chemotaxis was histamine; nevertheless this matter is not yet settled, because studies employing different techniques and different hosts have given inconsistent results or have required concentrations of histamine or eosinophils that may not characterize the living host (Felarca & Lowell 1968; Clark *et al.* 1975). A possible source of confusion is that the mast cell, which is the principal source of histamine, probably also liberates one of the most active chemotactic substances.

## THE RATE OF EOSINOPHIL PRODUCTION IN BONE MARROW

So far, I have focused on the behaviour of eosinophils in tissues. We know from clinical experience that tissue eosinophilia is often accompanied by blood eosinophilia, which in turn must be a reflection of accelerated production of the cells in the marrow. Until recently, nothing was known of the connection between these phenomena—that is, of the nature of the mechanism which increases the rate of production of eosinophils. This subject was investigated by several of us at Oxford between 1967 and 1973. Our experimental model was the blood and marrow eosinophil response to challenge of rats or mice by the nematode *Trichinella spiralis*. Inasmuch as that work has never been summarized, the main findings will now be listed.

In trichinosis the parasite, though encysted in striated muscle, causes prolonged eosinopoiesis in the bone marrow. The stimulus must be transmitted in the blood. Our first step was to look for evidence of a humoral eosinopoietic factor which would stimulate eosinophil production when injected into normal recipients. All tests gave negative results.

Clear evidence was then obtained that circulating lymphocytes played a part in the eosinophilic reaction: (*a*) eosinophilia was obliterated or greatly diminished by injection of antilymphocyte serum, prolonged thoracic duct drainage, or neonatal thymectomy; (*b*) thoracic duct lymphocytes from an animal in the intestinal phase of trichinosis caused eosinophilia when injected into syngeneic recipients; (*c*) the eosinophilic response involved cooperation of lymphocytes and bone marrow cells. Rats given whole-body irradiation and

then reconstituted with lymphocytes alone, or with bone marrow cells alone, failed to develop eosinophilia when challenged; whereas animals reconstituted with both lymphocytes and bone marrow cells did develop eosinophilia when challenged (Basten & Beeson 1970).

In collaboration with A. J. S. Davies and his group, it was shown that thymus-deficient mice, which give normal neutrophil responses to acute infection, showed markedly defective eosinophil responses to trichinosis. Controls reconstituted by grafts of thymus tissue gave normal eosinophil responses to trichinosis (Walls *et al.* 1971).

Much of our work employed a test system in which muscle-stage *Trichinella* larvae, too large to pass through a capillary bed, were injected intravenously. This led to the formation of granulomatous inflammatory lesions containing numerous eosinophils wherever parasites were trapped in the lungs (Boyer *et al.* 1971). In this situation the parasite does not survive, and can be seen to be disintegrating within a day or two. An increase in blood eosinophils is detectable on the second or third day, reaches its peak about the seventh day, and disappears by the tenth to the fourteenth day. This method of producing a sharp burst of eosinophil production by a single injection of foreign material has advantages over previously used systems, nearly all of which involved repeated injections of antigen. It enabled us to examine the bone marrow eosinophil response with tests analogous to those used to study antibody formation. We found, for example, that an enhanced response resulted from a second injection of larvae, resembling a secondary antibody response. Immunological specificity was indicated by the absence of cross-reactivity between the eosinophilic response to dextran beads and that to the parasite larvae (Walls & Beeson 1972*a*).

Another kind of study permitted by the model of intravenous injection of muscle-stage larvae was to test the effect of immunosuppressive agents on the eosinophil response. The results fell in a pattern consistent with that known to characterize the antibody response to a single injection of antigen (Berenbaum 1967). Certain immunosuppressants, such as cortisone and antilymphocyte serum, were effective when given a few hours before the intravenous injection, but had no effect if given 24 hours later. Other agents, such as methotrexate and cyclophosphamide, ineffective before antigen administration, suppressed the eosinophil response if given 24 hours later (Boyer *et al.* 1970). Here was evidence of interference with sequential steps leading to accelerated marrow production.

There was no eosinophil response to intravenous injection of the larval material if the parasites were first ground in a tissue grinder, reducing them to fragments small enough to pass through the pulmonary vessels, presumably to

be arrested by fixed macrophages elsewhere in the body. This refutes an oft-made suggestion that the eosinophilia of parasitic infestations is attributable to some unique constituent of parasites. Our findings, on the contrary, provided evidence that a key factor in the genesis of eosinophilia is the processing of foreign material by cells attracted to its site of lodgement in tissues—that is, in areas other than the reticuloendothelial system (Walls & Beeson 1972*b*).

Kinetic studies of the bone marrow response were made by Spry (1971), who counted labelled mitoses and the relative numbers of labelled cells making up the recognizable stages in marrow development. This revealed quickening of all phases of production, beginning with the earliest detectable precursors. After parasite challenge the mean cell cycle time in the marrow was shortened from 30 to 9 hours. Spry calculated that over a period of six days this could result in an output of mature eosinophils 64 times greater than would be produced in normal unstimulated animals.

Evidence linking the lymphocyte to eosinophil production or function has since come from other laboratories (McGarry *et al.* 1971; Ponzio & Speirs 1974; Schriber & Zucker-Franklin 1975). Colley (1973) has described a lymphokine which stimulates migration of eosinophils in a semi-solid medium. He used an immune system produced by schistosomal infection of mice. Similar results were reported by Kazura *et al.* (1975), also employing schistosomal infection.

## HYPOTHESIS

As things stand at the moment, I feel that the most attractive scheme to link local concentrations of eosinophils with increased blood levels would be one which postulates a feedback system:

Chemotactic factors produced in tissues, from pre-existing cell components or formed during immunologically mediated inflammatory reactions, cause local deposition of circulating eosinophils in the affected tissues. The presence or destruction of excessive numbers of eosinophils affects lymphocytes so that they release a lymphokine which acts in the bone marrow to accelerate eosinophil production. This in turn causes the number of circulating eosinophils to rise.

## THE EOSINOPHIL AND THE MAST CELL

The interrelation between the eosinophil and the tissue mast cell appears to to be of central importance. They are often found in association; the mast cell

elaborates a potent eosinophil chemotactic agent; the eosinophil ingests mast cell granules, and neutralizes histamine, a mast cell product. Very probably another mediator, slow-reacting substance of anaphylaxis (SRS-A), which has been shown to be a product of a close relative of the mast cell, the basophil (Lewis *et al.* 1975), is also part of this functional interrelationship. The mast cell is a source of heparin, and it has been claimed that eosinophils contain plasminogen (Barnhart & Riddle 1963), so they may also collaborate in tissue processes involving coagulation.

Particularly impressive is the evidence that the tissue mast cell can liberate a powerful eosinotactic agent. The chemical structure of this eosinophil chemotactic factor of anaphylaxis (ECF-A) has now been identified by Goetzl & Austen as a tetrapeptide (1975). These workers have also demonstrated ECF-A in the blood basophil. Mann has published photographs of eosinophils clustering about degranulating mast cells and ingesting mast cell granules (1969). The human disease called urticaria pigmentosa is characterized by excessive numbers of mast cells in the subcutaneous tissue. Sufferers are liable to develop urticarial wheals after slight injury to the skin. Serial biopsies in such patients have shown massive influx of eosinophils occurring within fifteen minutes of light stroking of the skin (Prakken & Woerdeman 1952).

The special relationship between eosinophil and mast cell is also emphasized by the occurrence of blood eosinophilia following measures which cause mast cell degranulation. Hungerford (1964) and Fernex (1968) showed that repeated injections of Compound 48/80, a mast cell degranulator, will evoke marked elevation of the blood eosinophil level after four days. The timing and extent of the elevation are consistent with an increase in rate of marrow production. A comparable phenomenon is observed when rats are acutely deprived of dietary magnesium, a procedure which also causes mast cell degranulation. The animals develop a distinctive syndrome within one or two days, characterized by reddening of the skin, obvious itching, and high blood and urine levels of histamine (Bois *et al.* 1963). Within four days they exhibit blood eosinophilia (Hungerford & Karson 1960). Again we appear to be observing this chain of events: mast cell degranulation → eosinophil chemotaxis → accelerated eosinophil production.

## THE EOSINOPHIL AS A MODULATOR OF IMMUNE RESPONSES

Regardless of whether histamine exerts a chemotactic influence, there is good evidence that eosinophils can exert some protection against its toxic effects (Kovacs 1950; Vercauteren & Peeters 1952; Lee 1969). Hubscher (1975) has evidence that the eosinophil accomplishes this by elaborating prostaglandins.

$E_1$ and $E_2$. Much study has also been made of another factor, released during IgE-dependent immune reactions: SRS-A. This agent, which contracts smooth muscle and alters vascular permeability, is inactivated by arylsulphatase, a component of the eosinophil (Wasserman *et al.* 1975). So, a case is developing that the eosinophil can be attracted to a locality where an IgE-mediated immune reaction has occurred, and that it may be capable of modulating the actions of some products of the allergic inflammation. One of its 'functions', then, may be to act as a homeostatic agent, preventing excessive and continuing spread of an inflammatory reaction.

## THE EOSINOPHIL AND TISSUE INJURY

It is not surprising, considering its content of proteolytic enzymes, that the eosinophil seems to be capable of damaging the host under certain circumstances. An interesting example of this was found in the so-called 'Gordon test' introduced in the 1930's, and regarded for a brief period as a specific test for Hodgkin's disease (Gordon 1933). When homogenized lymph node material from patients with that disease was injected intracerebrally in rabbits or guinea pigs, the animals developed a distinctive encephalitis, mainly due to destruction of Purkinje cells of the cerebellum. Subsequent investigation revealed that the test was not specific for Hodgkin's disease, but was simply an indicator of the presence of eosinophils in the lymph nodes, since the same phenomenon could be demonstrated by injecting buffy coat material from patients with blood eosinophilia (Seiler *et al.* 1969).

In clinical medicine, tissue destruction may be observed in sites where eosinophils seem to be the principal invaders. Examples are eosinophilic gastroenteritis, eosinophilic granuloma of bone and Löffler's endomyocarditis. In the last disease there is endocardial thickening due to fibrosis and eosinophilic infiltration, most prominent on the left side of the heart. The destructive process extends into the adjacent myocardium. Reports of endomyocardial fibrosis associated with prolonged high blood levels of eosinophils due to different causes have now appeared (Ive *et al.* 1967; Roberts *et al.* 1969; Borer *et al.* 1973; Brockington & Olsen 1973; Blatt *et al.* 1974; Frenkel *et al.* 1975). The cardiac lesion has been likened to that of carcinoid heart disease where an agent produced by the carcinoid tumour causes endocardial fibrosis (Zucker-Franklin 1971).

## SUMMARY

We seem to be emerging from the period when the role of the eosinophil was

called an enigma. There is a good basis for looking on this cell as a component of the immune system, recruited by lymphocytes in response to increased tissue utilization. One of its function seems to be to act as a modulator of inflammatory responses, notably those involving IgE and mast cell degranulation. In some instances of marked eosinophilia associated with neoplasia or certain other clinical states, the cell may be responding to a 'false signal', due to chance development of chemotactic factors, or of lymphokines which act directly on the bone marrow. A high and prolonged concentration of eosinophils can probably cause local tissue injury.

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## Discussion

*Lachmann:* You discussed evidence of mast cell factors being neutralized by eosinophil factors. Frank Austen has recently added a further factor by reporting that eosinophils contain phospholipase D, which would destroy the phospholipid platelet-activating factor (Kater *et al.* 1976). It is a novel report, since this enzyme is normally associated with cabbages and spinach rather than with mammalian tissue!

Perhaps you or Dr Ogilvie would comment on the work of Anthony Butterworth and his colleagues (1975) showing that the cell that kills the schistosomula of schistosomiasis in allergic reactions is in fact the eosinophil? If this is so, then even if the IgE antibodies and the mast cell are not directly involved in worm expulsion, perhaps they are acting indirectly by attracting eosinophils. Dr Beeson has said that their capacity to produce eosinophilia may be drawing an important effector cell to the site of infection.

*Ogilvie:* It is fair to mention that the first suggestion that schistosomules

die as the result of eosinophil attack was published in histological studies of the fate of *Schistosoma japonicum* in the skin of monkeys made immune by several infections of irradiated cercariae (Hsü *et al.* 1974, 1975). Butterworth *et al.* (1975) in *in vitro* studies of immunity to *S. mansoni* in man found that peripheral leucocytes from people without helminth infections would kill *S. mansoni* schistosomules coated with IgG antibodies from infected individuals, and the functional cell appears to be the eosinophil. These studies are supported by the work of Mahmoud *et al.* (1975*a*) who showed that the immunity of mice to invading *S. mansoni* was obliterated by giving the mice anti-eosinophil antiserum at the time of a reinfection. This phenomenon may be important in many helminth infections. The original observation of eosinophil adherence to helminths coated with IgG antibodies was made by Higashi & Chowdhury (1970) in Bombay, working with human antiserum and the invading stage of the filarial nematode parasite *Wuchereria bancrofti.* Dr C. D. Mackenzie and I became interested in this recently, and we are working on a model using eosinophils and other inflammatory cells from normal rats. Dr Mackenzie has been able to show that there is attachment of rat eosinophils to the surface of various helminths; this attachment is mediated by specific antibody and does not seem to be dependent on complement. A sequence of changes in the morphology of the eosinophils parallels cell attachment and also various forms of damage to the parasites, particularly to *S. mansoni.* It seems from this work that there is selective antibody-mediated attachment of this particular cell type which is deleterious to the parasites. The class of antibody involved in this reaction is of course of much interest; however, so far the antibody has not been identified, but it is unlikely to be IgE (C. D. Mackenzie, unpublished results 1976).

*Beeson:* That is true *in vitro*, but not in tissue sections. We were never impressed that the eosinophil was in direct contact with the parasite, in sections.

*Ogilvie: T. spiralis* may not be a good parasite with which to observe this phenomenon *in vivo.* Recent studies with *S. japonicum* show large numbers of eosinophils around parasites invading the skin of immune monkeys (Hsü *et al.* 1974, 1975).

*Lachmann:* Do you think the eosinophil is what is dislodging *Nippostrongylus*?

*Ogilvie:* As far as the adult stages found in the lumen of the gut are concerned, I think expulsion is not by direct attack as can be shown *in vitro* but perhaps indirectly by, for example, the release of prostaglandins (Hubscher 1975). I believe it is possible that the tissue stages of helminths may be attacked directly by eosinophils/macrophages, etc.

*Rosen:* It needs emphasizing that in Butterworth's system immune serum

must be present for the eosinophils to kill the schistosomula, which raises the question of the demonstration of the Fc receptor on the eosinophil: I believe such has been made?

*Lachmann:* Fc receptors on guinea pig eosinophils have been demonstrated in Robin Coombs' laboratory (Butterworth *et al.* 1976) and on human eosinophils by Tai & Spry (1976). They do indeed have very nice Fc receptors, but they are rather more particular than neutrophils about the species of Ig which they bind. Thus the guinea pig eosinophils do not react with rabbit IgG whereas they do with pig IgG. Normal human eosinophils behave similarly, whereas the immature eosinophils found in patients with eosinophilia have Fc receptors more similar to those of neutrophils and do react with rabbit IgG.

*Rosen:* Does the receptor have the same specificity that the human monocyte has for IgG1 and IgG3?

*Lachmann:* Tai & Spry (1976) have found that it does.

*Rosen:* The human monocyte Fc receptor also has considerable species specificity.

*Pepys:* Human eosinophils and basophils also have C3 receptors (Pepys & Butterworth 1974).

*Ogilvie:* One of the surprising things we have found is that not only do eosinophils (and macrophages and neutrophils) but also mast cells adhere very firmly to the surface of parasites (C. D. Mackenzie, unpublished 1976). Do you think, Dr Beeson, that eosinophils exist in what one might call an 'activated state', as macrophages do?

*Beeson:* There is growing evidence that even in the circulation, in hypereosinophilic states from any cause, eosinophils appear to have been activated, as judged by degranulation and vacuolization. Tai & Spry (1976) and others (Connell 1968; Saran 1973) have been finding in many different kinds of eosinophilia evidence that the cells in the circulation have been engaged in some activity.

*Rosen:* In the work John David did with Butterworth they were able to show that cytochalasin would stop the schistosomicidal activity of the eosinophil. Inhibitors of prostaglandin synthesis or of protein synthesis did not prevent this activity. Obviously, therefore, granule release is necessary for the eosinophil's K cell-like activity.

*Lachmann:* How similar is the schistosomula to *Nippostrongylus* in its structure?

*Ogilvie:* They are totally different. Whereas the surface of a nematode has an amorphous collagen-like structure, the surface of the schistosomula is an ordinary plasma membrane.

*Booth:* Another situation in which eosinophilia is found is fungal infection,

particularly fungal endocarditis. I don't know how that fits in, in relation to the morphological differences between the infecting organisms.

*Beeson:* That is the exception. In terms of what are commonly thought to be the infections, fungal infections and even tuberculosis may sometimes be the cause of an eosinophilic response.

Our studies in animals and reports in the clinical literature suggest that a granulomatous type of response is one of the keys. The pathology associated with eosinophilia is a granuloma. It is an occasional response to fungal infection, just as it is an occasional response to cancer. Out of ten cases of coccidioidomycosis, only one will show marked eosinophilia, and the rest will show none.

*Mayrhofer:* It is possible to adsorb antigens onto particles such as bentonite, inject those and produce granulomata in the lungs, and I wondered whether this produced eosinophilia and whether it would be a good model for demonstrating specificity?

*Beeson:* Schriber & Zucker-Franklin (1975) have done exactly that with better-defined antigens than we were using. They used human gamma globulin on a latex particle large enough to be arrested in the lung and obtained an eosinophilic response, a secondary response to a second injection, and no secondary response to a different antigen.

*Ogilvie:* Mahmoud *et al.* (1975*b*) have shown in studies of the granulomata that occur in the lungs of animals given an intravenous injection of schistosome eggs that about half the cells are esoinophils. Both eosinophils and enormous numbers of mast cells or basophils are also found at the site of ectoparasite attachment on the skin. Allen (1973) and B. Bagnall (personal communication 1975) showed that guinea pigs infested with ticks have massive reactions consisting of eosinophils and basophils at the site of the bites, and the ticks seem to die of an overwhelming basophilic diarrhoea!

*Beeson:* Humans with scabies also show eosinophilia.

*Evans:* I did not agree with your inclusion of necrotizing vasculitis among the conditions which do *not* produce eosinophilia, Dr Beeson. It is not uncommon to see cases of Wegener's granulomatosis who have both necrotizing vasculitis and a severe degree of eosinophilia.

*Beeson:* If you look at all cases of necrotizing vasculitis, only those with pulmonary involvement develop eosinophilia. Patients with peripheral neuropathy and other manifestations are not likely to have an associated eosinophilia.

*Lehner:* Other granulomas, such as histocytosis X and eosinophilic granuloma, are very rich in eosinophils. We don't know the aetiology of histocytosis X at all. What part do these large numbers of eosinophils play?

*Beeson:* I can only postulate that some eosinophilotactic factor is being produced as the initial event. Histocytosis X is a completely mysterious disease.

*White:* I am impressed by the large number of new factors you mentioned which attract eosinophils. Where does C5a stand now? Is this just a general granulocyte chemotactic factor?

*Beeson:* Other complement fractions have been shown to have some effect but C5a seems to be the strongest chemotactic factor.

*White:* It is not particularly specific for eosinophils; it also attracts granulocytes.

*Beeson:* In the Boyden chamber, yes. Neutrophils come through, but C5a seems to have a preferential attraction for eosinophils.

*Gowans:* What is the latest information on how lymphocytes tell the bone marrow to make more eosinophils? Is the idea that large T lymphocytes or their progeny migrate into the bone marrow and exert their effect locally?

*Beeson:* Spry (1970) looked with labelled large lymphocytes. They were found in bone marrow, but not preferentially. We put a large number of thoracic duct lymphocytes in a millipore chamber in the peritoneum of a rat. This appeared to stimulate eosinophilia in a normal rat, as if the lymphocytes were liberating a lymphokine, but we could never find it in the blood in a sufficient quantity to be recognizable.

*Gowans:* Do you find increased numbers of T lymphocytes in the bone marrow in cases where there is either a profound eosinophilia, or an accumulation of eosinophils in the tissues?

*Beeson:* We have never looked for evidence of that.

*Davies:* How would one look for T lymphocytes in human bone marrow?

*Pepys:* There are considerable problems in demonstrating T lymphocytes in human tissue satisfactorily, since T cells in cryostat sections seem no longer to bind sheep erythrocytes. However, T cells have been stained with anti-T cell antibodies in sections of human gut and liver (Husby *et al.* 1975; Strickland *et al.* 1975; Meuwissen *et al.* 1976). Specific anti-T serum could therefore be used on smears of bone marrow cells, or cell suspensions from bone marrow might be prepared and tested for other human T cell markers.

*Rosen:* There are no E rosetting cells or PHA-responsive cells in human bone marrow (Geha *et al.* 1974).

*Seligmann:* You would probably need to exsanguinate the subject in order to be sure you were dealing with bone marrow cells!

*Pepys:* I would like to draw attention to the possible connection between the T-cell mediation of eosinophilia and the T-cell dependence of IgE antibody production. We have looked at the effect of complement depletion in mice on the induction of IgE antibody production (Pepys *et al.* 1976, 1977; Pepys 1976). Mice

primed at six weeks of age with alum-ovalbumin produced neither IgE antibody nor eosinophilia, but, after a booster dose of antigen four weeks later, they formed high titres of IgE anti-ovalbumin antibody and developed a considerable but transient eosinophilia. The eosinophilia was greatest at the peak of the IgE response. Animals which were depleted of complement for four days or so at the time of priming failed to produce IgE antibody after the booster and also did not develop eosinophilia. Since *in vivo* complement depletion suppresses T-dependent antibody responses without affecting T cell activation and activity (Pepys 1976; Rumjanek *et al.* 1976), this suggests that the T dependence of eosinophilia may reflect the T dependence of IgE antibody production, rather than a direct effect of T cells or a T cell lymphokine.

The mechanisms by which IgE antibody might mediate eosinophilia are not clear, but IgE-dependent antigen-specific mast cell and basophil degranulation release the eosinophil chemotactic factor of anaphylaxis (ECF-A) and other active substances. It is not known whether ECF-A or other mast cell products can stimulate eosinopoiesis as well as local eosinophil accumulation, but this is being studied (K. F. Austen, personal communication).

*Beeson:* Judged by studies done in Japan and elsewhere, one does not always find an elevated level of IgE in parasitic infestations (Takenaka *et al.* 1975).

*Gowans:* Dr Beeson made a distinction between agents that attract eosinophils into tissues and agents which tell the bone marrow to make more of them. They are not necessarily the same.

*Lachmann:* Dr Rosen, do children who are agammaglobulinaemic but have normal T cell function develop eosinophilia in response to parasitic infection?

*Rosen:* They do, and children with severe combined immunodeficiency who have no T cells also develop marked eosinophilia: it is one of the characteristics of the disease. But I cannot be certain that it is not due to transplacental passage of maternal T cells.

*Davies:* One should remember the example of the polymorph where, as Blanden and his colleagues have shown, certain kinds of neutrophilic infiltrations induced by pox viruses are T cell-dependent in the broad sense that an animal that has no T cells, or only a few, will not get granulocytosis (Blanden 1974). On the other hand, the *E. coli*-induced granulocytosis (Walls *et al.* 1971) is completely thymus-independent, in that an animal with no T cells will develop if anything a slightly better granulocytosis. Thus no firm statement can be made about the neutrophil, as it has many functional properties, some of which are T cell-dependent, and others not.

*Ferguson:* In humans with graft-versus-host disease after bone marrow transplantation, blood eosinophilia is a constant and striking finding. Presumably that is a thymus-dependent reaction.

*Rosen:* It is.

*Vaerman:* I recall that the major protein of the granules of eosinophils is very rich in sulphydryl groups.

*Beeson:* Gleich *et al.* (1974) describe it as a very basic protein that is rich in SH groups. The possibilities of ways in which this material, which is unique to eosinophils, may affect the functioning of other cells are many, and this is one of the exciting areas for future research.

*Lachmann:* The reaction of heparin and highly basic compounds like protamine has been shown by Gewurz and his colleagues (Rent *et al.* 1975) to be strongly complement-activating. This has tended to be regarded rather as a pharmacological reaction, but one can picture that a heparin molecule from the mast cell reacting with a highly basic protein from the eosinophil in the extravascular space may also be able to recruit the complement system in inflammatory reactions where this occurs.

*Brandtzaeg:* If I may revert to Dr Mayrhofer's experiments with regard to the mast cell's potential for producing IgE (pp. 155–175), there are mucosal mast cells in normal intestine in fair numbers. Could the sections be incubated with serum from infected animals?

*Mayrhofer:* One could try that, or attempt to show concentration of IgE into mast cells *in vivo* after intravenous infusion of myeloma IgE. I have not done either.

*Soothill:* Dr Mayrhofer, can you give us an idea of how much IgE you are talking about in these tissues? It looks a lot in the micrographs. When a child develops allergy he becomes positive to prick tests before we can detect IgE in his serum. We postulated that all the IgE being made is taken up on the mast cells. I would like to have some idea how much IgE the mast cells take up and how long it stays.

*Mayrhofer:* I don't know the answer to that. The binding of IgE to the surface of skin mast cells sensitizes them maximally for antigen-induced degranulation for a week or ten days and then the sensitivity wanes over the course of weeks. I do not know how much IgE is present in these mucosal mast cells, except that their fluorescence is roughly comparable to that of the IgE-secreting plasma cells found in the mesenteric lymph nodes of infested animals.

*Vaerman:* Ishizaka & Ishizaka (1975) have claimed that mast cells are able to fix about $10^6$ molecules of IgE when fully saturated with myeloma protein.

*Soothill:* If we know how many cells, we can calculate the amount per gram of tissue.

*Vaerman:* The affinity of basophils or mast cells for IgE was very high.

*Lachmann:* I am worried about the idea that IgE passively taken up by mast

cells is then excreted, in view of the findings that IgE binding to mast cells persists, certainly in the skin, for weeks and that its affinity appears to be so high. Are you suggesting either that whole mast cells come out, or that there is some proteolytic process stripping the IgE from the cell surface?

*Mayrhofer:* I can see fluorescent mast cells between the epithelial cells of the gut and the respiratory tract. It is possible that these cells are merely shed into the secretions, liberating their IgE by autolysis or the action of digestive enzymes. However, if the IgE is actually contained in the cytoplasm, it seems possible that it might be released along with histamine and heparin when the cell encounters antigen.

*White:* It seems that Dr Mayrhofer has described a very particular mast cell. How do such cells relate to so-called globule leucocytes? They have been regarded as spent mast cells. Do you see eosinophils around globule leucocytes?

*Beeson:* Yes. Mann (1969) has beautiful pictures of five or six eosinophils around the degranulated mast cell.

*Mayrhofer:* The evidence that mucosal mast cells become globule leucocytes is not direct. The two cell types have similar staining properties. Globule leucocytes tend to contain less amines (Miller & Walshaw 1972) and under the electron microscope they tend to have more empty vacuoles than mast cells lying in the lamina propria (Murray *et al.* 1968) and for these reasons may be cells that have discharged some of their contents. However, there is no direct evidence that mast cells migrate from the lamina propria into the epithelium.

There is a third cell in the intestine, whose relationship with the two other types mentioned above is also unknown. Globule leucocytes are described in the epithelium of the crypts and the bases of villi, with very few near the villi tips. However, if one looks at the gut of normal rats, there are cells in the epithelial layer of the villus, extending to the tips, which look like intra-epithelial lymphocytes but which contain small numbers of Alcian-blue positive granules. The origin and fate of these cells is quite unknown.

*Vaerman:* Sometimes one sees eosinophils which phagocytose granules from the mast cells that have liberated these granules, and it has been said that this phagocytosis activates the eosinophil, which then liberates its factors, especially the arylsulphatase for the neutralization of SRS-A from the mast cell.

*Beeson:* This may be similar to the phenomenon that Shelley (1962) described of how to detect drug sensitivity by special stains of peripheral blood. He said one could find degranulated basophils as evidence of an immunological reaction going on.

*Lachmann:* Would anyone like to suggest what the physiological function of IgE is, particularly in the gut where all these IgE-containing cells are found?

*Ogilvie:* The original hypothesis suggested by Barth *et al.* (1966) that IgE

has a 'gate-opening' role still holds. There is no evidence against the idea that a function of IgE is to increase the passage of other immunoglobulins across the mucosal surface. There is evidence from other parasite systems that an anaphylactic reaction can sometimes exacerbate other immune responses. The classic example in helminths is the so-called 'self-cure' where, if an animal carrying a high burden of certain nematodes suddenly takes in a large secondary burden of that parasite, the previously established worm burden disappears within twenty-four hours. This reaction has all the characteristics of an immediate hypersensitivity reaction. In this situation, however, you do not always get protection, because very often the secondary load of parasites which apparently triggers the expulsion of those worms already in the host then takes the place of the expelled population.

*Booth:* That is an interesting contrast to what happens to bacteria in the gut. If you have an existing bacterial load which is non-adherent, for example in the stagnant loop syndrome, and you put in a particular strain, it is eliminated very quickly by the resident population (S. Tabagchali & S. L. Gorbach, unpublished observations).

*Soothill:* Dr Ogilvie, are you saying that you think self-cure may be mediated by IgE, but that you are not too sure how useful it is? I think that you have previously suggested that it is useful, but not an IgE effect.

*Ogilvie:* Unfortunately there are two quite different types of worm rejection, both of which have been described as a 'self-cure' reaction, but only one of them reflects the development of resistance to reinfection. What is known as classical self-cure was first described by Stoll in 1929 and in more detail by Stewart (1953) in sheep and it is in this situation that an incoming secondary infection may induce an anaphylactic reaction which expels established worms without inducing protection to further infection, as just described. Unfortunately, some authors also describe the termination of a *Nippostrongylus* infection as a 'self-cure' reaction too. In this case there is protection because the immunity generated during this process is very strong and long-lasting; the rats are immune for months. It is in this last mechanism that it has not yet proved possible to demonstrate a role for IgE antibodies.

*Booth:* How similar are parasites in the gut in terms of antigenicity? Are there large numbers of different antigenic determinants in different organisms, or in one particular parasite load are they all derived from the same original parasite so that they are all antigenically identical?

*Ogilvie:* In one particular parasite load, worms would not be derived from a single worm; in general, worms do not multiply within their host as bacteria, viruses and protozoa do. Different worm species have many antigens in common (Capron *et al.* 1968) and in fact it has proved difficult to detect

species-specific antigens, so that even now serological tests for helminth parasites are incapable of identifying a worm infection as far as the species level.

*White:* One wonders how far the proper 'self-cure' reaction in *Haemonchus* infestation is an effective contribution to immunity. Is it associated with inflammation in the gut wall?

*Ogilvie:* Yes; inflammation does occur in *Haemonchus* infection.

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*Immunology of the Gut*
Ciba Foundation


# Genetic and nutritional variations in antigen handling and disease

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*Abstract* Low function (deficiency), within the 'normal range', of each of five immunity functions is associated with immunopathological disease, and/or defective antigen handling. These are probably genetically determined, either polygenic or single gene, but environmental factors such as diet influence them greatly, and the vulnerability may be especially great in the newborn period. The relevant systems are those involved in the immune elimination of antigen (antibody and macrophages) and those possibly involved in the immune exclusion of antigen (IgA, the alternative pathway of complement, and cilial action). The gut has an especially complicated role in antigen-handling, and feeding influences its capacity to do so. Eczema was prevented by a regimen of neonatal antigen avoidance, which was largely breast-feeding, and it is likely that other immunopathological diseases result from antigen contact during periods of malnutrition. The mechanisms of such effects are likely to be complicated, but adjustment of the environment to suit the genetically vulnerable, particularly in the newborn period, can lead to the prevention of disease.

When immunity mechanisms react with antigen in the tissue, damage occurs, but usually antigen is eliminated and the reaction is terminated. The high incidence of immunopathological diseases in patients with immunodeficiency led to the concept that the mechanism which causes the damage may in itself be normal, but may be overstimulated by excessive antigen contact, because another mechanism had failed to eliminate or exclude the antigen (Soothill 1976*a*). This contrasts with the more orthodox theory that such disease results from primary overactivity of the damaging mechanism. There is now considerable evidence to support this view, and it leads to quite different approaches to prevention and treatment.

## MECHANISMS OF ANTIGEN EXCLUSION

Antigen exclusion is particularly important in the gut, but it also occurs in the

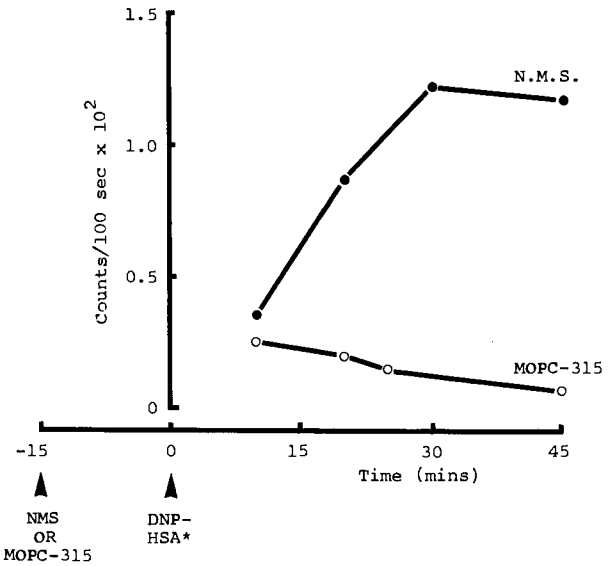


FIG. 1. Radioactivity in the serum of two rats injected into the trachea with $^{125}$I-labelled DNP-HSA (DNP-HSA*) at time 0. Fifteen minutes previously the rats had received (also by intratracheal injection) either mouse serum containing MOPC-315 IgA myeloma protein (○) or normal mouse serum (●).

respiratory tract and skin. Though the skin and mucus membrane barriers (cell layer, cilia and mucus) are the main mechanisms, the adaptive immune response plays a part. Lippard *et al.* (1936) showed that when a child first drinks cow's milk (at whatever age), first cow's milk antigen and then antibody to cow's milk is detected in his blood. This change could be an effect of either immune exclusion or immune elimination of absorbed antigen, but Walker *et al.* (1972) have shown by studies with everted gut sacs that the effect is partly one of immune exclusion. Extracts of gut wall will produce the effect (André *et al.* 1974) but the precise mechanism is not established in the gut; IgA, the immunoglobulin principally secreted in the mucus, can achieve immune exclusion in the respiratory tract (Stokes *et al.* 1975) (Fig. 1) but it is likely that other antibodies can do so too.

## MECHANISMS OF IMMUNE ELIMINATION

Antigen is eliminated by antibody, complement and phagocytes, and it is likely that defective function of one of these, primary (genetic) or secondary, could lead to defective elimination.

TABLE 1

'Immunodeficiency' within the normal range (>5% of the population) which contributes to vulnerability to immunopathological disease

| *Antigen specificity* | *Mechanism* | *Deficiency* | *Environmental influence* |
|---|---|---|---|
| Specific? | IR genes? | HLA-related | – |
| Non-specific | Antigen exclusion | IgA(transient) | Infant feeding, birth season |
| | | Complement (alternative pathway) | Infant feeding?, birth season? |
| | Antigen elimination | Low affinity antibody | Nutrition, infection |
| | | Macrophage clearing (PVP) | Nutrition, infection |
| | | Cystic fibrosis gene | |

## COMMON IMMUNODEFICIENCY UNDERLYING IMMUNOPATHOLOGY

Most of the recognized immunodeficiency diseases are rare and inherited by single abnormal genes, but these are a minority of the real problem. Immunopathology is so common (e.g. the highly familial phenomenon of atopy occurs in 16% of British children [Godfrey & Griffiths 1976]), that the variation underlying it is probably polygenic. We have studied the variation of certain immunity functions, selected because poor function would lead to defective antigen handling, and/or immunopathology. In Table 1 five such functions are listed, poor function of which occurs in more than 1 in 20 of the population (i.e. within the 'normal range'). These defects, whether of the adaptive immunity mechanisms or not, are all non-specific, in contrast to the IR (immune response) gene theory of Benacerraf & McDevitt (1972) which has led to the search for linkage between certain immunopathological diseases and certain HLA types. There is another fundamental contrast between these two approaches. Most of the systems that we are studying can be strongly influenced by environmental factors, so prevention is possible, whereas we cannot change our HLA type.

