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Volume 310
IMMUNOLOGY OF MILK AND THE NEONATE
Edited by Jiri Mestecky, Claudia Blair, and Pearay L. Ogra

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PREFACE

In the course of history, humans have attempted to interrupt the physiological and psychological bond formed between a nursing mother and her child by substituting breastfeeding with artificial formulas. A growing body of evidence indicates that breast milk, quite apart from its unsurpassed nutritive value, contains a large number of substances that protect the offspring from common infectious agents and allergens and promote the maturation of the gastrointestinal tract and the immune system. In addition to well described milk antibodies and soluble mediators of innate immunity, milk cells and pluripotent secreted factors – cytokines – are currently in the forefront of extensive research with respect to their importance in milk immunology. The purpose of this conference was to critically evaluate the current state of our knowledge concerning the protective role of immune agents found in milk, to provide up-to-date information of milk factors with respect to their role in the maturation of immunological defense systems in the neonate, and to reassess the importance of breastfeeding in the prevention of allergies in formula-fed infants.

We hope that the work presented by international participants will prompt many new ideas and stimulate further research in this important area.

This conference was sponsored primarily by the National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD. We would like to thank Drs. Sumner Yaffe and Delbert Dayton for their efforts with the organization, planning, and support of this conference. Furthermore, we would like to acknowledge the Carnation Cooperation, Procter & Gamble, Bristol-Myers, Ross Laboratories, and Stolle Milk Biologics for their financial support.

We would like to thank Ms. Arline Sanchez for her competent attention to many of the details that made it possible to bring this group of researchers together.

The organization of this meeting and publication of this book would not have been possible without the expert skills and dedication of Ms. Maria Bethune.

J. Mestecky
C. Blair
P.L. Ogra

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Lars Å. Hanson and Fehmida Jalil in collaboration with R. Ashraf, S. Bernini, B. Carlsson, J.R. Cruz, T. González, M. Hahn-Zoric, L. Mellander, Y. Minoli, G. Moro, F. Nave, S. Zaman, L. Mata, J. Karlberg and B.S. Lindblad

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INTRODUCTION

Most of us would believe that it is only in modern times that not all infants are exclusively breast-fed. However, even in ancient India and Europe[1] people were taught not to initiate breastfeeding at once, but to give other fluids and other materials, such as honey, that were likely to be contaminated. This is the custom still today in many traditional societies[2,3]. After breastfeeding has been started it is often incomplete ("partial breast-feeding"), but even then it may go on through the second year of life. There is information available that during previous centuries there was no breastfeeding at all in certain areas of Germany and Sweden. Infant mortality from diarrhea during the warm summer months in the early part of the 19th century was higher in such areas in northern Sweden than in adjacent regions where breastfeeding was the rule[4]. This presumably constitutes the first scientific evidence that breastfeeding can protect against infections.

Many studies claiming to demonstrate that breastfeeding protects against infection have been criticized for design and interference of confounding factors. However, other well controlled studies have been published which show protection from diarrheal disease[5,6,7,8,9], respiratory tract infections[8,9], and otitis media[11,12].

Many studies have demonstrated that most mothers in developing countries are breastfeeding, but little emphasis has been placed on determining the rate of exclusive breastfeeding, which is quite rare, with

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partial breastfeeding dominating in many developing areas[2,13]. Feachem and Koblinsky[5] noted that there were striking differences in the diarrhea morbidity and mortality in relation to the type of feeding. Morbidity and mortality were lowest among those infants who were exclusively breast-fed, higher among those partially breast-fed, and highest among those not breast-fed at all.

BREASTFEEDING AND NEONATAL SEPSIS

The effect of breastfeeding on the occurrence of neonatal sepsis was recently studied in Lahore, Pakistan[14]. For each 42 consecutive cases of neonatal sepsis (age 3-28 days), 8 age-matched controls were sought from the same socio-economic circumstances, living in the same part of the city. We managed to locate between 4 and 8, or as a mean 6.4, such controls per case and thus the statistical analysis had to be made for an unmatched case-control study. Only one of the controls was exclusively breast-fed and the majority was partially breast-fed. However, many more cases than controls were not breast-fed. An odds ratio of 18 was found, strongly supporting the capacity even of partial breastfeeding to protect against neonatal sepsis in this poor community ($p<0.001$). Intense microbial exposure of infants in this community directly from birth onwards results in a gut flora with several potential pathogens[13]. These can be kept within the gut by partial breastfeeding once it starts 1-3 days after delivery[2].

Confounding factors may easily confuse the outcome of investigations of this kind but in the present study of neonatal sepsis the cases and controls were comparable as to age, sex, birth order, place and mode of delivery, hygiene of birth attendant, time between rupture of membranes and delivery, instruments used for delivery, and care of the cord, etc.[13].

THE MODE OF FEEDING IN A TYPICAL DEVELOPING AREA

In preliminary analyses of poor populations in Lahore, Pakistan, it became obvious that to properly describe the extent of breastfeeding it was necessary to take in regard a number of confounding factors[14]. Thus, the season of the year, the population group and the area of living were important. In a new prospective study, we have followed longitudinally 1,476 infants born into a population of 4,000 families, 1,000 from each of four groups living in different areas, a village outside Lahore, a mud hut area and the old city slum of Lahore, with an upper middle class group for comparison[16]. It was found that exclusive breastfeeding was rare, occurring initially only among 18% of the mothers in the village and 10% in the mud hut area after 1 month of lactation. Partial breastfeeding was predominant among the three poor groups whereas artificial feeding was most common in the upper middle class group. This was also the only group where commercial formulas were used to any major extent. Again, we noted the effect of the hot season. Mothers believe that their infants need extra fluid then. This can be seen as more children getting extra fluid during the months of April-September. In parallel, fewer get breast milk, presumably because they suck less, being less

thirsty given the extra fluid. These fluids may often be contaminated adding to the risk of gastrointestinal infections that are more common during the hot season.

Since exclusive breastfeeding was so rare, we decided to try to inform 300 mothers about the advantages of exclusive breastfeeding, showing, e.g., an educational puppet show on video in their home, together with oral information, using a flip chart, booklets and posters at altogether three visits during pregnancy[17]. The team from the project, together with the local traditional birth attendant who had also been specially trained, visited the mother regularly from delivery on for follow up.

The motivation was quite successful, e.g., increasing the rate of exclusive breastfeeding at 1 month after delivery about 40%, both in the village and in the urban slum, compared to the prospective longitudinal study where no special motivation was applied. Actually, the rate of partial breastfeeding also increased by about 40% at 1 month after delivery. The onset of breastfeeding was influenced as well, so that the percentage of neonates starting already at 24 hours of age had increased from 5 to 90% in the village and from about 15% to around 75% in the urban slum.

THE MODE OF FEEDING IN RELATION TO INFECTIONS IN THE LAHORE STUDY

In the longitudinal follow up of the 1,476 children in the four population groups, it was quite clear that breastfeeding protected against diarrheal disease[18]. This was obvious for all the population groups in the ages 1-23 months. Breastfeeding is here defined as partial or exclusive breastfeeding, the latter constituting a small group as mentioned above.

The degree of protection was evaluated by comparison of the prevalence of diarrhea among the breast-fed and the non-breast-fed. As can be seen in Fig. 1, the efficacy of protection was between 60-80% in the three poor groups and close to 40% in the control group among the youngest infants. Protection decreased after 9-12 months, remaining at 10-25% even at 24 months of age. The protection was seen year around both for the youngest (1-3 months) and the oldest age group tested (19-21 months) (Figs. 2 and 3). This is of special interest since during the warm months of April-September diarrhea is much more common and the infectious doses may also be higher. Still, breastfeeding, mainly partial breastfeeding, seems to protect just as well during the diarrhea season.

To try to determine if fluctuations unrelated to the termination of breastfeeding could have influenced the results, they were analyzed by comparing the rate of infections at monthly intervals 1, 2, and 3 months prior to and 1, 2, and 3 months after the termination of breastfeeding. Diarrhea became more prevalent after breastfeeding was stopped in all the population groups including the controls (Fig. 4), in the warm as well as the temperate season and in the different age groups. Such a change was not seen for upper and lower respiratory tract infections, but further analyses are required for these infections.

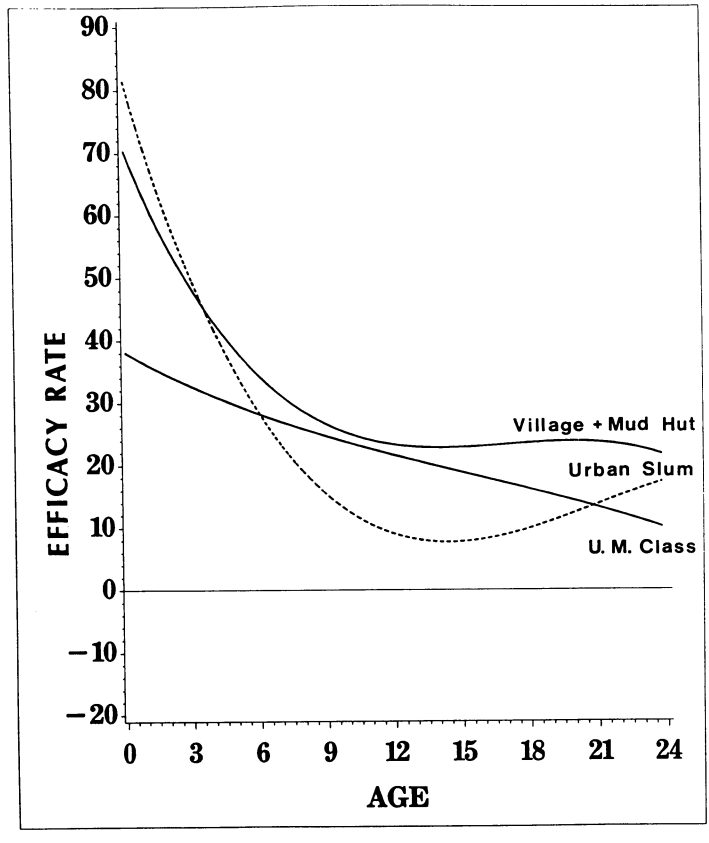

Figure 1. Efficacy of the protection of breastfeeding against diarrhea in three study areas. Efficacy, (E) is computed as the ratio between the incidence rate of diarrhea in the breast-fed infants (^{r}B) and in the non-breast-fed infants (^{r}C); $E = (1-{}^{r}B/{}^{r}C) \times 100$. The underlying observed values have been smoothed by a third degree polynomial function. Number of children for each area and age group is on the average 350 (range 23-935).

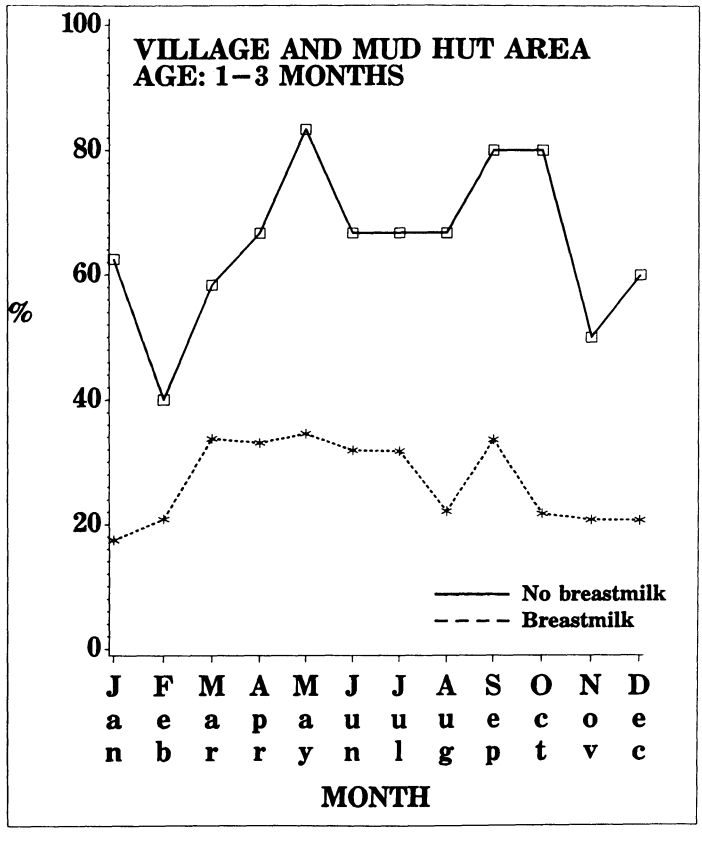

Figure 2. Incidence rate of diarrhea in the village and mud hut area analyzed per month of the year in the breast-fed and non-breast-fed infants, in the intervals 0-1, 1-2, and 2-3 months of age. About 40 observations/dot, range 6-195. April-June is the hot and dry season, July-September the hot and wet season.

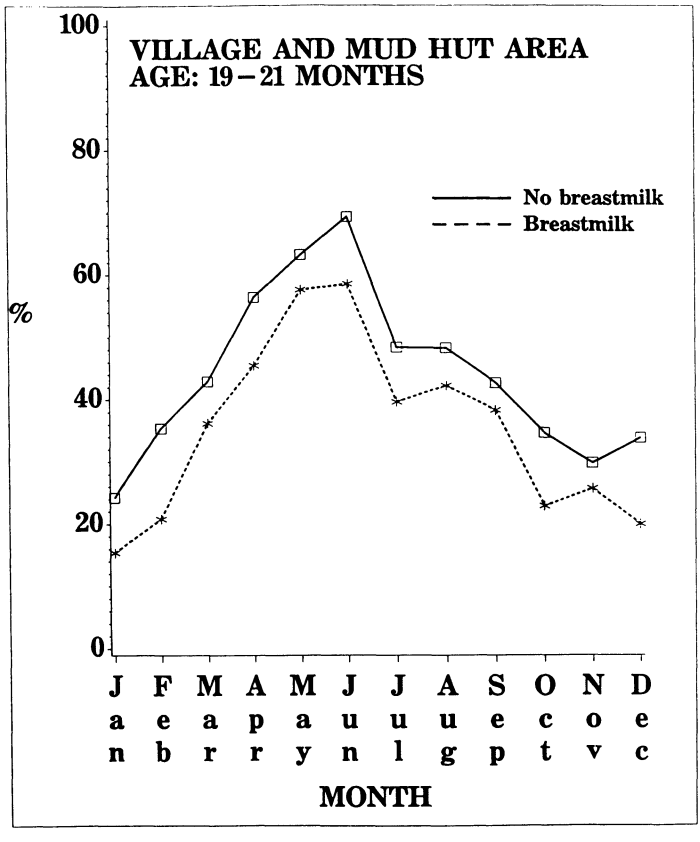

Figure 3. Same as Fig. 2 but for the age range 18-19, 19-20, and 20-21 months. Around 60-70 observations/dot, range 15-93.

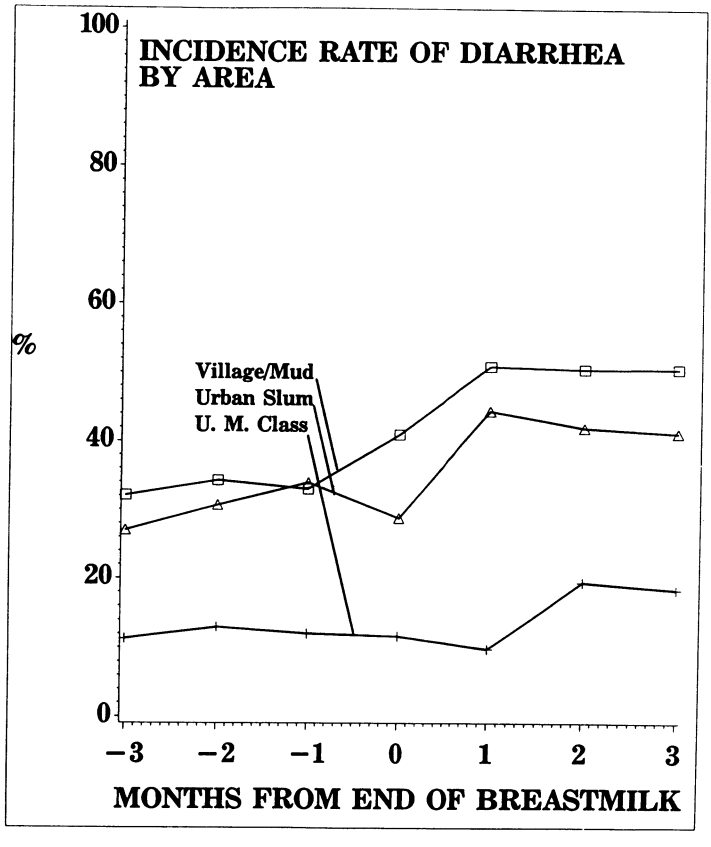

Figure 4. Diarrhea incidence rate by area of living, grouped monthly in relation to the time of termination of breastfeeding. Ages 1-24 months included. 300 observations/dot on the average, range 71-533.

CHARACTERISTICS OF HUMAN MILK ANTIBODIES

Few studies of the protective role of human milk have been able to define the important host defense component among the many present in the milk. Glass *et al.*[19] demonstrated that protection against cholera in breast-fed infants is related to the milk secretory IgA (S-IgA) antibody titres against the *Vibrio cholerae* enterotoxin and lipopolysaccharide. More recently, Cruz *et al.*[9,20] found evidence for the role of milk S-IgA antibodies in the protection against ETEC in breast-fed infants.

These findings illustrate the importance of the predominant antibody in milk and all other exocrine secretions, S-IgA, which was actually first found in and isolated from human milk[21]. The appearance of milk S-IgA antibodies after intestinal antigenic exposure was an early example, both in experimental animals[22] and man[23] of the homing mechanism of Peyer's patch lymphocytes to peripheral sites within the mucosal immune system.

Some abberations have been noticed, however, in the expected titer increase of human milk S-IgA antibodies after intestinal exposure. We have seen that whereas parenteral vaccination may boost milk IgA antibodies[24], peroral vaccines may have the reverse effect[25,26]. At this conference similar findings in lactating mothers with intestinal infections have been presented by Cruz *et al.*, adding to his earlier observation that a perorally given food protein may decrease pre-exposure milk antibody titers[27]. These findings suggest that "oral tolerance" as seen in experimental animals may not be a consistent phenomenon in man, especially since decrease in milk S-IgA antibodies after oral exposure was usually accompanied by serum antibody increase.

We have tried to use avidities, or the relative affinity index, of human milk antibodies to gain more information about the milk S-IgA response. Avidities were determined by elution of the milk antibodies in an enzyme-linked immunosorbent assay (ELISA) by different molarities of KSCN, determining the molarity which eluted 50% of the antibodies[28]. It was noted that Swedish mothers had significantly higher relative affinity index of milk S-IgA antibodies to *E. coli* O antigen and diphtheria toxoid than Pakistani mothers[28]. The reason for this is not known, but one possible explanation could be poorer nutritional status of the Pakistani mothers. In a recent study, however, we followed the titres and avidities of S-IgA antibodies in the milk of undernourished Guatemalan mothers before and after a food supplementation of 440 kcal/day for 3 months. No increases in titres or avidities were seen, suggesting that the differences noted between the Swedish and Pakistani mothers might be due to causes other than the nutritional status.

Surprisingly, it was found that vaccination of lactating mothers in Pakistan with a whole cell cholera vaccine increased titres, but not avidities of the milk S-IgA antibodies against *V. cholerae* endotoxin[29]. This may suggest that the milk antibody avidities were already high, unable to increase any further on immunization. It might be a sign that many of the milk antibodies are the result of mature immune responses originating from memory cells migrating into the lactating mammary gland. Accordingly, this would also explain how the milk at one time can contain antibodies against so many bacterial species and serotypes, many more than the mother can have met recently.

Table 1. Antibody Levels and Avidities of Milk IgA Antibodies to *E. coli* 06 Antigen in Costa Rican and Swedish Mothers During Lactation. Antibody Levels were Expressed as % of a Reference and Avidity as the Molarity of KSCN Eluting 50% of Antibodies

	Mothers	Colostrum	3-12 Months milk
Antibody levels median (range)	Costa Rican n=17	80[a] (20-165)	27 (6-148)
	Swedish n=10	72[a] (27-133)	41 (20-161)
Avidities median (range)	Costa Rican n=17	1.75[b,c] (0.48-2.90)	1.3 (0.15-2.8)
	Swedish n=10	2.5[c] (1.1-3.05)	1.45 (0.9-1.95)

[a] Colostrum significantly higher ($p<0.001$) than 3-12 months milk

[b] Costa Rican mothers significantly lower than Swedish ($p<0.043$)

[c] Avidities significantly higher in colostrum than in mature milk, Costa Rican mothers $p<0.001$, Swedish mothers $p<0.0001$

High avidities were also found by Sennhauser *et al.*[30] for S-IgA antibodies to *E. coli* in milk from mothers of premature babies. Presumably, these antibodies are also not part of primary responses.

In a recent study, we compared the antibody levels and avidities of milk S-IgA antibodies to soybean protein in Japanese and Indian mothers[31]. The Japanese mothers eat much more soybean protein than the Indian mothers who were well nourished. Still, the Indian mothers had significantly higher titres ($p<0.01$) and lower avidities ($p<0.01$) of their milk IgA antibodies to the 7S soybean protein than the Japanese mothers. Obviously, we do not yet fully understand the development of avidity in mucosal immune response; further studies in experimental animals might be most helpful.

Recent analyses of milk samples from normally nourished Costa Rican women showed the same decrease from colostrum to mature milk in IgA antibody levels against *E. coli* 06 as Swedish mothers (Table 1) and as has been seen previously. However, the avidities against this single O antigen decreased significantly as well, both for the Swedish and the Costa Rican mothers. The avidities in the colostrum samples of the Costa Rican mothers were significantly lower than those of the Swedish mothers (Table 1). This is presumably not due to differences in nutritional condition. Neither are differences in microbial exposure or racial background obvious explanations.

BREASTFEEDING MAY ENHANCE VACCINE RESPONSES IN THE OFFSPRING

In a recent preliminary study, we compared the serum and secretory antibody responses to oral poliovirus and parenteral diphtheria and tetanus toxoids in breast-fed infants and infants on either of two isocaloric diets, one high (1.5 g/100 ml) and one low (1.1 g/100ml) in protein[31]. The S-IgA antibodies in saliva against the three vaccines were significantly higher in the breast-fed group than in the two formula-fed groups that did not differ and were therefore combined (Table 2). The fecal IgM antibodies to the tetanus and poliovirus vaccines were also significantly higher in the breast-fed infants, as were the S-IgA stool antibodies. The latter, but not the former, could have come from the mother's milk. The serum antibody responses in the breast-fed and the combined formula group did not differ significantly after the first two doses. However, 21-40 months later the breast-fed group had significantly higher IgG titres against diphtheria toxin and neutralizing antibodies against poliovirus, than the combined formula group (Table 2).

Table 2. Significantly Higher Antibody Level Increases in Breast- Compared to Formula-Fed Infants Against Oral Poliovirus and Parenteral Tetanus and Diphtheria Vaccines

Antibodies	Age (months)	After vaccine dose no	p-value
Salivary IgA			
against tetanus toxoid	4	2	<0.01
diphtheria toxoid	4	2	<0.01
poliovirus	4	2	<0.05
Fecal IgM			
against tetanus toxoid	3 and 4	1 and 2	<0.05 and <0.05
diphtheria toxoid	3 and 4	1 and 2	not sign.
poliovirus	3 and 4	1 and 2	<0.01 and <0.05
Serum IgG			
against tetanus toxoid	21-40	3	not sign.
diphtheria toxoid	21-40	3	<0.01
poliovirus	21-40	3	<0.001

Antibody determinations by ELISA

Recently, we have also compared the avidities of the serum antibodies after the vaccination. The avidities of the serum IgG antibodies to poliovirus increased significantly in all groups, but were not significantly higher in the breast-fed than in the two formula-fed groups (Table 3). The avidity of the serum IgG antibodies to diphtheria toxin also rose significantly in the breast-fed group and the low protein formula group. The antibody avidity 20-40 months after the 3rd dose was significantly higher in the breast-fed group

Table 3. Avidity Indexes of Serum IgG Antibodies after Vaccination in Relation to Mode of Feeding. Avidity Index Expressed as Molarity of KSCN Eluting 50% of the Antibodies

Mode of feeding	Before 1st dose	Before 2nd dose	2w After 2nd dose	20-40 Mo after 3rd dose
	Antigen: poliovirus type 1			
Breast-fed n=11	1.2[a] (0.2-2.9)[b]	0.5 (0.1-2.2)	0.4 (0.3-1.1)	2.0 (1.3-3.4) p<0.001
Low protein formula n=10	1.3 (1.1-2.4)	0.6 (0.3-1.2)	0.5 (0.3-0.8)	1.9 (0.9-3.0) p<0.001
High protein formula n=10	1.6 (1.0-3.2)	0.6 (0.2-0.8)	0.5 (0.3-0.7)	1.4 (1.2-2.9) p<0.001
	Antigen: diphtheria toxoid			
Breast-fed n=7	1.1 (0.4-2.6)	0.6 (0.2-1.9)	0.5 (0.2-1.4)	1.8[c] (1.4-3.2) p<0.005
Low protein formula n=9	0.8 (0.2-1.4)	0.5 (0.2-1.1)	0.5 (0.3-1.1)	1.5 (0.4-3.0) p<0.02
High protein formula n=5	1.4 (0.6-1.7)	0.9 (0.4-1.9)	0.4 (0.3-1.4)	0.9[c] (0.4-1.9) n.s.

[a] median
[b] range
[c] $p<0.05$ between breast-fed and high protein formula group

than in the high protein group (Table 3). However, the groups are small and this preliminary study must be confirmed by more extensive investigations.

The reason for this suggested enhancement of the vaccine responses in breast-fed compared to formula-fed infants is not clear. We propose that it may be due to the effects of anti-idiotypic antibodies present in human milk (Table 4A and B), just as we have proposed that maternal idiotypic and/or anti-idiotypic antibodies to poliovirus reaching the fetus via the placenta may

induce the S-IgA and IgM antibodies to poliovirus found in newborn saliva[33]. These antibodies are present in Swedish newborns, although wild or vaccine poliovirus strains are not normally present in Sweden due to vaccination of 99% of the population with inactivated poliovirus vaccine only. We have found such antibodies in the offsprings of mothers with IgA deficiency or hypogammaglobulinemia as well, thereby excluding the possibility that these secretory antibodies came from the mothers[34]. The immunoglobulin preparation given prophylactically to the hypogammaglobulinemic mothers contained anti-idiotypic as well as idiotypic antibodies to poliovirus[35],

Table 4A. Direct Binding of Presumed Anti-Idiotypes Against Poliovirus Type 1 in 4 Human Milk Samples to Solid Phase-Bound Monoclonal and Polyclonal Antibodies to Poliovirus Type 1

Maximal binding in OD, by ELISA

Milk sample no.	Monoclonal antibodies to poliovirus type 1			Pool of monoclonals nos I-IV	Polyclonal antibodies to polio-virus type 1
	no I	II	III		
1	0.8	0.7	0.7	0.8	1.6
2	0.7	0.8	0.7	0.6	0.9
3	0.5	0.3	0.4	0.2	0.5
4	0.5	0.4	0.4	0.2	0.9

Table 4B. Competitive Inhibition of Poliovirus Type 1 Antigen-Binding to Solid Phase-Bound Monoclonal and Polyclonal Anti-Polio Virus Type 1 with Presumed Anti-Idiotypes in 4 Human Milk Samples

Maximal inhibition in % by ELISA

Milk samples	Monoclonal anti-poliovirus type 1	Polyclonal calf anti-poliovirus type 1
1	100	39
2	100	40
3	100	32
4	100	63

presumably providing transplacental stimulus in the fetuses of the mothers with hypogammaglobulinemia. Both the idiotypic and anti-idiotypic antibodies were present in the cord blood of the newborns of the antibody deficient mothers[34].

In newborn mice, Stein and Söderström[36] showed that anti-idiotypes to an *E. coli* K antigen given to newborn mice directly or via the milk primed them to respond with protective immunity when vaccinated with the bacteria. At that age they would otherwise not respond to a capsular K polysaccharide. This observation is also in agreement with recent work from Ogra's group, showing a response with specific antibodies to respiratory syncytial virus in newborn mice after they had been given milk with virus antibodies from their mothers who had been immunized with idiotypes or anti-idiotypes[37].

If our assumption of the role in the fetus and neonate of idiotypes and/or anti-idiotypes is correct, it means that breastfeeding may not only protect by passive transfer of antibodies but even actively prime the immune response of the offspring.

THE ROLE OF BREASTFEEDING FOR INFANT MORTALITY AND BIRTHRATES

In countries with high infant mortality the main cause of death is infections. It is obvious that partial, or better, exclusive breastfeeding may help prevent or ameliorate many of these infections, reducing infant mortality. This result of breastfeeding is very important *per se*, but there may be even more important repercussions. It has been claimed, and the background data are rather convincing, that decreasing infant mortality is followed within a relatively short time span[38] by decreasing birth rate. In fact, it seems that family planning programs can enhance this connection once it exists, but they may not function well in the face of high infant mortality. To prevent the on-going population explosion it seems therefore mandatory to use all means to decrease infant mortality. In countries that have successfully done so recently, such as Costa Rica, a decrease in birth rates soon occurred.

The connection between infant mortality and birth rate is multifactorial, but one common and obviously major link is breastfeeding which when frequent and persistent, has a clear anti-conceptional effect[39].

Against this background it seems equally important to study the immunological capacities of human milk, to determine its favorable effects on the infant, and to promote exclusive breastfeeding, especially in developing countries.

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T CELL DEVELOPMENT IN THE FETUS AND NEONATE

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INTRODUCTION

Properties of T Cells

T lymphocytes (T cells) play a central role in the immune response acting both to mediate specific immune functions and to modulate the function of all other aspects of the immune system. T cells mediate these effects both through direct cell-cell interactions and through the production of soluble mediators known as lymphokines.

T cells recognize antigen through a specific receptor, which is a heterodimeric structure similar to immunoglobulin[1]. Most T cells (~95%) express a T cell receptor (TCR) composed of an α and a β chain; these chains associate to form the antigen recognition portion of the TCR. The selective recognition of antigens is determined by variable portions of the molecules that are located in their amino-terminal half. The TCR associates on the cell surface with the pentameric CD3 complex, which links the TCR to other cellular components needed to mediate transduction of signals from the receptor to the cell interior[2,3]. A much smaller fraction (3-5%) of T cells express a TCR composed of a γ and a δ chain; in humans the fraction of T cells expressing the $\gamma\delta$ form of the TCR in neonates was similar to that in adults in one study[4] and less in another[4a]. Most $\gamma\delta$ TCR expressing T cells do not express either the CD4 or CD8 molecule. In contrast to T cells bearing the $\alpha\beta$ TCR, the function of T cells bearing the $\gamma\delta$ form of the TCR is not yet clear, although it has been suggested that they may play a role in defense against mycobacteria, in removal of damaged cells expressing heat shock proteins and in autoimmune disease[5,5a]. Virtually all T cells bearing the $\alpha\beta$ TCR also express on their surface either the CD4 (T4) or the CD8 (T8) molecule. These molecules act with the TCR to endow the T cell with the capacity to recognize specific antigen[1,6,7].

T cells recognize antigen in a way that is fundamentally different from the manner in which B cells (and immunoglobulin, which is the B cell antigen receptor) recognize antigen. Immunoglobulin binds to free antigen, often recognizing secondary or tertiary structure. T cells generally recognize relatively short (~8-9 amino acids) peptides, derived by the processing of larger

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proteins, and only do so when the peptide is bound to a major histocompatibility (MHC) molecule (known as HLA antigens in humans). The CD4 and CD8 molecules determine whether a T cell will recognize antigen bound to class II MHC molecules (HLA-D in man) or bound to class I MHC molecules (HLA-A, -B, and -C in man), respectively[6]. The co-recognition of antigen in association with self MHC molecules is central to the role of T cells in self/non-self discrimination.

Development of T Cells from Hematopoietic Precursors

T cells are derived from hematopoietic precursors, which migrate to and colonize the human embryonic thymus at approximately 8 weeks of gestation[8-10]. In the thymus, prothymocytes acquire the TCR and the CD3 complex and initially express on their surface both the CD4 and the CD8 molecules; because they express both the CD4 and the CD8 molecules these cells are known as double-positive thymocytes. Based on studies in the mouse, it has been determined that double-positive thymocytes undergo a dual selection process[6]. Negative selection removes cells that react strongly with self-antigens, and positive selection permits the maturation of cells that are capable of responding best to foreign antigens in the context of host MHC molecules. Negative selection appears to result either in deletion of thymocyte clones before they can mature into T cells or the establishment of a state of anergy so that the mature T cells that do develop are incapable of responding to the self-antigen that they recognize. Positive selection appears to allow the thymocyte to mature efficiently into a functional T cell. During positive selection, the expression of the TCR on the surface of double-positive thymocytes increases. In the case of cells that recognize antigen in association with class II MHC, CD4 expression is maintained and CD8 expression is lost; the reciprocal relationship holds for cells that recognize antigen in association with class I MHC[6,11]. These $CD4^+$ or $CD8^+$ cells are now mature thymocytes.

As indicated by the expression of TCR and of each of the developmental stages described above, human thymic differentiation appears to be complete by 18-20 weeks of gestation[8-10,12]. However, whether the diversity of TCR present in the mature host is present during fetal life is not yet known in the human. Because TCR diversity determines the range of antigens to which the individual is capable of responding, lack of diversity early in development could be a limiting factor in antigen recognition. There is evidence to suggest that the B cell repertoire is limited in the human fetus[13] and that the frequency of T cells capable of responding to certain antigens (e.g., herpes simplex virus) is extremely low in human neonates[14,15]. Recent studies examining the diversity of the receptors expressed by T cells bearing $\gamma\delta$ TCR, suggest that V region usage is different and more limited in the fetus than in the neonate. This is true both in man and in mouse[15a]. Diversity is further limited by usage of a single δ chain D region, and little or no addition or deletion of nucleotides at the junctions of the segments of these genes[15a,15b]. Although data strongly suggest that the $\gamma\delta$ TCR bearing T cells are a separate developmental lineage, it is likely that limitations in diversity also occur in the $\alpha\beta$ TCR expressing T cells. More studies to directly examine TCR diversity in the human fetus and neonate are needed.

Mature thymocytes are the immediate precursors of peripheral T cells and have acquired many of the functional attributes of T cells. They are capable of producing interleukin-2 (IL-2), expressing IL-2 receptors and

proliferating in response to mitogens[16,17]. However, their surface phenotype differs somewhat from that of T cells from adults (e.g., mature thymocytes express the CD38 molecule, whereas, resting T cells from adults do not). In addition, the capacity of mature thymocytes to mediate certain functions (e.g., production of interferon-γ) is less than that of adult cells[17-18].

It has recently been found that following release from the thymus, CD4 or CD8 T cells undergo additional differentiation in the periphery. This differentiation is associated with changes in the expression of isoforms of the CD45 molecule, also known as the leukocyte common antigen (Fig. 1), from the high molecular mass form CD45RA to the low molecular mass form CD45RO. Approximately 50-60% of circulating T cells (both in the CD4 and in the CD8 subsets) of adult humans express the CD45RA isoform and the others express in a mutually exclusive fashion the CD45RO isoform[17,19,20]. These isoforms can be identified by monoclonal antibodies including the 2H4 or 3AC5 (CD45RA) and the UCHL1 (CD45RO) antibodies. The CD45RO T cell fraction appears to contain most if not all functional memory T cells capable of responding to specific recall antigens[19,20]. Further, after activation *in vitro*, virtually all CD45RA T cells are converted to a CD45RO phenotype, while the CD45RO T cells continue to express this isoform; a similar process appears to occur *in vivo*. It has been proposed that cells expressing these different isoforms represent different maturational stages of a common cell lineage. The CD45RA T cells are thought to be naive – having never previously encountered the antigen which they recognize; the CD45RO T cells are thought to represent memory T cells – those that have previously been primed by encounter with their cognate antigen. For the remainder of the discussion the CD45RO cells will be referred to as memory T cells and to the CD45RA T cells as naive T cells.

Properties of Neonatal T Cells

Interestingly, T cells from human neonates share certain features with mature thymocytes and with naive peripheral T cells from adults, having a surface phenotype (>95% $CD38^+$ and $CD45RA^+$) that one might predict to be an intermediate in this differentiation process (Fig. 1). Neonatal T cells also differ in function from T cells from adults. Functional differences that appear to be of importance include diminished capacity to provide help for or actual suppression of immunoglobulin production by B cells[21], diminished generation and activity of cytotoxic T cells[22-27], and decreased capacity to activate macrophages[28]. T cell derived lymphokines appear to play a key role in mediating or modulating these functions[29,30]. Accordingly, we have sought to determine if immaturity in production of certain lymphokines might be a factor in the diminished function of neonatal T cells and to determine if this is a reflection of their incomplete differentiation, antigenic naivete or both. The postnatal acquisition of cells of the memory phenotype is discussed further in the presentation by Dr. A. Hayward.

METHODS

T cells were purified from adult peripheral blood and umbilical cord blood of neonates by conventional separation on Ficoll-Hypaque followed by removal of non-T cells with a commercial preparation of monoclonal antibodies and complement (T-Lymphokwik, One Lambda, Los Angeles) as

described[28]; some residual NK cells ($CD16^+$) remain in these preparation, however, similar results have been obtained with preparations further depleted of these cells. Isolation of the CD4 and CD8 subsets and of the $CD45RA^+$ and $CD45RO^+$ subsets of CD4 T cells was done by negative selection with monoclonal antibodies and complement and by indirect panning, respectively, as described[17,31]. These preparations were >95% pure. T cells were activated with the indicated agents as described[17,28,31,32]. Details of the methods for RNA analysis, nuclear run-on assays, *in situ* hybridization, and quantitation of lymphokine proteins have been described[17,28,32,33].

RESULTS AND DISCUSSION

Selective Immaturity in Lymphokine Production by Neonatal T Cells

T cells from neonates exhibited a selective immaturity in the production of certain lymphokines following activation by mitogens under conditions designed to induce maximal stimulation (Table 1). As determined by analysis of lymphokine protein in culture supernatants, neonatal T cells produced as much or more IL-2 and lymphotoxin, approximately 50% as much tumor necrosis factor (TNF) and GM-CSF, but 10% as much interferon-γ (IFN-γ) and IL-4 as did adult T cells.

As shown for lymphotoxin and for TNF, qualitatively similar results were obtained using whole blood mononuclear cells rather than purified T cells and with other stimuli (e.g., PHA), although the amounts of

Table 1. Lymphokine Production by Neonatal vs Adult T Cells

Lymphokine	Stimuli	Amount (x ± SD) Adult T cells	Neonatal T cells	Ref.
IL-2(U/ml)	ConA + PMA[a]	13793 ± 6910	18713 ± 6376	28
IFN-γ(U/ml)	ConA + PMA	1360 ± 261	122 ± 37	28
IL-4(pM)	Iono + PMA	30.5 ± 13.2	<3.5	31
Lymphotoxin (U/ml)	ConA + PMA PHA (MC)[b]	873 (n=2) 168 ± 154	1293 (n=2) 284 ± 280	32
TNF(pg/ml)	ConA + PMA PHA (MC)	13412 (n=2) 2160 ± 519	10809 (n=2) 996 ± 839	32
GM-CSF (pg/ml)	ConA + PMA	5275 ± 1962	2577 ± 1006	34

[a]PMA = phorbol myristate acetate; PHA = phytohemagglutinin
[b]Whole blood mononuclear cells (MC) were used.

lymphokines produced under these conditions were generally lower; this was also observed for the other lymphokines, including the markedly lower production of IFN-γ and of IL-4 by neonatal T cells[31]. Marked differences in IFN-γ and IL-4 production also were obtained when T cells were stimulated with the calcium ionophore ionomycin and PMA. These results show that the differences in IL-4 and IFN-γ production can not merely be explained by differences in signalling through the TCR-CD3 complex, which is bypassed by ionomycin and PMA[28,31]. These data also indicate that the major factor in the decreased production of IFN-γ by neonatal T cells is intrinsic to the T cells themselves; immaturity in the capacity of macrophages from neonates to help neonatal T cells produce IFN-γ[28,35] may be an additional but relatively minor factor in the differences observed.

Diminished Production of Lymphokines is Due to Diminished Transcription of Their Cognate mRNAs

The differences between adult and neonatal T cells in production of IFN-γ and of IL-4 and the relative amounts of these two lymphokines in culture supernatants were paralleled by differences in mRNA abundance, as determined by Northern blots, and mRNA transcription, as determined by nuclear run-on assays[31,33]. In an occasional experiment, small amounts of IL-4 mRNA were detectable in activated neonatal T cells but this was always much less than in activated adult T cells. These results indicate that the differences in IFN-γ and IL-4 production between adult and neonatal T cells primarily reflected differences in transcription of these genes in response to T cell activation.

Others have attributed diminished IFN-γ production by neonatal T cells to suppression, one group suggesting that suppression was mediated by the CD8 subset[37] and the other group suggesting that it was mediated by the CD4 subset[38]. Accordingly, we sought to determine if diminished lymphokine production by neonatal T cells was also seen with the isolated CD4 and CD8 subsets. In addition, to determine if diminished IFN-γ production by neonatal T cells reflected a reduced level of lymphokine production by individual cells, a reduced number of T cells producing the lymphokine or both, we examined individual cells for lymphokine mRNA by *in situ* hybridization. In unfractionated T cells from adults and from neonates, ~30-50% contained detectable IL-2 mRNA after activation with calcium ionophore and PMA. In contrast, detectable IFN-γ and IL-4 mRNA were found in 41% and 3% of adult T cells but in only 3% and <1% of neonatal T cells, respectively. When T cells from adults were fractionated into CD4 and CD8 subsets, IFN-γ mRNA was found preferentially in CD8 T cells (58%) as compared to CD4 T cells (34%) and IL-4 mRNA was found preferentially in CD4 T cells (4%) as compared to CD8 T cells (≤1%). However, although the same subsets of neonatal T cells were enriched for these mRNAs as in adults, only 4% of CD8 T cells were IFN-γ–positive and <1% of CD4 T cells were IL-4-positive[31]. Comparable results were obtained by RNA blot analysis and by analysis of supernatants for IFN-γ and IL-4 content[31]. These results do not exclude the possibility that a population of neonatal T cells found both in the CD4 and the CD8 subset suppresses expression by both subsets; however, a more direct explanation is that reduced capacity to express these lymphokines is intrinsic to neonatal T cells. This is further supported by the lack of inhibition of IFN-γ production by adult cells

when they are cultured in the presence of blood mononuclear cells from neonates[28].