In a prospective study of the development of allergy in infants of allergic parents we showed that the development of infantile eczema and positive prick tests to common antigens was preceded by transient IgA deficiency (Fig. 2) (Taylor *et al.* 1973; Soothill *et al.* 1976). Perhaps the allergy results from defective antigen exclusion by IgA, perhaps from another immunodeficiency associated with but independent of IgA, or perhaps from differences of gut flora (see below, p. 231). However it works, since the deficiency is transient it is possible that avoidance of the damaging environment in the brief period of

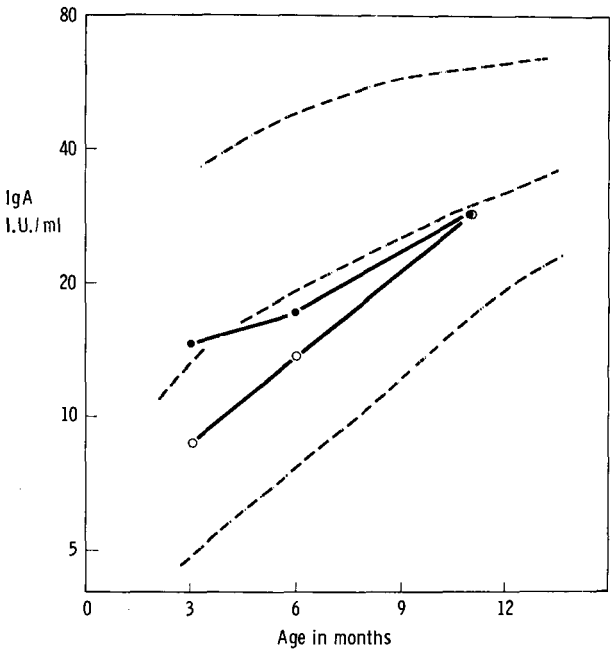


FIG. 2. Log mean values of serum IgA during the first year of life in infants of reaginic parents. ○, those with evidence of atopy. ●, those without. Means ± 2 S.D. for healthy British children reported by Hobbs (1970) are approximately indicated.

deficiency, and therefore of presumed vulnerability, might prevent disease; the results of a prospective study of this are encouraging for the prevention of eczema (preliminary results quoted by Soothill 1976*a*). If IgA works by activating the alternative pathway of complement, it is not surprising that a defect of opsonization for yeast phagocytosis (Miller *et al.* 1968), related to defective activation of the alternative pathway of complement (Soothill & Harvey 1976*a*), is associated with allergy (Soothill & Harvey 1976*b*). It was first thought to be rare, but we find this function defective in about 5% of the population and it contributes considerably to recurrent infection. The defect appears to be inherited in an unusual dominant way, and most affected individuals have only minor symptoms, so it is likely that environment can influence the effects greatly, perhaps particularly in the neonatal period. The effects of the defect, including severe diarrhoea, can be greatly improved by plasma infusion. The other surface defect, also resulting from a single gene defect, is heterozygosity for the cystic fibrosis gene, which includes again about 5% of people. Their sera have a factor which disrupts ciliary function, so we thought that this might be associated with defective antigen clearance, and so

allergy. The incidence of allergy is significantly raised (Warner *et al.* 1976), confirming the importance of surface antigen handling in immunopathology, though the nature of this defect does not point to preventive treatment.

Though it is possible that defective antigen elimination may underly the high incidence of immunopathology in patients with defects of individual complement components, none are common enough to be included in our list of defects within the 'normal range'; it is possible, however, that the defect in the alternative pathway of complement may operate at this level, rather than by immune exclusion. We have established two such systems in mice at other stages of the process, which are interrelated but perhaps partly independent. Inbred mice strains differ in the affinity of their antibody response to unadjuvantized soluble protein antigens on an antigen non-specific basis (Soothill & Steward 1971), and this is related to their capacity for immune elimination of antigen (Alper *et al.* 1972) (Fig. 3). Breeding suggests that this characteristic is transmitted by polygenic inheritance (Katz & Steward 1975). This is a different system of variation from the polygenically inherited variation of titre of agglutinating antibody, demonstrated by Biozzi *et al.* (1975) by genetic selection; it is uncertain how much variation in agglutinating antibody response there is in unselected individuals, but presumably it does vary. It is interesting that the low responders in this system survive longer than the high responders.

Animals making low affinity antibody with antigen in saline, produce high affinity antibody and achieve effective immune elimination when immunized with antigen in adjuvant (Fig. 3), so we suspected that the relevant function is the cooperation system—presumably macrophages and/or T lymphocytes. Carbon clearance and blockade studies confirmed this link (Passwell *et al.* 1974*a*) and a new macrophage function test, the clearance of polyvinyl pyrrolidone (PVP), showed identical ranking with affinity of antibody (Morgan & Soothill 1975) (Fig. 4). Though, for each strain, immunized mice clear PVP faster than immunized mice, an immunized CBA mouse clears PVP no faster than an unimmunized Ajax mouse, and the effect of immunization on the latter is far greater (Fig. 5). A method of measuring antibody affinity suitable for applying to man has still to be developed, but PVP clearance can be measured in man, and such differences are found.

So genetically determined differences in antigen exclusion and elimination exist, and some which can be tested are related to immunopathological disease. It is likely that all are, and that they contribute to local gut damage produced by any of the mechanisms listed by Coombs & Gell (1975). This theory would anticipate their participating together, which indeed they do. There is evidence that gut damage may be mediated by IgE, by complement activation (Matthews & Soothill 1970), and perhaps by cell-mediated responses (see Ferguson &

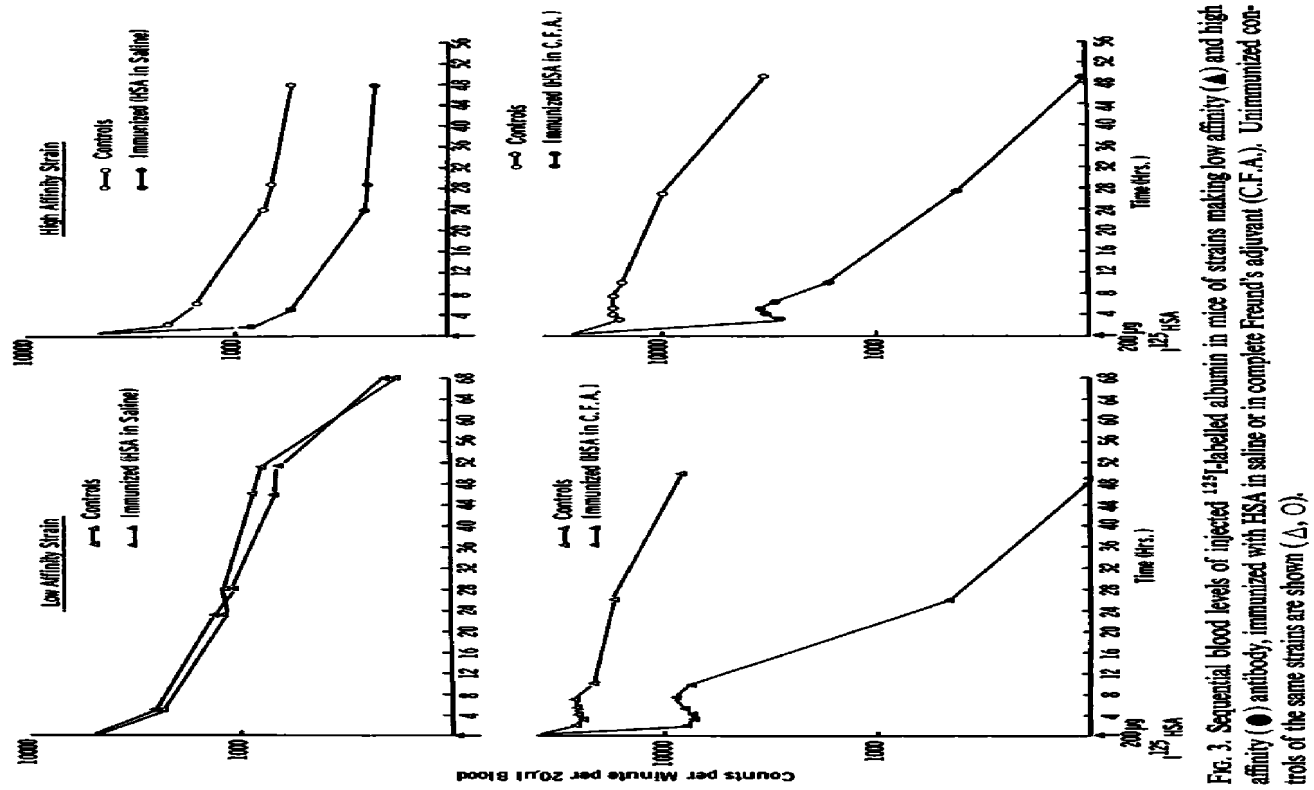


FIG. 3. Sequential blood levels of injected $^{125}$I-labelled albumin in mice of strains making low affinity (▲) and high affinity (●) antibody, immunized with HSA in saline or in complete Freund's adjuvant (C.F.A.). Unimmunized controls of the same strains are shown (△, ○).

MacDonald, this volume pp. 305–319). Dissemination of absorbed food antigens or complexes also leads to distant damage (e.g. eczema). The normal mechanism of avoiding such damage may be not only effective immune exclusion and

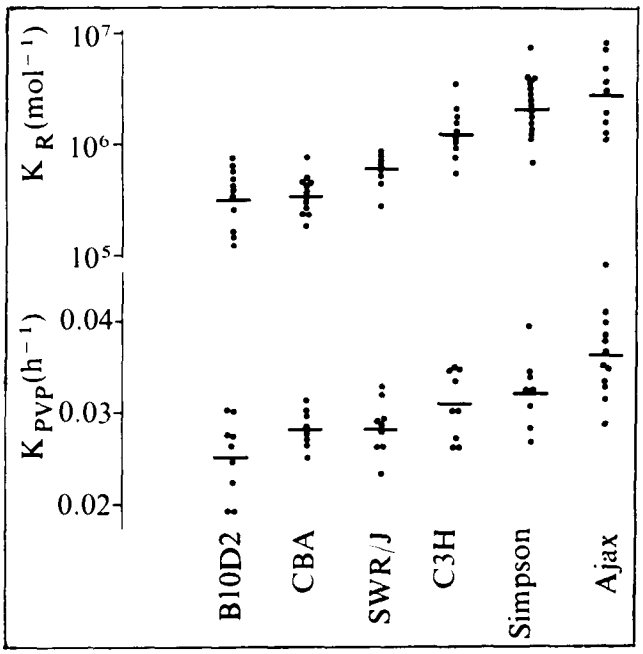


FIG. 4. Clearance of polyvinyl pyrrolidone ($K_{PVP}$) in mice of six inbred strains, compared with affinity ($K_R$) of antibody for HSA.

elimination of antigen, but also the partial tolerance sometimes induced by eating antigen (Chase 1946; Thomas & Parrott 1974). Disease would be expected to result from defects in any of these functions.

## ALIMENTARY INFLUENCES ON ANTIGEN HANDLING

We undertook our study of the role of transient IgA deficiency and of artificial feeding in the development of childhood allergy with the immune exclusion hypothesis in mind, but there are many other possibilities. Perhaps the transient IgA deficiency parallels a transient deficiency of suppressor T cells, or might lead to an uncontrolled, relatively adverse flora which might affect the response to swallowed antigens in a relatively immunodeficient child. The faeces of breast-fed infants grow mainly bifidobacteria, and those of bottle-fed babies mainly *Escherichia coli*. Perhaps *E. coli* growth is more uncontrolled and adherent to the mucosa of the relatively IgA-deficient infant than that of the healthy infant, so that damage is done, and endotoxin gets in, to adjuvantize

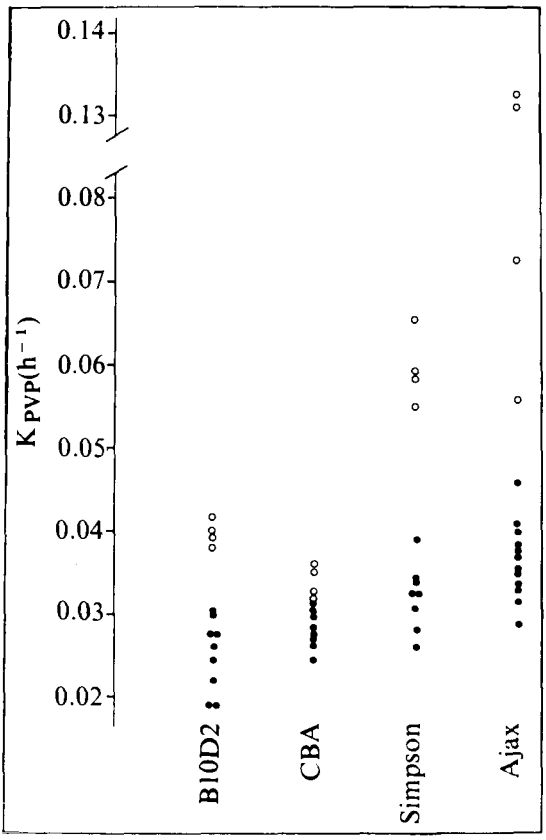


FIG. 5. Clearance of polyvinyl pyrrolidone ($K_{PVP}$) in normal mice (●) and mice immunized with 1 μg PVP (○).

absorbed swallowed antigens. The development of allergy in the infants with the opsonizing defect may result from any of these systems, or they may fail to activate the alternative pathway of complement by endotoxin, and therefore to eliminate it quickly too.

Apart from damage to the genetically vulnerable individual by suboptimal early feeding, there are other ways in which feeding can influence the relevant immunity functions. Many immunity mechanisms are defective in malnutrition (Chandra 1976; Soothill 1976*b*). Isocaloric protein or individual amino acid deprivation reduces the affinity of antibody (Passwell *et al.* 1974*b*), and the macrophage function, as measured by PVP clearance (Coovadia & Soothill 1976) (Fig. 6). The effect of protein malnutrition on macrophages is rapidly reversed by refeeding, as is the transferrin deficiency—also of immu-

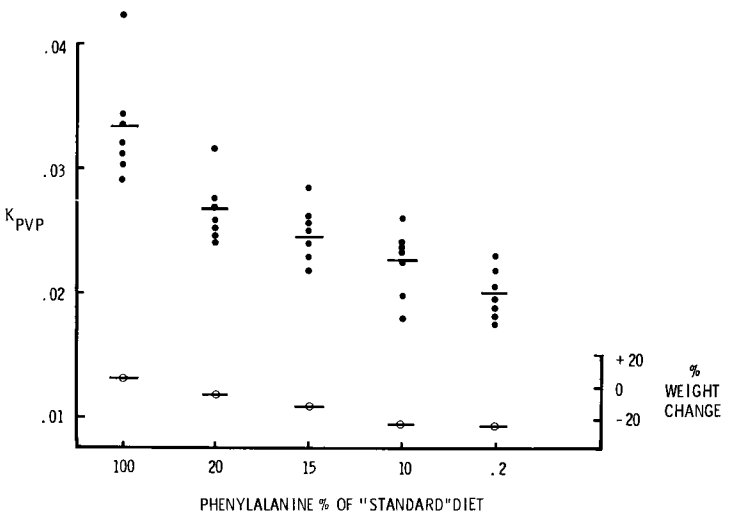


FIG. 6. Effect of dietary phenylalanine restriction on polyvinyl pyrrolidone clearance ($K$) and weight change in male Ajax mice.

nological significance (Antia *et al.* 1968). But the T cell deficiency persists for years (Chandra 1976), and may well influence cooperation in the antibody response, and so immune elimination of antigen. We are at present investigating another possible sustained effect of transient malnutrition on antigen handling. Animals genetically prone to produce low affinity antibody to antigen in saline produce high affinity antibody when their primary immunization is with antigen in adjuvant (Soothill & Steward 1971): if they are given antigen in saline first, they continue to make low affinity antibody even after subsequent administration of antigen in adjuvant—the 'doctrine of original sin' (Steward *et al.* 1974). We are investigating whether first contact with antigen at a time of protein deprivation has a long-term effect on the affinity of the antibody response to it, even after a return to a normal diet. If it is so, it has important implications for infant feeding, and possibly for the effects of secondary malnutrition due to gut disease.

## IMPLICATIONS FOR GUT DISEASE AND ALIMENTARY ALLERGY

It is likely, therefore, that variation in the capacity to exclude or eliminate food antigens occurs, and that it contributes to both local and generalized disease. The incidence of allergy to foods is uncertain, and the only definite basis for diagnosis is the three-fold cycle of remission and exacerbation with

withdrawal and reintroduction of the food described by Goldman (1976), which is not always applicable. Food allergy is particularly a problem in young children, who often grow out of it (presumably by the deficient mechanism maturing), and contributes to such very common symptoms as eczema, diarrhoea and infantile failure to thrive, though it is not known how often this occurs.

The role of primary malnutrition in a wide range of infections is recognized, and it probably contributes to immunopathological disease too. But demonstrated sensitization to food antigens is not necessarily damaging. Chandra (1976) has shown a high incidence of precipitating antibodies to cow's milk antigens in the sera of children with kwashiorkor, and they get better if fed cow's milk. Presumably, though exclusion was defective as a result of secondary immunodeficiency, elimination was adequate. But it is likely that in the genetically predisposed, damage might occur. Perhaps as well as malaria and other infections, malnutrition contributes to the high incidence of nephritis in developing countries, and better feeding will prevent much of it before malaria eradication is possible. The same factors could operate for absorbed food antigens.

Secondary malnutrition in chronic gut disease leading to defective antigen handling in the genetically predisposed could contribute to the distant manifestations of inflammatory bowel disease—arthritis, vasculitis, and so on—as well as the development of anti-food antibodies (Taylor & Truelove 1961). But the complexity of such hypotheses is illustrated by the problem of ulcerative colitis. Acheson & Truelove (1961) reported that patients with ulcerative colitis had been on artificial feeding in infancy at a time when this was unusual, but this was later discounted when their hypothesis of cow's milk allergy, based on the detection of anti-milk antibody, became less clear, when it was shown that such patients have antibodies to many food antigens. It is possible that the food antibodies are an irrelevant effect of both primary and secondary defects of immune exclusion of antigen, and that the disease results from a damaging gut flora becoming established in an infant with transient relative immunodeficiency, whose gut flora was poorly controlled because of artificial feeding in infancy. Perhaps a damaging cross-reacting auto-allergic sensitization, such as that described by Perlmann *et al.* (1967), occurs as a result of a poorly controlled *E. coli* flora, due to artificial feeding in an infant with transient immunodeficiency. Since immunodeficiency is associated with so many effects, the pathogenetic mechanism causative in each particular disease cannot be established retrospectively. Only prospective studies such as ours in infantile allergy (Taylor *et al.* 1973; Soothill *et al.* 1976; Soothill 1976*a*) can answer the questions and establish how to prevent the diseases. Since I think that infant feeding underlies so many chronic late effects, we intend to look for these, as

well as the more acute effects of infantile allergy and infection, in a large prospective study that we are planning.

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## Discussion

*André:* I have some data on transient IgA deficiency in adult allergy. It is known that infection of the gut with *Giardia lamblia* results in a reduction in IgA synthesis by the plasma cells associated with a reduction in the number of IgA plasmocytes in jejunal lamina propria and in the external secretion of IgA (Popović *et al.* 1974). I confirmed that fact in five patients (C. André, unpublished work 1975), using Dr Brandtzaeg's method (Brandtzaeg *et al.* 1974), and observed that in such patients there is a decrease in the density of IgA plasma cells and an increase in the density of IgE plasma cells. After treatment, the number of IgA cells increased and that of IgE cells decreased. One of these patients was suffering from asthma and after treatment for *Giardia lamblia*, this trouble disappeared.

*Brandtzaeg:* I did not know about this general phenomenon of *Giardia* suppressing local IgA production. I have only had the opportunity to look at one case and the gut of this patient was crowded with IgA-producing cells.

*Evans:* According to your hypothesis, Professor Soothill, would you not expect a variation in the incidence of hay fever according to birth date? Presumably if a child is born after the antigen has gone he is not exposed to it until he is about a year old.

*Soothill:* Table 1 (p. 227) did in fact claim an effect of birth season. It is apparently not pollen that matters but dermatophagoides (Soothill *et al.* 1976). There is variation of incidence of allergy with birth date and if you want to avoid allergy, and are an allergic family, take the contraceptive pill for Christmas and the New Year! Allergy is most likely with birth in September and October. Is this an effect of early contact with dermatophagoides or is it an effect of meeting respiratory syncytial virus at the time of the physiological trough in IgG, at three months? I don't know, but whatever the mechanism, using the pill at the right time would reduce the incidence of the disease.

*Brandtzaeg:* There are two questions in relation to the mechanism of antigen exclusion by IgA. In your patients with transient IgA deficiency, you apparently think that there is a strict relation between serum IgA, which you measure, and secretory IgA.

*Soothill:* The concentration of secretory IgA at three months was not related to the subsequent development of allergy, but secretory IgA levels rise more quickly than those of serum IgA and I believe we should have measured it in the first few days of life. From the clinical point of view, I do not mind what the mechanism is. The point is that the deficiency preceded the illness. Is it IgA deficiency which matters or is the transient IgA deficiency related to another transient deficiency, perhaps of a suppressor T cell population, for example? That is an equally good explanation of the phenomenon. I am not committed to the immune exclusion hypothesis as an explanation of the phenomenon; but we have shown that allergy follows, and therefore presumably results from immunodeficiency, and that the effect of that deficiency can be manipulated by appropriate handling, namely breast-feeding.

*Brandtzaeg:* There are data indicating that rather mature intestinal IgA levels are reached as soon as 1–2 months of age (Haneberg & Aarskog 1975).

*Soothill:* Yes. We probably just looked too late.

*Brandtzaeg:* On the model of immune exclusion in mice in the respiratory tract, you specified IgA, but I think you indicated that IgG may do the same thing. The specific feature of IgA is that it gets out, to do the job.

*Soothill:* Exactly. We are interested in what IgA does because it is in the right place. It *can* achieve this exclusion; whether it is important, I do not know.

*André:* I have used the same experimental model in the gut of BALB/c mice as you use in the respiratory tract of the rat (Stokes *et al.* 1975). The absorption of DNP-conjugated $^{125}$I-labelled human serum albumin was measured *in vivo* and *in vitro* on everted gut sacs. MOPC-315 IgA myeloma protein which binds to DNP prevents the absorption *in vivo* as it does *in vitro* when used at equivalence. Forty-fold less MOPC-315 than equivalence interferes with the absorption of DNP-albumin *in vitro* but not *in vivo*. I wonder if better results could be obtained *in vivo* by using secretory myeloma protein. The efficiency and resistance of MOPC-315 protein would be different in the respiratory tract, where you don't have proteolytic enzymes.

*Soothill:* We chose the easy route first; E. C. Swarbrick & C. R. Stokes are struggling with the gut now. I am encouraged that you can produce these effects. They think that they can too, but they have found the dose problem a real one.

*Pepys:* What do you think about Dr André's idea that IgA might mediate tolerance and that might be its role rather than simple immune exclusion (André *et al.* 1975)?

*Soothill:* I am very happy with that idea, but it does not make any difference to my pursuit, as a physician, of avoiding adverse contact with antigens in the vulnerable period. There are many possible explanations. I am merely using the low serum IgA as a marker for the deficiency.

*White:* You described work with mice that have a deficient function of their macrophages. You stated that the graded affinity of the antibody can be corrected by adjuvant. Do you mean Freund's complete adjuvant, and is it therefore the mycobacteria that correct the defect?

*Soothill:* Freund's complete or incomplete adjuvants work, and so does pertussis vaccine.

*White:* So it does not depend on mycobacteria. The difference between a simple antigen injection and Freund's incomplete adjuvant is I imagine due to long stimulation.

*Soothill:* You find macrophages in the site of the granuloma, however. We turned to measuring the macrophage function because we felt that the adjuvant was probably giving the macrophages the strongest encouragement.

*White:* Could I turn it round the other way? You are looking at a mouse which you regard as 'deficient'. Can I suggest that the mouse producing the high affinity antibody is deficient? If you give a long-continued antigenic stimulus, this then corrects the deficiency in avidity. In other words, the normal immune response produces a limited stimulation of the animal, since feedback mechanisms come in to stop the process and you would normally produce low affinity antibodies.

*Soothill:* It is known that the immune response matures. We gave four doses of antigen at weekly intervals, and bled the mice two weeks later. We looked at it over a considerable variation of time. The affinity of the antibody increases and the antibody disappears. This is a widely recognized phenomenon, so I do not fully understand your point.

*White:* I am suggesting that your mice correspond to Biozzi's low-responder mice (Biozzi *et al.* 1971), which also are regarded as being macrophage-deficient. This means that they produce antibodies for a short period of time, but such poor producers were the more successful survivors under natural conditions. You could regard them as the animals with more efficient feedback mechanisms, which stop antibody production.

*Soothill:* This interpretation is possible, but I think unlikely, since the poor macrophage function is clearly a 'deficiency' and dietary protein deprivation reduces macrophage function and affinity of antibody—it can hardly be expected to improve a response. I think that antigen elimination is a main function of antibody, and the low-affinity responders are certainly poor at this. And the combined effect is a big one. In all the animals we immunized with polyvinyl pyrrolidone, the clearance was increased. But even an immunized CBA mouse (a slow clearance strain) clears PVP less quickly than an unimmunized Ajax mouse. In the immunized Ajax mouse the clearance goes right up.

*White:* Do you see differences in the shape of the immune response between your low and high macrophage function groups?

*Soothill:* If by 'shape' you mean the duration of precipitating or agglutinating antibody, we have not measured this since they are only poorly related to the amount of antibody (Abt) and affinity. Abt is not obviously related to affinity.

*White:* In Biozzi's system, the low macrophage function group produce a lower level of antibody for a shorter period of time, whereas in the strain with high macrophage function, he found a high and prolonged antibody production.

*Soothill:* Biozzi selected on two systems: on the agglutination function of antibody, which is related both to the amount of antibody and to affinity, and on the clearance of carbon particles. You are talking about the former, selection for agglutination.

*Lachmann:* Katz & Steward (1976) have shown that the genetic control of the affinity and of the quantity of antibody made by mice are distinct, so they are selecting for different genetic parameters. On the other hand, Professor White is right, because in Biozzi's system the mice producing high antibody titres survive less long.

*Lehner:* Are you suggesting that there is a cause-and-effect relationship between the increased affinity in the mice and the increased macrophage

processing? This may of course be due to another cause, because one could envisage an increase in macrophage processing increasing the antibody titre but not in the same sense increasing the affinity of these antibodies.

*Soothill:* Titre does not give a precise description of antibody response, but agglutination titre depends partly on affinity, so I do not quite understand your distinction. I am suggesting that the high affinity response to antigen in saline results from active and efficient macrophage function, optimally presenting antigen to B cells.

*Lehner:* In order to get increased affinity, you presumably select out, as Andersson (1970) has suggested, cells secreting high affinity IgG antibody. How do macrophages do this?

*Soothill:* I don't know, but I gave you the information we have so far. As I described, manipulation, hormonal, nutritional, or by blockade, affects antibody affinity and macrophage clearance together, and you can lower the affinity of the antibody response by blockading the macrophages.

*Cebra:* Did you look at the IgG1:IgG2 antibody ratio ordinarily produced in these strains to your injected antigens? Secondly, in transient IgA deficiencies in children, have the effects of B cell mitogens in possibly stimulating the maturation of IgA plasma cells from peripheral blood lymphocytes been examined?

*Soothill:* This has been done in patients with no detectable serum IgA and normal numbers of lymphocytes with surface IgA (Cooper *et al.* 1971). We haven't done this in the transient cases. I have assumed it would work. We have not looked at the G1:G2 ratios.

*Rosen:* The transient cases have normal IgA fluorescent B cells. I don't know that anyone has stimulated them with B mitogens.

*Cebra:* Is there an effect on clonal expression caused by suppression of the IgA cells such that IgE cells may proliferate out and express their product to a greater extent than usual? Do you think that there is some reciprocal relationship of IgA to IgE antibody expression?

*Soothill:* It is attractive to link these two ideas, but we have not pursued this.

*Porter:* Is the diarrhoea you mentioned associated with nutritional antigens or is it due to bacterial involvement?

*Soothill:* I don't know. Dr Rosen will be referring to the problems of diarrhoea and immunodeficiency. It is a major feature in these children with defective yeast opsonization, but it is still essentially unexplained.

*Porter:* You claimed that giving plasma spontaneously cleared up the diarrhoea.

*Soothill:* Yes, it is a very impressive effect. And both functions are restored—the opsonization of yeasts and the activation of complement by inulin.

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*Immunology of the Gut*
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# Gastrointestinal complications of immunodeficiency syndromes

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*Abstract* Patients with B cell deficiency have a high incidence of prolonged *Giardia lamblia* infection of the gastrointestinal tract that causes symptoms of malabsorption with villus flattening. The changes are reversible with therapy directed against *Giardia*. There is a high incidence of pernicious anaemia in patients with agammaglobulinaemia. Those with abnormal B lymphocytes tend to develop lymphoid nodular hyperplasia. Gastrointestinal disease is rare in boys with X-linked agammaglobulinaemia when compared with adults with the 'acquired' or common variable form of the disease. T cell deficiency results in intractable diarrhoea and monilial infection of the gastrointestinal tract.

The primary immunodeficiency diseases are a prolix collection of syndromes which cannot be readily classified on the basis of aetiology, genetics or any other factor. It has appeared suitable and workable to classify these syndromes on the basis of their respective T or B cell deficiencies. The working classification of the World Health Organization Committee on primary immunodeficiencies is given in Table 1. The association between immunodeficiency and gastrointestinal abnormalities is well known and sheds light on the role of the immune system in maintaining normal gastrointestinal function. The gastrointestinal disorders which have been described in association with the primary immunodeficiency syndromes are listed in Table 2. It is obvious from this list that most of the abnormalities of the gastrointestinal tract occur in patients with common variable agammaglobulinaemia. Among these patients 20–50% develop gastrointestinal disease. On the other hand it is extremely rare to encounter gastrointestinal complications in X-linked agammaglobulinaemia. Of all the B cell abnormalities, gastrointestinal disease is thus seen most frequently, but not exclusively, in those patients with common variable agammaglobulinaemia.

TABLE I

Primary immunodeficiency disorders

| *Type* | *Suggested cellular defect* | | | *Inheritance* | | |
|---|---|---|---|---|---|---|
| | *B cells (Circulating Ig-bearing B lymphocytes)* | | *T cells* | *X-linked* | *Autosomal recessive* | *Other*[a] |
| | *Absent or very low* | *Easily detectable or increased* | | | | |
| X-linked agammaglobulinaemia | × | (×)[b] | | × | | |
| Thymic hypoplasia | | | × | | | × |
| Severe combined immunodeficiency | × | × | × | × | × | × |
| with dysostosis | × | ? | × | | × | |
| with ADA[c] deficiency | × | | × | | × | |
| with generalized haematopoietic hypoplasia | × | | × | | × | |
| Selective Ig deficiency | | | | | | |
| IgA deficiency | ? | × | (×) | | | × |
| Others | | ? | | | | × |
| X-linked immunodeficiencies—increased IgM | | × | | × | | |
| Immunodeficiency with ataxia telangiectasia | | × | × | | × | |
| Immunodeficiency with thrombocytopenia and eczema (Wiskott-Aldrich syndrome) | | | × | × | | |
| Immunodeficiency with thymoma | | × | × | | | × |
| Immunodeficiency with normal or hypergammaglobulinaemia | × | × | (×) | | | × |
| Transient hypogammaglobulinaemia of infancy | | × | | | | × |
| Variable immunodeficiencies (largely unclassified and very frequent) | × | × | (×) | | (×) | × |

[a] Implies multifactorial or unknown genetic basis or no genetic basis.
[b] Some cases with circulating B lymphocytes without detectable surface Ig have been found.
[c] Adenosine deaminase.

TABLE 2

Primary immunodeficiencies and gastrointestinal disease

| *Immunodeficiency syndrome* | *Gastrointestinal disorder* |
|---|---|
| 1. X-linked agammaglobulinaemia | Unusual |
| 2. X-linked immunodeficiency with increased IgM | Gastrointestinal malignancy |
| 3. Transient hypogammaglobulinaemia of infancy | Diarrhoea |
| 4. Selective IgA deficiency | 5% develop gastrointestinal disease, mainly gluten-sensitive enteropathy; isolated reports of inflammatory bowel disease |
| 5. Secretory IgA deficiency | Intestinal candidiasis |
| 6. Common variable agammaglobulinaemia | Giardiasis (most common)<br>Lymphoid nodular hyperplasia<br>Atrophic gastritis leading to pernicious anaemia<br>Bacterial overgrowth<br>Disaccharidase deficiency<br>'Flat villus' lesion (not gluten-sensitive) |
| 7. Thymic hypoplasia | Moniliasis |
| 8. Severe combined immunodeficiency | Chronic diarrhoea; usually idiopathic, occasionally salmonella or *E. coli* |
| 9. Ataxia telangiectasia | Increased α-fetoprotein<br>Reticuloendothelial malignancy |
| 10. Wiskott-Aldrich syndrome | Increased incidence of malignancy |

A persistent and constant finding amongst patients with T cell deficiency is intractable diarrhoea. Almost all patients with profound T cell deficiency develop intractable, watery diarrhoea, frequently caused by salmonella or shigella or enteropathogenic *Escherichia coli*. In patients in whom the T cell deficiency has been corrected by bone marrow or thymic transplants there has been a prompt cessation of the diarrhoea. The reversal of diarrhoea by the establishment of T cell chimaerism seems to indicate the importance of T cells in preventing this gastrointestinal catastrophe. In our discussion of gastrointestinal disease we follow the anatomical arrangement of the gastrointestinal tract, rather than discussing the gastrointestinal complications of each disease entity.

## GASTROINTESTINAL ABNORMALITIES ASSOCIATED WITH IMMUNE DEFICIENCY

### *1. Oral cavity and oesophagus*

Stomatitis and oesophagitis secondary to moniliasis, herpes simplex and cytomegalovirus commonly occur in patients with T cell deficiency. *Candida albicans* is perhaps the most common pathogen in patients with severe combined immunodeficiency.

### *2. Stomach*

The association between pernicious anaemia and agammaglobulinaemia has been well described (Twomey *et al.* 1970; Conn *et al.* 1968; Gelfand *et al.* 1972). Patients with pernicious anaemia and hypogammaglobulinaemia differ from those with classical Addisonian pernicious anaemia. They develop their symptoms at a much earlier age than patients with Addisonian pernicious anaemia. Antibodies to intrinsic factor, parietal cells and thyroglobulin, which are well described in patients with classical pernicious anaemia, are not found, and this provides evidence that circulating antibodies may play no role in the development of pernicious anaemia. A complete absence of plasma cells is noted in gastric mucosal biopsies, as compared to the abundant numbers of plasma cells in patients with atrophic gastritis with Addisonian pernicious anaemia. Many agammaglobulinaemic patients with pernicious anaemia also have diarrhoea or malabsorption. Gelfand *et al.* (1972) reported identical twins with pernicious anaemia and hypogammaglobulinaemia. Despite the absence of antibodies to intrinsic factor, cell-mediated hypersensitivity to intrinsic factor was shown in a macrophage inhibition factor (MIF) assay. An increased incidence of gastric carcinoma is also reported in agammaglobulinaemic patients (Hermans & Huizenga 1972).

### *3. Small bowel*

A. *Giardia lamblia.* Infestation with *Giardia lamblia* is by far the most common gastrointestinal abnormality in patients with common variable immunodeficiency (Fig. 1). *Giardia lamblia* was first discovered by Antoni Van Leeuwenhoek in 1681 when he recovered it from his own stool. Giardiasis has been well described in association with malabsorption but the mechanism of malabsorption is unclear. Some authors (Hoskins *et al.* 1967) have described marked mucosal changes characterized by villus flattening in immunologically

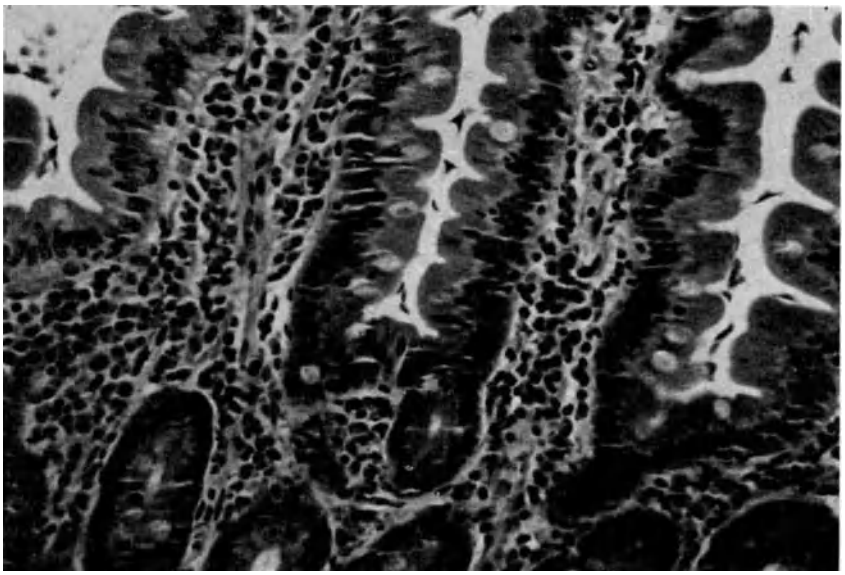

FIG. 1. View of the jejunum from a patient with common variable immunodeficiency. *Giardia lamblia* organisms are abundantly evident in the crypts. No villus abnormality is noted. × 120.

normal patients, while Brandborg *et al.* (1967) and Morecki & Parker (1967) have described mucosal invasion by the parasite with normal villus structure. Erlandsen & Chase (1974) have shown with electron microscopy studies that *Giardia* attaches to the microvillus border of the mucosal cell and that the subsequent abnormalities may be a result of direct injury to the microvillus border in combination with mechanical blockade of the mucosal surface. In patients with normal immunological function *Giardia* does not usually cause marked villus flattening although inflammation of the lamina propria is usually evident. In immunodeficient patients, however, Ament *et al.* (1973) have demonstrated a patchy intestinal lesion with varying degrees of villus flattening (Figs. 2 and 3). Treatment with metronidazole or atabrine has resulted in complete reversal of the villus lesion with alleviation of symptoms in most patients. Thus it would appear that there is a difference in the effect of *Giardia* infestation on the intestinal mucosa between immunodeficient and immunologically competent patients. It should also be stressed that the diagnosis of *Giardia lamblia* infection of the intestinal tract cannot be made easily on examination of the stool; the stool is far less revealing than examination of the duodenal fluid, mucosal imprinting and intestinal biopsy for the definitive diagnosis of giardiasis.

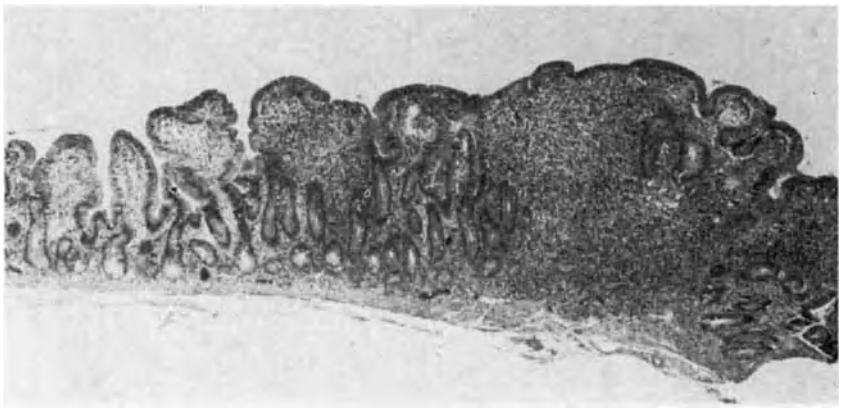

FIG. 2. Patchy villus flattening in the jejunum from a patient with common variable immunodeficiency. No evidence of giardiasis. × 27.

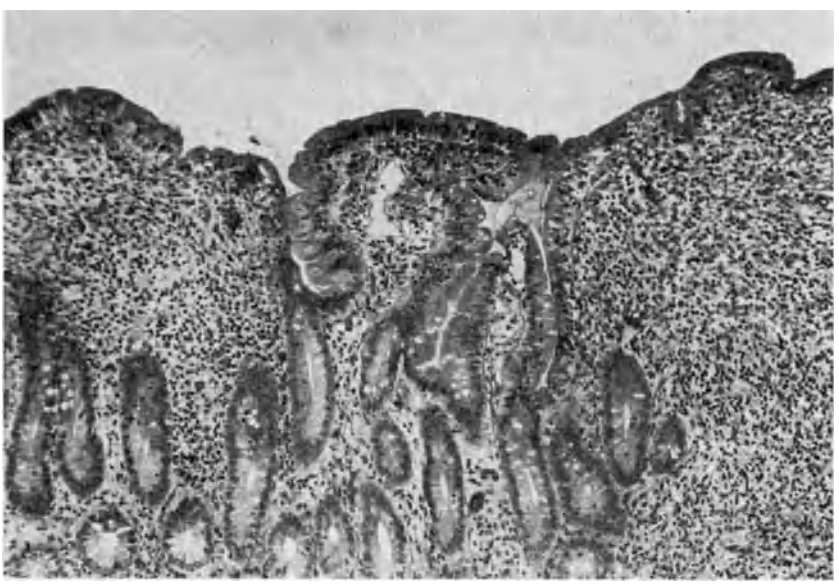

FIG. 3. High-power view of area of villus flattening from the jejunum of a patient with common variable immunodeficiency. Although there are abundant lymphocytes in the lamina propria, no plasma cells are seen. × 133.

B. *Lymphoid nodular hyperplasia.* Nodular lymphoid hyperplasia in the small intestine is associated with immunoglobulin deficiency states (Hermans *et al.* 1966) (Fig. 4). It is often associated with other gastrointestinal mani-

FIG. 4. An X-ray of the small bowel of a patient with common variable immunodeficiency showing nodular lymphoid hyperplasia extensively throughout the bowel.

festations of immunodeficiency, most notably giardiasis. There is a marked heterogeneity in B cell number in patients with acquired or common variable agammaglobulinaemia. In 19 patients with acquired agammaglobulinaemia, Geha *et al.* (1974) found markedly depressed numbers of circulating B cells in four patients, normal numbers in ten patients and an increase in number in five patients. The latter patients, with an increased number of B cells, had prominent lymph nodes with microscopical evidence of germinal centre hyperplasia. All patients with nodular lymphoid hyperplasia and agammaglobulinaemia have B cells, and the lymphoid hyperplasia of the intestinal tract is not encountered in agammaglobulinaemic patients without B lymphocytes. In the X-linked form of agammaglobulinaemia, where there is almost always complete absence of B cells in the bone marrow and peripheral blood (Geha *et al.* 1973),

no patients have been described with nodular lymphoid hyperplasia of the intestinal tract. Lymphoid hyperplasia of the terminal ileum is a normal finding in children and young adults and lymphoid nodular hyperplasia of the large bowel is a rare finding not associated with agammaglobulinaemia.

C. *Villus flattening.* There are many known causes of villus flattening in addition to that seen in gluten-sensitive enteropathy. In primary immunodeficiency the most common cause of villus flattening is giardiasis (Ament *et al.* 1973). Selective IgA deficiency in association with villus flattening as originally described by Crabbé & Heremans (1967) is a gluten-sensitive lesion, whereas villus flattening associated with common variable agammaglobulinaemia is not usually due to gluten sensitivity. In gluten-sensitive enteropathy (coeliac sprue) serum and mucosal immunoglobulin changes have been extensively described. The serum IgA concentration is elevated during the active phase and serum IgM is low (Hobbs & Hepner 1968). These values return to normal on gluten restriction. Intestinal mucosal studies by Falchuk & Strober (1974) have revealed increased IgA and IgM values which return to normal on gluten withdrawal. In patients with selective IgA deficiency and gluten-sensitive enteropathy, immunofluorescent studies have revealed increased IgM-producing cells in the lamina propria, probably a compensatory mechanism for the lack of IgA. It is extremely important to realize that none of the patients with IgA deficiency and gluten-sensitive enteropathy has been subjected to a gluten challenge to see whether the lesion is reproducible. This step is essential for the diagnosis of gluten sensitivity, as many types of the 'flat gut lesion' will respond to gluten withdrawal.

An *in vitro* organ culture model of gluten-sensitive enteropathy has been described in which the surface epithelial cells of jejunal biopsies of patients with active disease improve after 24 hours of incubation in a gluten-free environment. This improvement is inhibited in the presence of gluten protein (Falchuk *et al.* 1974). The addition of corticosteroids to the medium completely abolishes the inhibitory effect of gluten. We are now using this model to investigate the 'flat gut lesion' in patients with agammaglobulinaemia. Preliminary observations in two patients with villus flattening and agammaglobulinaemia reveal that the lesion improves in a gluten-free environment, and this improvement is not inhibited by the addition of gluten to the medium. We have as yet not had the opportunity to study the mucosa of a patient with IgA deficiency and gluten-sensitive enteropathy.

D. *Bacterial overgrowth.* Although significant numbers of aerobic and anaerobic organisms are present in the proximal jejunum of patients with

agammaglobulinaemia (Parkin *et al.* 1972; Ament *et al.* 1973) there appears to be little or no correlation with the severity of the gastrointestinal symptoms and prolonged antibiotic therapy has not been of value. Many of these patients with bacterial overgrowth have achlorhydria and atrophic gastritis and some have pernicious anaemia.

In immunocompetent individuals, gastric acidity and intestinal motility are important factors in preventing bacterial overgrowth. The mechanism of steatorrhoea in this instance appears to be related to bile salt deconjugation and/or direct mucosal damage. The presence of achlorhydria in these patients probably predisposes to bacterial overgrowth, but why no clinically significant symptomatology occurs is unknown.

E. *Disaccharidase deficiency.* Disaccharidase deficiency with villus flattening has been described in a number of patients with immunodeficiency (Dubois *et al.* 1970). In this series, however, no evidence of giardiasis was found, which is unusual. Ament *et al.* (1973) found four cases with abnormal lactose tolerance tests—three patients with common variable immunodeficiency and one infant with infantile X-linked agammaglobulinaemia, all of whom responded to lactose withdrawal. That the association between disaccharidase deficiency and primary immunodeficiency is specific remains to be clarified; it seems possible that most cases of disaccharidase deficiency in these conditions are secondary to mucosal disease.

### *4. Large bowel disease*

Colonic or rectal disease is unusual in patients with immunodeficiency. There have been isolated reports of ulcerative colitis and Crohn's disease, and one case of gluten-sensitive enteropathy and ulcerative colitis has been described (Falchuk & Falchuk 1975).

### *5. Dissociation of intestinal and circulating B lymphocytes*

Broom *et al.* (1975) and McClelland *et al.* (1976) have reported finding B lymphocytes in intestinal biopsies of patients with common variable immunodeficiency who have no circulating B lymphocytes. B cells with surface immunoglobulin, and *in vitro* synthesis and secretion of immunoglobulin by these cells, have been demonstrated. The pathophysiological importance of these findings is not clear at the moment, but they may explain differences among patients in their susceptibility to giardiasis and other gastrointestinal complications.

*6. Liver disease*

The association between immunodeficiency and liver disease of any kind is rarely reported in the literature. We have observed four patients with X-linked agammaglobulinaemia with persistent hepatitis B antigenaemia, with no development of chronic hepatitis. Three patients with the Wiskott-Aldrich syndrome developed hepatitis B antigenaemia but none developed chronic hepatitis or the carrier state. The natural history of hepatitis B in immunodeficient patients needs to be studied further in view of increasing evidence that the persistence of hepatitis B antigen and the subsequent development of chronic liver disease is related to abnormalities in immune function.

*7. Gastrointestinal malignancy*

It has been established that there is an increased incidence of neoplasia in patients with immune deficiency. Thirteen of 110 patients with immune deficiency seen at the National Institutes of Health over a period of less than ten years developed malignant tumours (Waldmann *et al.* 1972). Lymphoreticular malignancy is the commonest type of neoplasia found in these patients.

The development of lymphoma and leukaemia has been reported in patients with X-linked agammaglobulinaemia and we have three brothers with sex-linked agammaglobulinaemia, all of whom developed B cell lymphomas at 10–16 years of age in the terminal ileum. None of them had any evidence of gastrointestinal involvement before the malignancy developed. In common variable hypogammaglobulinaemia lymphoreticular malignancies are also common, and there is a considerable incidence of gastric carcinoma. Gastrointestinal malignancy occurs in association with nodular lymphoid hyperplasia in a high percentage of patients who develop cancer. Ten per cent of patients with ataxia telangiectasia have been reported to develop malignancy, usually of the lymphoid tissue. The Wiskott-Aldrich syndrome is characterized by a very high incidence of malignant disease, also usually of reticular endothelial tissue. It is of interest to note that in patients with intestinal lymphangiectasia who have protein-losing enteropathy, lymphopenia and consequent abnormalities in cellular immunity, three of 50 patients developed malignancy, two, lymphoma and one, reticular cell sarcoma of the stomach (Waldmann *et al.* 1972). In patients with immunodeficiency with helper IgM the gastrointestinal tract, including the liver and gall bladder, become infiltrated with IgM-bearing cells. The infiltrate can undergo malignant degeneration and has been fatal in several cases.

*8. Protein-losing enteropathy*

Studies of labelled plasma proteins have allowed the demonstration of excessive gastrointestinal protein loss as a major pathophysiological disorder leading to hypoproteinaemia and hypogammaglobulinaemia. This condition has been described in association with over 80 disorders. Some of these disorders are also associated with lymphopenia. A classical example of this type is intestinal lymphangiectasia. Many patients with agammaglobulinaemia and gastrointestinal disease also have gastrointestinal protein loss and therefore it may be difficult to differentiate them from patients with protein-losing enteropathy alone.

## SPECIFIC IMMUNE DEFICIENCY SYNDROMES

*Selective IgA deficiency*

Selective IgA deficiency occurs in one in 500–700 of the general population. The majority of these cases occur spontaneously, but there is an increased incidence in families with hypogammaglobulinaemia. Many of these patients may be asymptomatic or develop recurrent upper respiratory infections. In a review of 205 patients Ammann & Hong (1971) reported a high incidence of autoimmune disease but only ten patients had gastrointestinal disease, eight of whom had gluten-sensitive enteropathy and two, inflammatory bowel disease. It would thus appear that patients who lack both serum IgA and IgM have a much higher incidence of gastrointestinal disease than patients with selective IgA deficiency. Bienenstock (1975) has demonstrated that under special conditions IgM antibodies may become associated with secretory piece and assume similar characteristics to IgA in secretions. This may explain the protective effect of IgM in selective IgA deficiency and the relative paucity of gastrointestinal disease.

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# Discussion

*Ferguson:* I have had the impression that diarrhoea is not a feature where a

patient or animal has T cell deficiency but normal antibody production. This has certainly been my experience from one child with thymic aplasia, and a substantial number of patients with Hodgkin's disease and impaired cell-mediated immunity. I thought that it was when T cell deficiency was combined with antibody deficiency that the severe diarrhoeal syndrome occurred.

*Rosen:* That is not completely true. Children without T cells have severe diarrhoea, whether or not they have B cells. The term 'combined immunodeficiency' was coined because most of these children lack T and B cells, but some have B cells. It is not clear whether those who have B cells make antibody, but the presence or absence of B cells does not alter the severe diarrhoea.

*Pierce:* I am interested in the influence that T or B cell deficiency may have in predisposing children to infection with specific types of diarrhoea-causing organisms. I would expect invasive organisms, such as viruses, shigellae, salmonellae and invasive *Escherichia coli*, to predominate as causes of diarrhoea in children with T cell deficiency, since T cells presumably contribute to defence mechanisms within the mucosa. I would also expect non-invasive pathogens, such as *Vibrio cholerae* and enterotoxigenic *E. coli*, to cause disease in children with B cell deficiency, since antibody appears to be a major means of defending the mucosal surface. However, you say that diarrhoea is uncommon in B cell deficiency. Since the non-invasive enteropathogens such as *V. cholerae* or enterotoxigenic strains of *E. coli* are not very prevalent in the USA your patients may not be encountering them and thus may not be revealing their susceptibility. Is it known whether persons with only B cell deficiency have more trouble with diarrhoea in underdeveloped countries where these enteropathogens are more common, or whether pure B cell deficiency is a rare disorder in those parts of the world because they do not survive infancy?

*Rosen:* I cannot answer that. I suspect such children do not survive in the tropics.

*Lachmann:* There is evidence in rabbits that to get diarrhoea from shigella organisms you need an allergic response to the organism itself in the first place (Matsumura 1962). If you are not capable of mounting the response the infection may, I suppose, destroy the bowel altogether, but you might not get the typical diarrhoea syndrome.

*Pierce:* I have always thought that the dysentery syndrome was due to invasion of the colon, while the diarrhoeal syndrome is now thought to be due to an effect of enterotoxin on the small bowel with resultant water and electrolyte secretion.

*Booth:* Could I make a comment on selective IgA deficiency and coeliac disease? Another way of looking at the data is to see how many coeliac disease cases in a big series are IgA deficient, which gives a better picture to compare

with your one in five hundred of the normal population who have selective IgA deficiency and many who may be completely asymptomatic. The figure in my own series of about 200 patients is that one in fifty coeliac patients is IgA deficient. There clearly is an association but I do not know what it means.

Secondly, there is a report from Cambridge and my department of a pair of identical twins (Lewkonia *et al.* 1976). We showed that they were discordant: one was IgA-deficient and the other was not.

*Rosen:* The genetics of IgA deficiency is not clear. There is all kinds of conflicting information about its inheritance. It has been said to be inherited as a dominant; it has also been said to be X-linked, and even an autosomal recessive. The only convincing evidence is that of van Loghem (1974) that the inheritance of the defect is not linked to the Am locus, which is a structural gene for IgA corresponding to the Gm locus of IgG.

*Booth:* This presumably implies something environmental that switches on IgA production in the neonate. I have always assumed that the antigenic stimulus must be bacterial, in that this 'switch on' does not happen in the germ-free animal, who is receiving normal dietary antigens.

*Rosen:* It can certainly be switched on *in utero* in natural infection with syphilis or *Toxoplasma* or cytomegalovirus.

*Porter:* Husband & McDowell (1975) have done some work in the fetal lamb, giving *E. coli* antigens *in utero*. As early as fifteen days before parturition IgA cells were seen in the lamina propria. IgM cells predominated at birth. Germ-free animals certainly show no response. We have maintained pigs for up to six weeks in the germ-free state without the appearance of IgA.

*Rosen:* The normal human newborn can be looked on as a germ-free animal. There are a normal number of B cells in cord blood, by fluorescence and erythrocyte-rosetting markers.

*Gowans:* You mentioned a class of B cell deficient patients whose B cells do not respond to T cell signals and who develop nodular hyperplasia of lymphoid tissue. Is there anything wrong with them clinically? How are they picked up?

*Rosen:* They are susceptible to pyogenic infections, like other people with agammaglobulinaemia. They usually present with respiratory infection. In our hands, with the tetanus toxoid system we cannot get these patients' T cells to activate their own B cells, *in vitro*, but some of them have a striking germinal centre hyperplasia, in Peyer's patches and lymph nodes.

*Pepys:* Most of the patients whom we see with nodular lymphoid hyperplasia are referred to us with undiagnosed diarrhoea. We find that they have common variable immunodeficiency. Many are not infected with *Giardia* and don't respond to metronidazole therapy. We have not had any autopsies so we do not know whether the nodules are in Peyer's patches, but they are far

more frequent than the lymphoid aggregates found in the normal intestine. One can find two in one peroral jejunal biopsy. Some of the cells in these nodules have been shown *in vitro* to have B cell markers (M. B. Pepys & A. C. Dash, unpublished work).

*Booth:* You can find them all over the colon too, so it is not limited to the small intestine. It might not be a Peyer's patch phenomenon.

*Seligmann:* Do you imply that in your experience, Dr Rosen, most immunodeficient patients with nodular intestinal hypoplasia have enlarged peripheral lymph nodes with giant follicles and that, conversely, patients with enlarged peripheral nodes have nodular intestinal hypoplasia? This is not our experience.

*Rosen:* In some cases they are associated.

*Soothill:* The hyperplasia is not necessarily in the gastrointestinal tract, and it can be startling. It may be looked upon as a tumour. Clinically, it is important to make the distinction. I have protected several patients from dangerous anticancer treatment: where respiratory obstruction results, such minor local treatment as low dose X-ray will clear it, and they do fine.

On the question of *Giardia lamblia*, we have looked for it in many immunodeficient children with diarrhoea, largely the common variable type, and have only occasionally found it. I have put some patients on to metronidazole therapy because of the report that it may affect for instance bacteroides, and some of the patients in whom we have not found *Giardia lamblia* in faeces or biopsy have apparently improved with the treatment, so I am concerned with the clear identity of these phenomena.

*Rosen:* I think any pathologist used to these things can tell the difference. There is an age difference distribution of *Giardia lamblia*. What were the ages of your patients? That could explain the difference.

*Soothill:* These were mainly children aged 4–14 years.

*Booth:* Can we ask the parasite immunologists why these patients should specifically get this parasitic infection and nothing else?

*Ogilvie:* Nothing is known. The only intestinal protozoan parasites that have been studied in any detail immunologically are the coccidea.

*Booth:* Is it fair to say that *Giardia* is a more anaerobic parasite than any other?

*Ogilvie:* I don't think one can answer that.

*Davies:* Is this parasite causing any harm, or is it just there?

*Rosen:* You can get a sprue-like syndrome from it—malabsorption, diarrhoea, weight loss and steatorrhoea. If you treat with metronidazole or atabrine all these symptoms disappear.

*Evans:* Does the bowel recover as well?

*Rosen:* The villous flattening is not commonly seen but when it is, it goes away with the treatment.

*Ferguson:* We have evidence that the enteropathy associated with *Giardia* infection is due to the immune response, and I shall touch on this point later (p. 315). In normal people there probably is an immune response to *Giardia* which involves both antibodies and T cells. In hypogammaglobulinaemic individuals, the blocking or modulating properties of antibody will be absent, thus allowing enhanced or excessive T cell response and T cell-induced enteropathy. We have seen this in an antibody-deficient mouse model. *Giardia* infection has measurable but small effects on mucosal architecture in normal mice, but in antibody-deficient animals, giardiasis causes villous atrophy and crypt hyperplasia (A. Ferguson, G. Paul & T. T. MacDonald, unpublished 1976).

*Davies:* In that case if you treated the T cells, presumably the disease would also be cured.

*Rosen:* That would be rather drastic, however.

*Davies:* You are using Flagyl, an imidazole compound which is almost certainly active against lymphocytes as well as against the parasite. One has no real precedent for treating a parasitic infection in an immunosuppressed patient.

*Brandtzaeg:* Is there any information on T cell function in coeliac disease patients with selective IgA deficiency?

*Rosen:* I am not aware of any information.

*Bienenstock:* Ogra *et al.* (1975) have described a recent and retrospective study of cot death and an apparent deficiency of secretory component, and other cases have been reported (Strober *et al.* 1976). They are not all the same diseases, but one can't leave the subject of immunodeficiency without considering secretory component deficiency.

*Rosen:* Strober's case, which presented clinically with *Monilia* enteritis, had a normal serum IgA level, a low number of IgA-bearing cells or synthesizing cells in the gut, and a very small amount of secretory IgM, which is relevant to the earlier discussion of the role of secretory piece and the homing of B cells. Dr Brandtzaeg raised some valid objections to that report, since no biosynthetic studies had been done of secretory piece (p. 101).

*Soothill:* The terms cot death, or sudden infant death syndrome, describe the children who die suddenly in the first year of life from no clear cause, a surprisingly frequent event. There is doubt whether this is a disease, or a syndrome. It has been suggested that it is an immunopathological phenomenon. Such evidence includes the work of Robin Coombs and Mavis Gunther on the high incidence of anti-cow's milk antibody in the children (Parish *et al.* 1960). Ogra *et al.* (1975) reported that post-mortem tracheal washings and immunofluorescence studies of the bronchial mucus membrane were deficient either in secretory IgA deficiency or in secretory piece, or both. I believe that others have looked in a similar way and not found this.

*Bienenstock:* The important point was that Ogra suggested, on the basis of immunofluorescent studies of the presence of anti-viral antibody, that respiratory syncytial virus antigen was present in those patients, and that perhaps the defence mechanisms of the mucosa had allowed another antigen in, and that was the cause of the sudden death.

*Soothill:* The observation of viral antigens, especially RSV, is a confirmation of the work of Gardiner (1974) in the UK, who supplied Ogra with the antisera. How it links with the possible secretory piece deficiency and IgA deficiency is not yet clear.

*Lachmann:* I think it is fair to say that there are still as many hypotheses about the aetiology of cot deaths as there are groups working on it!

*Bienenstock:* I am simply interested in the question of secretory component in immunodeficiency, because therein would lie clues to the functioning of IgA at the mucosal surface.

*Brandtzaeg:* There was a comment in *The Lancet* on Ogra's report by Williams *et al.* (1976). They made salivary gland extracts from ten cot death patients. They found secretory component in all of them. Incidentally, virus infection of the glandular epithelium may reduce secretory component synthesis. Ogra's finding might be secondary to the effects of a virus infection. With regard to Strober's report, another similar case was published by the same group describing a 52-year-old male, as I discussed (p. 101), but in both cases neither immunofluorescence nor synthetic studies of secretory component were made. However, a finding in both these patients was a 20-fold increase of the IgM level in the intestinal secretions. This was not commented on by the authors. It seems similar to what happens in selective IgA deficiency. Perhaps there was a true glandular secretion of IgM with bound secretory component. This possibility was apparently not investigated.

*Soothill:* Turner *et al.* (1975) reported raised IgE levels and IgE antibodies to dermatophagoides and other inhaled antigens in a group of children who died suddenly. Buried in their data was the fact that they were also IgA deficient in a way absolutely superimposable on the data in our prospective study of the development of allergy. This has encouraged me to believe that there is some truth in the idea of immunodeficiency underlying sudden death in infancy, but allergy and susceptibility to viral infection may both be independent effects of immunodeficiency, and which causes the death is still not clear (Turner & Soothill 1975). I have more confidence in Turner's information than in many other reports because it was a large series systematically run. We are running a comparable series in the UK at the moment.

*Seligmann:* Are there any documented cases where there is lack of or an extremely low level of secretory IgA with normal or almost normal serum

IgA? Since it has been claimed that such a situation can occur, some of you may have seen such cases.

As a comment, you can see intestinal nodular hyperplasia in patients with selective IgA deficiency, which is interesting in respect of the way IgA-committed cells home into Peyer's patches.

*Rosen:* I have not seen any cases of absent secretory IgA with serum IgA present, but this has been reported. IgA-deficient patients have B cells bearing surface IgA.

*André:* A young man studied by Dr J. P. Revillard and myself in Lyons was completely agammaglobulinaemic. When we used immunofluorescence to look for plasma cells in his bone marrow, we found absolutely no plasma cells; there were some in the lamina propria. He doesn't secrete any immunoglobulin in saliva or in intestinal secretions.

*Pepys:* Both we (M. B. Pepys & A. C. Dash, unpublished work) and others (Broom *et al.* 1975) have made the same observation in a number of patients with common variable immunodeficiency, some of whom have low levels of immunoglobulin in the serum and secretions, but have apparently normal staining of populations of plasma cells in the lamina propria. So they have immunoglobulin-containing plasma cells which don't seem to secrete, at least at the same rate as in normal individuals.

*Booth:* There seem to be several types of selective IgA deficiency. There are some with no IgA cells. The more common type seems to have just a few. In other cases there is an antibody to the IgA and the IgA-secreting cells are normal.

*Brandtzaeg:* Savilahti (1973) showed a relationship between serum IgA levels and secretory IgA cells in the lamina propria. He found that serum IgA levels have to be less than 20% of normal before there is a change in the intestinal immunocyte population. The best sign of what is happening there is that the proportion of IgM cells increases. With regard to the immunoglobulin-secreting cells in the lamina propria of hypogammaglobulinaemic patients, Dr Pepys, when you say that there sometimes are apparently normal cell populations do you mean numerically normal?

*Pepys:* We have not counted, but we have a number of patients with common variable immunodeficiency, low serum immunoglobulin levels and normal-looking lamina propria plasma cell populations.

*Ferguson:* McClelland *et al.* (1976) have compared immunoglobulin synthesis by *in vitro* cultured intestinal biopsies and the numbers of plasma cells detected by immunofluorescence in the same specimens. They studied patients with hypogammaglobulinaemia and found considerably more immunoglobulin synthesis by biopsy fragments than would be anticipated from the numbers of immunoglobulin-containing cells there. They suggest that some of the in-

testinal lymphoid cells can synthesize but cannot secrete or store immunoglobulins.

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*Immunology of the Gut*
Ciba Foundation


# Immunobiology and pathogenesis of alpha chain disease

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*Abstract* Alpha chain disease, the most frequent of the heavy chain diseases, is a proliferative disorder of B lymphoid cells involving primarily the small intestine and mesenteric nodes. The characteristic immunoglobulin, whose detection by immunochemical techniques may present some difficulties, consists of incomplete $\alpha$ chains devoid of light chains. The deleted portion of the $\alpha$ chain is located in the Fd segment and involves both the variable and first constant domains. In both of two proteins for which structural data are available, normal sequence resumes at the beginning of the hinge region. The absence of L chains is due to a failure of synthesis. Alpha chain disease appears to proceed in two stages. The early stage is characterized by a possibly non-malignant diffuse and extensive plasma cell infiltration which may be reversible after administration of antibiotics. The later stage is characterized by overt neoplasia (immunoblastic lymphoma). The socio-geographic distribution of the digestive form of alpha chain disease shows a clear predilection for underprivileged populations living in areas with a high degree of infestation by intestinal pathogens which play presumably a crucial role in the pathogenesis of the disease.

Alpha chain disease is a proliferative disorder of B lymphoid cells affecting mainly young patients and involving primarily the IgA secretory system, in which plasma cells produce a population of immunoglobulin (Ig) molecules consisting of incomplete $\alpha$ chains devoid of light chains. Since the first description of this new immunoglobulin abnormality (Seligmann *et al.* 1968) in a young Syrian patient affected with malabsorption and diffuse plasmacytic infiltration of the small intestine (Rambaud *et al.* 1968), more than 100 cases have been recognized to our knowledge. Alpha chain disease is thus the most frequent of the heavy chain diseases. In three of these patients alpha chain disease was apparently confined to the respiratory tract (Stoop *et al.* 1971; Faux *et al.* 1973; Florin-Christensen *et al.* 1974). All the other patients were affected with the digestive form of the disease which is mainly localized in the

small intestine and the mesenteric lymph nodes. We wish to discuss here some of the basic problems raised by this condition, many of which are closely related to the subject of this symposium.

The main methods used for the immunochemical (Seligmann *et al.* 1969, 1971), cellular (Seligmann *et al.* 1969; Buxbaum & Preud'homme 1972) and structural (Wolfenstein-Todel *et al.* 1974) studies have been previously described in detail.

## NATURE OF IMMUNOGLOBULIN ABNORMALITY

The diagnosis of alpha chain disease (α-CD) relies entirely upon the detection of the pathological protein. In half of the 80 cases studied in our laboratory, the α-CD protein was not noticeable on the serum electrophoregram. When detectable by electrophoresis, it showed an abnormal broad band usually in the α2 or β region. The diagnosis is usually suspected or established by the immunoelectrophoretic analysis of the serum of these patients. In many cases the protein abnormality has escaped detection by routine immunoelectrophoresis using polyvalent antiserum to normal human serum, and analysis with monospecific antisera to IgA is essential. The abnormal component usually gives an abnormal precipitin line either extending from the α1 globulins to the slow β2 region or showing a faster electrophoretic mobility than normal IgA. However, in a few patients the α-CD protein had a slow electrophoretic mobility. The anomalous component does not of course precipitate with antisera to light chains. It should however be emphasized that this lack of precipitation with anti-κ and anti-λ antisera is not a sufficient criterion for the diagnosis of α-CD since a number of IgA myeloma proteins, even though they contained light chains (mainly λ chains), failed to precipitate with most such antisera. We have found that selected antisera to IgA which contain antibodies related to the conformational specificity of the Fab region—i.e. precipitating only with α- and light chains combined—are very useful for the diagnosis of α-CD by immunoelectrophoresis or the Ouchterlony technique. The immunoselection plate method of Rádl has also been used (Doe *et al.* 1972). In all doubtful cases the pathological protein should be purified, reduced and alkylated, and the lack of light chains should be demonstrated directly.

The striking and unexpected electrophoretic heterogeneity of these presumably monoclonal α-CD proteins is certainly due in part to the heterogeneity of their *N*-terminal sequences, as discussed below. It may also be related to two other features, the high carbohydrate content of most α-CD proteins and their high tendency to polymerize. Indeed on ultracentrifugation α-CD proteins appear to consist of dimers with a 3-4S sedimentation constant and, in most instances,

of larger polymers of various sizes. J chain was found in all purified α-CD proteins so far studied.

The concentration of the α-CD protein in the urine is usually very low. In most patients, however, it could be detected in concentrated urine and had the same electrophoretic and immunochemical characteristics as that in the serum. Bence-Jones proteinuria was never found. The pathological protein was also found in significant amounts in jejunal fluid, in patients with the digestive form of the disease, as expected from the involvement of the intestine, whereas the secretory IgA in the parotid saliva of these patients was normal.

The serum levels of normal IgA, IgG and IgM molecules are usually depressed. These decreases are not solely due to a protein-losing enteropathy, as shown by the disproportionate depression of serum Ig levels relative to the serum albumin concentration. Deficiencies of humoral immunity as well as cellular immunity have been demonstrated in a few patients. No functional tests of the secretory immune system have been performed in these patients to our knowledge.

The molecular weight of the monomeric polypeptide subunit of various α-CD proteins was found to vary between 29 000 and 34 000. The length of these chains is thus more than half but less than three-quarters of that of normal α1 heavy chains. Antigenic analysis and chemical studies indicated that the entire Fc fragment was present in α-CD proteins, that their *C*-terminus was identical with that of normal α1 chains and that the heavy-light peptide was missing. The hinge region was shown to be present in all eight proteins so far studied. In view of these results and of the molecular weight data, the missing portion of the chain is located in the Fd segment and involves both the $V_H$ and $C_1$ regions.

The *N*-terminal sequences of several α-CD proteins were shown to be heterogeneous. Even for those proteins with a single *N*-terminal amino acid, marked heterogeneity became apparent after two steps in degradation. Attempts to obtain the *N*-terminal sequence on an automated sequencer were unsuccessful. The *N*-terminal residues were different from those found in any of the subgroups of the variable regions of normal heavy chains. The most likely explanation of this heterogeneity is that it is the consequence of a limited intracellular proteolysis occurring after synthesis. The fact that the *N*-terminal residues found in the seven proteins studied were valine and/or isoleucine suggests that the degradation stops at this level for some reason, which could possibly be enzyme specificity, steric hindrance or the presence of a carbohydrate moiety. An analogous limited intracellular post-synthetic proteolysis of the NH2-terminus of an incomplete protein has been described for a non-sense mutant of alkaline phosphatase produced by *Escherichia coli* (Natori & Garen 1970).

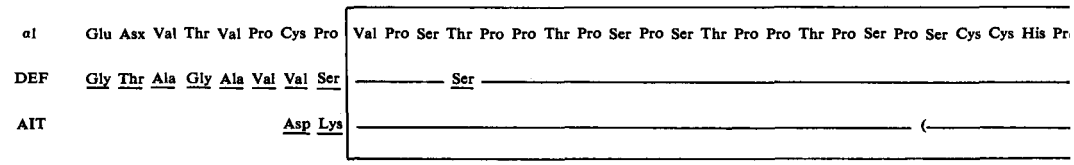


FIG. 1. Comparison of the hinge region of a myeloma α1 chain and of α-CD proteins DEF and AIT. Identical residues are in the box. Non-homologous residues are underlined. Line in the box indicates the sequence identical to that shown in the top peptide. (From Wolfenstein *et al.* 1975).

The demonstration of a large internal deletion in a γ-heavy chain disease protein (Frangione & Milstein 1969) led us to postulate that in α-CD we were dealing with a similar primary deletion followed and obscured by a secondary limited proteolysis (Seligmann *et al.* 1971). This hypothesis has been supported to some extent by biosynthetic and structural studies but has not yet been completely proved. Structural studies were performed in Dr Franklin's laboratory and in our laboratory on two α-CD proteins, DEF and AIT (Wolfenstein-Todel *et al.* 1974, 1975). The amino acid sequence of the hinge region of these two proteins, compared with that of a normal α1 chain, is shown in Fig. 1. Normal synthesis resumes in both proteins at a valine residue in the hinge region just preceding a segment which contains a partially duplicated fragment and the inter-heavy chain disulphide bonds. Starting with this valine residue at position 9, and with the exception of a substitution of threonine for serine at residue 12 of protein DEF, the sequence is completely identical to that of a normal α1 chain. It is of interest that this valine residue at position 9 of this tryptic hinge peptide could represent the counterpart of glutamine at position 216 of γ chains, the site where normal synthesis resumes in several γ-CD proteins with internal deletions (Frangione & Franklin 1973; Franklin & Frangione 1975). Fig. 1 indicates that the first eight residues of the tryptic peptide of protein DEF and the first two residues of the peptide of protein AIT differ from the sequence which normally precedes the valine in α1 chains. These short segments were thought to correspond to the variable region. If this assumption proves to be correct, the primary defect in these α-CD proteins would be an internal deletion encompassing most of the $V_H$ and $C_{H1}$ domains. It is, however, not excluded that the segment preceding the valine which marks the beginning of the normal sequence is not a portion of the normal V region but rather corresponds to an unusual repair of broken DNA or to a non-cleaved polypeptide (? precursor; ? viral genome). It appears

therefore essential to obtain *N*-terminal sequences of several α-CD proteins, despite the heterogeneity which precluded success in previous attempts.

All 60 α-CD proteins which have been typed so far in our laboratory belong to the α1 subclass. The absence of a single case of α2 heavy chain disease in this series is probably not accidental since 10% of the normal serum IgA and 30% of the normal secretory IgA molecules belong to the α2 subclass (Grey *et al.* 1968). The fact that all the molecules of α-CD protein in a given patient belong to only one IgA subclass and the finding of a single amino acid substitution in position 12 of protein DEF (Fig. 1) (which could however possibly represent an allotypic difference) strongly suggest that α-CD proteins are monoclonal. However, this assumption cannot be confirmed because an essential criterion for monoclonality, namely structural homogeneity of the variable region, could not be demonstrated. All attempts to raise individually specific ('idiotypic') antibodies to several α-CD proteins have failed in our laboratory.

## CELLULAR STUDIES

Cultures of jejunal biopsy specimens from α-CD patients in medium containing $^{14}$C-labelled amino acids have established that the α-CD protein is synthesized *in vitro* by the proliferating cells. Radio-immunoelectrophoretic analysis of protein synthesized *in vitro* by cells teased from mesenteric nodes also demonstrated the production of labelled α-CD proteins. Immunofluorescent studies of intestinal mucosa and mesenteric nodes revealed variable and sometimes weak staining in the cytoplasm of cells composing the cellular infiltrate. No membrane-bound immunoglobulin was usually detectable on the surface of α-CD protein-synthesizing cells.