The Absence of Memory T Cells May be a Major Factor in the Neonates Immaturity in IFN-γ and IL-4 Production

We and others[17,19] have shown recently that most of the IFN-γ and essentially all of the IL-4-producing T cells from adults are detected in the population of memory T cells defined by the surface expression of the CD45RO isoform of the leukocyte common antigen. In contrast, IL-2 production is approximately equal in naive and memory T cells from adults. To determine if the greater production of IFN-γ and of IL-4 adult T cells is attributable to the memory T cell population, which is essentially absent in neonates (see above), adult CD4 T cells were fractionated into CD45RA+ (naive) and CD45RO+ (memory) subsets. CD4 T cells were analyzed because they produced both IFN-γ and IL-4 and were more easily isolated in numbers sufficient both for supernatant and for RNA analyses. As shown by a representative experiment (Table 2), IL-4 was not detected in supernatants of CD45RA+ adult or neonatal CD4 T cells but was enriched in adult CD45RO+CD4 T cells. Similarly, in supernatants of adult CD45RO+CD4 T cells IFN-γ was > 10-fold and >100-fold more abundant than in supernatants of CD45RA+ adult and neonatal CD4 T cells, respectively. Results parallel these obtained by *in situ* hybridization (Table 2) and by RNA blots[17,31]. Although not shown, the small amount of IL-4 produced by adult CD8 T cells appeared also to be derived from the CD45RO+ subset.

Table 2. IL-4 and IFN-γ Production by CD4 Cells

Cells	% Cells + for mRNA		Supernatant concentration pM	
	IL-4	IFN-γ	IL-4	IFN-γ
Adult CD4	3	34	28	3340
Adult CD4 CD45RO+	8	51	58	4895
Adult CD4 CD45RA+	ND	ND	<4	335
Neonatal CD4	<1	3	<4	30

These results suggest that a deficiency in memory T cells may be one determinant of the neonates immaturity in production of IFN-γ and of IL-4. Two additional observations suggest that the absence of memory T cells is likely to be a major factor, at least for IFN-γ. First, both the number of CD45RO+ T cells and the capacity of infants' T cells to produce IFN-γ increase gradually with age, although a direct cause and effect relationship between these two events has not been established[4,39,40]. Second, if the absence of memory T cells is a major factor in the immaturity in IFN-γ production by neonatal T cells, one would predict that once a detectable antigen-specific T cell response develops, then IFN-γ production in response to that antigen should soon approximate that of T cells from adults responding to the same

antigen. In longitudinal studies of neonates and adults with primary herpes simplex virus (HSV) infection performed by Burchett *et al.*[41], this was the case. Although blood mononuclear cells from infected neonates made much less IFN-γ in response to HSV antigen than those from adults in the first 30 days of infection, there was no difference by 2 months, by which time HSV-antigen responsive memory T cells were detectable in lymphocyte-proliferation assays in these infected neonates as well as in the infected adults. Interestingly, when assayed at ≥ 2 months after onset of infection, production of IFN-γ by the infants' cells in response to HSV antigen was nearly equal to that produced in response to the mitogen Con A, whereas adult cells continued to produce much more IFN-γ in response to Con A than to HSV antigen. Although this observation may be interpreted in other ways, it is consistent with the presence of many additional memory T cells primed by previous exposure to antigens other than HSV in the adults that are not yet present in the more immunologically inexperienced infants. In preliminary experiments, we have also found that T cell lines derived from neonates, which have been propagated *in vitro* for one month or more by stimulation with mitogen, allogeneic cells and IL-2 , appear to produce IFN-γ as well as do similar lines of adult T cells; such lines express CD45RO and not CD45RA (not shown).

These findings suggest that the acquisition of memory T cells may be a major determinant of maturation for IFN-γ production. However, it is important to note that the results of the studies done *in vitro* may not necessarily fully reflect what occurs *in vivo*. As shown in Fig. 1, it is not known if neonatal T cells, which are not only $CD45RA^+$ but also express CD38, are able to mature directly into cells of the memory phenotype ($CD45RO^+$). It is possible that such cells progress through an intermediate stage in which their phenotype is identical to that of naive T cells in adults. Alternatively, the $CD38^+$ cells may be short-lived cells not capable of differentiating into memory T cells *in vivo* and may not be released from the thymus postnatally; in this scheme, cells exiting the thymus into the periphery postnatally, which are the precursors of memory T cells, would not express CD38. As a consequence of any of the above schemes, the derivation of memory T cells in response to an antigen challenge might be less efficient or more slow to occur in neonates than in mature individuals. Consistent with this prediction, slower acquisition of a detectable antigen-specific T cell response (and presumably therefore antigen-specific memory T cells) was observed in the studies of neonates with HSV infection described above[41]. This lag in acquisition of a detectable antigen-specific response is also consistent with the smaller fraction of HSV antigen-reactive precursor cells in neonates noted by Hayward and his colleagues[14].

Potential Mechanisms for Decreased Transcription of IFN-γ and of IL-4 by Neonatal and Adult Naive T Cells

Production of most lymphokines, including IFN-γ and IL-4, by T cells occurs only following activation. T cell-activation occurs as a consequence of engagement of the TCR by the antigen-MHC complex and is mimicked by mitogens. This process results in the transmission of a signal from the plasma membrane, which subsequently results in the activation of transcription of a series of genes in a contingent and orderly process (reviewed in 42,43). Transcription both of IFN-γ and of IL-2 (also presumably

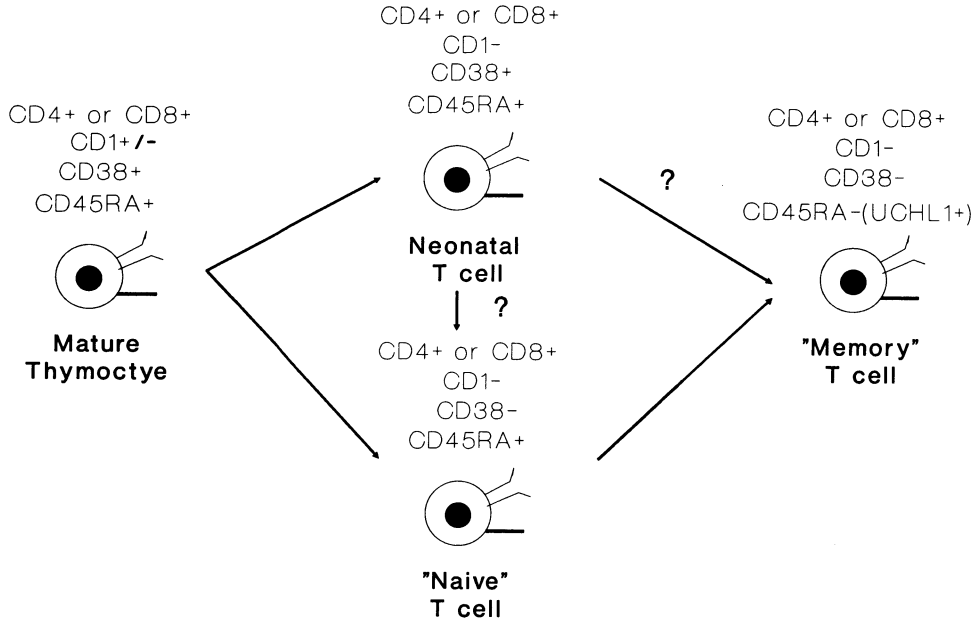

Figure 1. Post-thymic differentiation of T cells.

of IL-4) and other early activation genes begins within 1 h following stimulation, following the induction of the immediate, early genes. The exact process by which the signal from plasma membrane is converted to a change in the nucleus resulting in transcription of these or other genes is not fully understood. However, within the first min following activation a number of events occur. Phosphorylation of cellular proteins on tyrosine may be the first event, since it is detectable within 5 sec[44]. Soon thereafter increased generation of inositol phosphates is detectable and this is associated with an increase in intracellular calcium and activation and translocation to the plasma membrane of protein kinase C. Subsequent to these events there is a modification of proteins that bind to and activate a series of genes, ultimately including the lymphokine genes[43]; these proteins are often referred to as *trans*-acting factors because they are not directly linked to the gene(s) they affect but do modulate the gene's expression; that is, they act in *trans*.

The differences in lymphokine gene transcription noted above may be mediated by differences at any of the steps in this pathway. Many of the initial steps in this pathway appear not to differ in neonatal and adult naive T cells from those in adult memory T cells. Since induction of proliferation and of many of the lymphokine genes, e.g., IL-2, is similar in response to lectin mitogens, alloantigens, and to the superantigen[45] staphylococcal enterotoxin A[28], transduction of signals from the plasma membrane to the nucleus in response to mitogens is sufficient to mediate these processes. Further, since direct induction of an increase in intracellular calcium by ionomycin and direct activation of protein kinase C by PMA does not enhance IFN-γ and IL-4 by naive T cells, it is unlikely that deficits in these events account for the observed differences. We have also found that

translocation of both of the isoforms of protein kinase C, which are known to be expressed in T cells (βII and α), in response to ConA + PMA is similar in neonatal T cells, adult naive T cells, and adult memory T cells. Similarly, expression of the tyrosine kinases *lck* and *fyn*, which are believed to play a role in proximal events of T cell activation[46-48]), appears to be similar in memory T cells from adults as compared to naive T cells from adults or neonates. These results do not exclude other potential differences in signal transduction mechanisms between adult memory T cells and naive T cells from adults and neonates. In fact, the diminished proliferation of these non-memory T cell populations in response to stimulation with antibodies to CD3 or CD2[21,49,50], whereas, responses to other mitogens are similar to memory T cells, suggests that some differences in proximal signal transduction do exist. Nevertheless, such differences do not appear to be sufficient to account for the selectively diminished production of IFN-γ and of IL-4, which are seen with a wide variety of stimuli, including those which induce normal proliferation and Il-2 production.

Accordingly, we are currently focusing on factors in the nucleus that act directly to mediate transcription of the IFN-γ gene. We are testing the hypothesis that differences in *trans*-acting factors binding to and affecting transcription of these genes mediate the developmental differences in IFN-γ and IL-4 expression. To do so, we have initiated studies to map in detail the regulatory regions controlling IFN-γ transcription. Regions of the IFN-γ gene have been linked in plasmid vectors to the bacterial β-galactosidase gene and used in transient transfection of T cells. Initial results using the Jurkat T cell line, suggest that many of the sequences important for activation specific gene expression in T cells are located within 540 base pairs 5' of the transcription initiation site. These preliminary results are consistent with recently published data from another group[51,52]. More detailed mapping of this and other putative regulatory regions is needed to guide studies designed to identify *trans*-acting factors binding to these regions and particularly to identify those which account for the greater production of IFN-γ by memory T cells.

Immunological Consequences of the Neonates Immaturity in Lymphokine Production

The functions of IFN-γ and of IL-4 have been reviewed recently, as have other aspects of host defenses in the neonate[29,30,53,54]. IFN-γ and IL-4 both play important roles in the modulation of antigen-specific and non-antigen-specific immune responses. Accordingly, the immaturity in production of these lymphokines by neonatal T cells may be an important factor compromising the neonates resistance to infection. In certain cases, both IFN-γ and IL-4 have similar or synergistic effects on immune function. For example, both act to enhance the development and function of cytotoxic T cells[55,56]. Both also act to increase the expression of MHC class II molecules (HLA-D in the human) on may cell types (IFN-γ) or on B cells (IL-4). Increased expression of class II MHC molecules acts to enhance the antigen-recognition phase of specific immunity by increasing the presentation of antigen to CD4 (helper) T cells. IFN-γ many further enhance this phase of the immune response by increasing the production by macrophages of IL-1 and the surface expression of important intracellular adhesion molecules including ICAM-1[57,58]. Under appropriate conditions, both act to enhance B cell

proliferation[36,59,60]. Similarly, IL-4 and IFN-γ synergistically enhanced production of secretory component by an intestinal epithelial cell line *in vitro*; if this also occurs *in vivo* it may increase the translocation of polymeric IgA into secretions[61].

In certain situations, IFN-γ and IL-4 may play important roles in differential regulation of the immune response. This is clearly the case for the regulation of the isotype of immunoglobulin produced by B cells. IL-4 augments the production of IgE, an effect antagonized by IFN-γ, both *in vitro* and *in vivo*[62-66]. In addition, IL-4 appears to modulate many of the effects of IFN-γ on macrophages. IFN-γ markedly enhances the capacity of macrophages to kill or inhibit the growth of many microbial pathogens and to release pro-inflammatory mediators and cytokines (reviewed in 53). IL-4 antagonizes macrophage colony-formation, superoxide-production, and the release of IL-1 and chemo-attractants[67-69]; IL-4 may also inhibit the antiviral activity of IFN, although this latter effect has only been shown in murine cells[70].

Thus, a combined deficiency in IFN-γ and IL-4 production might act to impede the efficient induction of specific immune responses both by T cells, and B cells, impair the generation of antigen-specific cytotoxic T cells and might interfere with the normal modulation of the efferent phase of specific and non-specific immunity. Many of these effects are characteristic of the neonates immune response. These include delayed induction of antigen-specific immunity, impaired generation of cytotoxic T cells, limitation in the diversification of immunoglobulin isotypes produced, and impaired defenses against intracellular pathogens (reviewed in 53,54). In regard to the latter, recent data strongly suggest that the immaturity in production of IFN-γ is likely to be a critical factor in the unduly severe disease produced by *Toxoplasma* infection in the fetus. IFN-γ appears to be the critical mediator derived from T cells that allows the host to effectively contain infection with this intracellular parasite, probably in large part due to its capacity to activate macrophages to kill *Toxoplasma* and in other cells to inhibit its intracellular replication. Similarly, data from Kohl[71] indicate that IFN-γ is the only cytokine yet evaluated that allows human neonatal mononuclear cells to adoptively transfer protection against HSV to neonatal mice, which, like their human counterparts, are highly susceptible to fatal, disseminated infection; in contrast, mononuclear cells from adult humans can transfer adoptive immunity when either IFN-γ or IL-2 is added, reflecting the ability of adult but not neonatal T cells to produce IFN-γ in the presence of IL-2. The mechanism by which IFN-γ acts to confer protection is not clear but may include its ability to enhance NK function, directly inhibit viral replication, contribute to the development of cytotoxic T cells and stimulate macrophages to inhibit viral replication.

CONCLUSIONS

T cells in the fetus and neonate are phenotypically immature and are also naive – lacking previous exposure to exogenous antigens. This antigenic naivete may be an important factor in the functional differences between T cells from neonates and those from adults. Compared to memory T cells from adults, T cells from neonates are deficient in several functions, among which is the capacity to produce certain lymphokines. The most striking

deficiency is in the production of IFN-γ and of IL-4. Diminished T cell function appears to be an important factor in the overall immunological immaturity of the neonate. Studies to elucidate the molecular and cellular bases for these differences are important to further our understanding of normal development. Further, such information is necessary if we are to intelligently develop means to enhance the neonate's resistance to infection through passive or active immunological intervention.

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GROWTH FACTORS AND THE DEVELOPMENT OF NEONATAL HOST DEFENSE

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INTRODUCTION

Following birth, the gastrointestinal tract of the newborn undergoes important adaptive changes to the extrauterine environment. These changes include the development of an effective mucosal barrier against the penetration of antigens and antigenic particles presented to the intestinal surface by way of ingestion of milk or soy proteins, bacterial colonization of the gut, and formation of toxic byproducts of bacteria and viruses. Immaturity of this mucosal barrier may result in clinical disease states to which the newborn is specifically susceptible such as necrotizing enterocolitis, toxigenic diarrhea, and intestinal allergy. The content of breast milk is postulated to be an important modulator of the infants adjustment to the external environment.

THE DEVELOPMENT AND FUNCTION OF THE INTESTINAL MUCOSAL BARRIER

Macromolecules present in the intraluminal environment may traverse the intestinal mucosal surface either via specialized M cells overlying intestinal lymphoid tissue, endocytosis by the enterocyte or by peracellular migration through the zona occludens (Fig. 1)[1]. Attachment and penetration of macromolecules into the intracellular environment is limited by the gastrointestinal mucosal barrier which is composed of both immunological and non-immunological mechanisms (Table 1).

Non-immunological mechanisms. The non-immunological components of the gastrointestinal barrier represent the first line of defense to macromolecules by way of mechanical and chemical mechanisms. Intraluminal binding and breakdown by saliva, gastric acid, and digestive enzymes, contribute to the fragmentation and denaturation of intraluminal antigens.

The mucosal surface, consisting of the microvillus membrane and its overlying mucus layer, represent an important physical barrier to the attachment, uptake and transport of intraluminal antigens and bacteria from

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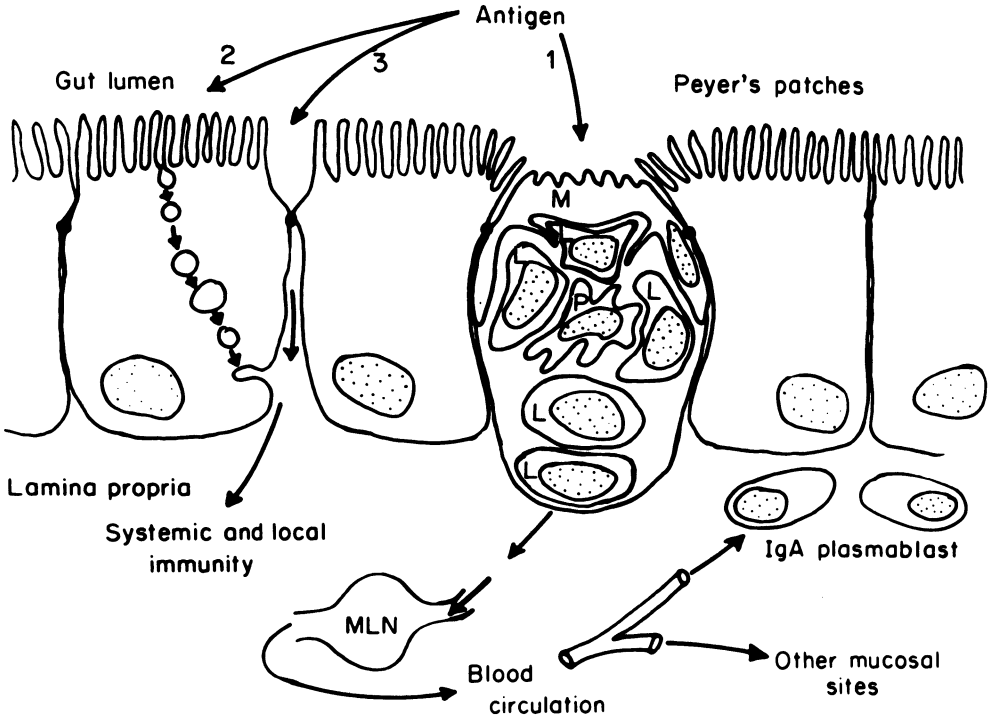

Figure 1. Schematic representation of the modes of entry of food antigens across the intestinal mucosa. The antigens in the gut lumen enter via (1) the specialized microfold epithelial (M) cells of the Peyer's patches in the normal intestine; (2) across the enterocytes in the immature intestine, and (3) the inter-epithelial tight-junction in the osmotically stressed intestine. Lymphocytes within the Peyer's patches are stimulated by antigens presented by macrophages (P). These lymphoblasts migrate to mesenteric lymph node (MLN) for further maturation and then enter the systemic circulation to redistribute along the submucosal sites where they mature into IgA-producing plasma cells (Reproduced with permission from N. Lyngkaran and M. Yodav, *in:* "Immunopathology of the Small Intestine," M. N. Marsh, ed., John Wiley & Sons, Toronto, p. 415, 1987).

Table 1. Components of the Mucosal Barrier

Non-immunologic factors:	
(1)	Intraluminal
	Saliva
	Gastric acid
	Gastric, intestinal, and pancreatic enzymes
(2)	Intestinal motility
(3)	Mucosal surface
	Mucus layer
	Microvillus membrane
Immunologic factors:	
(1)	Secretory IgA
(2)	Cell-mediated immunity

the intestinal lumen into the intracellular environment of the enterocyte. The biochemical composition and thickness of the mucus layer, influences the accessibility of antigens to the microvillus membrane. In the neonatal rat model, the mucus glycoproteins making up this layer, have a lower carbohydrate to protein ratio, a higher buoyant density, and a decreased content of fucose and N-acetylgalactosamine than the adult[2,3]. Studies suggest that the carbohydrate moieties of this mucus glycoprotein layer may act as binding sites or receptor inhibitors for ingested antigens, microorganisms and toxins[4,7].

The composition of the microvillus membrane of the intestine alters as the epithelial cell migrates from the crypt to the villus tip and as the animal matures. The microvillus membrane of the neonatal animal has a higher cholesterol and phospholipid content resulting in a higher lipid/protein ratio than that found in adult animals[8,9]. These differences may explain the decreased organization of the membrane and the increased membrane permeability noted in studies in developing animals[9]. Carbohydrate analysis of the microvillus membrane glycoconjugates have shown that the sialic acid content is higher and the fucose content is lower in the newborn rat[10]. Changes in the activity of the enzymes, sialyl transferase and fucosyl transferase, parallel carbohydrate levels during maturation[11]. As a result of these differences the accessibility of certain carbohydrate side chains and glycolipids within the microvillus membrane differ in neonatal and adult animals and may significantly influence the attachment of antigens, bacteria and toxins to the mucosal surface.

Immunological mechanisms. In the normal adult, antigens which traverse the mucosal epithelium induce a local immune response resulting in the production of dimeric immunoglobulin A (IgA) by primed IgA-producing plasma cells in the lamina propria. The specific IgA is then bound to the secretory component, an epithelial glycoprotein receptor, and transported across the epithelium to be released onto the mucosal surface. It is postulated that this specific IgA binds antigen in the intraluminal environment thus suppressing the transport of further antigen across the epithelium[12].