Biosynthetic studies with *in vitro* labelling excluded the possibility of the synthesis of a normal-size α chain followed by intracellular degradation to a smaller fragment after its release from the ribosomes (Buxbaum & Preud'homme 1972).

Immunofluorescent studies failed to detect any light chain production in the cells which secrete α-CD proteins. No free labelled light chains were found at radio-immunoelectrophoretic analysis of proteins synthesized *in vitro* by intestinal or mesenteric node proliferating cells. This failure of light chain synthesis has been confirmed by biosynthetic studies of nascent Ig subunits in such patients. The possibility remains, however, that the light chain is transcribed but not translated. Studies looking for the presence or absence of its messenger are warranted, since Cowan *et al.* (1974) have detected an inactive light chain messenger RNA in a non-secreting variant of a mouse myeloma

which contained an abnormally short heavy chain and failed to synthesize light chain.

## NATURAL HISTORY OF ALPHA CHAIN DISEASE

The natural history of α-CD is probably of utmost importance but is not yet fully elucidated. Its spontaneous course may be continuous but often proceeds as exacerbations separated by more or less complete and prolonged improvements. The main clinical features of the digestive form of α-CD are markedly uniform: severe malabsorption syndrome, chronic diarrhoea with steatorrhoea, abdominal pains, vomiting, massive loss of weight, finger clubbing.

The most important characteristic of α-CD is that the disease appears to proceed in two stages. The early stage is characterized by a diffuse and extensive plasma cell infiltration of the entire length of the small intestine, particularly in the upper part. Multiple biopsies taken during laparotomy or with a peroral capsule show a massive infiltration of the lamina propria by round cells which belong mostly to the plasma cell series, as confirmed by electron microscopy. The plasma cell infiltration causes wide separation and sparsity of the crypts and obliteration of the villous architecture without significant impairment of the integrity of the surface epithelium. In some instances the infiltrate extends to the deep submucosa. Mesenteric lymph nodes very often show the same cellular infiltrate which may lead to an obliteration of the normal architecture. In the absence of therapy, there is progressive deterioration usually culminating in frank malignancy with immunoblastic tumours which may lead to obstruction, intussusception or perforation of the small intestine. The lymphoma cells may form single or multiple circumscribed tumours. In some of these cases, the characteristic plasma cell infiltration of α-CD might not be recognized if intestinal biopsies were not performed at a site distant from the tumours. Noticeably, at any stage of the growth of the lymphoma, mesenteric lymph nodes show usually a higher degree of malignancy than the intestinal wall.

Patients with well-documented initial status and adequate long-term follow-up are still scarce. In two patients who were regularly followed in Paris until death, it was possible to observe the progressive passage from the mature plasmacytic proliferation of α-CD to overt malignant lymphomas (Bognel *et al.* 1972; Galian *et al.* 1977). Foci of large malignant cells first appeared in the deep mucosa and submucosa of the gut and all transitional forms between these cells and the plasma cells were observed.

Alpha chain disease is clearly a disease of the gut-associated lymphoid system. The extensive plasma cell infiltration almost always remains confined to the enteromesenteric area. Involvement of the large intestine or

stomach is rare. Hepatosplenomegaly is usually not observed. Peripheral lymphadenopathy is a very rare sign. Abdominal lymphangiography may reveal in some patients an involvement of the retroperitoneal lymph nodes. Even at the second stage, dissemination of the immunoblastic tumours outside the abdomen is a rare and late event of the disease.

Whether the plasmacytic phase of α-CD is 'pre-malignant' is open to question. The hypothesis of a benign process seems unlikely in those patients with high and rising serum levels of the pathological protein, if its monoclonal nature is confirmed. This explanation is also unlikely when the plasma cell proliferation penetrates deeply into the submucosa and possibly muscularis propria, or completely disorganizes the architecture of the mesenteric nodes and shows atypical forms with an admixture of large 'immunoblastic' cells. This pleomorphic invasive proliferation probably represents the early stage of the malignant lymphoma. However, in most cases, the plasma cells of the infiltrate appear to be mature, well-differentiated with scarce mitoses. This does not necessarily militate against malignancy since in other malignant lymphoproliferative disorders such as chronic lymphocytic leukaemia and Waldenström's macroglobulinaemia the proliferating cells are normal in appearance. Karyotypic studies of plasma cells taken from the intestinal infiltrate during the 'benign' phase may help to resolve this question. The truly benign nature of α-CD at its initial stage is a real possibility since apparently complete remission of the disease was achieved in several patients treated only with oral antibiotics (Rogé *et al.* 1970; Monges *et al.* 1975; Rambaud *et al.* 1976; B. Ramot, personal communication). Disappearance of the α-CD protein from the serum and intestinal fluid was noted in these patients together with a normal histological appearance and negative immunofluorescent studies. It should be emphasized that in one of these patients (Rogé *et al.* 1970) the complete remission had lasted for six years after withdrawal of the antibiotic therapy. It is also noteworthy that, in the three published cases, the initial serum level of α-CD protein was relatively low.

The overt malignant tumours which arise in the late course of α-CD were first classified as reticulum cell sarcoma or Hodgkin's disease. According to the present views of the pathologists, they should be considered as large cell 'immunoblastic' lymphomas. Several lines of evidence suggest that, if we assume that the plasmacytic proliferation of α-CD is monoclonal in nature, these large lymphomatous cells may be derived from the same B cell clone as the initial plasma cell proliferation. The two types of proliferation can be seen intermingled in the lymph nodes and in the gut (Bognel *et al.* 1972). Rough endoplasmic reticulum may be found by electron microscopy in the apparently poorly differentiated malignant cells (Doe 1975). All transitional

forms between the large undifferentiated malignant cells and the mature plasma cells may be found in some patients. Intracytoplasmic α-CD protein may be undetectable by immunofluorescence in the most malignant and undifferentiated cells (Bognel *et al.* 1972). However, α-CD protein has been identified in one patient in the perinuclear cisternae of the large lymphomatous cells found in the bone marrow (F. Reyes, personal communication). Furthermore, the study of membrane-bound immunoglobulins has provided conclusive evidence that the sarcomatous cells of morphologically similar lymphomas arising in two other immunoproliferative disorders, namely chronic lymphocytic leukaemia and Waldenström's macroglobulinaemia, originate from the same clone as the previous lymphoid proliferation (Brouet *et al.* 1975). Alpha chains were recently found in our laboratory on the surface of large lymphoma cells in one patient with α-CD.

The typical clinicopathological pattern of the intestinal form of α-CD may be associated with unusual immunoglobulin findings. In two patients, an entire IgA myeloma globulin was found in the serum and Bence-Jones protein was present in the urine of one of these cases (Chantar *et al.* 1974; Tangun *et al.* 1975). In another young girl who was under the care of Dr Stephen Bender in Frankfurt and who presented with the typical clinicopathological features of intestinal α-CD, we demonstrated a γ-heavy chain disease protein in the serum and in the intestinal biopsy. In a few patients with the usual clinicopathological pattern, we have been unable to detect the α-CD protein in the serum. Such a failure may reflect the insensitivity of the techniques used or the advanced undifferentiated stage of the malignancy. Careful prospective studies should include a systematic search for the abnormal protein in jejunal fluid and, in negative cases, at the intracellular level by immunofluorescence and biosynthetic studies, since non-secretory forms of α-CD may exist and immediate degradation of the incomplete α chain may occur. Such a study is necessary in order to establish whether the majority, if not all, cases of so-called 'Mediterranean lymphoma' represent the late malignant phase of α-CD. These Mediterranean lymphomas—that is, diffuse primary intestinal lymphoma associated with malabsorption and occurring in underprivileged young patients—were first reported in Israel (Ramot *et al.* 1965; Eidelman *et al.* 1966). These initial reports did not include immunoglobulin studies. A retrospective pathological study (Rappaport *et al.* 1972) revealed that the majority of these lymphomas begin as an apparently benign infiltration of the small intestine by plasma cells. Our hypothesis (Seligmann & Rambaud 1969) that many of these lymphomas are in fact α-CD, provided their definition is restricted to cases showing a diffuse plasma cell proliferation with or without superimposed sarcoma, has been confirmed by the study of serum immunoglobulins in numerous such patients.

PATHOGENESIS

TABLE 1

Geographic origin of 100 patients with alpha chain disease

| | | | |
|---|---|---|---|
| *Africa* | | *South America* | |
| Tunisia | 20 | Columbia | 1 |
| Algeria | 18 | North Argentina | 1 |
| South Africa | 2 | Mexico | 1 |
| Morocco | 1 | *Europe* | |
| *Middle East* | | Spain | 9 |
| Iran | 10 | South Italy | 5 |
| Israel | 8 | Turkey | 6 |
| Lebanon | 2 | Yugoslavia | 2 |
| Syria | 1 | Greece | 2 |
| Libya | 1 | Portugal | 1 |
| Iraq | 1 | Finland | 1 |
| *Far East* | | Netherlands | 1* |
| Pakistan | 2 | Great Britain | 1* |
| Cambodia | 1 | *North America* | |
| India | 1 | USA | 1* |

* Respiratory forms of the disease.

The geographic distribution of α-CD patients is very peculiar (Table 1). There now appears to be a wide spectrum of racial or ethnic origins and cases have been found in many parts of the world. It should be emphasized that the patients from the Netherlands, Great Britain and United States were those with the respiratory form of the disease and without detectable intestinal involvement. The digestive form of the disease appears to be extremely rare in western 'developed' nations and there is a clear predilection for underprivileged populations. These findings suggest that environmental factors providing a local and protracted antigenic stimulation may play an important role in its pathogenesis (Seligmann *et al.* 1971). One common factor among susceptible populations of various ethnic origins is their exposure to an environment of poor hygiene in areas with a high degree of infestation by intestinal pathogens. Studies conducted in affected populations have shown that chronic gastrointestinal infection and diarrhoea are common and serial intestinal biopsies in healthy people have shown an increased lymphocytic and plasma cell infiltration within the lamina propria of the small bowel. Since orally ingested microorganisms are known to be a powerful proliferative stimulus to the secretory IgA system, the early phase of α-CD could represent an aberrant humoral immune response following sustained topical antigenic stimulation of the intestinal mucosa. The specific

or non-specific nature of the postulated stimulating microorganisms is open to question. Limited bacteriological, parasitological and virological studies have not revealed evidence for a specific agent associated with α-CD. However, the postulated antigenic stimulation may have occurred many years before α-CD became clinically manifest. The clinical onset of the disease has occurred in some patients more than ten years after withdrawal from the environmental factors. Microorganisms involved in the pathogenesis of α-CD may be present only during infancy or childhood and absent in identifiable form years later at the time of diagnosis. Unfortunately, the absence of Fab in α-CD protein precludes its use for identifying putative antigenic stimuli. These environmental factors could trigger the clonal proliferation directly. Alternatively, they may only be predisposing factors causing a non-specific stimulation of immunocytes which could potentiate the oncogenic effect of a virus interfering with genes controlling IgA synthesis (Rambaud & Matuchansky 1973).

The postulated environmental antigenic stimulus might be associated with an underlying immunodeficiency. This could be a defect rendering the host more susceptible to infection with oncogenic organisms or a basic defect of the feedback mechanisms controlling the cellular proliferative response to stimulation. Immunodeficiency could be due to malnutrition, especially in early infancy, or to genetic factors. In fact, the role of environmental factors does not exclude the possibility of predisposing genetic factors. Although limited family studies have failed to reveal consistent Ig abnormalities, a search for genetic markers may help to identify predisposed subjects. Raised serum levels of the intestinal iso-enzyme of alkaline phosphatase were reported in patients with α-CD and Mediterranean lymphoma and in their healthy relatives (Ramot & Streifler 1966; Doe *et al.* 1972; Lewin *et al.* 1976).

It is remarkable that the plasma cell proliferation resulting from the postulated antigen stimulation appears to lead to α-CD rather than to myeloma. This fact suggests the following hypotheses (WHO Meeting Report 1976). An abnormal B cell clone synthesizing the α-CD protein could be produced in the gut through a series of recombinant events during embryogenesis. Another possibility is that a somatic mutational event gives rise to a cell producing the α-CD protein, permitting it to enter the gut-associated lymphoid system and to home into the lamina propria. In either case, the abnormal clone would overgrow in abnormal microenvironmental situations and could for instance be susceptible to the proliferative stimulus of bacterial lipopolysaccharide in the intestinal lumen. In addition, the abnormal clone could have a selective advantage for proliferation because of the lack of antibody activity of its immunoglobulin product, possibly resulting in the suppression of a feedback mechanism.

Alpha chain disease is characterized by a continuous sequence of events ranging from an apparently benign hyperplastic process reversible by the administration of antibiotics to an overt neoplastic proliferation. The elucidation of this sequence of events, as well as continued structural and cellular studies, may offer a revealing insight into the development of lymphomas in the gut-associated lymphoid system.

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## Discussion

*Davies:* Your Table 1 (p. 271) showing the geographical origin of the cases recorded a number in Algeria. Were they diagnosed in France or in Algeria?

*Seligmann:* A number were diagnosed in France; subsequently some were diagnosed in Algiers. I went there soon after we found the first cases and retrospectively we found a number of cases there. Interestingly enough, some of these patients were thought to have peritoneal tuberculosis, which is not surprising from the clinical pattern of the disease. Many of the 20 or so cases in Tunis were diagnosed there. When the local clinicians know how to detect the disease, the percentage of cases in which we find the alpha chain disease protein in the sera sent to us becomes very high.

*Booth:* The geographical distribution is interesting, because the question is whether it is in fact geographical or racial. The vast majority of the cases are from that Mediterranean area in which the Arabs lived at some stage in their history. That applies to Southern Italy, Greece and all the countries you mentioned. The only one that does not fit is Finland. Among the Israeli cases, no case has been recorded in the Ashkenazim. Going further East, I don't know of any case recorded in China, and there is no case yet from Black Africa. If it is an infection or a question of reaction to a recurrent infection, the obvious place to look is Australia among the aborigines, because the incidence of gut infection in children particularly is very high. I have corresponded with Australian friends and no case has yet been found there. So it could be genetic rather than environmental in causation.

In Africa, the cases in Cape Town described by Novis and his colleagues (1971) are interesting. I have seen some of them, and they call them Cape Coloured people. There was a lot of slave trading down that area by the Arabs for a long period and one wonders if Mediterranean blood didn't get mixed in at some stage.

*Seligmann:* This reminds me of letters I have had from Spain where they say that the patient is pure Spanish without a drop of Arabic blood, which is totally impossible! My guess would be that this disease is probably frequent in Central and South America, which would probably solve the problem of a genetic or geographic correlation. The disease has not been looked for there very much, but in Mexico City when they began to look one case was found very quickly. A similar case has been published from Texas. I should add that one of the cases recently studied in Paris by Rambaud was a French person who was born in Algeria and had lived there for a number of years.

As you say, there is no case from Black Africa, although we asked friends to look carefully for it in Dakar and the Ivory Coast. We hypothesized that if

our views on the pathogenesis of alpha chain disease were right, one might expect that in these Black African countries, in view of the kind of parasites common there, you would find μ-chain disease. In fact, two cases of μ-chain disease were found in the Ivory Coast, once I had asked people to look for it (Bonhomme *et al.* 1974; Danon *et al.* 1975). So this is possibly something similar in pathogenesis.

*Davies:* Your suggestion that because there is a lack of the appropriate Fab fragment, you are failing to get a feedback mechanism operating, is very interesting. It perhaps indicates that if you took normal serum IgA from people in the same area and gave it to the patients, you would get this feedback operating. That is to say, you are presumably missing a particular kind of antibody. Presumably the disease is a rare manifestation of a fairly common infection. If so, you might be able to treat it by simply giving IgA.

*Seligmann:* This is possible. I agree that treatment in the first stage of the disease in addition to the administration of oral antibiotics may be the proper immunoglobulins, some of them having the right antibody activity. In fact Dr Rosen has told me that in his case of the respiratory form of alpha chain disease in a young boy, the patient is doing well with progressively decreased levels of the alpha chain disease protein. He gave some antibiotics but the main treatment was gammaglobulins.

*Rosen:* The child no longer has his abnormal α-chain but remains very hypogammaglobulinaemic. He is doing well on replacement therapy.

*Booth:* On the question of tumours in the gut, one can get intestinal obstruction with big lumps of tissue. The lesson of this meeting has been that these IgA cells must be turning over rapidly. If the half-time of an IgA cell is normally 4.7 days, what is the doubling time or half-time of these cells or tumours in the intestine? Is there a normal circulation going on? I presume there is, because our first case was a patient in whom we found primitive-looking plasma cells in the blood (Doe *et al.* 1972). So presumably there is an enormous circulation. How one maintains that circulation and at the same time develops a tumour big enough to block the intestine baffles me completely.

*Gowans:* It might be the reverse: perhaps the cells are abnormal and have a long life and that's why accumulations develop.

*Seligmann:* I have no answer to this question about the lifespan of these cells. In the circulation, in the first stage you don't usually see plasma cells in the blood; in the second malignant stage they may be found. Even in the first stage you may see a few α-chain-containing plasma cells in the marrow (Seligmann *et al.* 1969).

*Booth:* In myeloma how long do plasma cells survive?

*Gowans:* Mattioli & Tomasi (1973) studied the lifespan of IgA-secreting cells

by giving continuous thymidine infusions to mice. They found a half-life of 4.7 days, although some labelled cells persisted for 7–8 weeks.

*White:* If you produce plasma cell proliferation in a rabbit by hyperimmunization, three weeks later it's completely back to normal. The plasma cells cannot therefore have a half-life of more than a few days.

*Evans:* Are the plasma cells that you see in the first stage capable of division, or do they originate from lymphoid precursors which are not obviously plasma cells?

*Seligmann:* Mitotic figures are rare in the first stage of plasmacytic infiltration in this disease. Divisions are seen more often in the second stage with the tumours. I cannot answer the question more precisely.

*Evans:* Are the peripheral blood leucocytes normal, in terms of their surface cell markers?

*Seligmann:* In the few cases where this has been studied they were normal, but I should add that in the few cases where membrane-bound immunoglobulins have been studied in alpha chain disease, no heavy chain was usually detected on the surface of the proliferating cells.

*Booth:* It is sometimes difficult to demonstrate immunofluorescent staining on the gut containing all these plasma cells.

*Seligmann:* We often had faint staining for intracytoplasmic $\alpha$ chains (Seligmann *et al.* 1969), but our technique was not as good as that of Dr Brandtzaeg! He tells me that in the case he studied he found bright staining.

*Brandtzaeg:* We also checked for the J chain, and this is relevant to the secretory IgA system origin of this disease. The J chain is present in the cells although its synthesis seems to be defective.

*Booth:* Do you find the alpha chain?

*Brandtzaeg:* Yes, but in the Finnish boy Dr E. Savilahti and I studied the cells seemed to be very immature.

*Booth:* The Finnish case was predominantly ileal as opposed to jejunal disease, as well as in the large intestine, so this case was somewhat different from those described by Dr Seligmann.

*Seligmann:* It is interesting that this is the only digestive case recorded in Northern Europe. Earlier (p. 38) it was suggested that the IgA system in the large intestine may be different from that in the small intestine. The Finnish case could be more like the respiratory forms.

*Beeson:* Would you expand on the antibiotic therapy? Were you aiming at anaerobes or aerobes; what drugs did you use; and did you have any failures?

*Seligmann:* Treatment is purely empirical. The antibiotics given in cases where remission was complete were usually tetracyclines. In no case so far have antibiotics been given according to the sensitivity to antibiotics of the intestinal

flora, if something abnormal was found. The shortest delay between the beginning of treatment and complete remission was 2–3 months and the longest delay to complete remission was one year. As far as I know, the number of patients with well-documented complete remission, including serum studies, biopsies and so on, is four or five so far.

There were some failures. One problem is that many of these patients did not have systematic laparotomy and in my opinion and that of my gastroenterological colleague, J. C. Rambaud, the first thing you should do in alpha chain disease without overt malignant tumours is a laparotomy and multiple biopsies to ascertain whether the patient is at the stage of purely plasmacytic infiltration or whether there are lymphomatous tumours and immunoblastic proliferation. It is difficult to say that antibiotics have failed in patients who have not undergone systematic laparotomy, because at the sarcomatous stage one would expect antibiotics to fail. The patients with a good follow-up are rare. Many have been studied in countries where a good follow-up is difficult and there are only a few cases, but the data are enough to say that in some patients, who are presumably at the first stage, you can get a complete remission.

*Booth:* J. C. Rambaud's and your practice of staging seems logical when you compare it with lymphomas.

*Brandtzaeg:* I don't think we can presume that the cells are different, even though the site of the lesion is. On Dr Davies' suggestion of treating these patients with IgA, I think it must be colostral IgA from the mothers in the geographical region, because this is a defect of the secretory IgA system.

*Rosen:* Dr Seligmann, is it possible that you haven't detected an IgA2 heavy chain because the chain isn't covalently held together and you may be getting degradation because of the structural anomaly?

*Seligmann:* This is a possibility which we have suggested, but we have no way to prove or disprove it at present. In fact, cellular studies with antisera specific for subclasses should be done in cases without detectable α-chain protein in the serum.

*Cebra:* Do any of the structural studies suggest that any part of the V gene message is being translated to give rise to any of your proteins? Since you don't find any surface product representing part of the α chain, and yet you suggest a compartmentalization of these cells, presumably the lodging properties shown by these cells and probably by normal IgA immunoblasts are not attributable to surface IgA? One possibility to explain lodging of IgA cells is that glycosyltransferases that build oligosaccharides onto IgA might be involved in changes in membrane glycoproteins or glycolipids of B cells. Have any of your structural studies concerned the oligosaccharides of these heavy chain proteins?

*Seligmann:* We have very few data on the variable part of these proteins,

since the only sequences we have are eight residues in one case and two residues in the other! These eight residues don't resemble much of what is known in V regions. For this reason, one hypothesis is that it could be something else than the variable region, which could be still more exciting. It could be a precursor or a viral genome or something else. We can't be sure that it is the V region that is defective.

The absence of membrane-bound chains on these cells is not a constant finding. Recently we had a patient in whom α-chains were detected on the surface of large sarcomatous cells for the first time. In our experience, the absence of heavy chains on the membrane of the cells in many but not all cases is common in all kinds of heavy chain diseases. We found the same in some cases of γ- and μ-chain disease. The carbohydrate content of α-chain disease protein is high (Seligmann *et al.* 1971) but the oligosaccharide moiety has not been studied.

*Rosen:* There must be several of them, to get PAS-positive staining.

*Gowans:* Are the Peyer's patches hypertrophic in the early stages of the disease, on the assumption that the cells would be originating there?

*Seligmann:* I have no personal experience here, as very few patients have been studied *post mortem.*

*Gowans:* At laparotomy, are the patches grossly hypertrophic?

*Seligmann:* No.

*White:* When one produces myeloma experimentally by giving a stimulus like paraffin oil, does one ever see this two-stage process? In other words, do you see a stage of diffuse plasma cell infiltration without obvious malignancy, or is it a kind of tumour right from the start?

*Seligmann:* I have discussed this many times with Mike Potter. The answer is that there is not a long-term 'pre-malignant' phase.

*White:* The question is whether there is diffuse plasma cell infiltration. It seems you do get this and at one focus you get a neoplasm. That isn't the picture that I have derived from descriptions of experimental myeloma production.

*Evans:* Human myeloma cases sometimes show diffuse infiltration of marrow; after melphalan treatment such cases may develop soft tissue tumours of morphologically undifferentiated but immunoglobulin-producing cells. This may be comparable with the observations in alpha chain disease.

*Seligmann:* The situation where you have chronic proliferation of B cells followed by an acute exacerbation with a B cell sarcoma is well known. We know this in chronic lymphocytic leukaemia, with or without treatment, and in macroglobulinaemia. It can also happen in myeloma and heavy chain diseases. So this is usual in the natural history of chronic B cell malignancies. We were

able to prove in some such instances that the blastic or sarcoma cells derive from the same clone as the previous chronic malignancy (Brouet *et al.* 1973, 1975), since we found the same Ig chains on both types of cells and the same antibody activity in the membrane-bound immunoglobulins in one case, which is proof of the same variable region. The special point about alpha chain disease is that the first stage may not be truly malignant, as opposed to macroglobulinaemia or chronic lymphatic leukaemia, which are usually viewed as chronic malignant processes.

*Lachmann:* How does this differ from the so-called benign monoclonal gammopathy which becomes myeloma after a variable period of years?

*Seligmann:* It differs in any case in the frequency in which malignancy occurs, because in alpha chain disease malignancy would probably occur in most patients after a few years, and this kind of evolution of benign monoclonal gammopathy is documented in very few patients and after a very long time.

*Lachmann:* In other words, the first stage may be longer than the life of the patient!

*Mayrhofer:* How does this pattern of events compare to that in those patients with coeliac disease whose illness takes a deteriorating course, eventually leading to development of an IgA-secreting lymphoma?

*Seligmann:* Our alpha chain disease patients have no past history of anything looking like coeliac disease.

*Booth:* It is really quite different. The lymphoma in coeliac disease is usually patchy and not a diffuse involvement of the intestine. It can also involve areas other than the gut.

*Mayrhofer:* Is it often associated with increased IgA levels?

*Booth:* In some cases but not all.

*Mayrhofer:* Does the tumour produce the IgA?

*Booth:* I don't think that's known.

*André:* Are there any modifications of the intestinal flora in patients with non-malignant alpha chain disease?

*Seligmann:* This has not been looked for carefully in many patients, but where it has, there has been no important abnormality. The anaerobic flora has not been well studied in many patients.

*André:* After tetracycline therapy, are there any obvious changes?

*Seligmann:* This has not been studied systematically yet.

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*Immunology of the Gut*
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# Immunological studies in inflammatory bowel disease

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*Abstract* Three aspects of immunological function were studied in patients with Crohn's disease and ulcerative colitis (inflammatory bowel disease): atopic status and serum IgE levels; serum concentration of C-reactive protein; and C3 activation. The incidence of atopy, assessed by prick testing with common allergens, did not differ in patients with inflammatory bowel disease from healthy controls. 12 of 39 patients with Crohn's disease and 5 of 20 with ulcerative colitis, among whom were some non-atopic subjects, had elevated serum levels of IgE. Serum levels of C-reactive protein in patients were significantly greater than normal, even in those in whom the disease was clinically quiescent. Symptomatic patients with Crohn's disease had significantly higher levels than similar patients with ulcerative colitis and in Crohn's disease the levels correlated well with an overall assessment of severity and disease activity. Although conversion of C3 was detected in fresh serum samples from inflammatory bowel disease patients and not controls, only minimal traces were present in just 7 of 89 samples of EDTA–plasma from 47 patients; this finding did not correlate with disease activity. However, there were low titres of immunoconglutinin in the sera of some patients, but not in controls, suggesting that complement activation may be occurring *in vivo*.

Ulcerative colitis and Crohn's disease are severe, chronic inflammatory conditions of the intestine, the aetiology and pathogenesis of which are unknown. They cause appreciable morbidity and may be fatal, and the incidence of Crohn's disease in England has tripled in the past decade or so (Miller *et al.* 1974). In both conditions the pathology of the lesions includes mononuclear lymphoid cells as well as other features which suggest that immunological mechanisms may be operative. A variety of immunological abnormalities have been described in these patients, but although certain phenomena are generally accepted

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there remains controversy about others (reviewed in Jewell & Hodgson 1976). It is not possible to formulate a generally acceptable hypothesis of aetiopathogenesis on the basis of existing observations, and all of the immunological findings could be secondary or epiphenomena. Even so, immunological mechanisms might be important in some of the manifestations of disease, and might also help to differentiate between these two conditions in which, despite a considerable clinical overlap, the prognosis and management may be quite different.

We report here the results of studies of three different aspects of immunological function in patients with inflammatory bowel disease.

## ATOPIC STATUS AND SERUM IgE LEVELS

Atopy may be considered in immunological terms as a predisposition to respond to common normal environmental antigens by the production of IgE antibodies (Pepys 1975). Abnormal environmental exposure can induce IgE antibody formation in non-atopic individuals (Pepys 1975). Atopic individuals and their atopic status may be identified by prick testing of the skin with extracts of the common, chiefly inhaled, allergens (Pepys 1975). Prick testing is a precise procedure which, if it elicits the characteristic weal and flare reaction, indicates the presence of reaginic antibodies which are generally of IgE class, although short-term mast cell-sensitizing IgG antibodies have also been demonstrated in man (Parish 1970).

There is a strong correlation between atopic status, defined by prick testing, and the occurrence of certain diseases, particularly extrinsic asthma, 'hay-fever' and infantile eczema. IgE antibodies are clearly implicated in the pathogenesis of the first two conditions. Quite apart from these diseases the atopic status of an individual, in terms of this propensity to produce IgE antibody, can modify profoundly the immunological and pathological consequences of abnormal exposure to antigens in the environment (Pepys 1969). Knowledge of the atopic status of patients with diseases of unknown but possibly immunological pathogenesis, such as inflammatory bowel disease, is therefore clearly of interest.

In the present study the atopic status of patients with Crohn's disease and ulcerative colitis was assessed using the following information: past and present history of asthma, 'hay-fever' or non-seasonal rhinitis clearly provoked by an extrinsic agent, and eczema; history of these conditions among first degree relatives; and the results of prick testing with a batch of common allergens (Bencard Ltd, Worthing) (Table 1). The food extracts were included though they are not as a rule helpful in identifying food allergens; no reactions

TABLE 1

Allergens used for prick testing

| | |
|---|---|
| Grass pollens | Cat fur |
| Tree pollens | Dog fur |
| Shrub pollens | |
| | Milk |
| *Dermatophagoides pteronyssinus* | Whole egg |
| | Fish |
| Moulds | Wheat |
| *Aspergillus fumigatus* | |

Allergen extracts were obtained from Bencard Ltd, Worthing.

to them were observed in any patients. Controls who were similarly assessed were matched approximately for age and sex with the patients but were otherwise randomly selected, and were members of the medical, nursing and technical staff of the RPMS. The results are shown in Tables 2 and 3.

Analysis of the results in Table 3 by the $\chi^2$ test demonstrated no significant differences between the three groups. The most meaningful observation is the incidence of one or more positive prick tests, namely about 26% in both disease groups. In large-scale testing of healthy subjects screened pre-employment for evidence of atopy the incidence is of the order of 30% (J. Pepys, personal communication 1976). Our control group had a higher than expected incidence of reactions, 50% giving one or more positive prick test reactions.

The incidence of asthma, 'hay-fever'/rhinitis and eczema was comparable to that reported by Jewell & Truelove (1972), and greater than that observed by Hammer *et al.* (1968). However, they both found a very low incidence among

TABLE 2

Atopic status in inflammatory bowel disease

| *Group* | *Prick tests negative*[a] | | *Prick tests positive*[a] | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *No family history of atopy* | *Family history of atopy* | *No atopic symptoms*[b] | | | *Atopic symptoms*[b] | | |
| | | | *1* | *1-3* | *>3* | *1* | *1-3* | *>3* |
| Crohn's disease | 17 | 14 | 2 | 2 | 0 | 1 | 2 | 4 |
| Ulcerative colitis | 12 | 7 | 1 | 2 | 0 | 0 | 3 | 1 |
| Controls | 9 | 6 | 0 | 3 | 1 | 4 | 2 | 5 |

[a] Subjects were prick tested with the allergens shown in Table 1. No individuals with negative prick tests had a history or suffered from asthma, 'hay-fever' or eczema.
[b] Asthma, 'hay-fever' or eczema.

TABLE 3

Atopic status in inflammatory bowel disease

| *Group (No.)* | *Positive prick test(s)[a]: atopic symptoms[b]* | | *Positive prick test(s)[a]: total* | | *First degree relatives with history of atopic symptoms[b]* | | |
|---|---|---|---|---|---|---|---|
| | *No.* | *%* | *No.* | *%* | *No.* | *No. at risk* | *%* |
| Crohn's disease (42) | 7 | 16.7 | 11 | 26.2 | 23 | 184 | 12.5 |
| Ulcerative colitis (26) | 4 | 15.4 | 7 | 26.9 | 12 | 120 | 10.0 |
| Controls (30) | 10 | 33.3 | 15 | 50.0 | 15 | 109 | 13.8 |

[a] Subjects giving one or more positive prick test reactions with the allergens shown in Table 1.
[b] Asthma, 'hay-fever' or eczema.

controls, including normals and patients with other diseases. It is difficult to ascertain the true incidence of these atopy-associated or allergic diseases in the general population, owing to problems of definition and recognition. There are also differences in methods used in previous surveys and variations in incidence and manifestations with age, social and other factors. Perhaps the best survey is that of Blair (1974), who examined in detail 9145 members of a single general practice and found the incidence of asthma, 'hay-fever' and infantile eczema alone or in any combination to be 7.2%. This would suggest that the incidence in our patients and our controls may be high but the numbers of individuals are relatively small, and for the reasons stated interpretation of this sort of result should be most cautious. The firmest evidence remains the result of prick testing, an objective and well-validated procedure which, contrary to the essentially historical data in other studies of this question, does not support an association between atopy and inflammatory bowel disease.

IgE antibodies are induced under ordinary circumstances by mucosal exposure to antigens, and there is evidence that even transient deficiency of IgA can predispose to reaginic antibody formation (Soothill 1974). One possible mechanism is that impaired IgA responses may modify the mucosal barrier to antigen penetration and permit IgE production to be stimulated. Patients with inflammatory bowel disease have mucosal ulceration which may be expected to allow enhanced penetration of luminal antigens, and it is therefore of some interest to examine their IgE responses. Knowledge of patients' atopic status is essential in these studies for, although total serum IgE levels do not correlate particularly well with severity of clinical allergy in atopic subjects, they do nonetheless tend to be elevated above the very low levels (mean 36.3 units/ml, Nye *et al.* 1975) found in a strictly non-atopic population.

We measured total serum IgE by the relatively insensitive modified radial

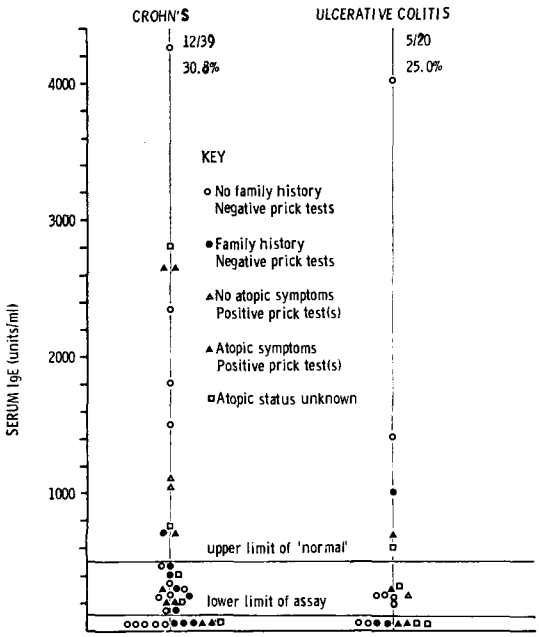


FIG. 1. Serum IgE levels in patients with inflammatory bowel disease. Family history and atopic symptoms refer to asthma, 'hay-fever' and infantile eczema. Prick tests were made with the allergens listed in Table 1. The proportions shown for each group are the patients with IgE levels above normal.

immunodiffusion method (Rowe 1969) which does not detect less than 100 units/ml. The levels among 'normal' individuals, who do not suffer from any clinical allergic symptoms, do not exceed 500 units/ml, shown in Fig. 1 as the upper limit of 'normal'. The present technique was not sufficiently sensitive to provide information about the lower range of IgE values; furthermore, the significance of the values recorded between 100 and 500 units/ml is not clear. These might be more frequent than would be found among healthy strictly non-atopic subjects, but a more discriminating assay is required to elucidate this. However, a proportion of patients with Crohn's disease or ulcerative colitis have definite elevation of total serum IgE, and some of these patients are non-atopic by all criteria.

There have been two previous studies of serum IgE in inflammatory bowel disease (Bergman *et al.* 1973; Brown *et al.* 1973), in neither of which were raised levels found, but IgE antibodies have a high affinity for the surface of mast cells and basophils, and it is these cell-bound molecules which are biologically important rather than the material free in the blood. The significance of

the present findings is not clear, though they do suggest that there may be enhanced stimulation of IgE formation in at least some patients with inflammatory bowel disease in the absence of any evidence of atopy. It would be of interest to know the antigen specificity of their IgE, and studies of this point are under way.

It is conceivable that IgE antibody directed against dietary or microbial antigens in the lumen could play a role in perpetuating damage caused by an initial insult which weakened the mucosal barrier to antigen. Also IgE-mediated immediate hypersensitivity reactions are known to play a permissive role in the development of IgG-mediated Arthus type reactions (Cochrane 1971). The lesions of both Crohn's disease and ulcerative colitis contain greatly increased numbers of IgG plasma cells (Baklien & Brandtzaeg 1975), and it has been suggested (Brandtzaeg 1974) that breakdown in the normal secretory IgA barrier at the mucosa can permit penetration of antigen and induction of local IgG antibody responses with an enhanced capacity for the mediation of tissue-damaging hypersensitivity reactions. It is of interest that a beneficial effect has recently been observed in ulcerative colitis of sodium cromoglycate, a drug which blocks IgE-dependent hypersensitivity reactions (Mani *et al.* 1976).

## C-REACTIVE PROTEIN

C-reactive protein (CRP) is a normal plasma protein which occurs in trace amounts, usually less than 2 $\mu$g/ml, in normal healthy individuals but which increases dramatically in a wide variety of pathological processes including trauma, infection, collagen disease and malignancy. It was initially detected by its interaction with pneumococcal C-substance, but in recent years it has been found to have a calcium-dependent binding capacity for a large number of different substances which are present in the body (Siegel *et al.* 1975) (Table 4), many of which may be expected to be more 'exposed' in damaged than in healthy tissue. Interaction between CRP and some of its substrates efficiently activates the classical pathway of complement (Siegel *et al.* 1974, 1975), and furthermore CRP has been reported to bind selectively to T lymphocytes and to inhibit some of their functions (Mortensen *et al.* 1975). CRP could therefore be an important modulator of inflammatory and immunological responses.

We have studied CRP in patients with inflammatory bowel disease in an attempt to correlate serum levels with diagnosis and disease activity, and in order to initiate an assessment of whether CRP might play a part in pathogenesis of the lesions or of other associated abnormalities. CRP was isolated from pathological plasma by absorption onto agar (Ganrot & Kindmark 1969), elution with EDTA, absorption with insolubilized anti-normal human serum

TABLE 4

Properties of C-reactive protein

| | |
|---|---|
| Normal plasma protein | Mol.wt. ~ 120 000 |
| Acute-phase reactant | |
| Calcium-mediated binding to: | |
| Pneumococcal C-substance, lecithin | |
| Polycations: histones, leucocyte cationic protein, myelin basic protein, protamine | |
| Polyanions: heparin, nucleic acid, dextran sulphate | |
| Reacts with low molecular weight substances: phosphoryl choline, mononucleotides, $SO_4^{2-}$ | |
| Fixes complement by classical pathway | |
| Binds to T cells *in vitro*; inhibits some of their properties | |

antibodies, and finally precipitation of residual impurities with 20% sodium sulphate. Anti-CRP serum was raised by immunization of a rabbit with the purified protein and used to measure the serum concentration of CRP by electroimmunodiffusion. The assay was calibrated using purified CRP as standard and it could detect 2 $\mu$g/ml with an error of $\pm$ 7% in replicate samples.

The patients studied were: 41 with Crohn's disease involving small, small and large or large bowel alone, diagnosed by clinical, radiological and where possible histological criteria; 43 with ulcerative colitis or proctititis diagnosed by clinical and radiological features and in whom Crohn's disease had been excluded as far as possible. The controls were 70 healthy members of the medical, nursing and technical staff of the RPMS whose age and sex distribution approximated to that of the patient populations. Venous blood samples were allowed to clot for 1–4 hours at room temperature before separation of serum and storage at —20 °C. One sample was tested for each control subject, whilst a total of 500 samples taken at different times from patients were examined.

There are considerable difficulties in assessing the severity and disease activity in inflammatory bowel disease, and there have been several attempts to quantitate clinical observations so that patients can be allocated to various categories. For the presentation of results here we tried to obtain groups suffering from ulcerative colitis and Crohn's disease of comparable overall clinical severity and we have therefore separated them only into the three broad categories, severe, moderate and quiescent, which are defined in Table 5. If an individual remained within a given category for more than one CRP measurement, the mean of all the levels (which were normally distributed) was used in plotting the results shown in Fig. 2. The statistical significance of differences between the various groups is shown in Table 6.

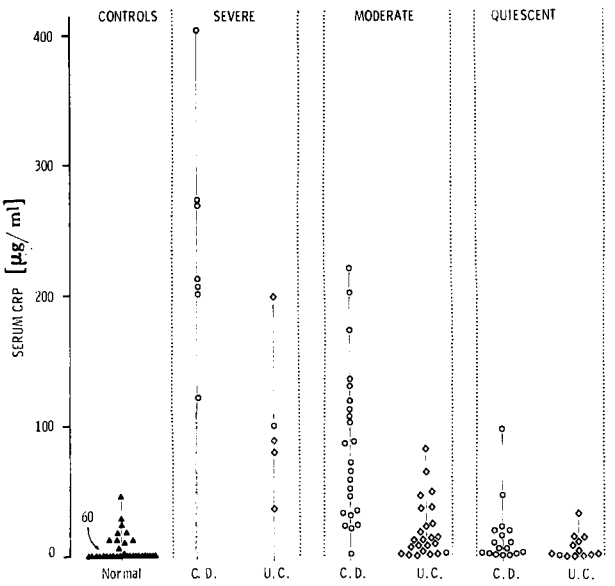


FIG. 2. Serum C-reactive protein levels in patients with inflammatory bowel disease. Each point represents a single individual. C.D., Crohn's disease; U.C., ulcerative colitis. For full details see text.

TABLE 5

Categories of disease activity in inflammatory bowel disease

| |
|---|
| *Severe* |
| Acute symptoms and signs necessitating immediate hospital admission |
| Abnormalities of ESR, Hb, WBC, serum albumin, rectal histology |
| *Moderate* |
| Variable symptoms, marked or mild, treatable as out-patients or by elective admission |
| Abnormalities among ESR, Hb, WBC, serum albumin, rectal histology |
| *Quiescent* |
| No clinical symptoms. Occasional abnormalities of ESR, rectal histology |

On the basis of this analysis and the longitudinal study of many patients during the course of illness and therapy three main interpretations are possible:

1. Among symptomatic patients those with Crohn's disease had significantly higher CRP levels than those with ulcerative colitis, and in pairs of individuals closely matched for disease activity the level was always greater in Crohn's disease. This measurement may therefore be useful in distinguishing between

TABLE 6

Statistical analysis of serum levels of C-reactive protein in inflammatory bowel disease
(1) Kruskal-Wallis one way non-parametric analysis of variance $P<0.001$
(2) Wilcoxon tests for differences between pairs of groups[a]

| | *Normal* | *Severe C.D.* | *Severe U.C.* | *Moderate C.D.* | *Moderate U.C.* | *Quiescent C.D.* | *Quiescent U.C.* |
|---|---|---|---|---|---|---|---|
| Normal | – | | | | | | |
| Severe C.D. | $P<0.01$ | – | | | | | |
| Severe U.C. | $P<0.01$ | $P<0.01$ | – | | | | |
| Moderate C.D. | $P<0.01$ | $P<0.01$ | N.S. | – | | | |
| Moderate U.C. | $P<0.01$ | $P<0.01$ | $P<0.01$ | $P<0.01$ | – | | |
| Quiescent C.D. | $P<0.01$ | $P<0.01$ | $P<0.01$ | $P<0.01$ | N.S. | | |
| Quiescent U.C. | $P<0\,01$ | $P<0.01$ | $P<0.01$ | $P<0.01$ | N.S. | N.S. | – |

[a] Statistical tables do not indicate significance greater than 1% level.
U.C., ulcerative colitis; C.D., Crohn's disease.

the two diseases in cases where the diagnosis is in doubt. Even life-threatening colitis did not produce very high CRP levels. For example two patients, whose levels on admission to hospital and before treatment are shown in Fig. 2 in the severe ulcerative colitis group, underwent emergency colectomy because of failure to respond to medical therapy within days. Comparable CRP values were frequent in moderately symptomatic out-patients with Crohn's disease.

2. In Crohn's disease the CRP levels correlated well with disease activity, whereas in ulcerative colitis the levels were appreciably greater in symptomatic than in asymptomatic patients only when the disease was extemely severe. In most individuals with either disease the CRP level tended to fall towards normal with remissions, either spontaneous or induced by therapy.

3. Even in the clinically quiescent state the CRP levels of the group as a whole differed significantly from normal, and a few patients with Crohn's disease in whom there were few or no symptoms had appreciably elevated levels. We have insufficient evidence to know whether measurement of serum CRP concentration can predict a clinical relapse, but it nonetheless seems to be a clinically useful aid in the assessment of activity as a basis for management.

Precise correlation of CRP concentration with other laboratory indices of disease activity is currently under way, but it is already clear that the CRP level does not just reflect the erythrocyte sedimentation rate, and is a much more sensitive indicator than serum albumin concentration or white blood cell count. Other aspects of the results of CRP measurement which are of interest are that it is elevated even in cases of Crohn's disease in which the colon is grossly spared, and that none of the treatments used,

including salazopyrine, corticosteroids, ACTH, azathioprine or metronidazole, seem to affect it unless there is remission of disease activity by both clinical and laboratory criteria.

Elevation of serum CRP is a non-specific response to tissue injury, but is probably fundamental as it appears early in both ontogeny (Felix *et al.* 1966) and phylogeny (Baldo & Fletcher 1973). The stimulus to CRP production and the determinants of the serum level attained are not known. The higher values we have observed in Crohn's disease may just reflect the more extensive tissue damage than occurs in ulcerative colitis. It is, however, also conceivable that the raised concentration of plasma CRP might influence inflammatory reactions in a number of ways resulting from its capacity for binding to phospholipids ubiquitous in cell membranes, for complement fixation and for adhering to T cells and inhibiting some of their functions. In contrast to the reported binding of CRP to T cells during *in vitro* culture (Mortensen *et al.* 1975) we, using a fluorescent rabbit $F(ab)_2$ anti-human CRP reagent, have not detected any lymphocytes bearing CRP in the peripheral blood of patients with high serum CRP levels. This raises the possibility that interaction between lymphocytes and CRP might affect their recirculatory behaviour, for example by acting as a trapping mechanism in damaged tissue. Many of the infiltrating lymphocytes in Crohn's disease are T cells (Strickland *et al.* 1975; Meuwissen *et al.* 1976).

## COMPLEMENT ACTIVATION

Complement is an important mediator of inflammation both at the humoral and at the cellular level. It also participates in cellular aspects of induction of the allergic response, probably as a consequence of the interaction between fixed C3 and the C3 receptors of B cells and macrophages (Pepys 1976). We have detected mononuclear cells with C3 receptors in cryostat sections of biopsies of lesions and resected tissue from all of four patients with Crohn's disease who were studied (M. B. Pepys & A. C. Dash, unpublished observations). There have been reports of immune complexes in the serum of patients with both Crohn's disease and ulcerative colitis (Doe *et al.* 1973; Jewell & MacLennan 1973), and for all these reasons it was of interest to see whether there is any evidence of *in vivo* complement activation in inflammatory bowel disease. We have used two approaches, first, examination of plasma for C3 conversion products and, second, assay of serum immunoconglutinins, which are autoantibodies against fixed complement, chiefly C3 (Lachmann 1967).

Initially, using a modified Laurell technique (Laurell 1965) of two-dimensional immunoelectrophoresis to study EDTA–plasma freshly separated in the cold, we found only very occasional traces of C3 conversion (Table 7). Subsequently

TABLE 7

Plasma C3 conversion in inflammatory bowel disease

| *Method*[a] | *Group* | *No. of patients* | *No. of samples* | *No. with C3 conversion*[d] | |
|---|---|---|---|---|---|
| | | | | *Patients* | *Samples* |
| 'Insensitive'[b] | Crohn's disease | 37 | 134 | 3 | 4 |
| | Ulcerative colitis | 20 | 50 | 2 | 2 |
| 'Sensitive'[c] | Crohn's disease | 34 | 68 | 6 | 1 + 5 (trace) |
| | Ulcerative colitis | 13 | 21 | 1 | 1 (trace) |

[a] Two-dimensional immunoelectrophoresis.
[b] Well origin, 1:5 dilution of sample, 5-10V/cm.
[c] Slot origin, neat sample, 10-20 V/cm.
[d] No sample showed more than 5% conversion; most much less.

Teisberg & Gjone (1975) reported that C3 conversion was often to be found in sera of patients with inflammatory bowel disease, but not in controls, and that the degree of conversion correlated with the extent and activity of disease. We therefore repeated our study comparing serum and plasma samples taken at the same time from each patient, and in addition using a Laurell technique which was capable of detecting minimal traces of C3 conversion and was more sensitive than that used in our first study. Fresh serum was separated one hour after venesection into plain glass tubes kept at 21 °C, whilst plasma was obtained by taking blood into EDTA on ice and centrifuging at 4° C; both sorts of samples were then stored in liquid nitrogen before testing.

We confirmed that there was some C3 conversion in all the sera from patients but in none of the healthy controls (Table 8). However, the degree of conversion did not correlate with the extent or activity of disease, and there was no conversion in any of the plasma samples.

TABLE 8

Plasma and serum C3 conversion in inflammatory bowel disease

| *Group* | *No.* | *C3 conversion in:*[a] | |
|---|---|---|---|
| | | *Plasma/EDTA* | *Fresh serum* |
| Crohn's disease | 8 | 0 | 8 |
| Ulcerative colitis | 5 | 0 | 5 |
| Controls | 10 | 0 | 10 |

[a] Detected by sensitive two-dimensional immunoelectrophoresis.
No sample showed more than 5% conversion.

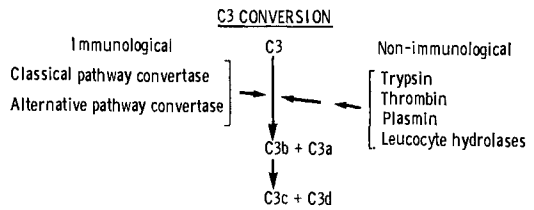


FIG. 3. C3 convertase enzymes.

C3 is known to be labile in serum as a result of its susceptibility to a variety of proteolytic enzymes (Fig. 3), and we had used EDTA–plasma freshly separated in the cold expressly to minimize this problem and to examine the *in vivo* C3 profile. It is difficult to ascribe C3 conversion in serum to *in vivo* complement activation, and we suggest that these findings may reflect acceleration of the normal *in vitro* conversion which occurs with clotting (Krøll 1970). This might result from a variety of factors which are absent in healthy controls, for example: polymorphonuclear leucocytosis, elevated CRP levels or alterations in other plasma proteins with biological activity in this respect. The conversion of factor B observed by Teisberg & Gjone (1975) in their patients can also be attributed to *in vitro* C3 conversion stimulating the C3b feedback loop of the alternative pathway (Lachmann & Nicol 1973).

We retested a number of EDTA–plasma samples by the more sensitive technique and found traces of C3 conversion in a small proportion (Table 7, p. 293). We conclude that this method does not provide very much evidence for *in vivo* C3 activation in the circulation in inflammatory bowel disease.

Immunoconglutinins in serum which had been heat-inactivated and absorbed with sheep erythrocytes were detected by agglutination of sheep erythrocytes sensitized with human complement. The sera of some of the patients contained low titres of immunoconglutinins, none greater than 1:4 (Table 9). The results on this small number of sera suggest that the occurrence of immunoconglutinin may be significant. Preliminary study of the levels of anti-C3 activity in parotid saliva did not reveal any difference between patients and controls.

The presence of immunoconglutinins in serum supports the idea that *in vivo* complement activation is taking place in inflammatory bowel disease, and accords with the finding of Potter *et al.* (1975) that there is an accelerated fractional catabolic rate of C3 in these patients with evidence for C3 consump-

TABLE 9

Serum immunoconglutinin in inflammatory bowel disease

| *Group* | *Number of individuals* | | | *Significance*[b] |
|---|---|---|---|---|
| | *Positive*[a] | *Negative* | *Total* | |
| Crohn's disease | 10 | 13 | 23 | $P=0.0135$ |
| Ulcerative colitis | 6 | 10 | 16 | $P=0.0536$ |
| Control | 0 | 11 | 11 | |

[a] Agglutination of complement-coated erythrocytes by inactivated absorbed serum. No serum titre was $> 1:4$.
[b] Fisher's exact test for difference from the controls.

tion at an extravascular site. There is thus some evidence suggesting that complement activation may be a feature of inflammatory bowel disease. More critical studies are required and, for example, a more sensitive assay for immunoconglutinin, which we are currently developing, might provide a clearer distinction between the levels in patients, with disease of varying extent and severity, and controls.

## CONCLUSIONS

The present studies shed some light on different aspects of immunological function in inflammatory bowel disease, although they do not favour any particular aetiopathogenetic hypothesis. From the practical point of view the results suggest that the measurement of serum CRP concentration may help to distinguish Crohn's disease from ulcerative colitis, and to assess disease activity in Crohn's disease.

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## Discussion

*Gowans:* I gather that these two diseases, ulcerative colitis and Crohn's disease, are very similar in certain respects. Can you elaborate on the similarities and the differences?

*Pepys:* Ulcerative colitis is a disease with acute and chronic inflammation in the colon; that is, with respect to the gut, it is restricted to the large bowel. It starts at the anus and may progress proximally to affect the whole colon. The lesions are always in contiguity. It presents a fairly homogeneous clinical picture. Crohn's disease may affect the bowel from the mouth to the anus and perianal skin. It is characterized by gross lesions in discontinuity. The histological marker present in many cases but not all is a granuloma which is not seen in ulcerative colitis.

*Gowans:* Are there any grounds for supposing that they have a common aetiology?

*Pepys:* When they affect the large bowel they may be clinically indistinguishable. There are some cases in which a diagnosis is never made, or is made only at operation or post mortem. There is a lot of overlap.

*Booth:* The fundamental pathological difference is that the pathology in the colon in ulcerative colitis is a mucosal ulceration that seems to involve the surface enterocytes as the primary lesion, whereas in Crohn's disease the lesion is a submucosal granuloma. Those are the extremes: in between are a whole range of changes.

*Evans:* I would not describe it as a *submucosal* granuloma; the mucosa is involved as well.

What concerned me, Dr Pepys, is the comparability of your groups. If you had compared cases of Crohn's disease with no obvious small bowel lesion, with cases of total colitis in ulcerative colitis, you might have a more valid comparison in terms of the volume of tissue involved. If you have included patients with ulcerative proctitis who may not have total colitis and compared them with patients with Crohn's disease who may have involvement of small intestine and the large intestine as well, it does not surprise me that there is a significant difference in an acute-phase reaction.

*Pepys:* As I said, the classification of patients with inflammatory bowel disease is difficult. It is often not possible to assess the overall extent of tissue damage even when all the clinical data are available. We tried to make the groups as comparable as possible in terms of overall clinical severity. For example, the 'severe' group contained patients with acute total ulcerative colitis and patients with acute total Crohn's colitis. Some of the ulcerative colitis patients went on to emergency colectomy because medical therapy failed. There were nonetheless significant differences in the C-reactive protein (CRP) levels between the two diseases, and this was the case even in less severely ill patients. On the question of small or large bowel involvement, our experience has been that patients with Crohn's disease affecting only the small intestine have CRP levels comparable to those seen in colonic disease.

*Lachmann:* On the whole, the measurement of acute-phase reactants has always turned out to be somewhat non-specific. Have you any evidence that if you regress the erythrocyte sedimentation rate (ESR) and the C-reactive protein levels in these two diseases, there are significant differences?

*Pepys:* The correlation coefficients ($r$) between serum CRP level and other clinical laboratory indices are shown in Table 1. No significant correlations were found among the ulcerative colitis patients for whom full data were

TABLE 1 (Pepys)

Relationship between serum concentration of C-reactive protein and other clinical laboratory tests

| | *ESR* | *WBC* | *Hb* | *Albumin* |
|---|---|---|---|---|
| *Crohn's disease* (44 patients) | | | | |
| Correlation coefficient ($r$) | 0.669 | 0.403 | —0.141 | —0.465 |
| Significance ($p$) | $<0.001$ | $<0.01$ | N.S. | $<0.01$ |
| $(1-r^2)\%$ | 55.2 | 83.8 | 98.0 | 78.4 |
| *Ulcerative colitis* (20 patients) | | | | |
| Correlation coefficient ($r$) | 0.435 | 0.274 | —0.122 | —0.247 |
| Significance ($p$) | N.S. | N.S. | N.S. | N.S. |
| $(1-r^2)\%$ | 81.1 | 92.5 | 98.5 | 93.9 |

available. Since $r^2$ represents the proportion of the variance of $y$ (CRP level) which can be attributed to its linear regression on $x$ (the other variables), the value of $1-r^2$ indicates the proportion of the variance of CRP which is independent of each of the other variables. Our results therefore suggest that measurement of serum CRP in Crohn's disease provides appreciably different information from that obtained by measuring ESR, WBC or serum albumin. This is borne out by sequential observations in individual patients.

*Lachmann:* Are you saying that the patients with Crohn's disease have discordantly high C-reactive protein levels compared to other parameters of the acute phase of inflammation, whereas ulcerative colitis patients have low C-reactive protein levels compared with the other parameters?

*Pepys:* All we can say is that for comparable abnormalities of ESR, WBC, etc., patients with Crohn's disease tend to have higher levels of serum CRP than patients with ulcerative colitis.

*Jones:* It is important to recognize that C-reactive protein may interfere with some of the tests used to detect immune complexes. Nevertheless, we have evidence in patients with coeliac disease, Crohn's disease and ulcerative colitis that a whole battery of tests are positive for immune complexes. For instance, we measure C3 and C4 levels, haemolytic complement, anti-complementary activity, Clq binding, and cryoprecipitability. Many of these tests may be affected by C-reactive protein, although I think that not all are. The question is of whether the levels of C-reactive protein are high enough to react with Clq.

We can confirm Bill Doe's evidence that coeliac disease patients when challenged with gluten develop immune complexes which aggregate platelets and that this activity subsequently fades (see Booth *et al.*, this volume, pp. 329–346). Perhaps the strongest evidence that at least some of this is due to immune complexes is that several of our patients with particularly high levels of anti-complementary activity and platelet aggregation have cryoglobulins, and it would be difficult to explain this phenomenon, which may be transient, in terms of C-reactive protein.

*Pepys:* The interaction of CRP with a variety of substrates activates the classical complement pathway via Clq very efficiently (Kaplan & Volanakis 1974; Siegel *et al.* 1975) and the concentration of CRP required is often exceeded in the sera of patients with inflammatory bowel disease. Some of the lipoprotein substrates with which CRP reacts are ubiquitous, and this may be a mechanism for the C3 conversion which we see in serum but not in plasma. I do not know whether the presence of CRP in serum can lead to Clq binding in your test system.

*Lachmann:* I imagine, Dr Jones, that you do your Clq precipitation in the

presence of EDTA. The reaction of C-reactive protein is calcium-dependent and should therefore not interfere.

*Jones:* That is possible. Do you know how C-reactive protein reacts with platelets in the platelet aggregation test for immune complexes? We find this test positive in some of our cases of Crohn's disease.

*Pepys:* CRP does not aggregate platelets on its own, but it inhibits the aggregation of platelets by either aggregated IgG or thrombin, and also the activation but not the activity of platelet factor 3 (Fiedel & Gewurz 1976). I do not know how this might affect your use of a platelet aggregation test for 'immune' complexes. With regard to the interaction between CRP and C1q it is worth noting that, although the binding of CRP to its substrates requires calcium ions, this may not be the case for CRP-C1q binding. Aggregated CRP might bind C1q just as aggregated IgG does.

*Jones:* The other point is that immune complexes are found most frequently in patients with colitis who have peripheral manifestations, such as joint involvement, vasculitis and pyoderma gangrenosa. Although C-reactive protein may be an important trap, there is sufficient evidence to suggest that immune complexes account for at least some of the peripheral phenomena in these conditions.

*Lachmann:* Would you agree that immune complexes in diseases where there may be breaches of the mucosal barrier allowing increased absorption of macromolecular material may not be of *primary* aetiological significance, even if they may help to cause the distant effects? The fact that immune complexes occur is not very surprising, in situations where abnormal amounts of food and bacterial antigens are absorbed.

*Jones:* Yes, I agree that they may account for epiphenomena. We have another observation on macromolecular antigens which may relate to that. We have been interested in circulating antibodies to bacteriophage $\phi$X174 (a phage of a non-pathogenic *E. coli* which is found in the stools of about one in five normal healthy individuals). We have only ever found circulating antibodies to this phage in three out of the 200 subjects that we studied. We have never found antibodies in patients with severe coeliac disease, where a leaky gut with access to intestinal antigens would be expected. We have found three subjects in whom an intravenous injection of $\phi$X174 produced a secondary response instead of the normal primary response. One had Crohn's disease, one had rheumatoid arthritis and one had chronic liver disease. This phage is a common gut antigen and it is therefore interesting and puzzling that antigens seem to be able to leak from the gut into the blood and produce circulating immune complexes, yet we don't find many people sensitized to this very immunogenic bacteriophage.

*White:* Have you proof that the IgA form of the antibody would work in your tests for phage neutralization?

*Jones:* Our bacteriophage neutralization test is extremely sensitive. We do have some evidence that circulating IgA neutralizes the bacteriophage.

*Lehner:* We have measured C-reactive protein in recurrent aphthous ulcers and in Behçet's syndrome (a condition in which patients suffer with oral and genital ulcers, skin lesions, often with iritis, arthrosis and thrombophlebitis). We find high levels of CRP, particularly in Behçet's syndrome, by the single radial immunodiffusion method (Adinolfi & Lehner 1976). Elevated CRP levels, however, are found in many conditions and it is unwise to attach too great a significance to this.

As CRP is one of the acute-phase proteins, have you assayed the others? We have found that while concentrations of CRP and C9 may be considerably increased, particularly in Behçet's syndrome, this does not apply to $\alpha$1 antitrypsin. I wonder which acute-phase proteins you are referring to, as there may be only a selective increase in some of them.

*Pepys:* We have not measured other acute-phase reactants in our patients although this has been done in inflammatory bowel disease (Weeke & Jarnum 1971). Our experience with C3 and C4 levels is that they tend to correlate with severity of disease, but in very severe or protracted illness they may remain low. This is not so with CRP, which remains elevated even terminally. I agree that the elevation of CRP concentrations is very non-specific and is a feature of almost any febrile or tissue-damaging illness irrespective of its aetiology. In the present context it is clinically valuable because it helps to distinguish between two overlapping diseases, and because it provides an index of disease activity in Crohn's disease. At a more fundamental level CRP is an interesting protein to study because of its rapid production, its dramatic increases in serum concentration, and its capacity to fix complement and to bind to and modify the activities of T lymphocytes.

*Soothill:* I find the absence of C3 conversion *in vivo* very pleasing and perhaps surprising. When we (Matthews & Soothill 1970) demonstrated this after feeding milk to milk-allergic patients I was worried that possibly it was not a direct effect of the milk, but an effect of other antigens getting in because of milk allergic damage to the bowel. Your patients had very damaged large intestines and some of your cases of Crohn's disease will have damaged small intestines too, so I was glad that you did not find C3 conversion.

Secondly, Ezer & Hayward (1974) showed that the serum of patients with Crohn's disease inhibited the C3-binding sites of normal B lymphocytes. We interpreted this as evidence of C3 activation *in vivo*, and possibly of immune

complex. This appears to be slightly incompatible with your essentially negative information.

*Pepys:* There are several problems in the interpretation of those observations. Firstly, since serum from patients with Crohn's disease was used the inhibition may have been due to *in vitro* phenomena following clotting rather than to changes occurring *in vivo*. It would be interesting to repeat the experiments with fresh EDTA-plasma. Secondly, substantial concentrations of fluid phase C3 conversion products are required to inhibit C3-dependent rosette formation on lymphocytes (Pepys & Butterworth 1974), so unless the major part of the serum C3 was converted and the serum was used neat, I am surprised at the extent of inhibition described.

Thayer has demonstrated reduced numbers of C3 receptor lymphocytes in patients with Crohn's disease and ulcerative colitis (Thayer *et al.* 1975). In more limited studies we have not seen this. I do not know whether CRP can block lymphocyte C3 receptors. According to Gewurz's group, CRP does not stick to B cells (Mortensen *et al.* 1975).

*Lachmann:* I am very interested to see your data on C3 conversion. Two patterns of C3 conversion products have been found *in vivo* under rather different conditions. Occasionally C3c (or possibly C3b) is found in freshly taken, warm plasma from patients with severe, presumably intravascular, complement activation; for example, in diseases like systemic lupus erythematosus. This is uncommon. C3c (and even more so, C3b) have a short half-life in plasma *in vivo* and one would not expect to find them unless they were being generated quite fast. A different C3 conversion product, C3d, is detected quite commonly in certain patients with renal disease. C3d is a long half-life fragment of C3 which appears to be generated extravascularly and to come back into the circulation (Charlesworth *et al.* 1974). There seems no reason to doubt that this C3d is really made *in vivo*. In the case of small amounts of C3c (or C3b) in fresh plasma, as have been reported by Versey *et al.* (1973), the possibility that this has been formed *in vitro* is, on the other hand, very real. It is known that in the acute phase of inflammation C3 levels, and levels of factor B of the alternative pathway, can be markedly raised. The high levels of these components potentiate the feedback mechanisms which give rise to C3 conversion. In 'acute-phase' patients even more than in others one can picture any one of a number of factors including for example the presence of slightly raised levels of cold autoantibodies to the patient's own red cells giving rise to minor C3 conversion. Do you take blood and clot it while warm in these tests, to make sure that this does not happen?

*Pepys:* The tests in which serum and plasma were studied in parallel were

all done at room temperature, because we wanted to replicate Teisberg & Gjone's (1975) experiment.

*Brandtzaeg:* Teisberg found that C3 conversion products disappear after a gluten-free diet is given and reappear after the introduction of gluten into the diet of coeliac disease patients. Does this not indicate that the technique is valid in showing *in vivo* complement activation?

*Pepys:* I have seen those data, but they were obtained on serum. Before ascribing C3 conversion to *in vivo* complement activation it is necessary to prevent the processes which are known to activate C3 during coagulation.

*Brandtzaeg:* Do you think the argument about an increased enzymic level in serum also applies to coeliac disease?

*Pepys:* I don't know. I would like to see the same study done on EDTA-plasma collected and separated in the cold.

*Brandtzaeg:* I understand that Dr Teisberg has had some technical problems using plasma instead of serum.

*Rosen:* I just wonder if these patients' lymphocytes can be infected with EB virus, since the C3 receptor may be identical or adjacent to the EB virus receptor of the B lymphocyte?

*Pepys:* I don't know. We have measured the concentration of C3 receptor lymphocytes in the peripheral blood of patients with inflammatory bowel disease and we do not find it to be low, as reported by Thayer *et al.* (1975). We have not studied it systematically because the isolation of mononuclear cells from the blood invariably distorts the proportions of the different lymphocyte populations (Brown & Greaves 1974), making it impossible to calculate their true circulating ratios and absolute concentrations. In addition monocytes also have C3 receptors. In an attempt to overcome these problems and to quantify precisely the absolute concentrations of circulating lymphocyte populations we are developing techniques for studying various markers in whole blood (Pepys 1976). In Crohn's disease and colitis we find some lymphocytes in the affected tissues which bear C3 receptors (M. B. Pepys & A. C. Dash, unpublished work).

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*Immunology of the Gut*
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# Effects of local delayed hypersensitivity on the small intestine

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*Abstract* There are many T and B cells in the small intestinal mucosa and local T cell immunity could have a role both in protective immunity and as a cause of disease (i.e. hypersensitivity). This latter aspect has been investigated by using several animal models to assess the effects of local delayed hypersensitivity on the structure and function of the small intestine.

Heterotopically transplanted grafts of fetal small intestine in mice (isografts and allografts) have been examined by conventional histology, scanning and transmission electron microscopy, by making direct measurements of villi, crypts, and lymphoid cell infiltrate, and by counting the number of mitoses per crypt. This cell-mediated immune reaction causes lymphocyte infiltration which is most marked in the lamina propria, hyperplasia of the crypts of Lieberkühn, increased cell loss with villous atrophy and a flat surface, but the individual enterocytes appear fairly normal.

Graft-versus-host disease causes exactly the same changes in structure and in cell kinetics as does rejection. However, crypt hyperplasia has been found to precede villous atrophy by several days. Preliminary experiments on local contact hypersensitivity suggest that intraluminal injection of oxazolone in the gut of sensitized mice also produces villous atrophy and crypt hyperplasia.

It is postulated that these effects are likely to be produced via lymphokines: by an 'enteropathic' factor which damages the lamina propria and basement membrane, and a factor which is mitogenic for crypt stem cells.

In mice infected with *Giardia lamblia*, crypt hyperplasia and lymphocyte infiltration of the epithelium are present and there is accelerated epithelial cell turnover. In rats infected with *Nippostrongylus brasiliensis*, the flat mucosa has been shown to be due to the thymus-dependent immune response and not directly to the damage produced by the parasite itself.

A common factor in the variety of conditions associated with villous atrophy and crypt hyperplasia may well be a local cell-mediated immune reaction to food, microbial, parasite or other antigens which causes changes in enterocyte turnover rate and malabsorption.

The small intestine has the unique and essential function of nutrient absorption, a function which resides in the sheet of enterocytes—columnar epithelial cells which cover the villi. However, large numbers of cells of the immune system are also to be found in the intestinal mucosa. These are dispersed singly or in small groups among the epithelial and connective tissue components of the intestine and they comprise T, B and null cells, together with mononuclear phagocytes, mast cells and eosinophils. Thus the gut has the cellular capacity for truly protective local immunity (e.g. antibody to enteric pathogens) and for entirely pathological, hypersensitivity reactions (e.g. food allergy). The situation can also be envisaged where a protective immune reaction will, of necessity, be accompanied by some tissue damage and in this paper we describe and discuss the harmful effects which may be produced by local delayed hypersensitivity reactions in the small intestine.

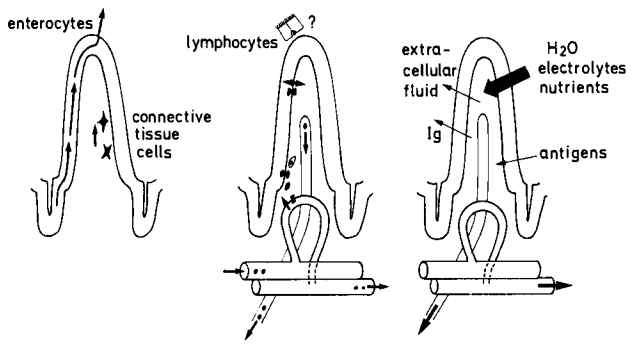


FIG. 1. Some of the components of the small intestine which are continually renewed or replaced.

The apparently regular arrangement of its crypts and villi is misleading when one considers the small intestinal mucosa as the backcloth for an immune reaction lasting several days. The epithelial, stromal and lymphoid cells are continually renewed or replaced, and the absorbed and exuded fluids will dilute, dissolve and wash away any soluble antigen in the vicinity (Fig. 1). Despite this, it is evident that some living and non-living antigens will persist for sufficient time to allow the evolution of a cell-mediated immune (CMI) reaction in the intestinal mucosa; these include parasites and viruses, particulate antigens and cellular or subcellular components of the tissue itself (Table 1).

## ALLOGRAFT REJECTION AS A MODEL OF DELAYED HYPERSENSITIVITY

When grafts of fetal mouse intestine are implanted heterotopically into adult mice of the same inbred strain, they take, grow and retain their normal morpho-

TABLE 1

Antigens which persist in the small intestinal mucosa for sufficient time to allow evolution of a cell-mediated immune reaction

*Microorganisms and parasites:*
- Lumen-dwelling
- Adherent
- In mucosa

*Non-living:*
- Attached to cells or stroma
- Precipitated
- Large particles

*Small intestinal tissue:*
- Histocompatibility antigens
- Autoantigens
- Organ-specific antigens

? Foods taken three or four times daily

logical appearances for several months (Ferguson & Parrott 1972). If donor and host are from different strains, grafts grow for a few days, then are infiltrated with lymphocytes and rejected (Ferguson & Parrott 1973). Such allografts of small intestine, implanted under the kidney capsule, provide a useful and inexpensive model of a local delayed hypersensitivity reaction. They have the additional advantage that, since the implanted tissue is fetal, there are no antigens within the lumen of the graft, no local immune reactions to foods or microorganisms, and any pathological features observed can be attributed directly to the rejection process. Rejection of allografts in this system is a thymus-dependent phenomenon (Table 2) (Ferguson & Parrott

TABLE 2

Thymus-dependence of small intestinal allograft rejection

| *Experimental design* | | *Number of grafts implanted ( > 10 days previously)* | *Number of grafts with complete mucosal destruction* |
|---|---|---|---|
| *Donor* | *Host* | | |
| CBA | → BALB/c | 46 | 45 |
| BALB/c | →CBA | 26 | 24 |
| BALB/c | →Thymus-deprived CBA | 32 | 5 |

Grafts of fetal small intestine were implanted under the kidney capsules of adult mice and subsequently examined histologically.

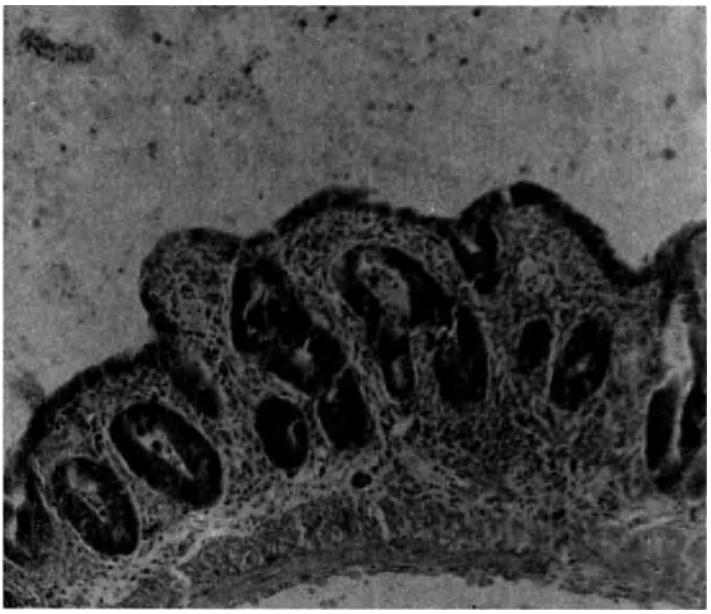

FIG. 2. Typical histology of rejection of a mouse small intestinal allograft.