Although the secretory component of the IgA complex is normally present in the intestinal mucosa by 22 weeks gestation, IgA-producing cells are not seen in the mucosa of the intestine until approximately 12 days of age[13]. Similarly, the concentration of IgA in saliva does not reach significant levels until at least 14 days of age[14]. It is postulated that this transient IgA deficiency may contribute to the increased transportation of macromolecules across the epithelium in newborn period[4]. This hypothesis is supported by studies in patients with selective IgA deficiency who have been shown to develop circulating immune complexes soon after consuming bovine milk and have higher titers of serum bovine milk antibodies than found in normal subjects[4,15].

Cell-mediated immunity and the production of other immunoglobulin classes in the intestinal lymphoid system also differ in the newborn and the adult animal. Studies in neonatal animals fed ovalbumin have demonstrated an enhanced cell-mediated immunity and IgG and IgE antibody production (or "priming"), while in contrast, adults and weanlings fed the same quantity of antigen showed significant suppression of specific responsiveness (or tolerance)[16,17]. The mechanisms responsible for the induction of tolerance in adults may be the result of a series of complex maturational events of the gastrointestinal and lymphoid systems. Experiments in neonatal mice have

shown that antigens either injected parenterally, or absorbed via the gastrointestinal tract, produce similar systemic concentration of antigens. It has therefore been suggested that immunoreactive antigens are taken up in greater quantities in newborns compared to adults due to the less effective mucosal barrier[4,16]. In addition, fragmentation of antigens during the digestive process may play a role in the induction of tolerance in adults. Denatured or fragmented proteins injected parenterally are capable of inducing systemic tolerance[18,19]. While these fragments may not be detected by traditional immunoassays which rely on the binding of specific antibody to the fragments, they have been shown to induce suppression of T lymphocytes *in vivo*. The production of suppressor T lymphocytes and inhibition of the response of specific helper T lymphocytes have been demonstrated in response to protein antigen or antigenic fragments *in vitro*[20-23]. Therefore, the character and degree of antigenic fragmentation in the gut may influence the type of stimuli presented to specific T and B lymphocytes within the lamina propria and may in part account for the differences in the immunological response to ingested antigens in the neonate and adult. Failure to develop T cell modulation of the B lymphocyte response may result in enhancement of the IgE and IgG production to specific antigen and may result in the development of IgE-mediated allergy or IgG complex disease[4]. Figure 2 depicts the enteromammary immune system, a very important and unique means of delivering maternal IgA to the infant gut to protect the mucosal surface until these immaturities are developed.

THE EFFECT OF FEEDING ON GASTROINTESTINAL DEVELOPMENT

Maturation of the gut is directly influenced by feeding. Piglets fed colostrum during the first 24 hours of life had a significantly greater increase in the size, weight, and DNA content of the small intestine compared with piglets fed water during this same period[24]. While artificial feeding causes growth of the small intestine, animal studies have shown that the increase in mucosal mass, DNA, and protein content of the small intestine is much greater in animals fed breast milk[25]. These studies strongly suggest that breast milk provides an important stimulus to the development of the neonatal intestine and may play a role in the development of the neonate's own intestinal host defenses.

In contrast to artificial milk, human and animal breast milk contain a number of factors including hormones, peptides, amino acids, and glycoproteins that may act to promote gastrointestinal maturation (Table 2)[26]. The duration of lactation appears to influence the degree of breast milk-stimulated intestinal growth and maturation. In the newborn rat, the concentration of DNA and the rate of DNA synthesis was greater in animals fed colostrum in comparison to animals fed mature milk (Fig. 3)[27]. This suggests that colostrum may contain growth factors not present in mature milk or present in higher quantities in colostrum.

Epidermal growth factor is an example of a polypeptide found in significantly higher concentrations in colostrum than mature milk[28]. It has been shown to increase DNA synthesis and mitotic activity and enhances brush-border membrane enzyme activity when injected into suckling mice[29-

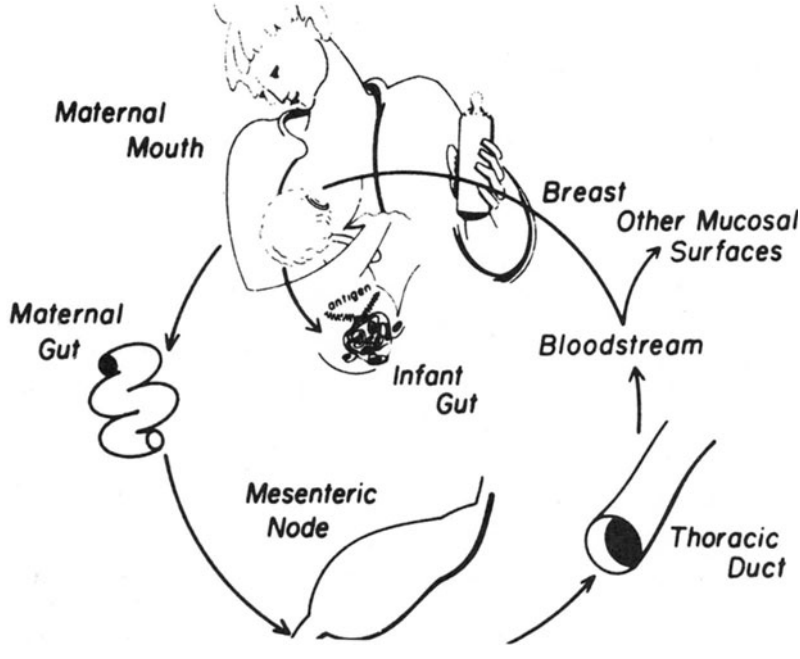

Figure 2. Dietary antigen entering the maternal gut reaches lymphoid follicles through specialized transport cells (M-cells). This antigen commits the lymphoblasts to specific IgA production, and these then migrate via mesenteric nodes and thoracid duct into the systemic circulation. During the periods of proper hormonal stimulation, such cells populate the breast and secrete S-IgA, which then is ingested by and functions in the infant. T cells, B cells, and macrophages are also the extruded into the breast milk and are immunologically active (Reproduced with permission from R. E. Kleinman and W. A. Walker, *Dig. Dis. Sci.* 24:876, 1979).

Table 2. Factors in Breast Milk that May Promote Gastrointestinal Maturation

Epidermal growth factor
Nerve growth factor
Somatomedin-C
Insulin-like growth factor
Insulin
Cortisol
Thyroxine
Taurine
Glutamine
Lactose
Amino sugars

[31]. Epidermal growth factor is resistant to acid denaturation and inactivation by trypsin and therefore is likely to be available in its active form in the neonatal small intestine[28] (Fig. 3). Although epidermal growth factor activity is still detected after a lactation period of 6 months, its ability to stimulate DNA synthesis decreases as the lactation duration increases[28].

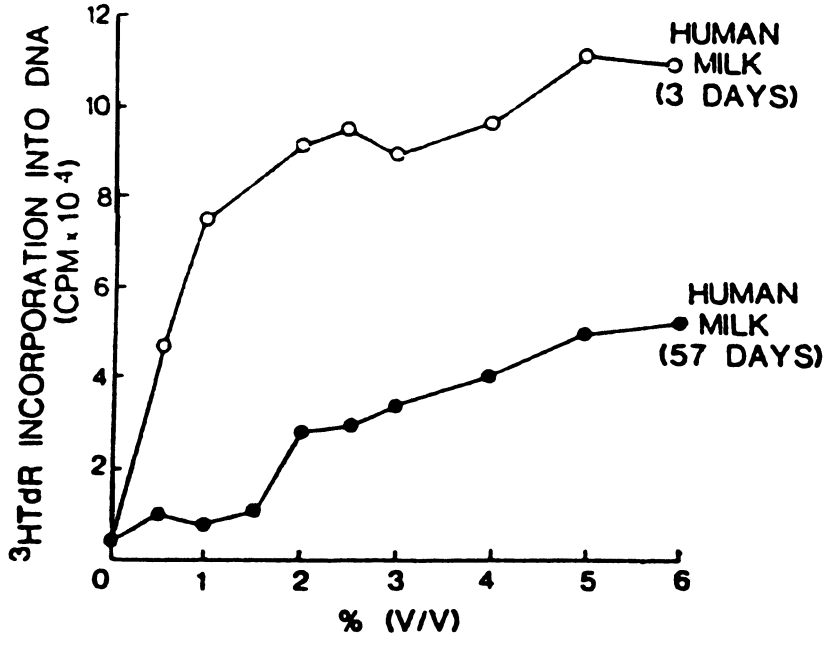

Figure 3. Stimulation of DNA synthesis by milk as a function of time in lactation period. Mitogenic activity is still present at 6 months but its ability to stimulate DNA synthesis decreases as time in lactation period increases (Reproduced with permission from D. Tapper, M. Klagsbrun, and J. Neumann, *J. Pediatric Surgery*, 14:803, 1979).

The influence of cortisone acetate in the maturation of the developing gut is well recognized. While hypophysectomy or adrenalectomy causes delays in gastrointestinal maturation, intraperitoneal injection of cortisol to suckling animals resulted in lengthening of the microvillus and enhanced brush border enzyme activity[32-34]. Acceleration of maturation of the intestinal mucosal barrier, as evidenced by decreased serum concentration of enterally fed ovalbumin, is found in newborn rats receiving cortisone during the

prenatal period[35]. Bacterial colonization of the mucosal surface was lower in rats treated with prenatal cortisone, perhaps as a result of alterations in glycoconjugates on the mucosal surface induced by cortisone. Translocation of bacteria to the liver was also less in rats receiving with prenatal cortisone suggesting that both the decreased number of bacteria attached to the mucosal surface and maturation of the mucosal barrier may play an important role in the prevention of necrotizing enterocolitis in neonates receiving prenatal cortisone[35]. While these studies report on the influence of intraperitoneally or parenterally administered cortisone, it is likely that cortisone presented to the intestine in breast milk may have similar effects on the maturation of the neonatal intestine[36].

Carbohydrates, such as lactose, present in breast milk have been shown to influence disaccharidase activity[37]. Long-term breast feeding prevents the decrease in lactase activity that is normally associated with weaning[38,39]. The role of glutamine has received recent attention in the preservation and repair of the mature small intestine[40-42]. Glutamine is also found in significant quantities in breast milk where it may provide an important fuel source for the growing intestine[43]. The amino sugars, N-acetylglucosamine and N-acetylneuraminic acid, are also found in significant quantities in human breast milk, especially during the first 5 weeks of lactation[44]. These sugars are important for the synthesis of surface proteins and lipids in the brain and intestinal epithelium. Changes in glycosylation of the microvillus membrane due to the incorporation of these amino sugars may influence the ability of microorganisms and antigens to attach to the mucosal surface.

In addition to hormones present in breast milk, the gastrointestinal tract endogenously produces at least 8 different peptide hormones. Within the first 4 days of life, feeding produces significant rises in serum levels of enteroglucagon, gastrin, and motilin[45]. The hormonal profile in six day old infants differs in infants fed breast milk or from those fed artificial milk[46]. While the changes in these hormone levels parallel gut maturation it is likely they also play a role in post-natal gut development.

CONCLUSIONS

The development of the gastrointestinal mucosal barrier represents an important adaptation of the neonate to the extrauterine environment. Maturation of the non-immunological and immunological components of this barrier enables the infant to protect itself against the attachment and penetration of potentially harmful antigens, bacteria, and toxins. The composition of breast milk may provide factors important for the stimulation of maturation of the gastrointestinal mucosal barrier and the development of the neonate's own host defenses.

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AMNIOTIC FLUID: THE FIRST FEEDING OF MUCOSAL IMMUNE FACTORS

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INTRODUCTION

Amniotic fluid (AmF) provides a protective environment for the unborn fetus. A number of immunologic factors typically found in mucosal secretions, including milk, are present in AmF. These include several innate anti-microbial substances such as lysozyme, lactoferrin, transferrin, peroxidase, and interferon-α[1-5]. The concentration of most of these factors increases in the AmF as the fetus matures, despite an overall decline in amniotic fluid protein concentration[2]. Specific immunologic factors in the AmF include maternally derived IgG and low levels of IgA and IgM[6]. The IgA2 in AmF has been shown to be of fetal origin based on the allotypic marker A2m[7]. Low levels of IgA, IgM and secretory component (SC)-positive antibodies specific for *Escherichia coli* and poliovirus are detectable in AmF from early (14-20 wks) and late (30-40 wks) gestational samples[8]. However, the frequency of detecting these antibodies in these samples did not seem to increase with gestational age.

The human fetus begins to express SC in various tissues by the seventh week of gestation[9]. The free SC (FSC) content and structural forms of secretory IgA (S-IgA) in AmF have been of recent interest in our laboratory[10]. The purpose of the present study was to examine the development of FSC and various molecular forms of S-IgA in AmF during gestation and to examine their relationship with several other mucosal immune factors in this fluid. The results suggest that the mucosal polymeric immunoglobulin transport system is functional in the fetus early in gestation. Its development is correlated with that of other immune factors in amniotic fluid during gestation, suggesting that the cells capable of transporting immunoglobulins also produce other immune factors. AmF may represent the earliest functional expression of the mucosal immune system in the fetus. Further, since AmF is swallowed by the fetus, this secretion may represent the first external exposure to several immune factors that will subsequently be provided by human milk.

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MATERIALS AND METHODS

Amniotic Fluid

Samples were obtained aseptically by abdominal amniocentesis for either fetal genetic-metabolic analysis or for phospholipid quantification. Gestational age was determined either by ultrasonography or by dates obtained by accurate maternal reports. For each sample, protease inhibitors were added (1 mM PMSF and 10 mM leupeptin), cells and debris were removed by centrifugation, and the samples were stored frozen at -20° C until analyzed.

ELISA for FSC, Lactoferrin, and Lysozyme

Each of these factors were quantified by specific ELISA's which have been described in detail elsewhere[10,11]. The values presented in the text represent the average of duplicate determinations for each sample tested. Two of 33 AmF samples were excluded from the analysis because their lysozyme values were well outside the range of the other AmF's from similar gestational age.

Western Blots

SDS-PAGE (7-10% polyacrylamide) was performed to separate the multiple forms of SC in amniotic fluid. The Western blotting technique has been described previously in detail[10]. Antibodies used to detect specific SC- and S-IgA related structures included: 1) rabbit polyclonal antibodies (anti-human SC, anti-human colostral S-IgA, anti-human α chain, and anti-human light chain, Dako, Carpinteria, CA), and 2) monoclonal antibodies produced in our laboratory and described elsewhere[12]: 5E8 (α chain-specific), 3F7 (SC-specific), 5G4 (S-IgA-specific) and 4B7 (κ chain-specific).

RESULTS

FSC Content in AmF

The FSC concentration in amniotic fluid samples taken at various gestational ages was determined by a quantitative ELISA. The ELISA technique we used is specific for the free forms of SC, since it is based on the specific binding to polymeric IgA-coated walls. The FSC bound to the plate was detected by specific polyclonal antibody to SC[10]. Fig. 1 shows the FSC concentrations of 27 different AmF samples. These results represent a subset of AmF reported on previously[10], for which we had adequate sample volume to measure lactoferrin and lysozyme. FSC was detected in all samples obtained from pregnancies of greater than 21 wks duration[10]. Lower concentrations of FSC were also detected in two out of three of the earliest samples tested (16 wks). FSC concentration in AmF appears to be directly related to gestational age. Because of the relatively small number of samples obtained for ages less than 26 wks, only the data from samples dating 28-40 wks was subjected to simple linear regression. The regression of FSC concentration against gestational age yielded a line with a slope of 1.026 and a correlation coefficient of 0.499, indicating that FSC concentrations in AmF increase with gestational age from 28-40 wks.

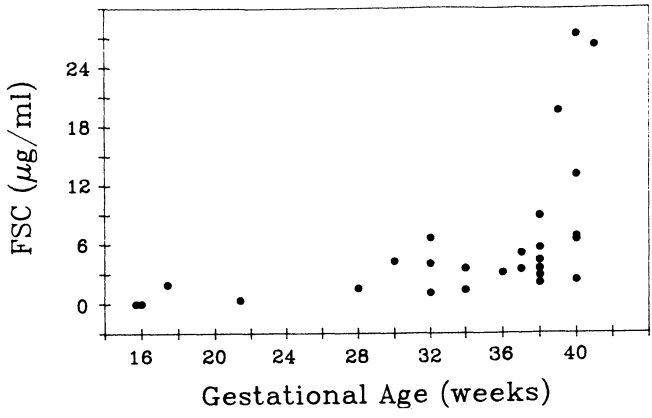

Figure 1. The effect of gestational age on the concentration of free SC in AmF. AmF samples taken at various gestational ages were assayed for FSC concentration by quantitative ELISA. Points on the scatter plot represent the average of duplicate determinations of FSC in individual samples. This data is a subset of data published previously[10].

Relationship of FSC to Other Mucosal Immune Factors in AmF

A number of anti-microbial substances have been described in AmF. We quantified two of these, lactoferrin and lysozyme, and compared their concentrations at various gestational ages to the quantity of FSC in the same AmF samples. Thirty three individual AmF samples were analyzed in the three specific ELISA's. Lactoferrin concentration increased with gestational age, attaining concentrations of 3-5 μg/ml by the end of normal gestation. Fig. 2 presents the relationship between the concentrations of FSC and lactoferrin in individual AmF samples. The r value of 0.583 indicates that a significant positive correlation exists between the FSC and lactoferrin concentrations in AmF.

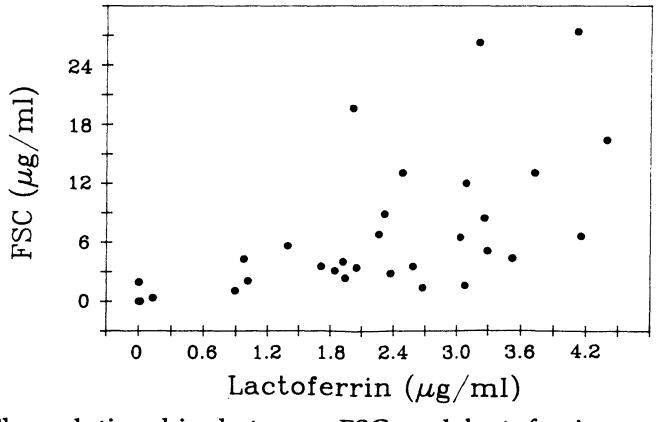

Figure 2. The relationship between FSC and lactoferrin concentrations in AmF samples at various gestational ages. FSC and lactoferrin concentrations were determined by quantitative ELISA's. Points on the scatter plot represent the average of duplicate determinations. The FSC values are the same as presented in Fig. 1.

Lysozyme concentration also increased with gestational age in parallel with that of FSC and lactoferrin. Fig. 3 shows that there is a direct relationship (r=0.399) between the concentrations of FSC and lysozyme in AmF. Fig. 4 represents the relationship between lactoferrin and lysozyme concentrations during gestation. A correlation coefficient of 0.7402 indicates a positive correlation between the concentration of these factors.

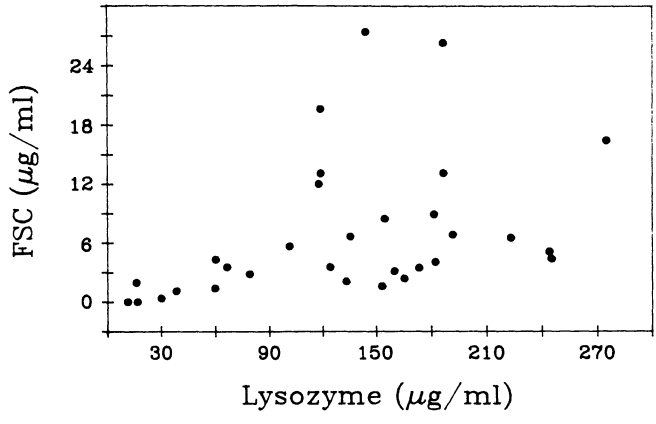

Figure 3. The relationship between FSC and lysozyme concentrations in AmF samples at various gestational ages (see legend to Fig. 2).

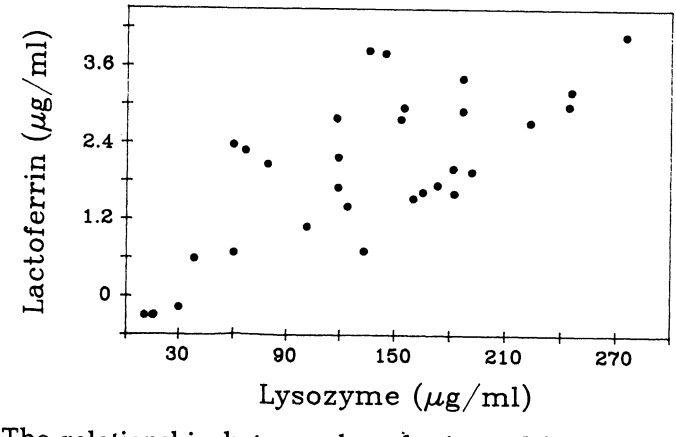

Figure 4. The relationship between lactoferrin and lysozyme concentrations in AmF samples at various gestational ages (see legend to Fig. 2).