1973) and, since destruction of the mucosa precedes the appearance of serum antibody by several days (Table 3) (Elves & Ferguson 1975), it is the cellular component of the thymus-dependent reaction which is implicated.

We have concentrated mainly on histopathological studies of the effects of rejection on the small intestine, and some of our findings have already been published (Ferguson & Parrott 1973; MacDonald & Ferguson 1976*a*) or are in press (Ferguson *et al.* 1976*a*; MacDonald & Ferguson 1976*b*). In all experiments, isografts (between animals of the same strain) have been compared with allografts (host and donor of different strains). Tissues have been examined by using conventional histology, scanning and transmission electron microscopy, direct measurements of villi, crypts and enterocytes, counts of intestinal lymphoid cells and studies of mitotic activity in crypt cells.

The most striking histological effect of rejection is seen before the mucosa is completely destroyed (Fig. 2). Grafts are infiltrated with lymphocytes, villi are short or absent and crypts of Lieberkühn appear abnormally long. This appearance is not unique to rejection of intestine in the mouse. Similar findings have been reported in dog intestinal allografts (Holmes *et al.* 1971) and in mice with graft-versus-host disease (Reilly & Kirsner 1965) where the histological lesion is accompanied by malabsorption (Hedberg *et al.* 1968; Palmer & Reilly

TABLE 3

Time-course and thymus-dependence of the humoral immune response to small intestinal allografts

| *Experimental design* | | *Proportion of host mice with serum antibody to graft strain antigens, at different times after graft implantation:* | | | |
|---|---|---|---|---|---|
| *Donor* | *Host* | *5 days* | *9 days* | *12 days* | *15 days* |
| CBA ⇆ | BALB/c | 0/12 | 0/12 | 12/12 | 12/12 |
| BALB/c → | Thymus-deprived CBA | – | 0/2 | – | 0/5 |

1971). These animal experiments produce changes very similar to those seen in intestinal biopsies in human coeliac disease and parasite infection (Ferguson 1975, 1976). Thus elucidation of the mechanisms whereby the local CMI reaction of rejection changes intestinal architecture may allow more rational interpretation of the significance of villous atrophy in clinical disease.

## VILLI IN ALLOGRAFTS OF SMALL INTESTINE

The enterocytes are formed by cell division in the crypts of Lieberkühn. They move up on to the villi and are shed from villous tips some 2–3 days later. It has become generally accepted that the shapes and height of villi are determined by the size of the population of enterocytes at any one time—if there are fewer mature enterocytes the villi change from finger-like or deltoid shapes to become ridges, then low convolutions. For a few days after implantation, the villi of allografts resemble those of normal intestine and of isografts. Once rejection is established the villi become shorter and the surface is ridged or flat; this feature is best seen by using scanning electron microscopy (Fig. 3). Yet despite the striking changes in the shapes of the villi, individual enterocytes appear remarkably normal during rejection, to both light and electron microscopic evaluation. Sheets of virtually intact columnar enterocytes can be seen in the lumen of many grafts (illustrated as Fig. 3 in Ferguson & Parrott 1973). We have found that the epithelium of allografts tends to separate very readily from the underlying basement membrane during fixation and cutting of sections, and in transmission electron micrographs abnormally wide spaces have been seen between the enterocytes lining the surface of the gut. One further point draws attention from the enterocytes themselves to the other tissue components. Large numbers of lymphocytes are found in the mucosa of rejecting grafts. These tend to be concentrated in the lamina propria, there are relatively few within the epithelium, and indeed intraepithelial lymphocytes

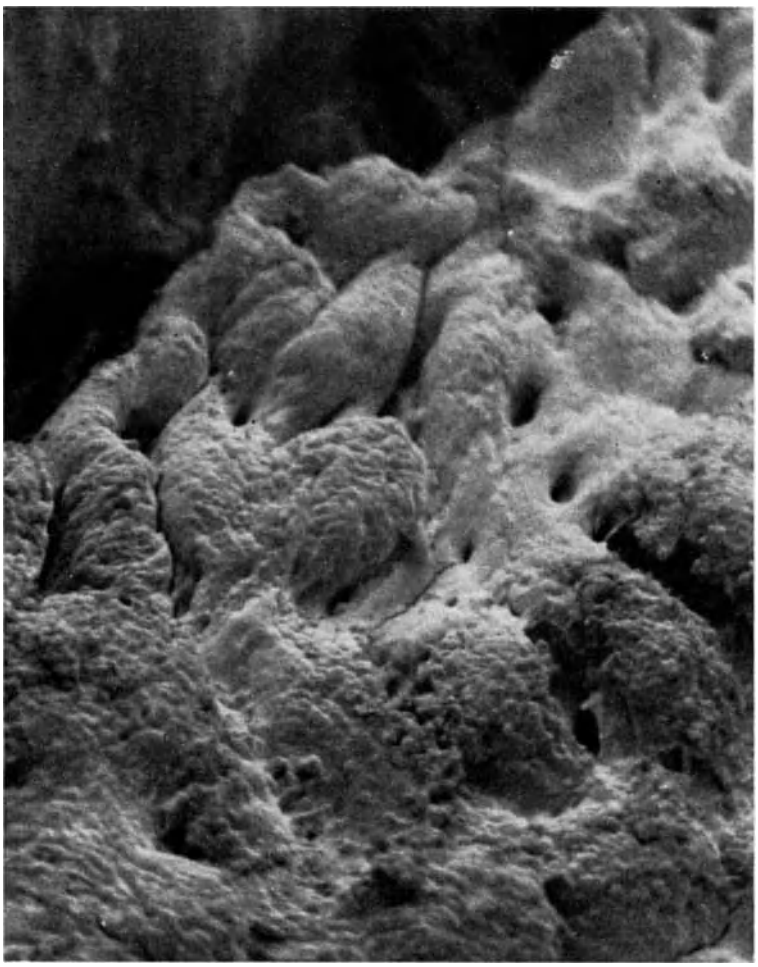

FIG. 3. Scanning electron micrograph of the luminal surface of a small intestinal allograft. (Supplied by Dr K. E. Carr, Department of Anatomy, University of Glasgow.)

were found in only 38 of 50 grafts with flat surface appearance (MacDonald & Ferguson 1976*a*). Thus the 'villous atrophy' and increased cell loss in rejection do not seem to be due to a direct result of early enterocyte death. We suggest that damage to or destruction of the underlying stroma—lamina propria and basement membrane—is a more likely mechanism (Fig. 4).

## CRYPTS IN ALLOGRAFTS OF SMALL INTESTINE

On subjective evaluation of rejecting allografts it appeared that the crypts of

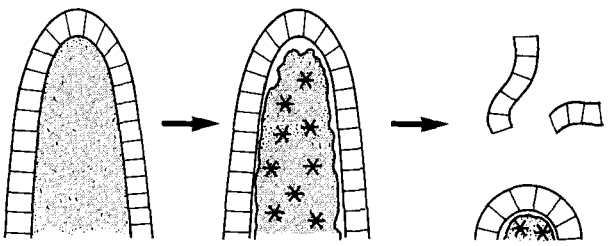

FIG. 4. A mechanism whereby the cell-mediated immune reaction of rejection, mainly confined to the lamina propria, produces villous atrophy.

Lieberkühn were longer than in isografts, and this was borne out by formal measurements of histological sections (MacDonald & Ferguson 1976*a*). Further to examine the significance of crypt hyperplasia, we used microdissection and metaphase arrest to examine the properties of single crypts. Mice bearing grafts were given colchicine two hours before they were killed (colchicine blocks mitosis in metaphase). Grafts were fixed in Clarke's fixative and Feulgen stained in bulk; groups of crypts were dissected out, squashed under a cover slip (Fig. 5), and the numbers of metaphases per crypt were counted. The results of this experiment are summarized in Fig. 6. In the crypts of normal small intestine and in isografts, there were some 2–4 mitoses per crypt per hour, whereas in allografts five days after implantation, 12.5 crypt cells entered mitosis hourly—crypt hyperplasia which had been considerably underestimated in direct measurements of crypt length. Between days 7 and 9, the cell production rate per crypt dropped significantly and this may be evidence of a late cytotoxic or ischaemic effect of the immune reaction on these rapidly dividing cells.

## TIME-COURSE OF CHANGES IN CRYPTS AND VILLI IN GRAFT-VERSUS-HOST DISEASE

Allografts provide only small pieces of tissue for examination, so we have used graft-versus-host (GVH) disease in order to examine more closely the time-course of changes in crypts and villi. GVH disease was induced in CBA $\times$ BALB/c F1 hybrid neonatal mice by injecting $25 \times 10^6$ parental spleen cells intraperitoneally, five days after birth. Mice were killed at intervals up to age 19 days and were given colchicine two hours before death. A detailed analysis of mucosal cytokinetics was obtained by measuring the various aspects listed in Table 4. The results of this study are published elsewhere (Ferguson & MacDonald 1976*b*) but the comparisons between villi, crypts and crypt

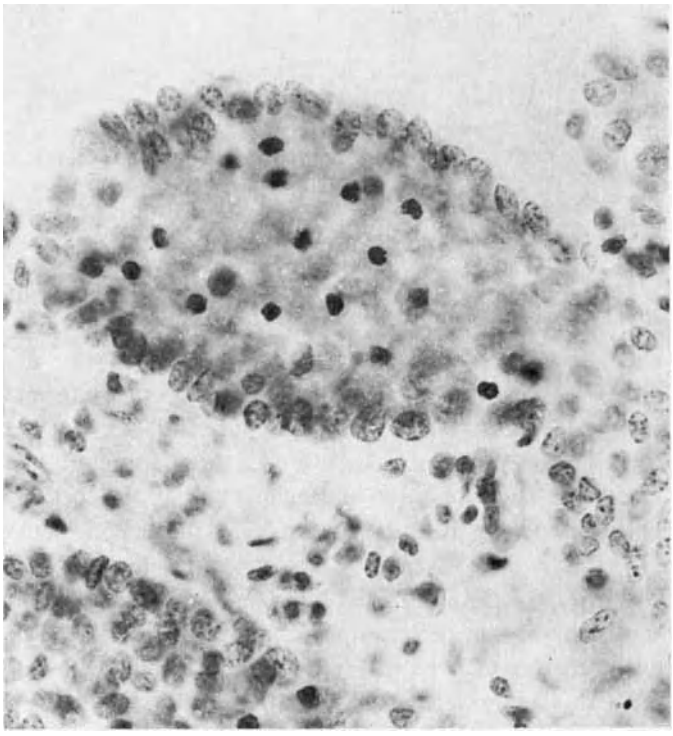

FIG. 5. Single crypts of Lieberkühn, dissected from a small intestinal allograft. The animal had been given colchicine 2 hours before death, so that mitoses are blocked in metaphase.

TABLE 4

| Measurements of small intestinal architecture and cytokinetics |
|---|
| Length of villi |
| Shapes of villi (finger-like in mouse) |
| Length of crypts |
| Number of duplicating crypts |
| Cell production per crypt per hour |
| Number of crypts associated with each villus (i.e. ratio crypts: villi) |
| Cell loss per villus per hour (calculated) |

mitosis in GVH animals and in their littermates are summarized in Fig 7. Striking crypt hyperplasia was found in the GVH mice aged 10 days when compared with their littermates, although the villi were the same height in both groups. Thus although crypt hyperplasia may in some conditions be a com-

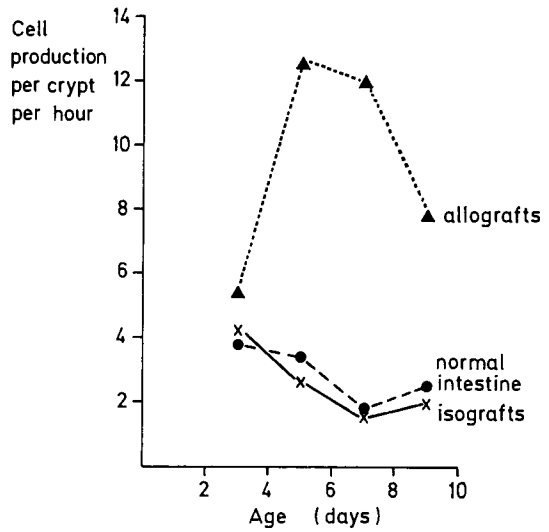


FIG. 6. The rate of cell production per hour (as assessed by metaphase arrest) in allografts (CBA → BALB/c) and isografts (CBA → CBA; BALB/c → BALB/c) of mouse jejunum, and in normal jejunum of the same age.

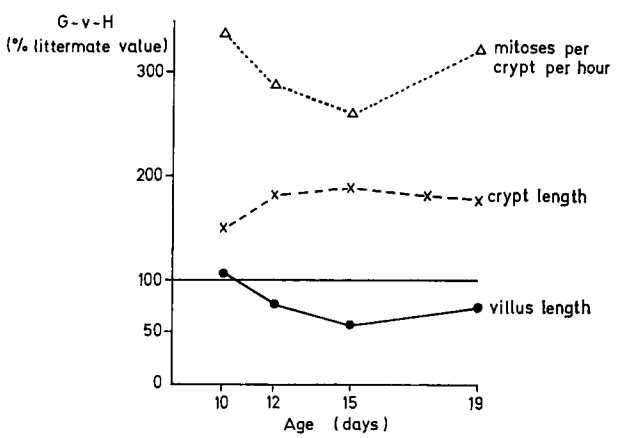


FIG. 7. Values for villous height, crypt length and mitoses per crypt per hour, in neonatal mice with graft-versus-host disease. Results are expressed as a % of the values for normal littermates of the same age.

pensatory response to loss of part of the mature enterocyte population, this cannot be the case in these experiments, since the 3–4-fold increase in crypt mitosis preceded villous atrophy by several days. We have therefore concluded that one of the direct effects of a local CMI reaction is stimulation of mitosis of crypt stem cells.

## CONTACT HYPERSENSITIVITY IN THE SMALL INTESTINE

Contact hypersensitivity (e.g. to tuberculin, dinitrochlorobenzene, oxazolone) is another experimental model of a local CMI reaction. There is a single report in the literature of dinitrochlorobenzene challenge, by feeding, of sensitized pigs (Bicks *et al.* 1967). This produced malabsorption, as assessed by xylose absorption, lymphocyte infiltration of the intestine, oedema and necrosis.

We have used a number of regimes in attempts to produce local hypersensitivity to oxazolone in sensitized mice. Five different schedules for oxazolone feeding were unsuccessful, but villous atrophy and crypt hyperplasia have been found in two groups of experiments—where oxazolone in olive oil was injected into a Thiry-Vella loop at laparotomy, and when oxazolone, dissolved in 50% alcohol, was injected into the lumen of the jejunum at laparotomy.

These preliminary findings indicate that crypt hyperplasia and villous atrophy occur even when a CMI reaction is directed against antigens other than those of the intestine itself. They support the theory that the tissue damage is mediated via lymphokines rather than by a direct toxic effect of T cells on the enterocytes.

## ENTEROPATHIC LYMPHOKINES?

A local CMI reaction may damage adjacent tissue by at least two mechanisms—direct T cell cytotoxicity, and via the action of the soluble factors, lymphokines. Light and electron microscopy have not yet provided evidence of a cytotoxic effect of the lymphocytes within allografts, but it seems likely that the two striking effects of local CMI—villous atrophy and crypt hyperplasia—are produced by 'enteropathic' lymphokines or factors. Two actions are postulated:

(1) destruction or distortion of the stroma of the lamina propria so that enterocyte adhesion is impaired—a truly enteropathic factor,

(2) stimulation of mitosis in the stem cells of the crypts of Lieberkühn—a crypt mitogenic factor.

These factors are as yet only theoretical. But as a first stage in their detection we have used several conventional assays in attempts to demonstrate secretion

TABLE 5

Secretion of lymphokines by isografts and allografts of mouse small intestine

| *Lymphokine* | *Assay system* | | *Isografts* | *Allografts* |
|---|---|---|---|---|
| Macrophage migration inhibition factor (MIF) | Migration of mouse spleen cells into planchettes filled with organ culture fluid | | 12% inhibition | 18% inhibition |
| Skin reactive factor (SRF) | Intradermal injection of organ culture fluid; sequential measurements of double skin thickness | | 0.12 mm peak increment | 0.11 mm peak increment |
| Chemotactic factor for macrophages (CF) | Distance migrated into Millipore filter by mouse peritoneal exudate cells | *Gey's fluid alone* 39±5 μm (mean ± S.E.) | 40 ± 3 μm | 41 ± 6 μm |
| | % chemotaxis | | 6% | 13% |
| Inhibition of macrophage chemotaxis (CIF) | As above; casein added to stimulate chemotaxis | 54±6 μm | 55 ± 7 μm | 45 ± 6 μm |
| | % inhibition of chemotaxis | | −2% | 60% |

of lymphokines by the T cells in allografts. (This work has been done in collaboration with Mr R. Russell.)

Isografts and allografts (6–8 days after implantation) were dissected from the kidney capsules of host mice, and were either cultured on the grids of disposable organ culture dishes for six hours (the culture fluid then being used for assay of macrophage migration inhibition factor, MIF, and skin reactive factor, SRF) or were placed directly in Boyden chemotaxis chambers. Results of the experiments are summarized in Table 5. There was no evidence of secretion by grafts of MIF or of SRF into organ culture fluids; however, allografts were found to secrete an inhibitor of macrophage chemotaxis.

## CMI AND THE 'ENTEROPATHY' OF PARASITE INFECTIONS

Immunity to parasites involves a range of immune reactions, and these affect the parasites at different stages of their life cycle. IgE and T lymphocytes are clearly important and probably in both cases effective protective immunity will of necessity produce some tissue damage. The nature of the immune response to *Giardia lamblia* is unknown. However, in children (Ferguson

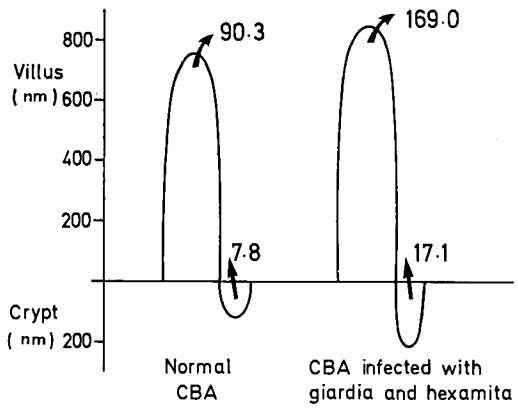


FIG. 8. Small intestinal architecture and cytokinetics in healthy CBA mice and in CBA mice infected with the parasites *Giardia* and *Hexamita*. The numbers indicate cell production per crypt per hour (measured directly) and cell loss per villus per hour (calculated).

*et al.* 1976*b*) and in mice (Table 6) infected with *Giardia* there are increased numbers of intraepithelial lymphocytes and several pieces of work suggest that the intraepithelial lymphocytes are T cells. Also in patients with hypogammaglobulinaemia but normal T cell immunity, *Giardia* infection is usually accompanied by partial villous atrophy and malabsorption (Ochs & Amen[illegible] 1976), findings which we would interpret as probably due to intense CMI reactions unmodulated by local antibody. Accordingly, we have made detailed studies of intestinal architecture in *Giardia*-infected mice, and have found that although there is no villous atrophy and no obvious crypt enlargement on conventional histological examination, crypt mitosis is doubled by *Giardia* infection (Fig. 8) and there is a more rapid transit of enterocytes up the sides of villi (Table 7). We have not had the opportunity to examine the effects of *Giardia* infection on the cell kinetics of the small intestine in congenitally athymic mice. On the basis of our work described in this paper it is likely that a lymphokine causes

TABLE 6

Influence of thymus and of *Giardia lamblia* infection on intraepithelial lymphocytes

| *Experimental group* | *No. of animals* | *Intraepithelial lymphocyte count (per 100 epithelial cells)* | |
|---|---|---|---|
| | | *Mean* | S.E. |
| CBA mice (6 wk) | 5 | 13.6 | 1.8 |
| *Nu nu* mice (6 wk) | 6 | 2.0 | 0.4 |
| *Sha sha* mice (6 wk), *Giardia*-infected | 7 | 21.8 | 3.0 |

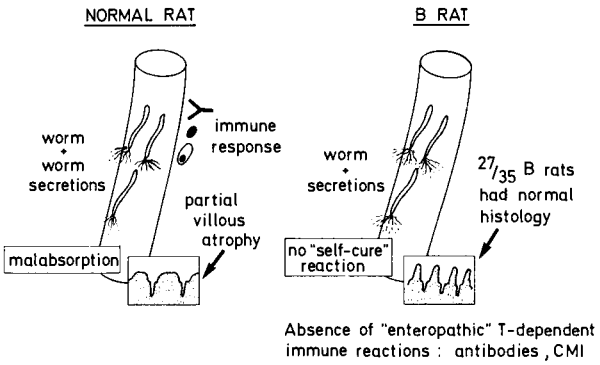


FIG. 9. An explanation for the lack of morphological damage to the small intestinal mucosa in thymus-deprived rats infected with the parasite *Nippostrongylus brasiliensis*.

crypt hyperplasia in this parasitic infection, so we would predict that thymus-dependent crypt hyperplasia would not be found in athymic mice infected with this or other parasites.

When rats are infected with *Nippostrongylus brasiliensis*, oedema, villous atrophy and crypt hyperplasia are found in those areas of the small intestine where the parasites are localized. We used T cell-depleted, B rats (thymectomy, 850 R irradiation, repopulated with autologous bone marrow) to determine whether these tissue changes are produced directly by parasites or are side-effects of the anti-parasite immune response (Ferguson & Jarrett 1975). The results are illustrated in Fig. 9. With the infective dose used (4000 larvae), changes in the appearance of the small intestinal mucosa were found in virtually all immunologically intact infected animals. However, villous atrophy and crypt hyperplasia were absent in 27 of 35 parasitized B rats. Thymus dependence of the worm-associated tissue damage has also been found by E. J. Ruitenberg

TABLE 7

Rate of transit of enterocytes in CBA and *sha sha* mice

| *Experimental group* | *No. of mice* | *Height of leading labelled cell (number of nuclei from villus–crypt junction)* | |
|---|---|---|---|
| | | *Mean* | S.E. |
| CBA mice | 6 | 23.8 | 3.0 |
| *Sha sha* mice | 6 | 44.3 | 3.9 |

Each mouse had been given 25 μCi[$^3$H]thymidine i.p., 25 hours before death. Autoradiographs of sections of jejunum were exposed for 18 days.

(personal communication) in his work on the immune response to *Trichinella* in normal and *nu nu* mice.

## HYPOTHESIS

Our experiments have shown that local CMI reactions, probably acting via lymphokines, can produce crypt hyperplasia and villous atrophy with ready separation of enterocytes from the basement membrane. However, CMI does not seem to damage the individual epithelial cells and does not correlate with the presence or absence of increased numbers of intraepithelial lymphocytes. Factors other than a local delayed hypersensitivity reaction are likely to be the cause of enterocyte changes and to influence the distribution of intestinal lymphocytes in diseases such as coeliac disease and acute gastroenteritis. However, although a variety of changes in small intestinal architecture can be produced experimentally, the flat mucosa with crypt hyperplasia and malabsorption is by far the most frequently encountered lesion in human and animal diseases. We suggest that a common factor in all of these conditions is probably a local CMI reaction to food, microbial, parasite or other antigens. This local hypersensitivity reaction results in crypt hyperplasia, fast epithelial cell turnover, reduced epithelial cell adhesion and villous atrophy, with the clinical end result of a malabsorption syndrome.

## IMPLICATIONS: LOCAL CMI REACTIONS AND MALNUTRITION

Areas of the world where nutrition is borderline or insufficient are also those areas where enteric infections and parasite infestation are endemic. It is to be hoped that effective bacterial, viral and parasite vaccines will be developed and used in the populations concerned, but if further work confirms that local CMI reactions can indeed cause malabsorption, then the nature of such vaccines and immunization schedules must be tailored to promote local immunity with a minimum of local delayed hypersensitivity.

## ACKNOWLEDGEMENTS

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## Discussion

*Bienenstock:* Functional MIF activity can apparently be found in high quantities in normal human intestinal secretions, according to Waldman (Gadol *et al.* 1976).

*Ferguson:* We have shown that a MIF can be secreted by small bowel biopsies from coeliac patients when cultured with gliadin, but we did not find MIF activity when biopsies from normal persons or from coeliac disease patients were cultured in the absence of antigen. Frederick & Bohl (1976) have found MIF secretion by suspensions of lamina propria cells of guinea pigs, but only when these cells were cultured in the presence of specific antigen (transmissible gastroenteritis virus in their experiments). We have not looked in normal human intestinal secretions but I would expect that many substances such as enterotoxin and trypsin would interfere with migration, so that it

would be difficult to show that the effect in question was indeed due to the presence of a lymphokine.

*Bienenstock:* Waldman has shown that this activity is compatible with the published molecular size of MIF. That does not prove that it is MIF. But if MIF *is* present normally, perhaps other lymphokines are there normally too? If so, this goes against your thesis that a lymphokine is responsible for villous flattening.

*Ferguson:* There are of course many T and B lymphocytes in normal gut. Their functions are still uncertain. We feel it is unlikely that, in normal animals which are not infected by parasites, local cell-mediated immune reactions influence the shapes of villi and crypts. The histology of the small intestinal mucosa in CBA, BALB/c and nude mice is very similar although there are substantial numbers of mucosal lymphocytes in the CBA and BALB/c strains but very few in the nude mice. I could not predict the source of MIF as secreted by normal uninfected gut, but I doubt if it would be from the T cells in transit through the tissue.

*Gowans:* On the point of the mechanism of destruction of the allografts, I was interested in your description of a gap developing under the epithelium and of the enterocytes falling away from the stump underneath. A gap suggests the development of oedema. I suspect that the lymphatic drainage of your grafts may be poor so that damage due to the allograft reaction may lead rapidly to an accumulation of fluid. Have you considered a simple mechanical shearing due to oedema? A second question: how quickly does blood supply fail in the allografts? Is this the crucial event determining graft destruction?

*Ferguson:* These grafts are attached to the capsule of the kidney and not to the underlying tissue, and in established grafts one sees apparently normal, although small, blood vessels. There are large lymphatic-like spaces at the edges of the grafts, but neither in isografts or allografts has oedema of the mucosa appeared to be a feature, and this point has been examined both by light and by electron microscopy. One reason why we think there is lymphoid cell traffic into and out of these grafts is that large lymphocytes can be seen in the lymphatic spaces draining allografts.

*Gowans:* That is why I thought they might be obstructed. I was recalling your own comment that the accumulations of lymphocytes in and around the grafts might have been due 'to obstruction of lymphatic drainage of the grafts' (Ferguson & Parrott 1972).

*Ferguson:* In the normal small bowel a considerable amount of water is absorbed across the epithelium. Some of this leaves the mucosa as lymph and I think it's likely that lymphocytes are passively propelled along in this stream of fluid, rather than that lymphocytes actively migrate along the lymphatics

from the gut to the lymph nodes. Certainly it's my experience of Thiry-Vella loops that the lymphatics within the villus cores often contain many lymphocytes, many more than one would expect in a segment of normal intestine that is actively absorbing jejunal fluids.

We have not examined the blood vessels by electron microscopy, but by light microscopy there does not seem to be any obvious capillaritis or arteritis. On rejection the grafts do not become black, as do rejecting skin grafts; they just slowly disappear, although fragments of the smooth muscle layer are recognizable for a long time. Nevertheless I would not be surprised if the final ulceration of the mucosa prior to complete rejection was partly due to a local anoxia.

*Gowans:* You mentioned that there may be a big traffic of lymphoid cells through the lamina propria of the normal gut. I am sure you will agree that histological studies will not tell us the answer. You have to go back over 40 years to find someone who has taken the trouble to cannulate individual lacteals in order to examine this point. Baker (1933) found in cats that prenodal lymph draining Peyer's patches contained many cells while that draining the intestine between patches contained many fewer cells. This should be an easy experiment to repeat employing modern methods of micromanipulation and until it is repeated the matter will remain unresolved.

*Booth:* It would be interesting to know whether one sees, in the electron microscope, endothelial cell swelling in the capillaries beneath the basement membrane. Do you get macrophages moving in? Are polymorphs seen? What sort of activity goes on in that subepithelial layer?

*Ferguson:* Polymorphs have not been seen in rejecting allografts. We have seen rows of cells with the appearance of lymphocytes, sitting under the basement membrane. With regard to the fine structure of the basement membrane, the electron microscopist, Dr K. E. Carr, has concentrated on the surface morphology and the enterocyte appearances up to now. In view of our present hypothesis on the significance of the basement membrane, she is now examining the lamina propria closely.

*André:* Is there a difference in graft-versus-host reaction if one grafts a piece of gut with or without Peyer's patches?

*Ferguson:* A baby mouse has tiny Peyer's patches which contain mainly T cells, and are presumably areas of T cell traffic. As far as I know the workers who have examined the small bowel in graft-versus-host disease have not looked to see whether the reaction is worse, or whether ulceration occurs more rapidly, around the Peyer's patches. Most of the grafts in our experiments will not contain a Peyer's patch, because the transplanted intestine is a length of only 5 mm, but we have noted that in areas where there is an obvious nodule

of lymphoid tissue in a rejecting graft, that area is crammed with blast cells. It does look as if this is a route of entry of T cells into the grafts. However, in other parts of the same graft a millimetre or two away from the presumed Peyer's patch but in the same section, the histological stage of rejection, and the nature of the lymphoid cell infiltrate, are identical to the appearances we find in other allografts at the same stage of rejection, in which no Peyer's patch has been present.

*Evans:* I want to ask a general question about the acceptability in immunological terms of a truly localized delayed hypersensitivity reaction in the gut which is not demonstrable anywhere else. Your models have been developed with coeliac disease in mind, and a major problem in coeliac disease is that you cannot demonstrate by skin testing any significant difference in terms of delayed hypersensitivity between coeliac patients and other patients, with wheat proteins. You can get skin reactions on biopsy but there is no real evidence that this is a delayed hypersensitivity reaction. Are you developing the thesis here that you get a truly localized delayed hypersensitivity reaction with no spillover into the other systems?

*Ferguson:* It was purely fortuitous that our early studies of the morphology of rejection showed a similarity to the small bowel in coeliac disease. If I had not worked as a clinical gastroenterologist before my period in laboratory immunology, I would probably have failed to appreciate the significance of this chance finding. At the moment I see no reason to postulate that the cells which can mediate a delayed hypersensitivity reaction to gluten are confined to the mucosal surfaces. In a disease like tuberculosis there are sensitized T cells throughout the body and a local reaction will occur at any site where an appropriate amount of antigen has been concentrated. Indeed this latter aspect is a great problem in trying to suggest that coeliac disease is due only to a delayed hypersensitivity reaction. I do not think that molecules of gluten will stay in the small intestine for a sufficient length of time to allow the evolution of a cell-mediated immune reaction. However, if gluten is precipitated in immune complexes, or if it has stuck to reticulin or to the surface of enterocytes, or if for some other reason coeliac patients retain gluten antigen in their gut, then I can envisage the evolution of a cell-mediated immune reaction.

If one does skin tests with soluble antigens, these diffuse into the tissue fluids and are no longer present at the site 12–24 hours later. Thus in order to demonstrate delayed hypersensitivity to gluten in coeliac disease it will be necessary to do skin tests by using particulate preparations of a range of gluten antigens, both in normal people and in coeliac patients.

*Evans:* Are you saying that the methods of skin testing for delayed hypersensitivity need an immobilized antigen in order to be valid?

*Ferguson:* Yes.

*Pepys:* It has been known for a long time that the antigens which produce delayed hypersensitivity reactions in the skin of man need to be antigens which fix, like tuberculin, and remain in the tissue for a long time. The tuberculin reaction can be modulated by agents which affect vasomotor tone and vascular permeability. Addition of histamine to the tuberculin causes the tuberculin to diffuse away and prevents the appearance of a positive reaction. Alternatively, the introduction of adrenaline together with a high dilution of tuberculin makes the antigen fix in a more localized area and may bring out a positive reaction which is not seen with that dilution of tuberculin in the absence of adrenaline (Pepys 1955).

Another reason for the failure to demonstrate delayed hypersensitivity reactions to gluten in the skin in coeliac patients may be that they have Arthus reactions which could act like histamine in 'diluting out' the antigen. Coeliac patients were found to have clear Arthus reactions on skin testing with gluten antigens which correlated with their serum titres of anti-gluten antibodies (P. Baker & A. E. Read, personal communication); on a gluten-free diet, the reactions went away as the levels of anti-gluten antibody fell.

*Pierce:* I am not certain how long an antigen has to remain in tissue to induce a delayed hypersensitivity reaction. Some of our studies of the response of rats to cholera toxoid suggest that soluble protein antigens may remain in the gut mucosa for several days after a single exposure (Pierce & Gowans 1975). This is suggested by the observation that a single gut booster induces a systemic traffic of IgA immunoblasts which home selectively to the lamina propria of the boosted portion of the gut 4–5 days after boosting. Since this homing is antigen-specific I presume it is due to antigen trapped in the mucosa at the time of boosting and persisting there for at least 4–5 days, though I realize other explanations are possible. My point is that there is no evidence in these circumstances of anything looking like a local delayed hypersensitivity reaction, and the rats do not sicken or die from apparent systemic delayed hypersensitivity reactions. Perhaps persistence of antigen for 4–5 days is not long enough.

*Ferguson:* Surely for antigen to persist for several days it would need to be intracellular. The macrophage is a suitable candidate which has been completely ignored in our discussions so far.

*Cebra:* Has the cholera toxoid lost all its tissue-fixing properties at the time you use it?

*Pierce:* Yes. It no longer binds to cell membrane as cholera *toxin* does.

*Lachmann:* These tests are also used the other way round. The retention of antigen can be used as a test for cellular immunity to it. If you inject radiolabelled tuberculin into the skin of a tuberculin-sensitive guinea pig and of a

normal guinea pig the rates of clearance are very different, the antigen being retained in the skin of the immunized animal.

*Bienenstock:* On this question of local cellular immunity, O'Neill in my laboratory has shown that in guinea pigs fed with BCG he can demonstrate MIF production specific for PPD from cells derived from Peyer's patches and to a lesser extent from the lamina propria, in the absence of skin-test positivity and in the absence of the turning on of splenic lymphocytes (M. O'Neill & J. Bienenstock, unpublished). So local feeding of antigen can give rise to the local production of MIF; whether it is B or T cell-derived is another issue.

*Cebra:* Since undifferentiated crypt cells have been implicated in the transport of secretory IgA out into the gut lumen, I wonder if an involvement of more of these cells in cell division to generate enterocytes, as you indicate occurs in certain situations, interferes with the secretory process. In your *Giardia*-infected mice have you measured the overall concentration of secretory IgA in the gut fluid to see whether there is a sharp fall in the level?

Secondly, it is difficult to disperse the cells in the lamina propria and recover intact and reasonably viable plasma cells. As Dr Bienenstock mentioned, you can get a fair number of small round cells out, and these could be looked at to determine the proportion of T cells. It would be even more interesting to use antisera against Ly1 or Ly2,3 to determine the proportion of suppressor T cells present in animals in which you suspect delayed hypersensitivity.

*Ferguson:* There is little information on the nature of protective immunity to *Giardia*, and I am unaware of any work on IgA or secretory IgA responses in this infection. With regard to the nature of the lymphocytes in the gut, Dr Bienenstock's department has reported that there are T cells, null cells and B cells in preparations of intestinal lymphocytes.

*Bienenstock:* There are very few B cells. In fact our methods are questionable, in the sense of whether they represent the cell population in the lamina propria (Rudzik & Bienenstock 1974).

*Pierce:* If one is drawing a parallel between host-versus-graft reaction and what takes place in parasitization with the introduction of foreign antigens, it is not clear to me why, during *Nippostrongylus* infection or other parasitic infections, the individual does not go on to reject the bowel completely, though obviously he doesn't.

*Ferguson:* In patients with coeliac disease there are many tiny areas of ulceration in the affected areas of the small bowel. In addition large deep benign ulcers have been reported in a small proportion of such patients. We have suggested that an established cell-mediated immune reaction will speed up the rate of cell turnover—a potentially protective although non-specific mechanism for the exfoliation of parasite-infected or virus- or bacteria-

infected epithelial cells. Perhaps it is only when there is an intense cell-mediated immune reaction, as is seen in rejection, that one finds changes in the general architecture, malabsorption and, in extreme cases, ulceration.

*Lehner:* You suggested two lymphokines as explaining some of your findings and I particularly wonder about the mitogenic factor, which has only been shown to cause mitosis in lymphoid cells and not in epithelial cells. Have you looked for evidence of the latter?

*Ferguson:* Assays of mitogenic factor are usually done with lymphocytes or lymphoid cell lines. However, in their work on leishmaniasis in the guinea pig, Bryceson *et al.* (1972) found that the thickening of the skin in leishmaniasis was a thymus-dependent reaction.

*Lehner:* You have shown chemotactic inhibition by means of casein attraction of cells. What is the mechanism?