Structural Analysis of AmF S-IgA by Western Blotting

Fig. 5 represents a Western blot identifying the various forms of SC (both free and IgA-associated) in AmF. An individual 40 wk gestation AmF sample was compared to purified dimeric IgA, S-IgA, and FSC standards. The blot was developed using a rabbit anti-human colostrum Ab preparation which contains Ab's specific for SC and α chain.

As demonstrated by ELISA in Fig. 1, AmF from late in gestation contained substantial amounts of FSC (78 kDa band in Fig. 5). However, a unique set of AmF proteins of 170-200 kDa also stained positively with the antibody to human S-IgA. (380 kDa). A very small amount of S-IgA of the typical (380 kDa) size was detectable in approximately 80% of the AmF samples tested. Thus the majority of AmF samples contained SC in both the free (78 kDa) and two IgA associated (170-200 kDa and 380 kDa) forms.

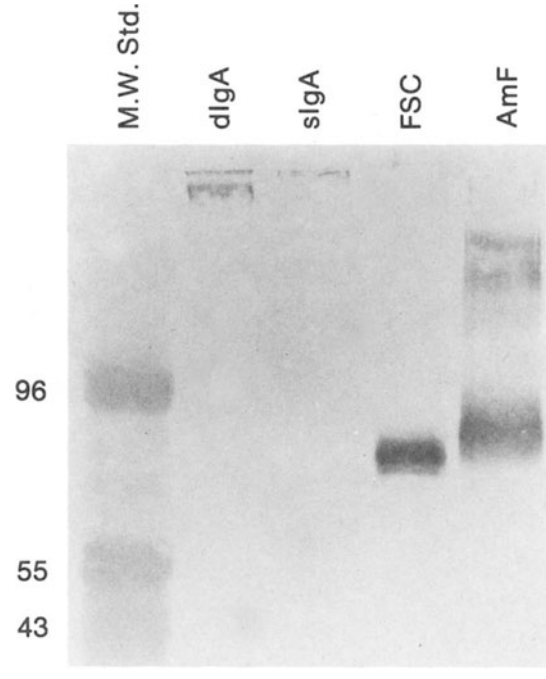

Figure 5. Molecular forms of SC in AmF as shown by Western blotting. Samples were separated by 10% non-reducing SDS-PAGE. After transblotting onto nitrocellulose, SC and IgA were detected by a polyclonal anti-colostral S-IgA antibody preparation. Lanes are as follows (from left to right): 1) M. W. Std., molecular weight standards, sizes for 96 kDa, 55 kDa, and 43 kDa are indicated along left border; 2) dIgA, purified human serum dimeric IgA (0.5 μg); 3) S-IgA, purified human milk S-IgA (0.25 μg); 4) FSC, purified human milk FSC (0.25 μg); 5) AmF, human amniotic fluid (20 μl) (used with permission from *J. Immunol.*)[10].

We have recently partially characterized the unusual forms of IgA in the 170-200 kDa range[10]. As shown in Fig. 6, these IgA forms stained with both polyclonal and monoclonal Ab's specific for the α chains, light chain, and SC. In lanes 2-4, the staining with polyclonal antibodies specific for SC, α, and light chain is shown. Staining of both the 380 kDa S-IgA and the 170-200 kDa forms was seen with all three antibody preparations. The staining with mAB's in lanes 5-8 substantiates the results found with the polyclonal preparations. These results demonstrate that the 170-200 kDa moieties not only contain SC, but also antigenic determinants of α and light chains. We have also found that the size of the major peptide chains (SC, α, and light) in the AmF IgA comigrate on reducing SDS-PAGE gels with those from typical S-IgA isolated from human milk[10]. Thus, the lower molecular weight forms of IgA in AmF seem to differ from typical S-IgA in the stoichiometry of the chains rather than in the length of each peptide.

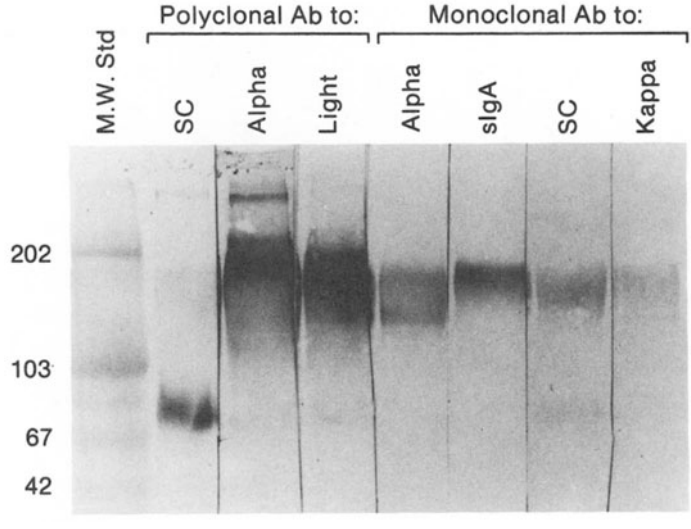

Figure 6. Western blots of amniotic fluid stained for SC, α, and light chains. The same amniotic fluid was appllied to each lane of a 7% non-reducing SDS-PAGE (20 μl per lane). After transblotting onto nitrocellulose, individual lanes of the tranfer were cut apart and stained separately with various monoclonal and polyclonal antibodies followed by the appropriate secondary antibody-HRP conjugates. The primary antibodies used on the individual lanes are as follows (from left to right): 1) polyclonal anti-SC, 2) polyclonal anti-α chain, 3) polyclonal anti-light chain, 4) monoclonal anti-α chain (5E8), 5) monoclonal anti-S-IgA (5G4), 6) monoclonal anti-SC (3F7), 7) monoclonal anti-κ (4B7) (used with permission from *J. Immunol.*)[10].

When samples of AmF from various gestational ages were compared by Western blotting, the proportion of the small and large molecular forms of IgA did not appear to be related to gestational age. Fig. 7 shows such a Western blot stained with polyclonal antibodies specific for IgA. Since the results of this assay are not quantitative, it is not possible to determine the relationship between the concentration of any of the IgA forms and gestational ages.

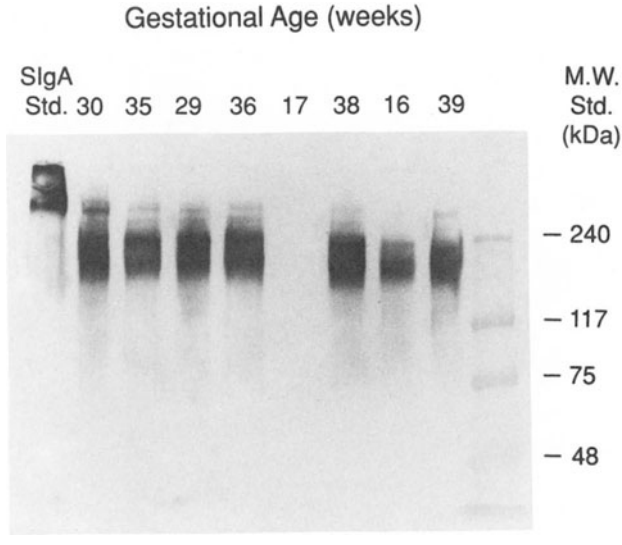

Figure 7. Western blot demonstrating the development of the various forms of IgA in AmF. AmF samples from various stages of gestation were separated on a 7.5% SDS-PAGE and transferred onto nitrocellulose sheets. The transfers were developed with a polyclonal antibody specific for α chains and an appropriate secondary enzyme-conjugated antibody. Lane 1 contains a set of molecular weight markers, the size of which is indicated in the right margin. Lanes 2-8 were loaded with 25μl of AmF from pregnancies, the gesttation age of which is indicated above each lane. Lanes 9 and 10 contain S-IgA and FSC purified from human milk.

DISCUSSION

We have recently reported that AmF contains a quantity of FSC that is easily detected by Western blotting and ELISA[10]. The concentration of FSC increases with gestation. During this same time period, the composition of AmF proteins is shifting toward fetal origin and away from maternally derived proteins. This finding suggests that the expression of the polymeric immunoglobulin transport system (SC production) of the fetus begins early in

gestation and increases as gestation progresses. By the end of normal gestation, the concentration of FSC in AmF is comparable to those of saliva and bile[10]. The fact that low levels of fetally derived S-IgA is also present in AmF suggests that the polymeric immunoglobulin transport system of the fetal-placental unit is functional beginning early in gestation.

Our studies confirm the finding that both lysozyme and lactoferrin in AmF increase with gestational age[2,5]. Our data for the development of lactoferrin concentration in AmF were similar to those described previously[4]. However, the values for lysozyme concentration, as determined in our ELISA system, were approximately 10 fold higher than those reported previously[3]. When we subjected some of the same AmF samples to an assay based on lysozyme enzymatic activity (*Micrococcus lysodeikticus* assay) we found levels (data not shown) similar to those reported previously. We have not found such large discrepancies between the results from the ELISA and enzymatic assay for lysozyme in other secretions. Our results suggest that most of the AmF lysozyme detected antigenically in our ELISA is enzymatically inactive.

We also found that the development of AmF FSC levels parallels those of both lactoferrin and lysozyme. These results suggest that all three of these proteins may arise from the same epithelium or that common regulatory mechanisms govern the production of epithelial antimicrobial factors and the polymeric immunoglobulin transport system. These innate immune factors, which are thought to play a role in mucosal immunity, may contribute to the antimicrobial activity of AmF. Such activities may help to protect the fetus against intrauterine infection. These same factors which are swallowed and inspired by the fetus may provide transient protection against the onslaught of microbes experienced soon after birth. Human colostrum and milk which contains a high concentration of these same factors may take over this function as soon as feedings are initiated.

We were able to confirm the presence of S-IgA in AmF as found by others[8]. However, the predominant forms of S-IgA in AmF have sizes of only 170-200 kDa. This heterogeneous set of proteins has antigenic determinants of SC, α heavy chain, and light chain. J chain content in AmF S-IgA has not yet been investigated. Further characterization of this unique set of proteins is underway. However, based on our current data, one might speculate that these proteins arise by reduction of interchain disulfide bonds of 380 kDa S-IgA. Studies of *in vitro* reduction of S-IgA indicates that the disulfide bonds between α and J chains, which hold the IgA dimer together, are the most susceptible to reduction[11]. Such reduction produces monomeric fragments of the S-IgA molecule which resemble the 170-200 kDa AmF moieties in molecular weight.

We have not yet quantified the various forms of IgA in AmF. However, preliminary analysis of Western blots suggest that relative to FSC, the concentrations of all of the molecular forms S-IgA in AmF are low and do not change dramatically during the gestational period we have studied (15-41 wks.). These results are consistent with those previously reported using a semi-quantitative IgA immunoassay[7]. The pattern of IgA development in AmF is clearly different from that of FSC, lactoferrin and lysozyme which increase substantially with gestation. This apparent difference suggests that the development of IgA-producing plasma cells limits the secretion of S-IgA into AmF.

Metabolic studies, using injection of radiolabelled proteins into AmF indicate that more than two thirds of the AmF proteins, including IgA are

cleared each day[12]. Thus, although the concentration of IgA in AmF is low, the synthetic rate of S-IgA must be substantial. The most likely function for S-IgA in the AmF is to provide a barrier between the fetus and microorganisms that could produce intrauterine infections, a major cause of preterm labor[2].

The precise site of clearance of the S-IgA from the AmF is unknown. However, at term, approximately one liter of AmF is swallowed by the fetus each day, while 250 ml per day is absorbed by other means (most likely across the fetal membranes)[13]. Clearly, both the respiratory and gastrointestinal tracts of the fetus are potential sites of deposition for the S-IgA and the innate anti-microbial substances found in AmF. These immunologic factors may help to protect the neonate during the critical transition to extra-uterine life. In this context, AmF may be considered the first form of mucosal immune factors to be ingested by the developing infant.

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ONTOGENY OF THE SECRETORY IgA SYSTEM IN HUMANS

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INTRODUCTION

Secretory immunoglobulins consist of IgA or IgM, secretory component (SC) and joining (J) chain. J chain is a 15 kilodalton polypeptide that participates in the intracellular polymerization of IgA and IgM and is found in plasma cells[1]. J chain was also found at earlier stages of B cell differentiation before the onset of immunoglobulin synthesis[2]. IgA or IgM is produced by plasma cells, polymerized intracellularly and secreted into the lamina propria of glandular tissues. SC, which is localized in the basolateral membrane of glandular tissues, functions as a receptor for J chain-containing polymeric immunoglobulins[3]. Ontogenically, it has been reported that SC and IgM are expressed at an early gestational age while IgA usually appears later[4,5]. However, the exact development of the secretory immune system in the human fetus has not been examined extensively.

To identify the developmental stage at which α, μ and J chains and SC gene expression begins in human fetal tissues, we have used the polymerase chain reaction (PCR) technique to determine the presence of corresponding mRNA because human fetal tissues are very small and evaluating synthesis of immunoglobulins, J chain, and SC by usual techniques would be impossible.

MATERIALS AND METHODS

Gestational Age

Nineteen aborted embryos and fetuses aged 6 to 36 gestational weeks were used in this study. Fetal liver, thymus, spleen, lung, and small intestine were taken and processed for the extraction of mRNA.

Determination of the gestational age was made by crown-rump length, fetal foot length, fetal palm length, morphological features of hand and foot surfaces, and the date of last menstruation of the mother [6,7].

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Preparation of cDNA

RNA was extracted from fetal tissues in an usual manner. Extracted RNA was boiled for 5 min and RNase inhibitor (20 units), 1.25mM dNTP's, oligo dT (1 μg/ml), 10 x PCR buffer [100mM Tris-HC1 (pH 8.3), 15mM $MgC1_2$, 500mM KC1, 0.01% gelatin], reverse transcriptase (5 units), and 3'primer (10 pmol) were added. Reaction mixtures were incubated for 60 min at 42°C, and heated at 94°C for 5 min and chilled on ice.

Amplification of cDNA by PCR

cDNA was diluted with 80μl of PCR reaction mixture (10 x PCR buffer, followed by the addition of 40 pmol of the 3' primer, 50 pmol of the 5' primer and 2.5 unit of *Taq* polymerase), the RNA-DNA hybrid was denatured at 95°C for 30 sec, the primer was annealed at 55°C for 30 sec and the primer extended at 72°C for 1 min. To prove that the assay detected RNA rather than contaminating DNA, we also amplified samples without reverse transcriptase.

Southern Blot Analysis

PCR products electrophoresed in 1.7% agarose gel were transferred to a nylon membrane. After pre-hybridization (50% formamide, 5X SSC, 0.01M Na_3PO_4, 0.5% non-fat dry milk, 0.1% SDS), the membrane was hybridized with ^{32}P-labeled internal probe at 42°C for 3 h, then washed three times with 6X SSPE and subjected to autoradiography. The intensity of the signal was determined by densitometric tracing.

Sequences of Primers and Probes

Sequences of primers and probes for J chain, μ and α heavy chains, and SC used in this study are shown in Table 1. Amplification of human J chain, μ chain, α chain, and SC sequences yields 208bp, 250bp, 380bp, and 258 bp products, respectively.

RESULTS

Sensitivity and Specificity of the PCR Assay

To evaluate the sensitivity and specificity of the assay, a dilution experiment was conducted. Total RNA from Daudi cells was diluted in steps from 1 to 6 orders of magnitude by using 10μg of K562 total RNA per ml as the diluent. The amplified products were analyzed by gel electrophoresis and visualized under UV light by ethidium bromide fluorescence. A 10^{-5} dilution of 1 μg of RNA from the J chain-positive Daudi cell line still provided an easily detectable signal in this assay. One microgram of RNA is roughly equivalent to the amount contained in the cytoplasm of the 100,000 Daudi cells (10pg of cytoplasmic RNA per cell). Thus, a 10^{-5} dilution represents RNA from about one Daudi cell. Since only 1/10th volume of amplified product was used for analysis, the positive signal represents the amplified product of less than one cell equivalent. This result demonstrates that detection is feasible even when expression of J chain occurs in very few cells.

To prove that the assay would detect RNA rather than contaminating DNA, we also amplified samples without reverse transcriptase. No band was observed in the gel, indicating that the RNA was not contaminated with genomic DNA.

Table 1. Sequences of Primers and Probe

Primer	Sequence	
J Chain		
IS4-1	CTGTAAAAAATGTGATCC	(sense primer)
IS4-2	TACGGACGATAGGACTG	(anti-sense primer)
IS4-3	CTGTTCCGGAAGCACTT	(internal probe)
μ Chain		
CS1	ATCACGTGCCTGGTGAC	(sense primer)
CS2	TGGACTTGTCCACGGTC	(anti-sense primer)
CS3	TAACTCTTGTTTCGGTAA	(internal probe)
SC		
SC1	AGCAAGGCCTTCGTGAA	(sense primer)
SC2	AATGGCTTTGTTCTCAAT	(anti-sense primer)
SC3	TAACTCTTGTTTCGGTAA	(internal probe)
α Chain		
α1	ATCCCCGACCAGCCCCA	(sense primer)
α2	TACGACGGTGGGGGCTG	(anti-sense primer)
α3	AGTGCCTAGCCGGCAAG	(α1 internal probe)
α4	AGTGCCCAGACGGCAAG	(α2 internal probe)

Expression of J, μ and α Chains, and SC

In the early fetal liver, we found a significant reactivity of J chain expression at the 6th gestational week while expression of the μ chain was not detected in the same RNA samples from fetal liver. Beginning at the 6th gestational week, the frequency of J chain expression increased rapidly by week 16. The expression of μ chain at week 7 laged behind that of J chain, but followed a similar rapid increase at the 16th gestational week. In other organs (spleen, thymus, lung, and small intestine), J chain and μ chain expression was detected at week 16 (Fig. 1,2).

Alpha chain expression was found at week 16 in the thymus, lung, and spleen, at week 24 in the small intestine and at week 36 in the liver (Fig. 3).

The expression of SC occurred at a different time in fetal organs. SC was detected at week 7 in the small intestine, at week 16 in the lung, at week 20 in the thymus, and at week 24 in the liver (Fig. 4). No reactivity indicating SC expression was found in the spleen.

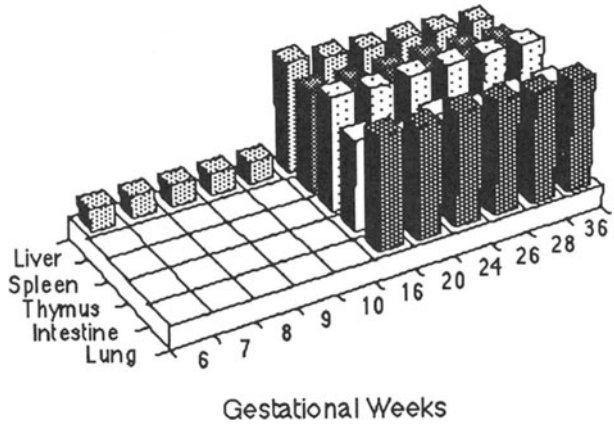

Figure 1. J Chain Expression.

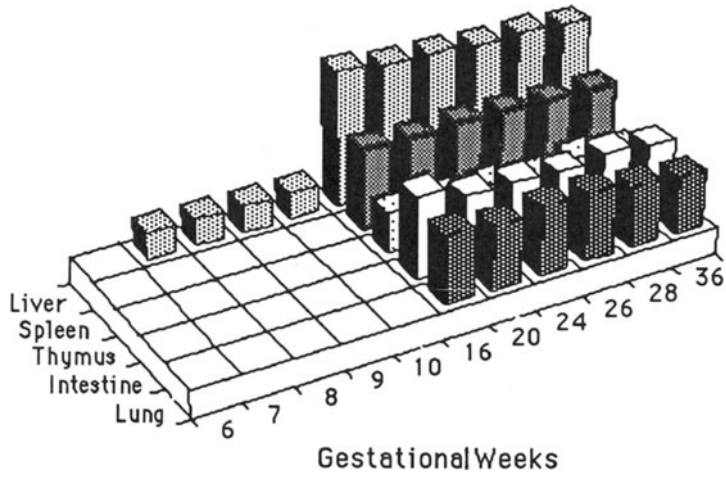

Fig. 2. μ Chain Expression.

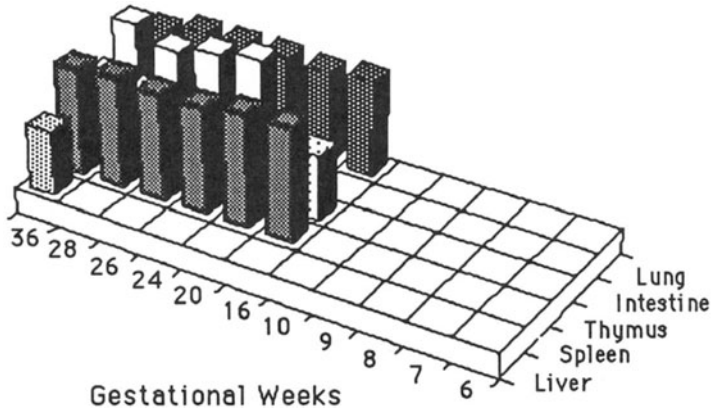

Figure 3. α Chain Expression.

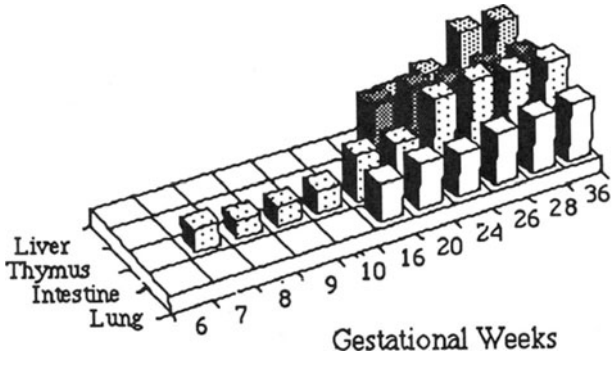

Figure 4. Secretory Component Expression.