*Ferguson:* Casein is used as a standard chemotactic agent. If one wishes to assay a factor which will inhibit chemotaxis, the cells are attracted through a filter by using casein in the lower chamber, and it is possible to see whether the substance under consideration inhibits the casein-induced chemotaxis.

*Lehner:* Casein has been shown to be a good B cell mitogen. I wondered if this would affect your results.

*Parrott:* I doubt whether the fact that casein is mitogenic would make any difference to its chemotactic effect. The concentrations at which mitogens such as Con A are chemotactic are much lower than those at which they are mitogenic (Wilkinson *et al.* 1976).

*Cebra:* How much physical association is there between the basal part of the plasma membrane of the epithelial cells and the basement membrane? It would seem that as cells are dividing out, they must slide over the basement membrane; that is probably not being constantly regenerated at the same rate as overlying enterocytes.

*Ferguson:* The region of the basement membrane and basal lamina is indeed formed continuously, but it is not known how much is produced by the epithelial cells and how much is a liquid or solid component of the ground substance of the lamina propria. In specimens of small intestine which have been allowed to autolyse overnight, the epithelium strips off and scanning electron microscopy can be used to examine the underlying basement membrane. There are holes in this basement membrane (Toner *et al.* 1970) and the numbers seem to correspond to the numbers of lymphocytes crossing the basement membrane in conventional sections. However, I think the epithelial cells move at a different rate from the basement membrane.

*Booth:* Marsh & Trier (1974) labelled the fibroblasts in the basement

membrane, in fact, and showed that they moved up the villus in the same way that the enterocytes do.

*Ferguson:* They also (Marsh & Trier 1974) showed that the fibroblasts moved more rapidly up the side of the villi than did labelled enterocytes.

*Cebra:* I am wondering whether the supposed T lymphocytes may have only one kind of lymphokine, the mitogenic factor, and the other effect is produced by a breaking of the tight junctions that join the epithelial cells together, so that in order to get a sheet cells off, all that is needed is to break it in a few places. It could be done mechanically by the lymphocytes forcing their way between the epithelial cells and breaking open junctions, rather than by an effect on adhesion to the basement membrane.

*Ferguson:* In coeliac disease a high number of intraepithelial lymphocytes is a constant finding in untreated patients (Ferguson 1974). This is not so for other conditions where the mucosa is flattened, for example *Nippostrongylus* infection. In fact there are fewer lymphocytes than normal between the epithelial cells in the flattened mucosa in worm infections (T. T. MacDonald & A. Ferguson, unpublished 1975). Neither are there increased numbers in acute gastroenteritis or in graft rejection (Ferguson *et al.* 1976; MacDonald & Ferguson 1976). Thus damage by intraepithelial lymphocytes cannot explain the villus atrophy.

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*Immunology of the Gut*
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# Immunopathology of coeliac disease

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*Abstract* Coeliac disease may be defined as a condition in which there is an abnormal jejunal mucosa with loss of villi, which improves morphologically after treatment with a gluten-free diet. Pathologically, there is damage to the jejunal enterocytes, with hyperplasia of crypt cells so that overall enteropoiesis is increased. On conventional or scanning electron microscopy the enterocytes are markedly abnormal. Histochemically, the normal punctate appearance of the lysosomes is lost and sensitive lysosomal enzyme assays on mucosal biopsy samples using isopycnic centrifugation techniques show that there is an increase in total lysosomal activity with reduction in lysosomal latency.

Studies following gluten feeding in patients whose mucosa has returned to normal after treatment with a gluten-free diet show that pathological abnormalities appear within 4–8 hours of gluten challenge. Complement together with extracellular IgM can be demonstrated in the lamina propria, suggesting the formation of immune complexes. In untreated coeliac disease there is a significant reduction in serum levels of C3 and C4. There is also evidence indicating the presence of immune complexes in the serum. Coeliac disease may therefore be an intestinal model of an immune complex disease, in which an antigen derived from gluten reacts with an antibody formed locally in the gut, fixing complement and causing damage to the enterocyte by activation of lysosomes.

Dicke (1950) was the first to show that coeliac disease is produced in some way by a normal dietary constituent, gluten, which is present in wheat and rye flour. Subsequent studies have repeatedly shown that there is a toxic factor present in gluten which damages the absorbing cells of the jejunal mucosa in subjects with the disorder, whether the condition presents in childhood or in adult life. The mechanism by which this toxicity occurs is not yet established with certainty but there is increasing evidence that coeliac disease can be considered as an immunological disorder in which there is a reaction between an antigen present in gluten and an antibody secreted by the immunocytes of the intestinal mucosa. This is associated with complement fixation and sub-

sequent cell damage. The purpose of this paper is to review the evidence which suggests that coeliac disease is at least in part an immune complex disease.

## DEFINITION

The term 'coeliac disease' is often used to describe a variety of conditions in which an abnormal jejunal mucosa is associated with malabsorption. For the purpose of this paper coeliac disease will be defined as an abnormality of the jejunal mucosa which improves morphologically after treatment with a gluten-free diet.

## MORPHOLOGY

In coeliac disease the appearances of the intestinal mucosa are strikingly abnormal. There are variations in the severity of the lesion in different patients (Stewart *et al.* 1967) but at low power under the dissecting microscope the mucosa is usually flat and devoid of villi. At higher power the mucosa can be seen to be divided by grooves into a mosaic pattern. The crypts of Lieberkühn, not being hidden by villi, open directly on to the surface of the mucosa and their orifices can be clearly seen. The histology of the flat mucosa reveals three main features. The enterocytes are no longer columnar but are flat, cuboidal, and reduced in number. The enteroblasts (or crypt cells) are increased, the crypt layer being markedly thickened compared with the normal, and there is an infiltration of the lamina propria with abundant lymphocytes and plasma cells. There is clear evidence that there is damage to the enterocytes, the hyperplasia of the enteroblasts being compensatory in an attempt to make up for damage to the surface cells, a situation analogous to haemolytic anaemia (Booth 1970). The appearances of the abnormal jejunal enterocyte improve dramatically when a gluten-free diet is given, the response being more rapid and more striking in children than in adults. These observations refer to the jejunal enterocyte, for the ileum in this condition is either normal or less severely involved than the jejunum (Rubin *et al.* 1960; Booth *et al.* 1962; Stewart *et al.* 1967), which would be expected if the intestine were being damaged by an orally ingested toxin.

## HISTOCHEMISTRY

The anatomical changes already described are associated with abnormalities in the histochemistry of the intestinal cell. Brush border enzymes are usually abnormal and frequently reveal a reduction of alkaline phosphatase as well as

diminished staining of other brush border enzymes (Riecken *et al.* 1966). Lysosomal enzymes can also be shown to be abnormal. In the untreated jejunal mucosa the lysosomal enzymes, for example acid phosphatase and esterase, are not present as discrete organelles as they should be in the normal situation, but are diffusely scattered throughout the disorganized enterocyte. As Wattiaux & de Duve (1956) have suggested, it is possible that this diffusion of intensely hydrolytic enzymes into the cytoplasm may contribute to cell damage. The changes shown are not specific to coeliac disease and probably simply reflect the gross damage that gluten causes to the susceptible enterocyte.

## CYTOCHEMICAL STUDIES

The cytochemistry of jejunal biopsies in untreated coeliac disease has been compared with the results in control subjects and in patients who have been treated satisfactorily with a gluten-free diet (Peters *et al.* 1975). As others have shown, there are gross reductions in the activities of the major brush border enzymes, but the results for the lysosomal enzymes have been shown to be quite different. Table 1 shows the specific activities of six acid hydrolases and of lactate dehydrogenase, a cytosol enzyme, in homogenates of biopsies from control subjects and from patients with coeliac disease. Mean activities of all acid hydrolases were found to be significantly higher in untreated patients than in control subjects. The increase in activity differed for each enzyme, with acid phosphatase showing the smallest rise. Enzyme activities in patients treated with a gluten-free diet decreased towards normal values. For acid phosphatase

TABLE 1

Activities of acid hydrolases and of lactate dehydrogenase in jejunal biopsies from control subjects and patients with adult coeliac disease

| *Enzyme* | *Control subjects* | *Untreated coeliac disease* | *Treated coeliac disease* |
|---|---|---|---|
| *N*-Acetyl-β-glucosaminidase | 2.12 ±0.15 (20) | 3.28 ±0.31 (9)$P<0.001$ | 2.71 ±0.25 (5)$P<0.05$ |
| α-Galactosidase | 0.185±0.012(20) | 0.402±0.021(7)$P<0.001$ | 0.301±0.063(7)$P<0.01$ |
| Acid phosphatase | 16.2 ±2.1 (21) | 22.5 ±2.1 (9)$P<0.05$ | 14.9 ±3.2 (6)N.S. |
| Acid β-galactosidase | 1.15 ±0.31 (19) | 2.20 ± 0.23 (7)$P<0.001$ | 1.65 ±0.39 (7)$P<0.05$ |
| β-Glucuronidase | 2.01 ±0.19 (19) | 4.41 ±0.24 (7)$P<0.001$ | 3.02 ±0.42(11)$P<0.05$ |
| Acid diesterase | 0.74 ±0.21 (8) | 1.74 ±0.31 (4)$P<0.01$ | 0.82 ±0.31 (4)N.S. |
| Lactate dehydrogenase | 4120 ±850 (16) | 1400 ±210 (7)$P$±0.05 | 3680 ±940 (7)N.S. |

Activities are expressed as mean values ± S.E.M. (munits/mg of protein) with the number of samples assayed in duplicate shown in parentheses. $P$ is probability that the difference from values in control subjects is due to chance alone. N.S., not significant.
(From Peters *et al.* 1975.)

and acid diesterase there was no significant difference between control values and values in treated patients. The other acid hydrolases showed significantly elevated activities in the treated patients compared with controls but the values were lower than in the untreated patients. The cytosol enzyme, lactate dehydrogenase, in contrast to the acid hydrolases, showed a significant decrease in untreated coeliac mucosa with a return to normal activity after treatment.

*Fractionation experiments*

Using fractionation techniques, it was possible to demonstrate the latent, sedimentable, recovered and released acid hydrolase activities in the fractionated mucosal biopsies obtained both from normal subjects and from the patients with coeliac disease before and after treatment. These results are set out in Table 2. The latent *N*-acetyl -β-glucosaminidase activity was strikingly reduced

TABLE 2

Latent, sedimentable, recovered, and released acid hydrolase activities in jejunal biopsies from control subjects and patients with adult coeliac disease

| *Enzyme* | *Control subjects* | *Untreated coeliac disease* | *Treated coeliac disease* |
|---|---|---|---|
| *N*-Acetyl-β-glucosaminidase: | | | |
| Latent | 73.6±1.19(13) | 59.7±1.99(7)$P<0.001$ | 68.2±1.56(6)$P<0.05$ |
| Sedimentable | 69.0±2.0 (10) | 56.9±1.5 (6)$P<0.001$ | 62.6±1.5 (5)N.S. |
| Recovered | 91.2±2.9 (13) | 82.2±4.3 (6)N.S. | 92.6±5.2 (5)N.S. |
| Released | 62.3±2.2 (11) | 69.4±5.1 (6)N.S. | 69.9±5.1 (5)N.S. |
| β-Glucuronidase: | | | |
| Sedimentable | 76.9±1.8 (12) | 68.8±2.8 (5)$P<0.05$ | 77.6±1.34(5)N.S. |
| Recovered | 92.1±4.6 (12) | 85.5±2.6 (5)N.S. | 87.0±9.2 (5)N.S. |
| Released | 56.5±3.6 (12) | 63.4±6.2 (5)N.S. | 59.5±6.1 (5)N.S. |
| Acid phosphatase: | | | |
| Sedimentable | 72.4±4.6 (5) | 60.4±1.3 (6)$P<0.05$ | 68.8±2.1 (5)N.S. |
| Recovered | 87.7±7.4 (5) | 72.6±2.8 (6)N.S. | 80.3±5.5 (5)N.S. |
| Released | 51.6±4.9 (5) | 72.9±4.9 (6)$P<0.05$ | 69.2±7.1 (5)N.S. |
| Acid β-galactosidase: | | | |
| Sedimentable | 69.3±2.8 (8) | 59.3±1.2 (4)$P<0.05$ | 68.3±4.2 (5)N.S. |
| Recovered | 82.3±2.6 (8) | 85.0±1.4 (4)N.S. | 86.8±3.5 (5)N.S. |
| Released | 52.2±2.8 (8) | 70.8±6.3 (4)$P<0.05$ | 50.9±3.5 (5)N.S. |

Latent, sedimentable, recovered and released activities, expressed as percentage, are defined in the text. Results are shown as mean values ± S.E.M. with the number of samples estimated in duplicate in parentheses. $P$ is probability that the difference from values in control subjects was due to chance alone.
(From Peters *et al.* 1975.)

in untreated patients but biopsies from treated patients showed activities nearer to those of control subjects. The patterns of change of the sedimentable, recovered, and released activities of the four acid hydrolases were very similar. Biopsies from patients with untreated coeliac disease showed decreased sedimentable acid hydrolases. This was most marked for *N*-acetyl -β-glucosaminidase but the other enzymes also showed a significant decrease. The sedimentable acid hydrolase activities (%) in the biopsies from treated patients were all within the normal range. No significant change in recovered enzyme activity was found for any of the four enzymes in the three groups of patients studied. The released enzyme activity (%) showed higher values for the biopsies from untreated coeliac disease than for the control subjects, but only for acid β-galactosidase and acid phosphatase were the differences statistically significant. For these two enzymes the activities returned to the normal range after treatment.

The interpretation of the increased fragility of lysosomes in the mucosa in coeliac disease is uncertain. First, it may indicate an increased permeability of the lysosomal membrane. Secondly, the changes in latent activity may reflect an increase in the size of lysosomes in the coeliac mucosa such as has been shown by electron microscopy (Padykula *et al.* 1961; Riecken *et al.* 1966; Rubin *et al.* 1966; Shiner 1967). The role of the lysosome in the pathogenesis of enterocyte damage is not completely established by these experiments but would be compatible with an immunological mechanism of cytolysis.

## IMMUNOLOGICAL ABNORMALITIES IN COELIAC DISEASE

There appears to be an overall reduction in lymphoreticular tissue throughout the body in untreated adult coeliac patients. This is indicated by the small spleen that may be found in adult coeliac disease and by the signs of splenic atrophy which are recognized in the blood, the presence of Howell-Jolly bodies being particularly striking. McCarthy *et al.* (1966) have shown that there is a reduction in peripheral lymphoid tissue and it has also been found that the lymphocytes in coeliac patients may show impaired transformation, whether they be circulating lymphocytes (Blecher *et al.* 1969) or lymphocytes teased out from mesenteric lymph nodes (Housley *et al.* 1969). All these findings suggest lymphoreticular dysfunction and this is supported by studies of the immunoglobulins in coeliac disease (Hobbs & Hepner 1968) which have shown reduced levels of IgM in 60% of untreated patients. This is a secondary phenomenon, since the levels return to normal after treatment with a gluten-free diet. The reduced level of IgM is not due to increased loss of IgM into the gut from damaged mucosa, because the overall rate of synthesis of IgM in coeliac

disease is reduced (Brown *et al.* 1969), probably reflecting the general depression of lymphoreticular function in this disease. It is tempting to speculate that this may be an important factor in untreated patients, in whom there is a greatly increased incidence of lymphoma and intestinal neoplasia (Gough *et al.* 1962; Austad *et al.* 1967; Harris *et al.* 1967).

The immunological response to gluten in coeliac disease is not mediated through reaginic sensitivity since, as Hobbs *et al.* (1969) and Asquith *et al.* (1969) have shown, serum IgE levels are normal unless coeliac patients also have atopic manifestations such as asthma or allergic rhinitis. Furthermore, immunofluorescent studies do not implicate IgE-containing cells in the gut mucosa and the concentration of IgE in the jejunal fluid is not apparently increased. Circulating antibodies to gluten fractions and other food substances, especially milk proteins, are often found in untreated coeliac patients, both in childhood and in adult life (Heiner *et al.* 1961; Alarcøn-Segovia *et al.* 1964; Kivel *et al.* 1964; Taylor *et al.* 1964; Bayless *et al.* 1967). The techniques used in these studies have varied considerably and it is therefore difficult to make comparisons between the work of different groups but it seems unlikely that any of these serum antibodies are specifically correlated with the mucosal damage of coeliac disease, since they can be found in other disorders, such as ulcerative colitis.

Histological studies of the jejunal mucosa show that there is a dense infiltration of the lamina propria with lymphocytes and plasma cells in coeliac disease. There is also an increase in the inter-epithelial lymphocyte (theliolymphocyte) population in the intestinal mucosa. The immunoglobulin classes of the plasma cells of the lamina propria have been studied by immunofluorescence by a number of workers. It is clear that the normal preponderance of IgA cells in the gut is also found in the mucosa of coeliac patients (Rubin *et al.* 1965). Crabbé (1967), in a single patient, however, reported an excess of IgM-producing cells in the untreated coeliac mucosa and subsequent studies on larger numbers of patients have confirmed this finding (Douglas *et al.* 1969; Søltoft 1970). In seven untreated coeliac patients Douglas and his colleagues (1969) showed that there was an excess of IgM-producing cells, an abnormality which persisted in all but two of fifteen patients treated with a gluten-free diet. The excess of these cells was localized to the jejunum, since examination of the rectum and bone marrow showed a normal distribution of immunocytes.

## COMPLEMENT IN COELIAC DISEASE

Studies of the complement system in coeliac disease suggest that activation of complement may play an important role in the pathogenesis of the mucosal

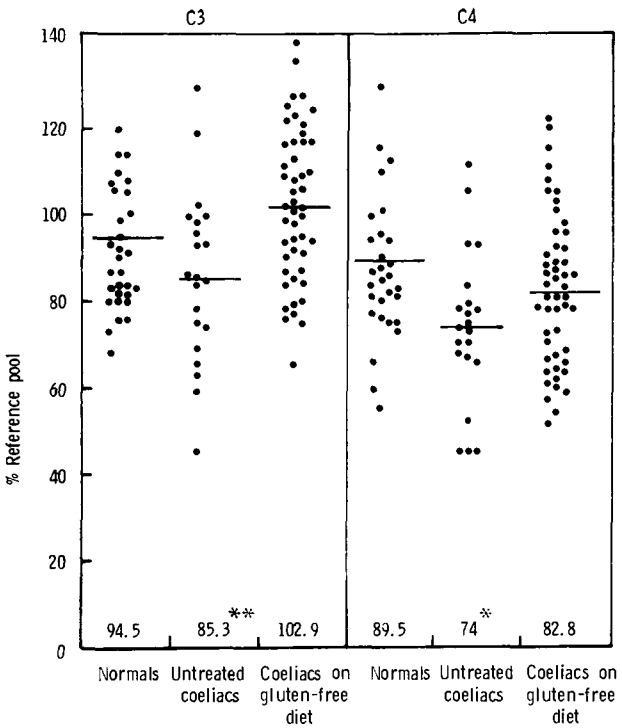


FIG. 1. Levels of C3 and C4 in plasma from normal subjects, coeliac patients taking a normal diet, and those who had been taking a gluten-free diet for over six months. Means are represented by horizontal bars. (From Doe *et al.* 1975.)

lesion. Deposits of the third component of complement have been detected in the jejunal mucosa of childhood and adult coeliac patients after they have received a single gluten challenge (Shiner & Ballard 1972; Doe *et al.* 1972*b*). Furthermore Rossipal (1972) has shown significant reductions in serum C3 after sustained gluten challenge in childhood coeliacs who have been successfully treated with a gluten-free diet. Studies of the levels of various complement components in the serum have been made by Doe *et al.* (1975). The results of studies of plasma levels of C3 and C4 in 30 normal subjects, 20 coeliac patients taking a normal diet and 53 coeliac patients taking a gluten-free diet for at least six months, are shown in Fig. 1. Mean plasma C3 was lower in untreated coeliac patients than in normal controls, but this difference was not statistically significant. A significantly higher mean C3 level was found in coeliac patients taking a gluten-free diet than in the untreated group ($P<0.001$). Mean C4 levels were significantly lower in untreated coeliacs than in controls ($P<0.01$), but while

TABLE 3

Relationship between gluten-free diet and presence of C1q precipitation in sera from adult coeliac disease patients

| *Adult coeliac disease patients* | *No. of patients* | *C1q positive* | |
|---|---|---|---|
| | | *No.* | % |
| Normal diet | 50 | 20 | 40 |
| Gluten-free diet for > 6 months | 77 | 8* | 10* |

*$P<0.001$ ($\chi_1^2 = 13.79$).
(From Doe *et al.* 1973).

the treated coeliac group had a higher mean C4 level, this difference did not achieve significance.

Further studies have utilized the C1q precipitation test for detecting complement-binding soluble antigen–antibody complexes in the sera of patients with coeliac disease (Doe *et al.* 1973). The techniques used were those described by Agnello *et al.* (1970). Studies were done in 50 patients with adult coeliac disease who were receiving a normal diet and in 77 who had had a gluten-free diet for longer than six months. The results are set out in Table 3. Of the 50 patients with adult coeliac disease on a normal diet, 20 (40%) showed a positive C1q precipitation test. Results after a gluten-free diet, however, were significantly different. In this group only 10% had a persistently positive C1q precipitation test. These studies, together with the observations previously referred to, suggest the possibility that there may be circulating immune complexes involving the binding of complement in the serum of patients with coeliac disease, and it is therefore important to know whether complement fixation occurs at the level of the intestine.

## IMMUNOLOGICAL ABNORMALITIES AFTER GLUTEN CHALLENGE

The possibility that gluten challenge might induce an antigen–antibody reaction in the jejunal mucosa in coeliac disease has been previously studied in children by Shiner & Ballard (1972) and ultrastructural changes have been documented by Shiner (1973). When gluten is given in a dose of 30 g to an adult patient whose mucosa has recovered after treatment with a gluten-free diet, the morphological changes can be sequentially studied by repeated jejunal biopsies. The data obtained in our laboratory from such studies in five adult patients with coeliac disease in the 48 hours after they had received a gluten challenge can be summarized as follows. Four hours after gluten had been ingested remarkably little change was found in the intestinal mucosa. There

was slight oedema in some biopsies and an occasional eosinophil could be seen but it was not until eight to twelve hours after gluten challenge that significant abnormalities occurred. As Shiner & Ballard (1972) and Shiner (1973) have pointed out, the earliest changes occur in the sub-epithelial region, endothelial cell swelling being noted in blood vessels in the lamina propria. By 12 hours, however, there is a diffuse cellular infiltration which in our studies was at this time due to infiltration with polymorphonuclear leucocytes, and was associated on immunofluorescent study with the deposition of complement and extracellular IgM. At 24 hours the mucosa had become frankly abnormal with stunted villi and grossly abnormal enterocytes. At this time, however, the infiltration of the lamina propria was predominantly due to mononuclear cells, active macrophages, and plasma cells. By 48 hours most of the abnormality had disappeared and the villi were showing signs of recovery. A similar sequence of pathological events can be demonstrated when gluten is directly instilled into the ileum (Rubin *et al.* 1962).

There were at the same time important changes in the inter-epithelial lymphocytes (Fig. 2, p. 338). These began to increase at four hours after gluten challenge in some patients and reached a peak level at 12 or 24 hours after the gluten was given (Ferguson 1975). The precise significance of these lymphocyte changes is not known but it is clear that even though there may be a reaction between antigen and antibody, with complement fixation occurring at the level of the lamina propria, there are at the same time important changes occurring in the lymphocytes. Since the majority of the inter-epithelial lymphocytes are in fact T lymphocytes, this observation may indicate an important contribution of cell-mediated immunity in addition to humoral immunity in the pathogenesis of cell damage.

## ASSOCIATION OF COELIAC DISEASE WITH CRYOGLOBULINAEMIA

There is additional evidence to suggest that immune complexes are concerned with coeliac disease, at least in some cases. Doe *et al.* (1972*a*) described four patients with coeliac disease, all of whom had cryoglobulinaemia. It has been suggested that mixed cryoglobulins represent circulating antigen–antibody complexes (Lospalluto *et al.* 1962; Meltzer & Franklin 1967) which may result in the deposition of immune complexes in vessels, causing arteritis and other features of immune complex disease. The four patients described by Doe *et al.* (1972*a*) all had vasculitis and cryoglobulinaemia. Three patients had extensive skin rashes and in the first patient the dermatological appearances were suggestive of cryoglobulinaemia. Two of these patients showed a morphological improvement in the jejunal mucosa when a gluten-free diet was given; one

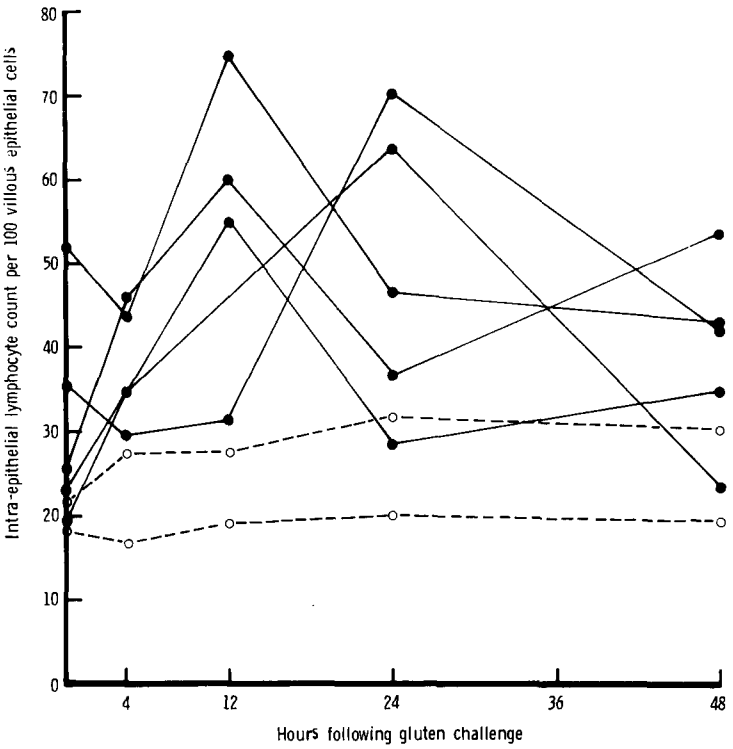


FIG. 2. Serial inter-epithelial lymphocyte counts in five adult coeliac disease patients who received an oral challenge of 30 g gluten after a prolonged period of gluten withdrawal. (From Ferguson 1975.)

patient had a history suggesting malabsorption which dated from childhood, and the other patient had originally presented with malabsorption which had responded satisfactorily to treatment with a gluten-free diet some years earlier. He had, however, then become unresponsive to gluten, and had developed a severe mucosal abnormality with dense collagen deposited under the basement membrane of the enterocytes. It is curious that this relationship between coeliac disease and cryoglobulinaemia has until now only been reported in one laboratory.

## CONCLUSION

The evidence presented in this paper suggests that coeliac disease may be due to a reaction between antigen and antibody occurring at gut level. If this is so, the question has to be asked whether there is an *abnormal* entry of large

amounts of antigen which then produces a *normal* immunological response of the immunocytes of the intestine. Alternatively, there might be a *normal* entry of whatever amounts of antigen can normally penetrate the small intestinal mucosa, with an *abnormal* immune response. Studies of the relationship between coeliac disease and HLA antigens have repeatedly indicated a close relationship between coeliac disease and HLA-B8 (Falchuk *et al.* 1972; Stokes *et al.* 1972) and more recently with HLA-DW3 (Keuning *et al.* 1976). Coeliac disease is known to be genetically determined, although the precise nature of its genetic background is not completely clear. The present evidence suggests that it is inherited as a dominant with incomplete penetrance (MacDonald *et al.* 1965). These two pieces of evidence would be in keeping with the idea that coeliac disease is due to an abnormal immune response gene, the basic abnormality being a genetically determined failure to clear antigens which enter the lamina propria of the gut.

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We thank the many colleagues at the Royal Postgraduate Medical School who have been associated with us in these studies during the past ten years. We also gratefully acknowledge the generous support of the Medical Research Council and the Wellcome Trust.

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## Discussion

*Rosen:* The gut epithelium has been demonstrated to be the site of C1 synthesis, which may be germane here. Is the kind of fluid loss that we see in gluten-feeding very like what we see in patients with hereditary angioneurotic oedema, where there is C1 activation in the gut, and where the major involvement is also jejunal, and only very rarely in the ileum or the colon?

*Booth:* I don't know the answer to that.

*Rosen:* It seems that C1q, C1r and C1s are all synthesized in the gut. But do these patients get massive fluid loss into the gut lumen?

*Booth:* I don't know. They certainly lose fluid if they are untreated.

*Rosen:* Falchuk (Katz *et al.* 1976) can also induce the lesion *in vitro*, by taking a biopsy of intestine from a coeliac patient in remission and adding gluten to the culture. He can prevent the change by adding steroids. What is the mechanism of that?

*Booth:* I think steroids are simply lysosomal stabilizers. If you study the response to steroids in coeliac patients histochemically, as Wall working with us has done, you can show the punctate appearance of the lysosomes becoming

clearly re-established (Wall *et al.* 1970). I would have said it was a non-specific response to steroids.

*Pierce:* It has been shown that gut biopsies from coeliac patients in remission challenged *in vitro* with gluten do not show an increased synthesis of IgA and IgM. How does this fit with what Dr Rosen has just said about induction of flattening *in vitro*? It seems either to be contradictory, or to exclude a role for antibody, since you are saying that antibody may play some role in mediating the response yet it cannot be induced *in vitro* by gluten challenge.

*Rosen:* But who knows what is in the biopsy material? There may be plenty of antibody there all the time, and you are adding the antigen.

*Evans:* Those findings are different from those originally reported by Falchuk. If he had a patient in good remission from coeliac disease and cultured a biopsy with gliadin he could not produce an impairment of brush-border height. If on the other hand he gave the patients a small challenge with gliadin beforehand, and then cultured their biopsy tissue, they were sensitive to gliadin. If he co-cultured a biopsy from a patient in complete remission with a biopsy from a patient with untreated coeliac disease, and then challenged with gliadin, there was shrinkage of the brush border in the biopsy of the patient in remission.

*Booth:* The technical differences between different groups are enormous here. The work David Evans is referring to is based on estimates of alkaline phosphatase activity as a measure of brush-border activity and when you do that, in terms of measuring tissue phosphatase or that in the surrounding fluid, you can get all sorts of results. Conclusions based solely on alkaline phosphatase activity are hard to interpret.

*Evans:* Falchuk has also stated that there are alterations in brush border morphology (see Katz *et al.* 1976).

*Rosen:* It comes back to the point that there is probably pre-existing antibody in the biopsy.

*White:* I am trying to compare your findings with nephritis induced by say serum sickness, Professor Booth. You have an antigen–antibody reaction and this leads to separation of the endothelial cells—that is, histamine or another agent separates them. Therefore antigen–antibody complexes are presumably let out and they sit on the basement membrane. You have evident damage to the basement membrane, which can be gross. That should mean that most biopsies ought to show complexes sitting on the basement membrane and easy to demonstrate. Are they?

*Booth:* No, they are not. This is the practical point. When you take a biopsy and look for immune complexes, in fact you are looking for antibody in

punctate form such as you would find in a renal glomerulus. Nobody has yet found that. I hope Anne Ferguson is going to do so!

*Ferguson:* One point we tend to forget is that intestinal biopsies are usually taken after a fairly long fast. The patient comes to the hospital without breakfast and the biopsy is obtained a couple of hours later. Thus the intestine of the patient has not been exposed to foods for 15–20 hours before the biopsy is taken—that is, it has been gluten-free for at least this length of time.

*Booth:* The problem remains that even when you challenge with gluten, it is hard to detect complement. In the Norwegian data on complement in the mucosal biopsies, can you see anything like an immune complex?

*Brandtzaeg:* I don't think we should expect to see immune complexes in tissues like the lamina propria, which cannot be compared with the kidney.

*White:* It is a bewilderingly constant thing that if you inject a small dose, say 1 mg, of a protein antigen into an animal it segregates antigen–antibody complexes through the walls of blood vessels throughout the body and they sit as circumscribed foci on the basement membrane.

*Rosen:* That is intravascular antigen and an intravascular model. This is different.

*Brandtzaeg:* I have one question about the pathogenetic immunoglobulin class. You suggested that it was IgM.

*Booth:* Mietens (1967) showed that anti-gluten antibodies in the lumen of the gut were IgM.

*Brandtzaeg:* In the lumen you may expect the antibodies to be IgM or IgA but in serum most people agree that they are mainly IgG, and this IgG must be distributed extravascularly in the lamina propria. In one patient we succeeded in picking out an antigen from gluten which could be used in the indirect immunofluorescence technique to show local antibody-producing cells. We found that of the IgG class, almost 6% of the cells were involved in antibody production against this gluten antigen. Only 1.5% of the local IgA cells showed this activity and almost none of the local IgM cells. The problem was that the particular antigen that we used successfully didn't work in other patients. This emphasizes the heterogeneity of the immune response to gluten antigens.

*Lachmann:* A study by Sikora *et al.* (1976) has shown that the B2 subfraction of Frazer's fraction III of a gluten digest gives impressive lymphocyte stimulation in patients with coeliac disease, more so than with crude antigens. One obviously needs to have the right antigen, both in looking for antibodies and in looking for cellular immune reactions in these patients.

*André:* To come back to *in vitro* biopsy cultures, Dr Ferguson has evidence that if the biopsy is challenged with α-gliadin, the lymphocytes in the biopsy produce MIF (Ferguson *et al.* 1975).

*Ferguson:* That was work which I have referred to as being only preliminary, and it has not yet been repeated.

*André:* The shortening of the villi not only occurs after challenging biopsies with gluten but also with casein. This has been shown by Jos *et al.* (1975) at the Enfants-Malades Hospital in Paris.

*Ferguson:* Jos *et al.* (1974) have shown that casein may cause damage to cultured jejunal biopsies from some children with coeliac disease. They have also shown that the lymphocytes of children with coeliac disease may be stimulated to mitosis by casein extracts.

*Porter:* Over a number of years there has been evidence in calves of an intestinal sensitivity to an antigen of soya. Dutch workers (Van Adrichem & Frens 1965) showed high levels of serum antibodies against undefined soya antigen in calves fed soya proteins in a milk-replacement diet. Recently Smith & Sissons (1975) showed that after feeding soya proteins to fistulated calves there were physiological changes in the gut in terms of flow rates. There was an increase in the time taken for markers to move between the duodenum and ileum, and yet there was a massive influx of electrolytes, so that the volume passing through the ileum was increased.

We have looked at this syndrome and have demonstrated in Thiry-Vella loop pigs that once animals are sensitized with the soya antigen, the action of the antigen within the loop produces a dramatic decrease in flow through the loop only while the antigen is present. Thus within the experimental model you could apply the antigen at any time and get an immediate decrease in flow rate, remove it and return to the normal rate.

*Booth:* It would be interesting to know what is happening to the mucosa.

*Porter:* Coming back to the immunopathogenic component, the immunoglobulins involved in the development of these responses are almost exclusively IgG1, which is both a precipitating and a complement-fixing antibody. I do not believe that that type of Arthus response is immediately responsible for the decrease in flow within the loop in response to the antigen. It is more likely to be an immediate type hypersensitivity mechanism.

*Booth:* A case has been recorded in man of soya flour sensitivity (Ament & Rubin 1972). I don't know the timing of the response.

*Porter:* It is very fast in calves. We do not get the physiological response without sensitization. It takes 7–10 days to achieve sensitization. I wonder if this could be a reasonable model system for studying gluten sensitivity?

*Pepys:* A significant proportion of patients with bird-fancier's lung, a form of extrinsic allergic alveolitis, have recently been found to have jejunal villous atrophy histologically indistinguishable from coeliac disease (Berrill *et al.* 1975). This raises the possibility that similar immunological mechanisms

directed against a variety of inhaled or ingested antigens might cause the intestinal lesion seen in coeliac disease.

*White:* Is there any evidence that villi can actively contract? Are there contractile cells or particular sorts of fibroblasts susceptible to the action of pharmacological agents?

*Booth:* The best cine pictures of this are in the dog and have been published by Verzar (Verzar & McDougall 1936). The villi can be seen pumping up and down. But it depends on the species: in the rat the villi are mostly leaf-shaped and do not pump. In the dog they do. Whether they pump in man has not been satisfactorily shown.

*White:* Perhaps smooth-muscle contractant drugs could flatten them.

*Ferguson:* Villi can readily be made to contract or shrink considerably. In an ordinary biopsy processed for conventional histology they contract by around 50% (A. Ferguson, A. Sutherland & T. T. MacDonald, unpublished work 1976). By altering various aspects of the fixation and embedding procedures it is possible to make the villi long and smooth or short and irregular. However, the number of cells along the sides of each villus remains the same. In the so-called villous atrophy under discussion here, the number of epithelial cells is only about a tenth that of the normal value.

*Booth:* I do not think contraction has anything to do with coeliac disease, but it is an interesting point whether human villi contract.

*Rosen:* What is the HLA association of coeliac disease?

*Booth:* The original observation made in 1972 was of an 80% incidence of HLA-B8, made simultaneously in the USA and in the UK. A recent paper from Holland (Peña *et al.* 1976) suggests that the true association is with the HLA-DW3. The difficulty is that the DW3 linkage is not absolute. You still have some cases without that particular lymphocyte configuration. It is an interesting association and suggests the possibility of an immune-response gene abnormality of some sort, which probably means that coeliac disease is a genetic reaction to the entry of the small amounts of antigen which get across the mucosa under normal circumstances.

*Rosen:* The association at D is no more firm that it was at B.

*Pepys:* There has been one report in which unique B lymphocyte surface antigens were found in almost all the patients studied with either dermatitis herpetiformis or with coeliac disease and were not found in any of the normal population (Mann *et al.* 1976). It is suggested that these B cell antigens are analogous to the Ia antigens in mice.

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# General discussion

## HOMING OF T BLASTS

*Parrott:* Until recently little emphasis has been placed on the functions of T lymphocytes in the gut mucosa but Dr Rosen (Katz & Rosen, this volume, pp. 243–261) has suggested that they have a protective function whilst Dr Ferguson has emphasized their destructive function (Ferguson & MacDonald, this volume, pp. 305–327). I will summarize the basic data on the location of T cells before describing our recent work on the homing of T blasts to the gut mucosa.

There is good evidence that T like B cells are sensitized to intraluminal antigen and that they become sensitized in Peyer's patches. For example, Müller-Schoop & Good (1975) demonstrated that after oral immunization of guinea pigs with live *Clostridia* or BCG, preparations of T cells from Peyer's patches responded by proliferation to *in vitro* stimulation with killed organisms.

There are clearly defined T and B areas in Peyer's patches and the preparation of T:B cells varies according to age and antigenic challenge (for literature review see Parrott 1976). The area of particular interest in Peyer's patches is that referred to as the 'dome', since this is immediately below the area of specialized epithelium which allows transport of antigen (Bockman & Cooper 1973). In germ-free thymus-deprived mice the dome is depleted of lymphocytes as well as recognized T areas (Parrott & Ferguson 1974). After staining with an anti-T antiserum (Lamelin *et al.* 1972) T cells can be demonstrated in both the dome and in the T areas (D. Guy-Grand, personal communication) and after infusion of [$^3$H]adenosine or [$^3$H]uridine-labelled B or T cells the one place in Peyer's patches where one finds intermingling of both cell types is in the dome (Parrott & Ferguson 1974). Perhaps this is where both cell types meet antigen and immune responses are initiated?

TABLE 1

The 24-hour localization of [$^{125}$I]iododeoxyuridine-labelled mesenteric lymph node cells (MLN) in the small intestine of normal NIH mice and mice infected with *Trichinella spiralis*[a]: a comparison of mesenteric node T and B blasts

| *Cell inoculum* | *Mean % injected dose* ± S.D. | |
|---|---|---|
| | *Uninfected* | *Infected* |
| Whole MLN[b] | 6.3 ± 1.0 | 9.9 ± 1.9 |
| T-MLN[c] | 6.4 ± 0.6 | 12.1 ± 0.5 |
| B-MLN[d] | 4.6 ± 0.6 | 5.3 ± 1.4 |

[a]Cells transferred four days before infection by oral intubation with approximately 450 larvae.
[b]Whole MLN contains 45% T cells.
[c]MLN separated on a nylon wool column contains more than 95% T cells as determined by anti-T antiserum cytotoxicity.
[d]From adult thymectomized, lethally irradiated, bone marrow-reconstituted mice; contains less than 15% T cells.
(Data from Rose *et al.* 1976*a*,*b*.)

After antigen-induced proliferation it would seem reasonable to suppose that the dividing T cells leave Peyer's patches and travel by the same mesenteric and thoracic duct routes as B immunoblasts (see Cebra *et al.* and Husband *et al.*, this volume, pp. 5–28 and 29–54) to reach the lamina propria. If one separates mesenteric lymph node cells so as to get an almost pure preparation of T cells and then incubates them with [$^{125}$I]iododeoxyuridine so as to label only immunoblasts, then these T immunoblasts home to the gut (Table 1) in the same purposeful way as has been described by Professor Gowans for B immunoblasts (Husband *et al.*, this volume, pp. 29–54). Sprent (1976) has recently shown that activated T blasts taken from the thoracic duct lymph also home to the gut. Guy-Grand *et al.* (1974) demonstrated by immunofluorescence that there are cells with a T cell surface antigen in the lamina propria and between the epithelial cells of the villi and I have shown by autoradiography (unpublished) that after infusion of labelled T blasts there are labelled cells in both these sites. T blasts therefore normally migrate to the gut, possibly to perform the protective functions described by Dr Rosen. In situations where the gut is inflamed after infection with *Trichinella spiralis*, increased numbers of T mesenteric blasts (but not B blasts) migrate to the gut (Table 1; Ogilvie & Parrott, this volume, pp. 183–195). The increased migration is coincident with villous atrophy and the onset of inflammation and it may be that this experimental model, like Dr Ferguson's allografts, will prove useful in studying the destructive effects of T cells in the gut, particularly in relation to the mechanism of villous atrophy.

I have been interested for some time in what promotes the extravasation of

TABLE 2

The 24-hour localization of [$^{125}$I]iododeoxyuridine-labelled mesenteric (MLN) and peripheral (PLN) lymph node cells in the small intestine of NIH mice infected with *Trichinella spiralis*

| *Cell inoculum* | *Mean % injected dose* ± S.D. | |
|---|---|---|
| | *Uninfected* | *Infected* |
| MLN | 7.0 ± 0.8 | 11.4 ± 0.6 |
| Ox-PLN[a] | 2.0 ± 0.4 | 6.1 ± 0.9 |

[a]Ox-PLN: blast cells from lymph nodes four days after the application of oxazolone (10 mg in acetone).
(Data from Rose *et al.* 1976*b*.)

lymphoid cells into the gut and skin and other epithelial tissues and whether cells destined for one site can be diverted to another. Normally blast cells taken from peripheral lymph nodes will not migrate to the gut (Griscelli *et al.* 1969; Guy-Grand *et al.* 1974; Parrott & Ferguson 1974). It seemed possible, however, that the inflamed or infected gut would prove to be more accessible to cells from other sources as well as those which directly drain the gut. We know that T blasts from sites draining a contact sensitizer such as oxazolone migrate in a non-specific way to any site of inflammation on the skin, whether induced by the contact sensitizer or an unrelated sensitizer (such as picryl chloride) or any inflammatory agent such as croton oil or turpentine (Asherson *et al.* 1973). Accordingly, we labelled the blast cells which are produced in lymph nodes 3–4 days after the application of the contact sensitizer with [$^{125}$I]iododeoxyuridine and injected them into normal mice and mice infected with *T. spiralis*. Such cells, which are almost all T blasts, do go to the gut which is inflamed by the parasite but not to normal gut (Table 2). If, however, infected mice are also treated with oxazolone on the ear skin, localization to the gut returns to normal and the blast cells are located in the treated ears and auricular lymph nodes (Rose *et al.* 1976*b*). Mesenteric lymph node blasts, even pure T blasts, cannot, however, be persuaded into inflamed skin (Rose *et al.* 1976*b*). Splenectomy (unpublished observations) does increase slightly the tendency of both peripheral and mesenteric blasts to migrate to the gut. Can the clinicians tell us whether the presence of an atrophied spleen, which occurs in some patients with coeliac disease, exacerbates the disease?

In summary, we have shown that T blasts of the mesenteric node normally migrate to the gut and that they go there in increased numbers when the gut is infected by the nematode *T. spiralis*. We have shown that peripheral blasts which go to sites of inflammation can be diverted to the infected but not to the normal gut. Now we must investigate whether this increased and diverted

migration of T blasts has a clinical significance not only in relation to worm expulsion but also in relation to non-specific exacerbation of inflammation in the gut.

Finally, to bring us back to the problem of what persuades blast cells to extravasate, Peter Wilkinson and his group (Wilkinson *et al.* 1976; Russell *et al.* 1975) in our laboratory have shown recently that activated lymphocytes and immunoblasts (T and B) will 'chemotax' in a Boyden chamber towards endotoxin, casein and altered human serum albumin (HSA). This would seem to offer a good explanation of how one finds lymphocytes as well as monocytes in an inflammatory site. Perhaps chemotaxis will explain why immunoblasts extravasate into the gut wall. In the normal gut endotoxin and perhaps casein would be present. This would not apply to the antigen-free gut grafts which blast cells nevertheless enter (Guy-Grand *et al.* 1974; Parrott & Ferguson 1974) but maybe in an antigen-free gut there might be some altered serum albumin.

*Bienenstock:* Delphine Guy-Grand & Claude Griscelli have mice of the C3H strain which have no epithelial lymphocytes at all in their guts (C. Griscelli, personal communication 1976). These animals live a normal life, apparently, and reach normal old age. Guy-Grand & Griscelli tried to find out whether normal blasts go into this gut epithelium, doing the same experiments as Delphine Parrott described, and so far have been unable to discover what is wrong with these mice. Therein may lie a key to what is going on.

*Gowans:* Is secretory IgA produced in this strain?

*Bienenstock:* The IgA is there, but no T blasts homing to the epithelium. However, T blasts from a syngeneic animal home to the fetal gut of these animals transplanted under the kidney capsule.

*Vaerman:* Professor Parrott, was the T antigen against which the antibody was made a surface antigen? I was surprised how brightly the sections of Peyer's patches stain. Some cells appear to have their cytoplasm stained—of course, the membrane can be there too. But recalling Dr Mayrhofer's pictures of mesenteric lymph node cells at the periphery of the node, which have surface IgE labelling which distinguishes them from those in the gut epithelium, I am surprised by the intensity of the staining and its appearance, for a surface antigen in sections.

*Parrott:* I am also surprised, but there are two previous papers (Lamelin *et al.* 1972; Matter *et al.* 1972) in which the same antisera were used to identify T cells and a different antiserum for B cells.

## THE INDUCTION OF TOLERANCE TO ORALLY ADMINISTERED ANTIGENS

*André:* A study of tolerance in mice was started several years ago with

Professor J. F. Heremans and others. Mice were immunized by the intragastric administration of sheep red blood cells. The response was studied in terms of plaque-forming cells in the spleen. This response peaked at about day 9. Plaque-forming cells of the IgA class predominated over a moderate IgM cell response and a smaller number of IgG cells. In the same mice, after a second intragastric immunization of red cells three months later, the response was similar to the first response: there was no memory. This experiment was repeated with two intragastric administrations of antigen only two weeks apart. There was almost no response in the spleen (André *et al.* 1973). One explanation of this result is that an immune inhibition of absorption of the antigen by the gut occurs (André *et al.* 1974). This does happen, but it wasn't the sole explanation for this phenomenon.

We then studied the reciprocal influence of parenteral and oral immunization. Mice were immunized with sheep red cells by the intraperitoneal route and two weeks later by the intragastric route. The response in the spleen was the same as that in mice primed only intragastrically, the difference being that the base-line level of plaque-forming cells of the IgM class was raised; this was a carry-over from the intraperitoneal administration 14 days earlier. The reverse is *not* true. When mice were first immunized intragastrically and two weeks later received an intraperitoneal injection of the same antigen, they failed to respond, either at the level of plaque-forming cells in the spleen or at the level of haemolytic IgM antibody in the serum.

We then collected serum from mice that had received antigen intragastrically, two weeks previously. Serum was injected intraperitoneally into virgin mice which eight hours later received a single intraperitoneal injection of sheep red blood cells or an intragastric injection of the antigen. The priming of pretreated animals by the oral or parenteral route did not induce any antibody, although a small number of IgM plaque-forming cells were found. The same results were observed by studying IgA antibody against sheep red blood cells, and IgA plaque-forming cells. So there seems to be a factor in the serum which induces immunological tolerance. No IgM antibody against sheep red blood cells was found in the injected sera, or any IgG, and only a small amount of IgA antibody (maximum 1:8 titre).

At that time Dr P. Masson in Brussels had developed a method for detecting immune complexes, using latex particles covered with human IgG which are normally agglutinated by C1q or by rheumatoid factor. Immune complexes can be shown by inhibition of this agglutination. No evidence of immune complexes could be demonstrated in our serum with C1q but such complexes were shown using rheumatoid factor. As IgA doesn't fix C1q, we concluded that the complex involved some part of the membrane of the sheep red blood cells and IgA.

We now turned to *in vitro* experiments. Mice were immunized intraperitoneally and their spleen cells were incubated four days later with serum—either normal mouse serum, or serum from mice immunized two weeks previously with sheep red blood cells, or serum from mice immunized with a large dose of sheep red blood cells one day earlier.

In such conditions the incubation of the spleen cells in normal mouse serum doesn't modify the IgM plaque-forming cell response. Serum from mice immunized only 24 hours previously doesn't alter the normal IgM response, so we concluded that the tolerogenic factor cannot be a tolerogenic form of the antigen itself. Using serum from mice immunized two weeks previously decreased the normal number of plaque-forming cells of the IgM type.

In our latest experiments done with Dr Vaerman the tolerogenic factor in this serum was removed by immunosorbents; one of these was rheumatoid factor, which completely abolished the tolerogenic properties of the serum *in vitro*. We also used as an immunosorbent antibody against IgA. By removing all IgA from the serum, and presumably all antigen—antibody complexes with IgA, the tolerogenic *in vitro* influence of this serum was also completely abolished. This was not found if we used antibody against IgM as an immunosorbent. The tolerogenic *in vitro* influence of the serum was also abolished when we used antibody against sheep red blood cells as immunosorbent (André *et al.* 1975).

*Lachmann:* Is it correct to call this tolerance? Tolerance usually describes the failure of *induction* of cells. You are taking cells and stopping them expressing their capacity to make antibody *in vitro*.

*André:* The *in vivo* process could be different from the *in vitro* system.

*Vaerman:* The *in vitro* system could be called effector cell blocking.

*Davies:* Was there a response to the original intragastric injections of antigen in the Peyer's patches and mesenteric lymph nodes?

*André:* I haven't looked at them.

*Davies:* We did some experiments which were in essence similar (O'Toole & Davies 1971). We gave intraperitoneal or intravenous injections of sheep red blood cells to mice and at various times later tried to stimulate, by injection of the antigen in the forepaws of the mouse, the lymph nodes draining the site of injection. We found an initial non-specific failure to respond; after four days we found a specific failure to respond which lasted for 14 or 15 days, and this is similar to what you found. This phenomenon was spleen-dependent. In other words, you need an active response in the first instance. If the spleen was removed within about a day of the first intraperitoneal or intravenous injection we didn't get the blocking in these lymph nodes.

In such experiments, having not found an antibody, one must be careful to

show that there is not enough antibody to block by passive immunization. You can take hyperimmune serum which contains IgG, dilute it beyond any detectable titre, and still find an almost absolute block on the capacity of a mouse to produce anti-sheep red blood cell antibody. This possibility has to be ruled out. This particular phenomenon was subsequently studied by others who confirmed our findings but did not elucidate the mechanism. The kind of result you have shown, suggesting that a complex may be involved, is the most interesting I have seen as an explanation of that kind of observation.

*Soothill:* What happens if you go on feeding the antigen to the mice and immunize while you are still feeding the antigen?

*André:* I have done only a few experiments of this type, and there was no response at all, but I was giving a large dose of antigen. In a similar experiment by Bazin *et al.* (1970) several years ago mice were immunized with sheep red blood cells in the drinking water. They observed a plaque-forming response in the spleen and in the mesenteric lymph node. The experiment went on for about three months, and there was a progressive decay in the IgA response in the spleen.

*Pepys:* We have been repeating these experiments of Dr André with essentially the same results, although we haven't done transfer or *in vitro* studies. We have looked at numbers of rosette-forming cells (RFC) to sheep erythrocytes in the Peyer's patches and mesenteric nodes at different times after the initial oral immunization (M. B. Pepys & A. H. L. Fielder, unpublished observations). We find a brief peak of RFC which precedes the plaque-forming cell (PFC) response in the spleen, but our results in the spleen are much more variable than André's and we don't find the regular peak of PFC in the spleen at day 9.

*Lachmann:* Is there a possibility that two weeks later the cells which you would like to find in the spleen making plaques are in the lamina propria not doing anything in particular?

*Gowans:* I would like to know whether, when you challenge intraperitoneally and get no response in the spleen, you nevertheless find a response in the mediastinal lymph nodes?

*André:* I don't know. However, on intraperitoneal challenge after intragastric immunization there is no response at the plasma level. As there is no IgM antibody at the plasma level, I think there is no response in the mesenteric and mediastinal lymph nodes.

*Lachmann:* Do the mice make antibody in the intestinal secretion instead?

*André:* I haven't looked there.

*Bienenstock:* Rothberg *et al.* (1973) showed a similar situation in bovine serum albumin (BSA) feeding experiments in rabbits. If he splenectomized the animals or gave them a big dose of BSA intravenously (100 mg), he found

lymphocytes in the circulation which were proliferating in response to that particular antigen, whereas before he couldn't show that. Have you given a big dose of antigen intravenously?

*André:* No, but I have done an experiment in rats using human serum albumin (HSA) as a soluble antigen. We found exactly the same situation. An intragastric administration of 200 mg of HSA was followed by an intramuscular injection of the same antigen. Anti-HSA antibody in sera was assayed by its antigen-binding capacity. By comparison with control animals there was a big reduction in the anti-HSA antibody titres of rats immunized by both routes.

*Pierce:* Are you referring to the local response?

*André:* In this experiment there was a local response dependent on IgA. In the serum there was antibody against HSA of IgA and IgM class. In this system, antigen–antibody complexes probably involved both kinds of antibody, because both our systems, based, on C1q and on rheumatoid factor, were inhibited.

*Pierce:* Did you fail to see a local response after the subsequent parenteral challenge?

*André:* There was a local response after oral immunization. I haven't looked for a local response after the parenteral challenge.

*Pierce:* The experiment is similar to one we have done with cholera toxoid in rats in which oral priming followed by intraperitoneal boosting does produce a local response (Pierce & Gowans 1975). If the systemic immune response to parenteral antigen is being modified by prior oral exposure, it may be that the gut immune response to the parenteral booster remains intact.

*Parrott:* In experiments with Howard Thomas we fed BSA to rats in large amounts (Thomas & Parrott 1974). We got a transitory, small amount of serum antibody to BSA but couldn't find any local production—that is, specific antibody-forming cells in the lamina propria or in the lumen of the gut—and neither did they respond to challenge with BSA in Freund's adjuvant given intramuscularly.

*Pierce:* Did you try a later oral challenge? Under these circumstances we have seen what appears to be inefficient oral priming but much more efficient oral boosting (Pierce & Gowans 1975).

*Parrott:* No, we didn't challenge orally. But normally you get a nice response to BSA in Freund's adjuvant given intramuscularly.

*Lachmann:* BSA is a very different antigen from cholera toxoid. When used in monomeric form at appropriate concentrations it induces tolerance so that one cannot get a subsequent response even to BSA in complete Freund's adjuvant (see Dresser & Mitchison 1968).

*Parrott:* We looked at the state in the serum of the small amount of BSA that had been absorbed. It wasn't the tolerogenic form of BSA. It was native BSA.

*Lachmann:* Were you getting the phenomenon that the antigen which comes through the liver is monomeric and tolerogenic?

*Parrott:* We thought we would get that, but we showed that we didn't.

*Brandtzaeg:* I am curious about your test system, Dr André. Are you suggesting that human rheumatoid factor has affinity for rat IgA and can be used to remove the IgA?

*André:* Yes. There is no animal species specificity, as P. Masson showed.

## IMMUNIZATION WITH ESCHERICHIA COLI HYBRIDS

*Schmidt:* I would like to describe briefly our attempts to immunize mice orally against salmonellosis with *Escherichia coli* hybrids. It is well known that living cells or live vaccines immunize more efficiently against salmonellosis than do killed cells. For oral vaccination we used a live vaccine consisting of avirulent *E. coli* hybrids which express the O antigens of *Salmonella typhimurium* or *Salmonella typhi.* (We introduced the genetic information for O antigen synthesis from a *Salmonella* Hfr donor into *E. coli* by conjugation. The *E. coli* recipient was a non-enteropathogenic strain isolated from human faeces.) The oral vaccination of adult mice with hybrids resulted in a rather low protection rate against *Salmonella* infections. We found that in adult mice the hybrids were eliminated from the intestine within two days. This may explain the poor efficiency of the vaccine.

In other experiments we vaccinated 4–5-day-old baby mice orally with a drop (containing about $10^8$ cells) of hybrid suspension. We found that the hybrids colonized the intestine and persisted for 14 days and in some cases longer. When we challenged these mice four weeks after immunization they were well protected.

To achieve protection of adult mice it appears necessary to start hybridization with *E. coli* strains which can colonize the adult mouse intestine. Experiments of that kind are in progress.

*Soothill:* Was the protection against oral infection by salmonella organisms, or did you also get protection against parenteral administration of salmonella?

*Schmidt:* Protection was measured by the rate of elimination of challenge bacteria from liver and spleen. As compared to untreated mice the numbers of challenge bacteria recovered from the organs were also very low in mice infected intraperitoneally.

*White:* Are there any factors such as the adherence of these *E. coli* organisms to the gut wall which are responsible for their success?

*Schmidt:* This is what we have to test now.

*Cebra:* It would certainly be advantageous to isolate an *E. coli* from the mouse for your immunization studies. Although you did succeed in colonizing the neonates, and one presumes that the bacterial organisms used were adherent to cell surfaces, a naturally occurring organism would more surely have been selected for adherence and successful colonization. Have you tried to clean up the gut of the adult mice before you try to colonize them with the hybrid by using antibiotics, in order to cut down competition from the resident bacterial flora?

*Schmidt:* We haven't done this.

*Mayrhofer:* If you used native *E. coli*, not hybridized, did it colonize?

*Schmidt:* The native *E. coli* colonized the intestine of very young mice, but not that of adult mice.

*Pierce:* It would be important to know whether the colonization you achieved in infant mice was in the small bowel or colon. If it were in the colon you might have induced local colonic protection and perhaps even systemic immunity without neccessarily protecting the small bowel. This could be very important in your attempts to immunize orally with a living hybrid strain, since the major site at which *S. typhimurium* invades the gut is probably the distal ileum (Carter & Collins 1974). Your measure of protection was the duration of survival of the challenge strain in liver and spleen, so systemic invasion had obviously taken place. If you had achieved small bowel protection by oral immunization, you might have prevented invasion by the challenge strain altogether.

*Schmidt:* It may be that in oral vaccination of baby mice the hybrids penetrate the epithelial cells of the intestine and spread through the whole organism, thus evoking systemic immunity. Whether intestinal immunity is also achieved remains to be tested.

*Lachmann:* Have you looked to see if you get anti-salmonella antibodies in the milk of the mice infected with the hybrid organism?

*Schmidt:* No.

*Lachmann:* One can see fascinating possibilities if you could parasitize a cow with hybrids of the enteric organisms of man and could use the milk to give passive immunity in humans.

## ANTIGEN ENTRY IN GUT

*Lachmann:* The question arises of how much antigen normally goes across the bowel mucosa. We have been told on the one hand that all orally given

antigen is digested; on the other hand, various contributors to the symposium have given us to understand that orally administered material, whether sheep red cells, bacteria or food, is adequately immunogenic and presumably does cross the mucosa. Can a quantitative statement be made about what macromolecular material, and where, goes across the gut?

*Gowans:* Can we add to your questions the possibility that, in pathological conditions, defects of digestion may lead to the chronic exposure of the gut to immunogenic residues?

*Bienenstock:* Rothberg showed that if rabbits were given 0.1 % BSA in the drinking water it did not produce systemic immunization or systemic antibody formation and the level of circulating antigen did not rise above 0.06 μg protein N per ml (Rothberg *et al.* 1970). These levels in the circulation were not immunogenic, as judged by subsequent attempts at parenteral immunization with those amounts. However, those quantities were never systemically infused.

*Porter:* When we were developing oral vaccination in pigs with *E. coli* we used heat-stripped bacteria which yield endotoxin. We measured the endotoxin in terms of haemagglutination inhibition, but we did a dilution study in which we fed 1 ml doses containing $10^{10}$–$10^{6}$ organisms. There was a definite threshold at about $10^{6}$ or $10^{7}$ for a local immune response. In none of those was there evidence of a systemic stimulus in terms of circulating antibody.

*Gowans:* The experiments I am thinking of are not studies on systemic antibody responses but measurements of the transport of immunogenic proteins from the gut into the intestinal lymph.

*Bienenstock:* Rothberg has looked at the molecular size of BSA in the circulation after oral feeding. Much of what goes across into the circulation is normal intact BSA; about 0.01 % of the administered dose gets across and a considerable portion of that is native BSA.

*Brandtzaeg:* We have an *in vitro* model in which the rabbit colon (the whole gut wall) is used as the membrane in a diffusion chamber (K. Tolo & P. Brandtzaeg, unpublished). After two hours at room temperature we recover in the order of 0.1 % of the antigenic material (HSA) applied on the epithelial side.

*Pierce:* One has to be cautious when interpreting results from a model like that, because the slightest handling of gut considerably alters the diffusion of macromolecules across the mucosa.

*Brandtzaeg:* Yes. There is a considerable difference between various membranes and various animals and *in vivo* the penetration through the gut epithelium would probably be much less than 0.1 %.

*Lachmann:* Is it clear that the amount absorbed is a percentage of the amount given rather than a function of the time available for absorption? Does the

amount absorbed show a dose–response relationship, with the amount present on the epithelial side and, if so, does the curve flatten out at higher concentrations?

*Brandtzaeg:* I don't know, but at the concentration we have used (30 mg/ml) the serosal concentration after two hours has been in the order of 0.1%.

*Soothill:* Lippard *et al.* (1936) observed the complement-fixing bovine milk antigen and complement-fixing anti-milk antibodies in normal children when they were weaned, at whatever age. First he detected the antigen and then antibody, after conventional feeding of normal children. I know of no faults in that study. The more recent work includes studies of inverted gut sacs (Walker *et al.* 1972), and the work of Heremans' group on antigen in mesenteric blood after feeding. Immunodeficient children, particularly those with primary immunodeficiency (Buckley & Dees 1969) and those with secondary immunodeficiency due to severe malnutrition, have a high incidence of anti-food antibodies in their blood, so they appear to have been sensitized (see Chandra 1976).

*Lachmann:* The formation of anti-milk antibodies in children is likely to be due at least in part to regurgitation of milk into the lung, where it should be fully immunogenic.

*Pepys:* Gruskay & Cooke (1955) gave infants one gram of ovalbumin per kilogram body weight as a 10% solution by gavage after 4–8 hours fasting. Venous blood was drawn one and two hours later and ovalbumin in the serum was measured by a quantitative precipitation technique. The normal absorption was found to be 0.02% of the ingested dose, whilst in children with various sorts of diarrhoea this increased to 0.1%.

*White:* Delphine Parrott mentioned the idea that antigen goes to and through the domes of the Peyer's patches (p. 347). We attempted to put antigens, not over Peyer's patches, but over the follicles of the caecal tonsils in the chicken, one caecum being used as the control of the other. We failed with several antigens to find penetration of antigen into these follicles, but the follicles have a beautiful dendritic network. This is shown not by the pattern of the antigen, which we couldn't demonstrate, but by using a fluorescent conjugate against 7S chicken immunoglobulin. This demonstrates a pattern very similar to what you see in Malpighian bodies in the spleen: that is, antigen–antibody complexes localized to the surface of dendritic cells scattered uniformly throughout the germinal centre.

*Bienenstock:* The chicken caecal tonsil is not the same as a mammalian Peyer's patch. Synthetic studies using radio-labelled amino acid precursors show that chicken caecal tonsils are characterized more by the production of IgG than IgA, whereas the bursa produces mainly IgM (Bienenstock *et al.* 1973).

It looks more like peripheral lymphoid tissue than would appear from its position in the bowel.

*Gowans:* Professor R. R. Wagner and I have recently examined the ability of viable bacteriophage R17 to pass from the intestinal lumen into the thoracic duct lymph. Since this phage is easy to assay by conventional plaquing techniques we could obtain quantitative estimates of the amount of 'undegraded' material which passed across the normal intestinal epithelium into the draining lymph. Phage was injected into the lumen of the duodenum of rats in which a thoracic duct cannula had been inserted on the previous day and, in a number of experiments, 0.03–0.1 % of the administered PFU were recovered in the lymph; most was recovered in the first hour. We do not know whether the phage penetrated the Peyer's patches or passed through the epithelium elsewhere. The total recovery was small but the experiments show that the small gut and the draining mesenteric nodes could not prevent some living virus from entering the efferent lymph and thus the blood. Whether the penetration of the gut by microorganisms and macromolecules occurs normally and on a scale which is potentially hazardous to the animal remains to be established.

*Porter:* The quantity is a question of the initial load. If you had fed only 0.1 % of what you in fact fed, would any have got across?

*Gowans:* I don't know. We certainly gave huge quantities of phage (about $10^{11}$ PFU) and the assay is very sensitive so the physiological significance of these observations is unclear. At the moment we are simply interested in finding out whether the phage passes through the Peyer's patches or elsewhere; our methods should enable us to do this.

*Lachmann:* If you think of eating a 12 oz steak, 0.01 % is about 34 mg, which could be a substantial dose of antigen!

*Gowans:* Presumably information on this point can be obtained by feeding radiolabelled antigens and assaying in the intestinal lymph. On the other hand, I don't know the extent to which fragments large enough to be immunogenic would be absorbed into the portal blood.

*Soothill:* There is the problem of digestion, leading to detection of the label on small peptides, and of free label sticking to endogenous proteins.

*André:* We have now measured the total human albumin transmitted by the gut in the rat (André *et al.* 1974). In this system we had evidence that local immunization of the gut with human albumin impairs its capacity to absorb the corresponding antigen and that immune intestinal secretion also blocks antigen absorption in non-immune rats. Quantitative data were obtained by Warshaw *et al.* (1971). They observed that around 0.01 % of the administered dose of horseradish peroxidase was transmitted into mesenteric lymph and portal blood of rats. But the same group (Warshaw *et al.* 1974) also observed

that a larger percentage of the administered dose of bovine albumin was absorbed.

*Lachmann:* Do you find whole proteins in venous blood?

*André:* Yes.

*Ferguson:* Warshaw *et al.* (1974) infused $^{3}$H-labelled BSA into the duodenum of rats and found that 2% was absorbed as intact BSA molecules. In other experiments they showed that 0.8% was transported away from the gut via the lymphatics and 1.1% via the portal vein (Warshaw & Walker 1974).

*Booth:* The liver also affects the amount absorbed; there is a difference in the antibody titres to dietary antigens in patients who have a portocaval shunt and those who haven't, suggesting a hepatic sieving of whatever goes up the portal vein. This is well-known for bacteria.

*Brandtzaeg:* In the *in vitro* system that I referred to (p. 357), the penetrability of the large bowel wall in the rabbit is at least 20 times greater than that of the sublingual oral mucosa. This shows the difference between the various mucous membranes.

*Lachmann:* This suggests that if people don't digest their food they might absorb more of it further down the gut!

*Booth:* Insulin given by mouth is absorbed.

## THE INTESTINAL BACTERIAL LOAD

*Booth:* The gut is far from sterile. The proximal intestine has broadly speaking an aerobic flora which reaches concentrations of $10^3$ to $10^5$ organisms per ml. The ileum has something between the jejunum and the colon, and you begin to find an anaerobic flora of various types in the ileum. In the colon the flora is predominantly anaerobic when grown under proper conditions. *E. coli* is less important here. But the flora is there, and the infection following perforation of the duodenum shows that very clearly.

*Pierce:* It is important to recognize that most of the laboratory animals we work with are quite different from humans. Mice and rats, for example, have a highly developed complex microflora with much larger numbers of bacteria in the small intestine than do humans. This is an important difference when one is considering the antigenic load or the microbiological environment of the small bowel in man, as compared with laboratory animals.

*Booth:* The so-called normal subjects studied in Calcutta had loads much more like those of experimental animals, in the 'normal' intestine. Western Europeans seem to have greater loads than patients in the USA. One assumes that the more extensive packaging of food in the USA is responsible for that difference.

*Gowans:* It is interesting that the bacterial load in the various parts of the intestine is not apparently reflected in the local density of IgA-secreting cells in the lamina propria: the density in the large and small gut of rats looks to me to be about the same.

*Booth:* In patients with huge bacterial loads due to jejunal diverticulosis, there is no evidence of an increase in IgA cells.

*Porter:* In the pig there is almost a reciprocal arrangement: the bacterial load increases towards the posterior region of the small intestine and the IgA and IgM cell population increases towards the anterior (see earlier discussion, p. 44).

*Pierce:* There are certainly non-immunological mechanisms which contribute to keeping much of the small bowel 'clean'. These include the 'acid barrier' of the stomach and the continuous distal propulsion of the gut contents. Their importance can be seen in the terminal ileum where stasis apparently contributes to the development of a colon-like microflora, even though the local immune system appears similar to that of the proximal small bowel. It should also be apparent that the 'clean' proximal bowel is repeatedly exposed to a wide variety of microbial and dietary antigens, sufficient to account for a vigorous local immune response even though persistent and heavy bacterial colonization is not seen.

*White:* Of course, measured loads refer to the content of the bowel and don't take account of bacteria sticking to the wall.

*Mayrhofer:* In small rodents, the mucus layer of the small intestine contains large numbers of bacteria and these are not necessarily of the same species as those that can be isolated from the luminal contents (Savage *et al.* 1968).

## GUT NOT A PRIMARY LYMPHOID ORGAN

*Lachmann:* One other topic is the possible role of the gut, particularly in the mammal, as a primary lymphoid organ. It hasn't been mentioned so far: is that because there is a general consensus that the idea is exploded?

*Gowans:* The evidence on this point can be found in the excellent Ciba Foundation Symposium on the Ontogeny of Acquired Immunity (1972). My own view is that the evidence for gut-associated lymphoid tissue having a role analogous to that of the bursa in birds is very weak. In any event we now have excellent experimental support for a special function for the Peyer's patches: to fire off cells into the intestinal lymph which migrate into the lamina propria and synthesize IgA.

*Lachmann:* Further, Max Cooper and John Owen have now shown that fetal liver is the source of B cells in mammals (see Owen 1972).

## FUTURE WORK

*Lachmann:* The clinicians here are surrounded by many members of the clone of immunologists interested in the secretory immune system. What models of investigation would they (the clinicians) like to suggest that could give more information about the problems exercising them? On the other hand, one could ask the immunologists to suggest appropriate investigations to apply to the clinical problems, ethical considerations being taken into account. Has any line of work become apparent during the symposium which could throw light on the problems of inflammatory bowel disease and of coeliac disease, or establish their immunological origin?

*Gowans:* And can you produce in experimental animals diseases whose pathology mimics the pictures seen in Crohn's disease and ulcerative colitis?

*Ferguson:* Many acute infectious diarrhoeas produce changes in the rectal mucosa which are virtually indistinguishable from the appearances of colitis. Also, D. B. L. McClelland & H. Gilmour (personal communication) have seen granulomata in the rectal mucosa in patients with salmonella gastroenteritis. If the mechanisms of tissue damage in infectious diseases are elucidated, this may throw some light on the idiopathic inflammatory bowel diseases such as ulcerative colitis and Crohn's disease.

*White:* Do you mean that a fully blown epithelioid cell granuloma with giant cells is found in someone who has had salmonellosis?

*Ferguson:* Yes: classical granulomas (with giant cells) are found in rectal biopsies from patients admitted to hospital merely for the clinical management of diarrhoea associated with proven enteropathogenic infection. These granulomas appear to be due to the infective process, although these patients have not yet been studied for long enough to exclude completely the possibility of underlying Crohn's disease.

*Parrott:* I am interested in investigating the consequences of the switching or diversion of cells destined for some experimentally induced lesion to the gut. Can Professor Booth tell me whether, when other lesions arise in association with ulcerative colitis—iritis, joint lesions and so on—the colitis gets better or worse, or are they in parallel?

*Booth:* They can be completed unrelated.

*Parrott:* And can you give me any leads with the atrophic spleen?

*Booth:* The spleen is not usually atrophic in children in coeliac disease; this occurs in about 20% of adult patients, if you measure spleen size using chromium-labelled heat-denatured red cells and scanning.

*Parrott:* Are the other lesions worse in patients with atrophic spleens?

*Booth:* I can't answer that.

*Rosen:* I would like to go back to a point of departure which had to do with IgA-producing cells. We didn't discuss the fact that if you look at a population of IgA-deficient children, they are at risk for a list of diseases that is too long to enumerate. The best studied are lupus erythematosus and rheumatoid arthritis. We have much information on the homing of IgA cells but we need more information on what they are doing physiologically, in terms of what their cell products are doing to enhance the barrier function of the gut.

*Lachmann:* Although these children are IgA deficient, we don't know what their primary defect is. It may lie on a biochemical pathway that has additional manifestations in functions other than IgA synthesis. There is no evidence that they have a defect in the structural gene for the $\alpha$ chain.

*Soothill:* There is good evidence that they haven't, since they have IgA on the surface of their B lymphocytes.

* * *

*Lachmann:* This is probably a good point at which to draw the discussion to a close, having got back to where we started from! We have studied three fairly distinct types of problem, starting with the physiology of the immune system as it affects the gut, mainly from the secretory immunoglobulin point of view but also from that of cell-mediated immunity; we have discussed infectious organisms, both bacteria and nematodes; and we have considered the immunopathology of inflammatory bowel disease and some other conditions. A great deal of information has been put about. Nevertheless, it is clear that answers to the immunological problems of gut disease are not yet forthcoming. We don't yet understand the aetiology or even in any detail the pathogenetic mechanisms in inflammatory bowel disease. Perhaps by the time the Ciba Foundation explores this topic again, this will all have changed.

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