DISCUSSION

In B cell ontogeny, it is well-known that pre-B cells first express cytoplasmic μ chain, and then differentiate into B cells having surface μ chain. In humans, pre-B cells are found in fetal liver as early as 7 weeks of gestation and surface IgM-positive B lymphocytes appear approximately two weeks later as determined by immunofluorescence techniques[2,8,9]. Similarly, the appearance of cytoplasmic μ chain-positive cells precedes that of surface μ chain-positive cells in mouse fetal liver[10].

As for J chain expression during the B cell differentiation pathway, McCune *et al.* provided evidence for J chain expression at the pre-B cell stage of differentiation using a human cell line[11]. Similarly, Hajdu *et al.* suggested that J chain expression actually precedes μ chain synthesis during B lymphocyte maturation[12]. Early studies of the ontogeny of J chain expression in chickens and pigs revealed that the J chain becomes detectable at different stages of embryonic development as determined by immunocytochemical techniques [13,14]. As previously described, Iwase *et al.* reported that a few J chain-positive cells in the human fetal liver were detected at the 16th gestational week by the immunofluorescence technique[15].

Our results clearly show that the expression of J chain in fetal liver represents a relatively early event in B cell ontogeny, and occurs earlier than that of μ chain by approximately one week.

Although a small amount of IgA is detectable in the human fetus, it is generally accepted that IgA synthesis occurs after birth[5]. A few IgA-producing cells have been found at the 26th gestational week in the small intestine and lung by immunocytochemical techniques[15]. In this study, α chain expression was detected at an earlier week (16th week) in the same organs by PCR.

The presence of SC in the developing human fetus has been reported at 8-12 weeks in the lung, and between 13 and 17 weeks in the small intestine[4,16]. We found that SC expression occurred at different gestational time periods in the human fetus in different organs. Thus, SC was detected at week 7 in the small intestine, at week 16 in the lung, at week 20 in the thymus, and at week 24 in the liver.

CONCLUSIONS

The expression of J chain in the fetal liver represents a relatively early event in B-cell ontogeny and precedes that of μ chain by approximately one week. In contrast, α chain expression was found at relatively late stages of fetal development. SC expression in the human fetus differed in its time course between various organs.

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IgA-SECRETING CELLS IN THE BLOOD OF PREMATURE AND TERM INFANTS: NORMAL DEVELOPMENT AND EFFECT OF INTRAUTERINE INFECTIONS

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INTRODUCTION

It is now well appreciated that human infants at birth usually have undetectable levels of IgA in the serum, which slowly rise over the first few years of life to attain adult levels by puberty[1,2]. Most newborns also lack secretory IgA (S-IgA) at birth, with IgA in the saliva or tears not detectable until the first wk. Increasing S-IgA levels are noted in the first few mo of life, sometimes being demonstrated before serum IgA (3,4, Cripps *et al.*, this volumn). As noted by Moro *et al.*, (this volume), as well as by others[5,6], the genetic and cellular mechanisms for the production of IgA responses are in place before birth, although induction of IgA responses would necessitate antigenic stimulation *in utero*. However, the subset of B cells involved in the production of serum or S-IgA is still unclear. The B cells found in several fetal organs and in the blood of the fetus and neonate are primarily of the $CD5^+$ rather than the $CD5^-$ subset; the latter represent the majority of B lymphocytes in human adults[7-9].

We have used the reverse enzyme-linked immunospot (RELISPOT) assay - developed recently in our laboratories to measure the number of circulating B lymphocytes secreting immunoglobulins (Ig) of different isotypes[10-12] – to determine the numbers of IgA-secreting cells (SC) in neonates soon after birth, in comparison to those found in older infants and in adults. After ascertaining the levels of Ig-SC in the blood of 48 normal neonates within the first 5 d of life, we tested over 250 other neonates with intrauterine-acquired syphilis, as well as various other suspected or proven infections, most of which were acquired intrapartum or postnatally. Comparisons of IgA-SC and serum levels of IgA were also made in the neonates and older infants. Finally, the results of the studies described here have been combined with those reported earlier[10-13] to provide a better perspective on the continuum of the development of IgA-SC, and $CD5^+$ and $CD5^-$ B lymphocytes, from birth to 2 yr of life.

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MATERIALS AND METHODS

Study Populations

Venous blood was obtained, with parental consent, from neonates of different gestational ages with a variety of suspected infectious conditions and from apparently uninfected newborns. It has been found that Ig-SC are not detectable in the first 5 d after an antigenic stimulus[14]. Therefore, blood from neonates was obtained within the first 5 d of life, a period during which any elevations of Ig-SCs would be expected to reflect intrauterine antigenic stimuli. Some of the infants were also studied at weekly intervals within the first mo of life. For the B cell subset studies, blood was obtained from normal subjects at birth (cord blood) and up to 2 yr of age, as well as from healthy adult volunteers. For comparison, IgA-SC assays performed in healthy individuals of different ages and reported elsewhere[10,11] are also included.

Reverse enzyme-linked immunospot (RELISPOT) assay for Ig subclasses

The assay employs the basic RELISPOT method[14], as adapted in our laboratory[10], with some variations for this study, as noted below. Solid phase antibody specific for the $F(ab')_2$ fragment of human IgG was used to capture all Ig molecules (mainly through the light chains), as they were released from the B cells in short-term culture. Local zones of captured Ig of different classes were then identified by the biotinylated IgA-, G-, or M-specific antibodies (for IgA1 and IgA2 subclasses, refer to earlier paper[10]).

The 96-well, nitrocellulose-membrane plate was pretreated by overnight incubation with 100µl per well of goat anti-human IgG-$F(ab')_2$, at 20µg/ml in PBS. After emptying the fluid content, the plates can be stored at -20°C for up to 3 mo. At the time of testing, the plate was washed with PBS (five times) and exposed to 100µl of 5% FCS in RPMI 1640 medium, for at least 30 min in a 5% CO_2 incubator. Peripheral blood mononuclear cells (PBMC) were isolated, by Ficoll-Hypaque gradient centrifugation, from the heparinized blood obtained from study subjects. The cells, suspended in 100µl of medium supplemented with 5% fetal calf serum and 50 µg/ml gentamicin, were added to duplicate antibody-coated wells and cultured overnight in 5% CO_2 at 37°C. The number of cells used in each well was 5×10^5 PBMC for newborns and infants <2 months and $1\text{-}2 \times 10^5$ PBMC for older individuals. The cells are removed with 10 washes of PBS, followed by 10 washes of PBS with 0.05% Tween (PBS-Tween). The wells were then exposed, for 2 hr at room temperature, to 100 µl of the biotinylated goat anti-human IgA, IgM, or IgG antibodies diluted appropriately in PBS-Tween supplemented with 5% FCS. After 10 washes with PBS-Tween, the plates were exposed to horseradish-conjugated avidin for 1 hr. After washing 10 times with PBS-Tween and 10 times with PBS, were added 100 µl/well of the enzyme substrate solution containing 1.5 mM 3-amino-9-ethylcarbazole and 10 µl of 30% H_2O_2 in 30 ml of 0.1M acetate buffer (pH 5.0). The reaction was terminated after 20 min by removal of the substrate and washing with distilled water.

The appearance of dark-red circular foci, or "spots" on the nitrocellulose membrane indicate the areas where Ig-SC were incubated. The characteristic spots have a homogenously stained center and slightly diffuse periphery. The size of the different spots in the same well varied significantly, reflecting the different quantity of Ig molecules secreted by the individual lymphocytes. Negative control tests, with omission of either PBMC or the

isotype-specific antibodies, consistently resulted in the absence of spots. Moreover, previous experiments had demonstrated that spot formation by Ig-SC can be inhibited by cycloheximide[15] and that $CD3^+$ T cells did not generate any spots[10]. These observations indicated that the production of spots in the assay is directly related only to Ig-SC. From earlier studies[14], the correlation between the number of input cells and the number of SC would suggest that each spot represents one Ig-SC of the isotype specified by the biotinylated antibody used.

Serum IgA Measurements

Total serum IgA levels were quantitated using the ELISA technique[16].

Analysis of Lymphocyte Subpopulations by Flow Cytometry

Mononuclear cell subpopulations were determined by two-color direct immunofluorescence, using a whole blood staining technique with the appropriate monoclonal antibody and flow cytometry. The following pairs of fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (Becton Dickinson and Coulter Immunology) were used: Leucogate (CD45/CD14) to verify the gating on leukocytes; simultest isotype control; FITC-Leu 1 (CD5) combined with PE-Leu 12 (CD19).

A single laser flow cytometer (Becton Dickinson, San Jose, CA), which discriminates forward and right-angle light scatter, as well as two colors, was used with a software package (Simulset BD). The gated cells were verified with simultest Leucogate (CD45/CD14) to ascertain that a minimum of 90% of the events within the gates were of lymphocyte origin. A minimum of 1.5×10^4 cells are analyzed. Relative percentages and absolute numbers of cells in each subset were calculated using the simulset software and absolute lymphocyte counts. Two-color flow cytometry was performed using a whole blood procedure. Briefly, 10 µl of appropriately labeled monoclonal antibody was pipetted to its corresponding labeled tube; 100 µl of whole blood with an anticoagulant was added to each tube. The tubes were vortexed and then incubated at room temperature for 15-20 min in the dark, 2 ml of the FACS lysing solution (BD) was added to each tube. The tubes were vortexed and incubated at room temperature for exactly 10 min in the dark. The cells were pelleted by centrifugation at 250 *g* for 5 min at room temperature. The supernatant was aspirated, and the cells were resuspended in 2 ml of cold wash buffer solution (PBS + 2% FCS + 0.1% sodium azide). The cells were spun down again and the pellet was resuspended in 300 µl of cold 1% paraformaldehyde. The prepared tubes were stored at 4°C in the dark until flow cytometric analysis was performed.

RESULTS

Establishing Normal Levels of IgA-SC in Neonates

We first established the base level of IgA-SC in the blood of 48 normal neonates within 5 d of life. As illustrated in Fig. 1, the 97.5 percentile for the number of IgA-SC was 8 per 10^6 PBMC. It is of interest to note that in the same neonates, the 97.5 percentile for IgG-SC and IgM-SC was higher than for IgA-SC - being 28 for Ig-SC and 44 for IgM-SC.

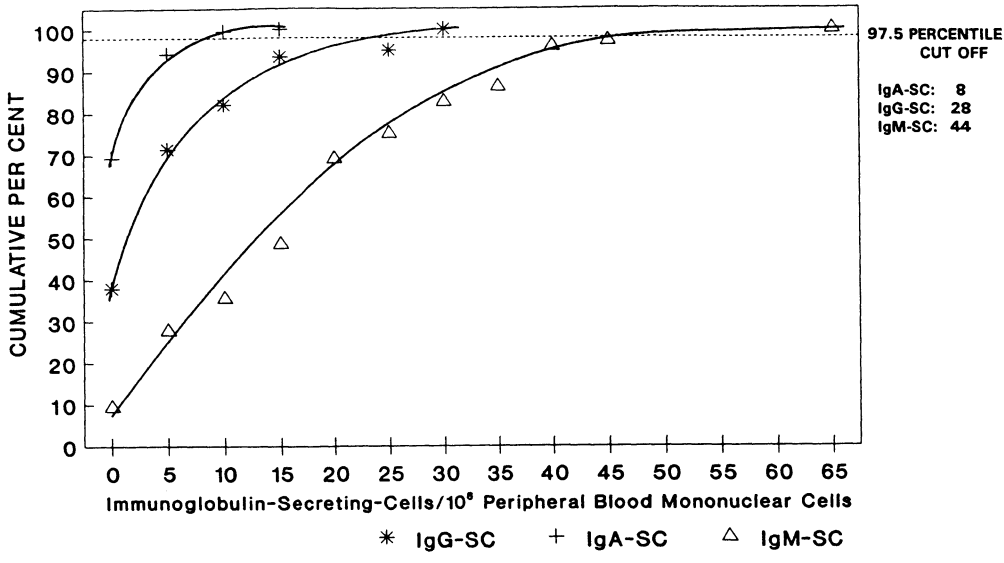

Figure 1. IgA-, IgG-, and IgM-SC/10^6 PBMC in 48 normal term and preterm neonates 1-5 d of age.

IgA-SC in Preterm and Term Neonates with Different Clinical Conditions

Neonates of different gestational ages with various clinical conditions were found to have levels of IgA-SC, IgM-SC, and IgG-SC above the 97.5 percentile for normals in different combinations (Table 1). Some neonates showed increased numbers of IgA-SC alone, or in combination with IgM-SC and/or IgG-SC; there were also others noted who had elevations of IgM-SC and/or IgG-SC without concomitant rises of IgA-SC. Thus, of the 254 neonates with different clinical conditions, 58 (23%) had elevations in the number of lymphocytes secreting one or more of the Ig isotypes. When any or all of the Ig-SC were increased over normal, only 4 of the 58 (7%) neonates showed elevations of IgA-SC alone; altogether IgA-SC were increased above normal in 25 (43%). When elevations of any Ig-SC occurred, IgA-SCs were elevated more often in preterm (19/34 or 56%) than in fullterm infants (6/24 or 25%). Whether IgA-SC were elevated or not, IgM-SC were almost always increased and IgG-SC less commonly.

More detailed observations on the 25 neonates with IgA-SC elevations are presented in Table 2. It can be noted that the numbers of IgA-SC/10^6 varied from 9 to as many as 554/10^6 PBMC. Elevations were noted in infants born with gestational ages as low as 25-30 wk and whose birth weights ranged from 600 to 1570 g. In fact, the highest numbers were found in the blood from babies below 37 wk, rather than in the full-term neonates, perhaps reflecting differences in antigenic stimuli.

Development of IgA-SC and B Cell Subsets Over the First Two Years of Life

A subgroup of neonates with <8 IgA-SC within the first 5 d were followed for the first mo of life. Although only small numbers of patients were followed, after the first 2 wk of life, elevations of IgA-SCs were

Table 1. Ig-SC in Premature and Term Neonates with Various Clinical Conditions

	Number of babies at given gestational ages with elevation of each isotype grouping[a]										
	Number of neonates tested	25-30 Weeks			31-37 Weeks			38+ Weeks			Total (%) positive for any Ig isotype
		IgA only	IgA and IgM &/or IgG	IgA & IgG	IgA only	IgA and IgM &/or IgG	IgM &/or IgG	IgA only	IgA and IgM &/or IgG	IgM &/or IgG	
a. Untreated maternal syphilis											
1. Baby symptomatic	11	0	2	1	0	3	1	0	2	0	9 (82%)
2. Baby asymptomatic	47	0	0	0	0	1	2	1	2	8	14 (30%)
b. Other bacterial infections[b]	108	1	3	2	0	6	4	0	1	6	23 (21%)
c. No apparent infection	88	1	0	1	1	1	4	0	0	4	12 (14%)
Total	**254**	**2**	**5**	**4**	**1**	**11**	**11**	**1**	**5**	**18**	**58 (23%)**

[a] In venous blood obtained within 5 d of life.

[b] Includes septicemia, meningitis, pneumonia, clinical sepsis and/or premature rupture of membranes; note that these infections are most often acquired at or after birth, rather then *in utero* such as congenital syphilis.

Table 2. Elevated Peripheral Blood IgA-SC Detected at 0-5 d in Premature and Term Neonates with Varying Clinical Conditions

Gestational age:	25-30 wks	31-37 wks.	38 wks
Weight range:	600-1570 g	830-2950 g	2290-3940 g
	Number of IgA-SC/10^6 PBMC		
Untreated maternal syphilis (n=9)			
Baby symptomatic	11, 35	20, 67, 229	29, 39
Baby asymptomatic	---	---	10^+, 30
Other bacterial infections[a] (n=11)	13, 19^+, 25, 26	9, 10, 31, 33 33, 57, 150, 554	18
No apparent infection (n=5)	15^+	9, 12, 14^b	---

[a] Includes septicemia, meningitis, pneumonia, clinical sepsis and/or premature rupture of membranes; note that these infections are most often acquired at or after birth, rather than *in utero*, such as congenital syphilis.

[b] Only IgA-SC elevated.

detected in the majority of the babies (Table 3). The slightly diminished IgA-SC responses in the lower gestational age babies might be due to their greater prematurity or to the impact of antibiotics. Antibiotics, which were given for prolonged periods to the smaller premature babies could have decreased the antigenic stimuli provided by the normal microbial gut flora. It should be noted that IgA-SCs were detected earlier in babies with congenital syphilis, and that the higher numbers of IgA-SC found in the first month of life were in those infants with intrapartum or prenatal infections[12].

To improve the overall perspective on the development of IgA-SC with age, the results which we have reported elsewhere[10,11] on other groups of infants, 1-48 mo of age, as well as on adults, are summarized in Table 4. The median number of IgA1-SC and IgA2-SC/10^6 PBMC was found to be higher in those infants between 1 mo and 2 yr than in adults. Not shown is the finding that the number of IgA-SC in both these infants and adults are usually higher than those for IgG-SC, and much higher than those for IgM-SC.

The total numbers of B cells, and those of $CD5^+$ and $CD5^-$ B cell subsets[13], are also included in Table 4 for comparative purposes. From 1-48 mo of age, the number of B lymphocytes is 2-3 x higher than that found in adults (as are the number of total lymphocytes and of T lymphocytes). Interestingly, $CD5^+$ B cells, which predominate at birth continue to increase in numbers during the first 2 yr of life. The numbers of $CD5^-$ B cells, which are

lower at birth, increase more rapidly to almost equal the number of $CD5^+$ B cells over the first 2 yr. Thereafter, the numbers of $CD5^+$ cells decrease and of $CD5^-$ B lymphocytes increase[13], so that by adulthood, the numbers of $CD5^-$ B cells are 3 or more times greater than the numbers of $CD5^+$ B lymphocytes.

Table 3. Changes in IgA-SC Over Time in Neonates of Different Gestational Ages with no Elevation of Ig-SC at Birth

	Numbers of neonates with elevations of IgA-SC/total number tested at each age group (% of total)			
Gestational age	0-5 d	6-14 d	15-21 d	22-31 d
25-30 wk	0/15	5/11 (45%)	5/11 (45%)	4/8 (50%)
≥ 31 wk	0/64	6/12 (50%)	3/4 (75%)	2/2 (100%)

Table 4. Development of IgA1- and IgA2-SC, B Lymphocytes and $CD5^+$ and $CD5^-$ B Cells in Neonates, Older Infants, and Adults

	0-5 d		Ages 1-48 mo		Adults	
	Range	Median	Range	Median	Range	Median
number/10^6PMBC	(n=19)		(n=48)		(n=32)	
IgA1-SC	0-8	0	428-2350	505	39-1199	243
IgA2-SC	0-8	0	16-450	121	28-332	86
number/mm3	(n=8)		(n=40)		(n=7)	
Total B lymphocytes	20-800	307	130-4000	1376	100-800	336
$CD5^+$ B cells	10-500	192	90-2400	746	20-200	84
$CD5^-$ B cells	10-600	140	30-1600	634	70-600	252

Comparison of IgA-SC and Serum IgA Levels

Of the 10 neonates with elevated numbers of IgA-SC, only 3 were found to have serum levels of IgA over 2 mg/dl. A more detailed comparison of IgA-SC and serum IgA levels in 32 infants 1-48 mo of age is presented in Fig. 2. No significant correlation was found between the IgA-SC numbers and serum IgA levels. Most often, the number of IgA-SC were elevated, with very low serum levels measured in the same individual.

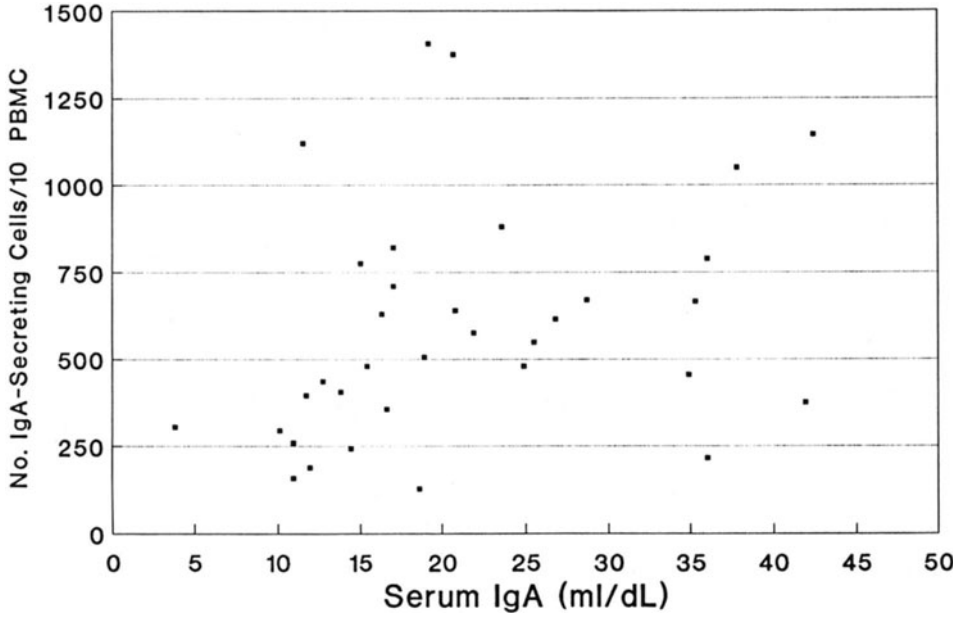

Figure 2. Serum IgA vs IgA-SC in children 1-48 mo of age.

DISCUSSION

As reviewed elsewhere[5,6] and at this symposium, the human fetus, within 20 wk of gestation, possesses the necessary genetic and cellular apparatus to express all Ig isotypes, including IgA1 and IgA2. Yet, with few exceptions, primarily as a result of infection[17,18], IgA has not been detectable in serum or secretions until a wk or more after birth, even in full-term neonates. In this report, we now provide evidence that, with the appropriate antigenic stimulus, such as that provided by congenital syphilis, neonates as early as 25 wk gestation have circulating IgA-SC cells often without detectable serum IgA. The numbers of IgM-SC, and less often, of IgG-SC, were more often found to be elevated above baseline than were those of IgA-SC. Of some interest was the observation that when elevations in the numbers of any IgSC occurred, the numbers of IgA-SC were more often increased in neonates below 38 wk gestation (56%) than in fullterm infants (25%). The higher number of IgM-SC than those for the other isotypes in apparently normal infants corresponds to the findings of serum IgM levels in many such neonates at birth[1,2]. Indeed, increased levels of serum IgM at birth have been suggested as a means to suspect *in utero* infections[17], although the presence of rheumatoid factor has actually proven to be a better marker[18]. The presence in the serum of transplacentally-transmitted IgG has not heretofore made it possible to measure readily neonatal IgG synthesis, until our current application of the RELISPOT methodology to this issue[12]. More specifically, in relation to the central theme of this symposium, the often undetectable levels of serum IgA at birth, even in newborns with intrauterine infections, has made enumeration of circulation IgA-SC of particular relevance.

The ability to demonstrate IgA-SC in neonates with intrauterine infections and other putative infectious stimuli, as well as the large number of IgA-SC found in infants 1-48 mo of life (Table 4), when serum IgA levels are usually low (Fig. 2), suggest that the lymphocytes detectable in the blood are primarily mucosal IgA cells in transit[19]. The numbers of IgA-SC were found to be higher in infants between 1 mo and 2 yr of age than in adults, in whom serum IgA concentrations are much higher. Nevertheless, in both infants and adults, the numbers of IgA-SC were often greater than those of IgG-SC, in contrast to the relative concentrations of IgA and IgG in the serum. These findings are consistent with the earlier calculations of Delacroix[20] that the daily production of IgA is greater than that for IgG. The comparatively higher numbers of IgA1-SC than IgA2-SC are also consistent with the ratios for IgA1- and IgA2-containing cells in several mucosal tissues[19]. The data presented here indicate that the ratio of IgA-SC to the number of circulating B cells in normal infants or adults ranges between 1 in 400 to 1 in 1000. More direct evidence that the majority of IgA-SC are indeed B lymphocytes in transit is currently being sought.

We also need more direct evidence to determine which subset of B lymphocytes is related to the IgA-SC detected in peripheral blood. Several reports have noted that the major B lymphocyte subset found in the fetus and neonates at birth are $CD5^+$, corresponding to the Ly1 subset in mouse fetuses[7-9]. Of particular relevance is that the lymphocytes found in the Peyer's patches of the human fetus are primarily of the $CD5^+$ subset. The $CD5^+$ B lymphocytes in humans, or $Ly1^+$ B cells in mice, have also been shown to secrete autoantibodies and rheumatoid factor and have been suggested to have a possible role in primary defense against the gut flora[7-9]. Although not performed in the same infants or adults as those in whom the Ig-SC assays were studied, the results in Table 4 support the view that $CD5^+$ B cells may be associated with S-IgA activity. Indeed, studies currently conducted by Peters *et al.*[21] indicate that many of the B cells secreting IgA in the gut are of the $CD5^+$ subset.

CONCLUSIONS

1. The normal pre-term or full-term neonate has <8 IgA-secreting cells (SC) per 10^6 peripheral blood mononuclear cells (PBMC), in contrast to higher number of IgM-SC (as many as $44/10^6$ PBMC) and IgG-SC (as many as $28/10^6$ PBMC).

2. Neonates, as early as 25 wk gestation or 600 g at birth, are able to respond to intrauterine stimuli, most often associated with infectious agents, e.g. syphilis, by demonstrating elevations of IgA-SC. Such elevations are usually associated with elevations of IgM-SC and/or IgG-SC, although there may be no rise in IgA-SC numbers when the Ig-SC of other isotypes, particularly IgM, are elevated.

3. Elevated numbers of IgA-SC are found in the peripheral blood of the majority of infants by 1 mo of age, with higher numbers being demonstrated in most infants 1 mo to 2 yr of age than in normal adults,

perhaps reflecting exposure to a larger number of new antigenic stimuli during infancy than adulthood.

4. Indirect evidence suggests that the circulating IgA-SC detected during the neonatal period (occurring most often as a result of infections, or during the first 2 yr of life in normal infants) resulting from various endogenous and microbial antigenic stimuli, represent mucosal B lymphocytes in transit. The increase in both $CD5^+$ and $CD5^-$ B lymphocytes observed during the first 2 yr of life also suggests that the IgA-SC may be related to both B cell subtypes.

ACKNOWLEDGEMENTS

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Note in Galley Proof: Zubler's group (*Clin. exp. Immunol.* 84:389, 1981) has recently shown that newborn B cells are competent to differentiate into high-rate IgM, IgG, and IgA secreting cells during a 10-day culture period in the presence of mutant EL-4 thymoma cells and T cell supernatants. In an editorial in the same issue (p. 373), Gordon reviewing also earlier work arrives at the conclusion, for which the current report provides further evidence, that neonatal B cells have the inherent potential for stimulation-approaching that of adult B lymphocytes.

DEVELOPMENT OF T CELLS WITH MEMORY PHENOTYPE IN INFANCY

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INTRODUCTION

Studies in mice give a reasonably clear view of the genes in he germline which are rearranged to give the αβ heterodimer of the T cell receptor for antigen. The role of the thymus in modulating the germline repertoire through positive and negative selection has been demonstrated in transgenic mice[1] and in the context of the mixed lymphocyte stimulatory (MIs) antigens, which result in the deletion of entire families T cells using certain β chain families for their V regions[2]. Comparable T cell selection events are likely to occur during the production of thymus cells in human infants. It is T cell maturation in he thymus which provides the fetus and newborn with a clonally diverse population of naive T cells which is available for a response to an environmental antigen. T cell responses to antigen are characterized by proliferation (synonymous with clonal expansion) and the production of memory T cells. The recognition that human T cells express different isoforms of the T200 common leukocyte antigen (CD45) depending on their prior proliferative responses has made the process by which the naive T cell pool is educated by antigen open to examination[3].

Differential RNA splicing of CD45 gives a total of 5 isoforms, depending on which exons are spliced out[4]. The two largest forms have molecular masses of 220 and 205 kDa and are bound by CD45RA antibodies such as 2H4[5]. The smallest version of CD45, CD45R0, has all 3 exons spliced out, a molecular mass of 180 kDa and is bound by the UCHL1 antibody[6].

T cells in the thymus with precursor function express CD45RA[7] and this is the phenotype of over 90% of the T cells of healthy newborns[8]. Mitogen-stimulated CD45RA T cells start to express CD45R0 after 2-3 days of culture and by 6 days most have ceased to express CD45RA[9]. The studies reported here were undertaken to determine whether the rate at which T lymphocytes with the memory cell phenotype (CD45RO) normally appeared in blood, and whether this would be influenced by environment.

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RESULTS

Development of CD45ROR T Cells in Healthy Infants

The percentage of memory T cells in healthy infants increases logarithmically during the first years of life[10], to plateau somewhat after puberty (Fig. 1). The percentage of CD8 cells with the CR45RO isoform is 10-20% lower than the CD4 subset, but the slope is essentially the same for both subsets and for T cells using αβ or γδ receptors (data not shown).

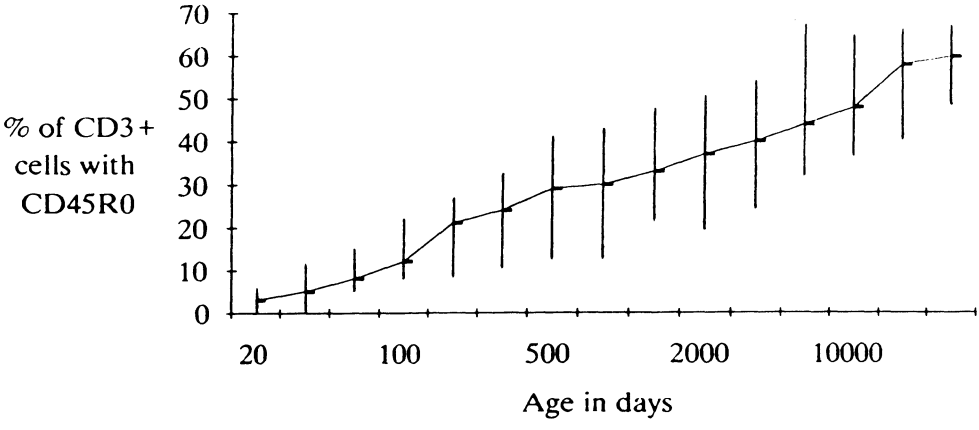

Figure 1. The percentage of T cells expressing CD45RO plotted against age for healthy controls. Ficoll-separated mononuclear were stained with FITC-CD3 and PE-UCHL1 and analyzed on an EPICS C to determine the percentage of double-positive cells.

To explore the effect of neonatal antigen stimulus on the appearance of CD45RO+T cells we performed phenotypic analysis of blood from a series of infants who received blood transfusions[11]. The results (Fig. 2) indicate that higher frequencies of CD45RO+ cells are found in this population, and that this increase occurs amongst T cells expressing CD4 or CD8 and using either of 2 Vβ families, Vβ5 or Vβ8 (data not shown). Overall these results argue for a diverse T cell response to the immune stimulus of an allogeneic blood transfusion.

T Cell Activation at a Single Cell Level

The relatively slow accumulation of CD45RO+ cells in the blood of healthy infants might reflect the dynamics of T cell output from the thymus, the development of peripheral lymphoid tissues and the response to antigen or it might be an indicator that naive T cells are relatively unresponsive to triggering. Evidence to support the latter view comes from Byrne *et al.*[12] and Sanders *et al.*[13] reports of a reduced response by naive T cells to CD3-mediated triggering. While these data are clearly reproducible (Table 1) they are difficult to reconcile with the ability of human newborns to respond well to certain immunizations, such as BCG or vaccinia.

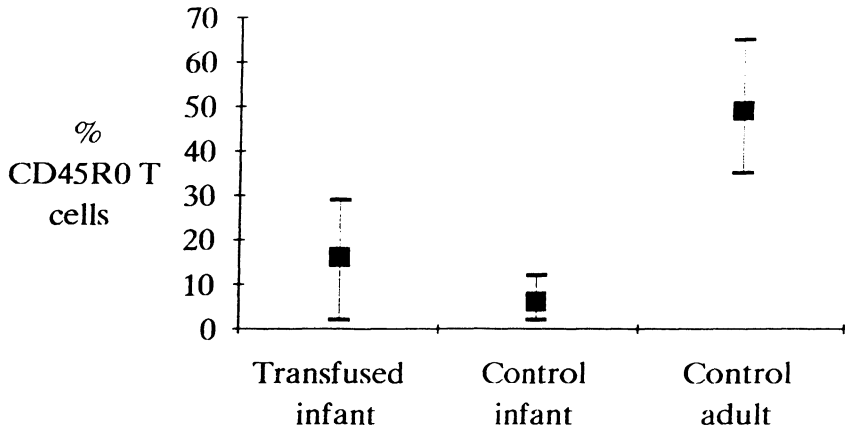

Figure 2. Mean and range of % of blood T cells expressing CD45RO for infants 1-14 weeks of age, control or transfused, and healthy adults.

Table 1. CD45RO+ and CD45RA+ Cells Respond Equally to Staphylococcal Enterotoxin B when Antigen-Presenting Cells are not Limiting

Stimulus[a]:	0	CD2[b]	CD3	SEB
Cells cultured:				
Unseparated	225±30	3747±120	64776±2320	22779±3310
$CD45RA^+$	277±33	11408±223	1994±188	24637±2900
$CD45RO^+$	360±46	3727±88	14337±2080	23445±2870

[a] results are expressed as thymidine uptake by 5×10^4 cells/well after 4 day culture

[b] CD2 was $T11_2$ and $T11_3$ at a 1:200 dilution, CD3 is UCHT1 at 1 μg/ml and SEB at 1 μg/ml

CD3 triggers an equivalent $Ca2^+$ flux by naive and mature T cells (Table 2) and the lack of proliferation is less apparent when other T cell ligands, such as Staphylococcal enterotoxins, are used (Table 1). A speculative interpretation is that the naive T cell proliferative response has a greater requirement for co-stimulator factors (such as IL-1 and IL-6) that the response of the $CD45RO^+$ subset. A recent report from Koulova *et al.*[14] identified a specifically unresponsive subset of the $CD45RA^+$ cells.

A subset of human T cells proliferates in tissue culture in response to IL2 alone. The frequency of IL-2 responsive cells is greater in the $CD45RO^+$ than the $CD45RA^+$ subset[15] but these data raise the possibility that some T cells may switch their CD45 phenotype as a consequence of by-stander activation, rather than from specific antigen triggering.

Infections, Immunization, and T Cell Responses During the First Year of Life

Effective antibody- and cell-mediated immune responses to immunizations given in the first weeks of life provide the strongest evidence for the immunological responsiveness of the newborn[16,17]. However, when the immune stimulus is in the form of a cytolytic virus the outcome can be different. Newborns with congenital rubella continue to excrete the virus for years and are slow to make immune responses to it[18]. Infants who have neonatal herpes simplex virus infections often develop cutaneous recurrences of the virus and the frequency of peripheral blood $CD4^+$ T cells, which

Table 2. Percent of Cells Showing Ca^{2+} Flux Following CD3 Activation[a]

	Subset tested	Percent of cells responding at time: 0	20	40	60	80
Adult	$CD45RA^+$	4±1	18±4	47±5	50±5	48±3
	$CD45RO^+$	7±2	17±4	48±8	52±8	50±6
Newborn	$CD45RA^+$	3±1	19±3	44±6	51±4	48±6
	$CD45RO^+$	4±4	15±2	37±8	49±5	45±6

[a] cells were loaded with Fluo 3 (Molecular Probes, Eugene OR), triggered with CD3 antibody and analyzed at 0.5-1 x 10^3 cells per sec. The time units shown are sec. Results are the mean ± 1 SE for 3 samples of each subset, which was identified as $CD45RO^-$.

proliferate in cultures stimulated by viral antigens remains low for months or years[19]. The reasons why T cell responses to rubella and herpes simplex virus fail to mature following neonatal infection are poorly understood. Perhaps viral antigen centers the thymus and interferes with the production of T cells with specificity for these viruses. The effect is clearly antigen specific in that normal immune responses are made to other antigens.[20].

Prematurity is a common problem and the studies of Bernbam *et al.*[21] and Koblin *et al.*[22] suggest that ex-premature infants may be slower than age-matched controls in initiating a response to diphtheria pertussis-tetanus toxoid immunization. We recently measured the antibody and T cell response of a group of prematures and controls to influenza virus immunization. The results for the IgG response to A/Taiwan strain of influenza virus, summarized in Fig. 3, suggest that the slow response of ex-prematures persists into the first year of life. Only 38% of the ex-premies achieved detectable frequencies of influenza virus-specific T cells in their blood, compared with 79% of the controls.

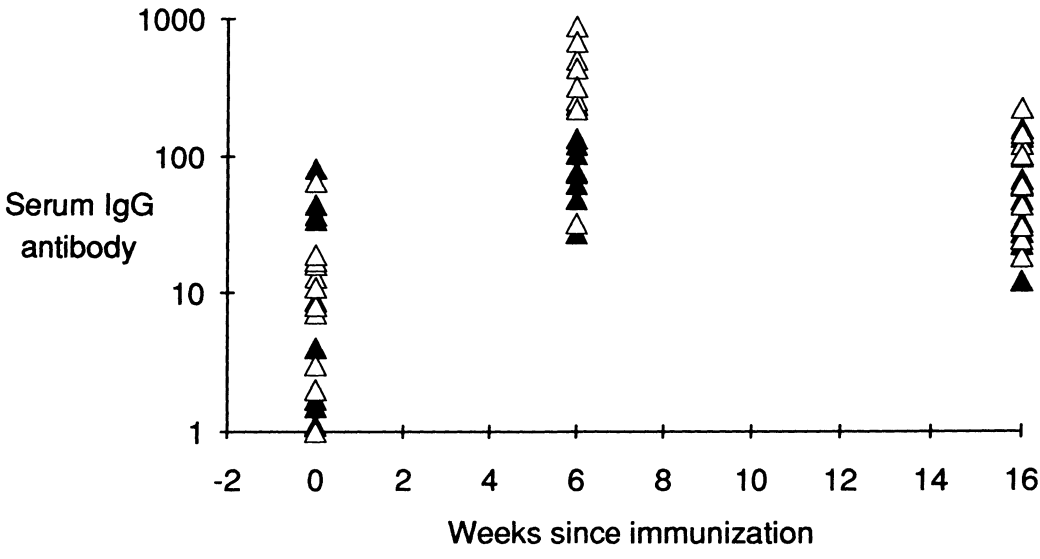

Figure 3. Serum IgG antibody to A/Taiwan before and after primary immunization. ▲ ex-premies with bronchopulmonary dysplasia; Δ healthy controls.

CONCLUSIONS

The switch from $CD45RA^+$ to $CD45RO^+$ phenotype appears to reflect an age-related accumulation of memory cells in the circulation. The phenotypic changes are associated with functional changes such as an increased proliferative response to IL-2 and an increased production of interferon-γ. Studies to date have necessarily focussed on positive (and so measurable) responses. Experiments in mice are starting to show that inactivation is an alternative outcome for the interaction of antigen with T cells, and that T cells which have become anergic to stimulation may persist in the circulation[23,24]. Whether similar events can occur in humans remains to be seen.

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THE EFFECT OF HUMAN MILK, PROTEIN-FORTIFIED HUMAN MILK AND FORMULA ON IMMUNOLOGIC FACTORS OF NEWBORN INFANTS

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INTRODUCTION

Malnutrition is a well known cause of secondary immunodeficiency[1]. Many immunologic abnormalities have been found in infants following intrauterine malnutrition[2] which may persist for months or years, leading to higher morbidity and mortality[2,3]. As far as we know, there are few reports in the literature describing the effect of early-protein post-natal malnutrition on the immune response. Post-natal malnutrition is more frequently encountered now with the survival of very low-birth weight (LBW) neonates and an extensive research has been directed towards their nutritional requirements. Fortification of human milk with proteins has been suggested during the last years in feeding LBW infants[4,5].

The aim of our study was to investigate and compare the effects of banked human milk, protein-fortified banked human milk, and a protein-fortified banked commercial formula for LBW infants on the development of certain immunologic factors in the newborn infant.

MATERIALS AND METHODS

Infants and Feeding

Forty-two LBW infants were studied whose birth-weights and post-natal ages at the beginning of the study are shown in Table 1. Eleven of them received human milk from the milk bank of our unit. This was early-lactation milk which was pasteurized at 52°C for 30 min and subsequently stored at -20°C. Twelve infants received banked human milk supplemented with a human milk fortifier (Eoprotin - 06) kindly offered by Milupa-Germany. The composition of Eoprotin - 06 is shown in Table 2. The protein consists of a mixture of purified bovine milk protein fractions. Three grams of this fortifier were added per 100 ml of human milk. The remaining 9 infants received a commercial formula (Prematyl) prepared for LBW babies. The main constituents in the three types of milk used are shown in Table 3.

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Table 1. Clinical Characteristics and Types of Feeding of LBW Infants

Type of feeding	No. of infants	Birth weight (g) (means+SD)	Post-natal age in days of oral feeding alone (means+SD)
Fortified banked human milk	12	1413±460	21±15,7
Unfortified banked human milk	11	1377±307	21,2±17,3
Formula	9	1520±220	13,1±6,8

Table 2. Composition of Human Milk Fortifier Eoprotin-06 (3.0 g)

Protein	: 0,6g
Carbohydrate	: 2,1g
Energy	: 11 Kcal
Na	: 23mg
Cl	: 12mg
Mg	: 2mg
Ca	: 39mg
P	: 28mg
Ca:P (weight ratio)	: 1.4
Vitamin A	: 30 µg
Vitamin C	: 20 µg
Vitamin E	: 200 µg
Vitamin K_1	: 0,2 µg

The infants received mainly total parenteral nutrition during the first days of their life; however, partial oral feeding was usually commenced within the first post-natal week. The study started on the day that the infants could tolerate and were covered with oral feedings alone. The mean ± SD post-natal age in days at which the study started in the 3 groups of infants is shown in Table 1. The study lasted for 3 weeks and none of the infants had any medical problems during that time.

Table 3. Composition of Unfortified, Fortified Human Milk, and Formula

	Unfortified human milk (HM)	Fortified human milk (FHM)	Formula (F)
Protein g/dl	2,1	2,68	2
Carbohydrate g/dl	7,7	9,7	7,7
Lipid g/dl	2,5-3	2,5-3	3,5
Ca mg/dl	19,6	41,4	70
P mg/dl	10,4	27,5	35
Kcal/dl	60-62	72-74	70

Growth and Laboratory Investigations

For the determination of optimal nutrition and its effect on immunologic factors the weight gain, head circumference, length and mid-arm circumference of the infants were determined on the 1st and 21st days of the study.

As immunologic factors, the immunoglobulins IgG and IgM as well as the third component of complement (C3) were determined. For the determination of the immunologic parameters, the solid phase of the enzyme-linked immunoassay (ELISA) was used[6]. We applied a 2 stage sandwich technique. We used rabbit anti-human IgG, IgM, and C3 antibodies (DAKO, Denmark). Conjugation was accomplished with the enzyme horseradish peroxidase (Sigma, St. Louis, MO, USA) by the periodate method. The sensitivities of the assays were 3 ng/ml for IgG and IgM and 1 ng/ml for C3.

As the values of the immune factors were not distributed normally, statistical analyses were performed after logarithmic transformation of the data. Comparisons were made using the Student's t-test and the paired t-test.

RESULTS

Growth

The weight gain during the 3 week study period in the 3 groups of babies is shown in Fig. 1. The infants who received fortified human milk (FHM) gained 652 $\pm$ 142 g, the ones with unfortified gained 501 $\pm$ 250 g, and those who received formula 572 $\pm$ 119 g. The difference in weight gain between those receiving fortified human milk and those receiving unfortified human milk (HM) was statistically significant ($p < 0.05$). However, it should be taken into consideration that the quantity of milk received differed in the 3 groups of babies. Thus, the infants with the FHM received in total during the study period 5.588 $\pm$ 1349 Kcal, the infants in the HM received 4.360 $\pm$ 1068 Kcal, and the infants fed with formula received 4.270 $\pm$ 114 Kcal. The weight gain/1000 Kcal did not differ statistically in the 3 groups of infants.

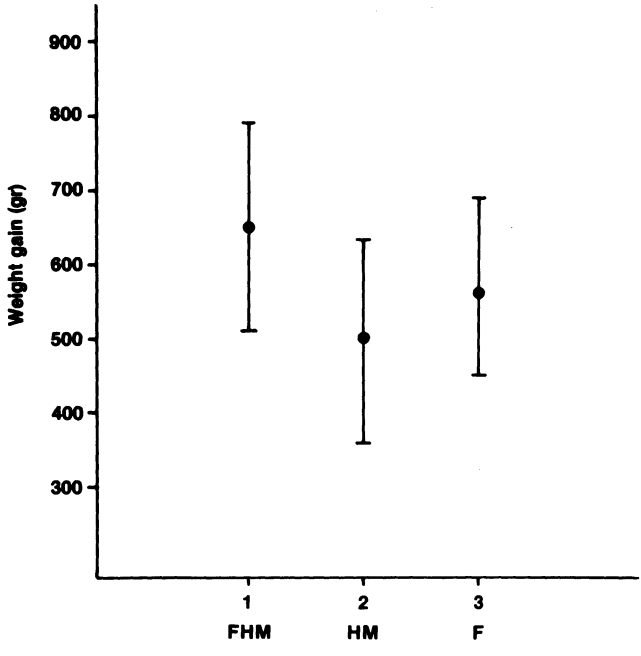

Figure 1. Weight gain (M±SD) in infants receiving the three kinds of milk. FHM, fortified human milk; HM, human milk; and F, formula.

There were no statistically significant differences in the growth of head circumference in the 3 groups of infants.

Regarding the length, infants who received FHM showed a better gain when compared to those who received HM ($p < 0.057$). No significant differences were found between the gain in length in formula fed infants when compared with the gain in the other two groups.

Mid-upper arm circumference values being similar on day 1 in the 3 groups of infants were significantly higher on day 21 in the babies fed FHM when compared to those fed HM ($p < 0.02$). In the formula-fed infants, mid-upper arm circumference values did not differ when compared with those of the two other groups on the 21st day of the study.

Serum Immunoglobulins

The serum means and ranges of the IgG immunoglobulins in the 3 groups of babies are plotted in Fig. 2 while the means ± SD are shown in Table 4. On the first day of the study, the values were similar between infants fed FHM and HM. The IgG values of the infants who received formula were significantly higher than those of infants fed HM ($p < 0.01$).

On the 21st day of the study, the IgG values in babies who received FHM were significantly higher than those of infants who received HM ($p < 0.01$). The values of the infants who received formula were similar to those of infants with FHM and significantly higher than those of infants who received HM. However, as the values of infants fed with formula were significantly higher on day 1 than those of infants fed HM, the comparison is not valid.

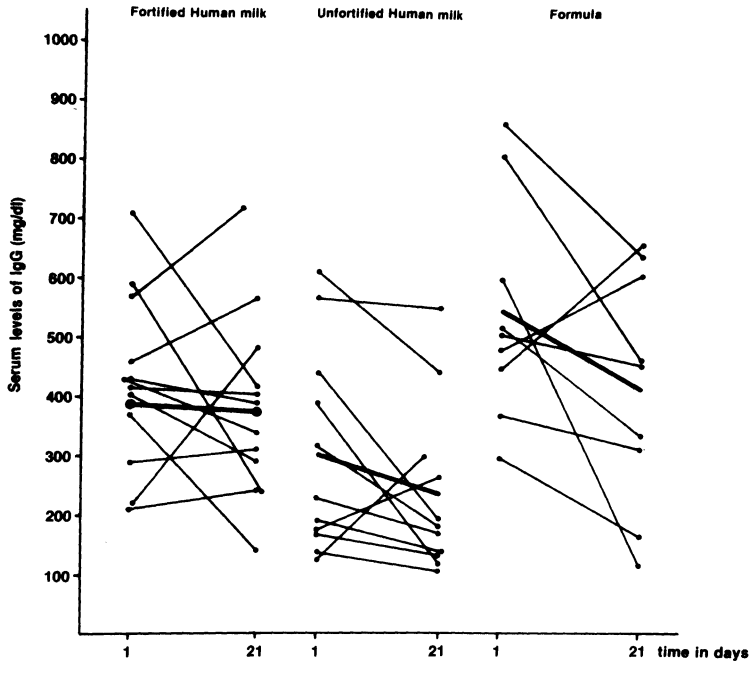

Figure 2. Serum levels of IgG (means and ranges) in low birth weight infants fed: fortified human milk (FHM), unfortified human milk (HM), or formula (F).

Table 4. Serum IgG Levels (mg/dl) in the 3 Groups of Infants (means±SD)

	Day 1	Day 21
FHM	381±172	379±157
HM	305±173	234±146
Formula	549±186	417±196

The paired t-test within each group of infants did not show any statistically significant differences. However, the trend of the IgG values to remain steady in the groups of infants who received FHM and to fall in the other 2 groups of babies is obvious (Fig. 2).

The serum means and ranges of the IgM immunoglobulins in the 3 groups of babies are plotted in Fig. 3 while the means ± SD are shown in Table 5. The IgM values in both groups of babies, those who received FHM and those who received HM, tend to rise and they are similar both on the 1st and 21st day of the study. The values of the formula-fed infants are higher both for days 1 and 21 when compared to those in infants fed with FHM or HM.

The serum means and ranges of the C3 levels in the 3 groups of infants are plotted in Fig. 4 and the means ± S.D. are shown in Table 6. There are not statistically significant differences between the values on days 1 and 21 in the 3 groups of infants studied. However, there is a very obvious trend of the levels of C_3 to rise in infants who received FHM and to drop in those who received HM, or formula.

Table 5. Serum IgM Levels (mg/dl) in the 3 Groups of Infants (means±SD)

	Day 1	Day 21
FHM	21±13	26±18
HM	22±7	29±16
Formula	40±26	47±24

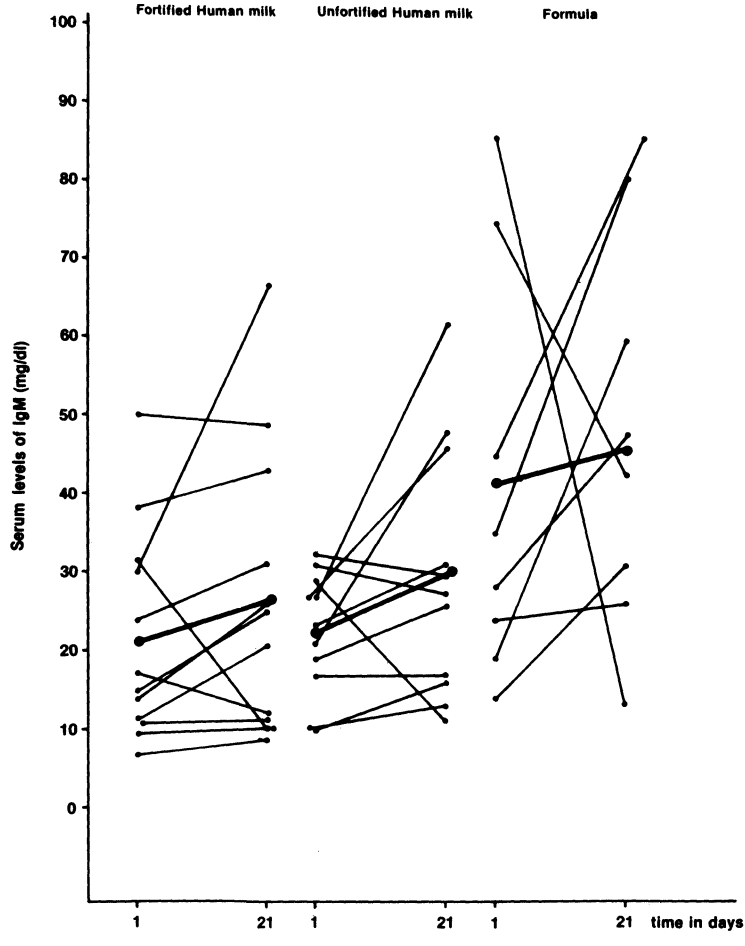

Figure 3. Serum levels of IgM (means and ranges) in LBW infants fed: fortified human milk (FHM), unfortified human milk (HM), or formula (F).

Table 6. C3 Levels (mg/dl) in the 3 Groups of Infants (means±SD)

	Day 1	Day 21
FHM	70±32	77±26
HM	111±73	79±26
Formula	79±27	70±22

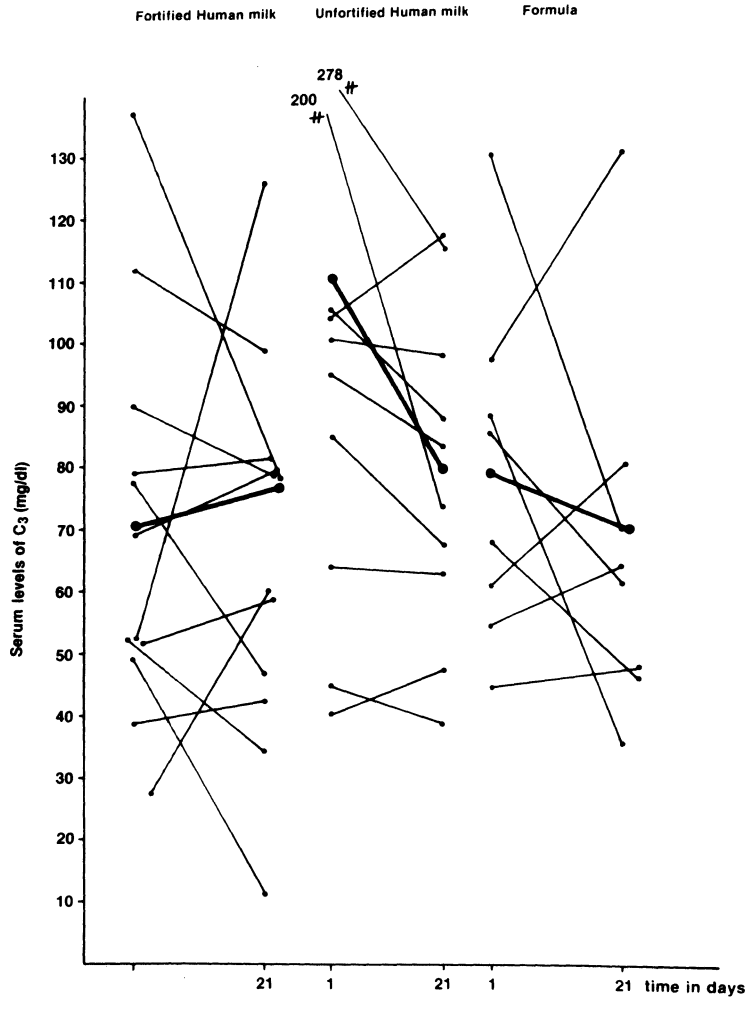

Figure 4. Serum levels of C3 (means and ranges) in LBW infants fed: fortified human milk (FHM), unfortified human milk (HM), or formula (F).

DISCUSSION

As we have seen, growth was much better in infants who received FHM than in those who received HM. Other investigators had similar findings and, furthermore, they showed that the weight gain composition in babies fed FHM was similar to that obtained by the fetus[7]. The infants fed FHM had a better weight gain than those on formula, as they tolerated larger quantities of milk. There is no doubt that LBW infants on FHM had the best nutrition and received optimal quantities of protein.

We found that IgG levels on day 21 were significantly higher in infants fed FHM when compared to those found in infants fed HM. The higher IgG levels found on day 1 in formula-fed infants could be attributed to the higher birth weight of these infants. Thus, values on the 21st day of formula-fed infants could not be compared with values of babies fed FHM or HM.

Studying full-term infants, Stephens *et al.*[8] found no differences between those receiving human milk and cow's milk. They do not mention the protein content of each type of milk. However, it is well known that full-term infants have higher levels of IgG at birth and a much better ability to produce it.

Studying premature infants 31-32/52 of gestation between 1 week and 4 months after birth, Savilahti *et al.*[9] found significantly higher IgG levels in the infants receiving human milk when compared to those receiving cow's milk. The difference in protein intake in the two diets was small. They suggested that LBW infants may absorb IgG from human milk. However, our findings do not support this proposal because infants in both groups fed FHM and HM in our study should have similar IgG levels.

Zoppi *et al.*[10] performed an interesting study: they applied two different diets to full-term neonates both supplying 100 K cal/g, but one contained 2 g and the other 4 g of cow milk protein. The infants who received the low protein diet had significantly lower IgG levels and increased morbidity.

We believe that both optimal protein intake and immunostimulating factors in FHM, like growth factors and lymphokines, may contribute to the faster production of IgG by the LBW neonate. It has been shown that very immature infants already in the first week after birth can produce IgG1, and IgG3[11] immunoglobulins.

No differences were found in the IgM serum levels in the 3 groups of babies during the study period, although values were, from the beginning of the study, higher in the formula-fed group of infants. Stephens *et al.*[8] found significantly higher levels of IgM antibodies to *E. coli* "O" antigens in bottle-fed compared to breast-fed infants.

The complement levels rose in the infants who received F.H.M. and fell in those who received HM, or formula. Zoppi *et al.*[10] reported low complement levels in infants who were receiving low protein diets.

From our findings, it appears that FHM elicits the best immunologic responses and it should be given to the vulnerable LBW infants during the critical first days of their life.

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ONTOGENY OF THE MUCOSAL IMMUNE RESPONSE IN CHILDREN

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INTRODUCTION

The theory of mucosal immunology is now well advanced and supported by experimental data. It is clear that mucosal immune responses can be manipulated and that individuals who can be defined as having a deficit in mucosal immune competence appear to be prone to chronic conditions such as recurrent infection, atopy and clinical asthma.

A deficiency in secretory IgA (S-IgA) at mucosal surfaces may facilitate the entry of allergens and pathogens through the gastrointestinal or respiratory mucosa. Mucosal S-IgA deficiency occurs in the neonatal period and the length of deficiency varies[1,2]. The incidence of allergic disease is much higher in IgA-deficient individuals[3] and in retrospective studies of children with atopy[4,5] or asthma[6,7] the salivary IgA levels have been reported to be lower than in control subjects. Transient serum IgA deficiency was reported to be associated with the development of infantile atopy[8] and a prospective study of children with a family history of atopy demonstrated significantly lower levels of salivary IgA in children at 4 and 8 months who subsequently developed atopy[9]. The data on whether respiratory infections in the first year of life predisposed to the development of asthma or atopy is conflicting. Several studies have reported a significant association between respiratory viral infection and the subsequent development of asthma or atopy[10-13] while other studies failed to show a difference with control groups[14,15].

Over the past decade we have conducted studies to assess the ontogeny of the mucosal immune system. In a longitudinal programme, 263 healthy full term children were studied from birth to 5 years of age. The mucosal immune status of the children was assessed using saliva collection at regular intervals. Two cross-sectional studies have also been conducted: a study of normal school children aged 5 to 13 years; and a study of Papua New Guinea highland children aged from birth to 5 years. Patterns of mucosal immune ontogeny have been established, factors which affect the ontogeny pattern investigated and markers of mucosal immunocompetence identified.

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THE ONTOGENY OF THE MUCOSAL IgA RESPONSE

Salivary IgA was not detected at birth and in the first week of life was present in only 14% of samples. Salivary IgA levels increased rapidly from the first week of life to peak at 6 weeks of age (median = 19 mg/l; 95% confidence interval (CI), [1.0, 87.6] mg/l). The concentration decreased to lower levels at 12 weeks (median = 13.5 mg/l; 95% CI, [2.7, 51.0] mg/l) and several infants (2-6% of cohort) had consistently low levels and transient absences of salivary IgA during the first 4 years of life (unpublished observations).

Up to 4 years of age the geometric mean of total salivary IgA remained below 20 mg/l. At 5 years of age the concentration of salivary IgA significantly increased ($p<0.05$; mean = 27.0 mg/l; 95% CI [20.2, 36.3] mg/l)[15]. In an independent cross-sectional study[15] of school aged children the mean salivary IgA level was 100.7 mg/l (95% CI, [81.5, 124.4] mg/l) at 5 years of age. The IgA levels in the school children decreased between 5 years and 7 years (mean = 49.3 mg/l; 95% CI [38.0, 64.0] mg/l). The IgA levels remained relatively constant after 7 years of age and were similar to the levels observed in adults (mean = 53.2 mg/l; 95% CI [38.9, 72.8] mg/l).

IgA antibody specific against *Escherichia coli* O antigen was measured to assess the pattern of development of specific immunity in the mucosal system to a common antigen[15]. *E. coli* has been shown to be an almost universal inhabitant of the intestinal tract of man and to colonize the gut soon after birth. Low levels of IgA antibodies were detected during the first 4 years of life. The levels increased 2-3 fold when children attended school (mean = 2.00 ELISA Units [EU/ml]; 95% CI [1.31, 3.03] EU/ml) and remained relatively constant to 8 years of age after which the levels increased towards adult levels (mean = 8.20 EU/ml; 95% CI, [5.33, 12.63] EU/ml).

FACTORS WHICH AFFECT THE PATTERN OF IgA ONTOGENY

Our studies have identified 5 factors which modify the pattern of IgA ontogeny, namely: the mucosal IgM response, mucosal permeability, feeding, environmental exposure and nutritional status.

The Mucosal IgM Response

The ontogeny pattern for total IgM was similar to that described for IgA[2]. IgM was absent in saliva at birth. It was detected in 15-20% of infants between 4 and 26 weeks of age and was observed in approximately 10% of children older than 26 weeks. The presence of IgM was significantly associated with IgA. The level of salivary IgA was significantly higher in infants with positive IgM measurements than for those with no IgM detected at almost all ages studied up to 4 years. This suggests that the appearance of both IgA and IgM in mucosal secretions is stimulated by exposure to "novel" antigens or to polyclonal mitogens and that in neonates mucosal IgA deficiency is not compensated for by IgM antibodies as is the case in congenital IgA deficiency in adults and children. The strong association between salivary IgA and IgM levels also supports the concept of local plasma cell secretion rather than serum transudation.

Mucosal Permeability

The presence and level of IgG and albumin in salivary secretions were considered markers of mucosal permeability[1,2]. IgG was detected in 75% of neonates studied on the first day of life. This declined to 10% by the end of the first week and to 2% by 26 weeks of age. However, after 1 year of age IgG was routinely detected in the saliva of 30% of children studied. The concentration of IgG in saliva at birth was high (90th percentile = 54.1 mg/l) and decreased rapidly to predominantly non-detectable levels by 16 weeks of age. After 34 weeks of age IgG was again detectable in saliva (90th percentile = 15.8 mg/l) and increased to adult levels by 2 years of age (90th percentile = 28.2 mg/l).

Albumin was also detected in saliva at high concentrations near birth (median = 29.0 mg/l; 95% CI, [9.0, 90.9] mg/l) and decreased in parallel with IgG to lower levels at 12 weeks of age (median = 10.0 mg/l; 95% CI, [4.0, 31.4] mg/l). The concentration showed only a slight increase with age after 26 weeks. The mean albumin levels in samples with detectable levels of IgG were significantly higher than in samples with undetectable IgG ($p > 0.025$). Spearman rank correlations were conducted. Strong correlation was observed between IgG and albumin in the first 26 weeks of life which suggested that the majority of IgG in saliva during this time results from serum transudation and not from local plasma cell production. A lesser but still significant association between IgA and albumin suggests that the changes in permeability may be associated with episodes of immune stimuli, resulting in the production of local IgA.

Feeding

Infants were grouped according to their feeding pattern, during the first month of life as: totally breast-fed; totally formula-fed; and mixed-fed if fed breast milk in conjunction with other food types. The different feeding regimes resulted in significant differences in the pattern of ontogeny of mucosal IgA responses, particularly in the first 4 weeks of life[2]. Formula-fed children reached peak levels of salivary IgA at an earlier age (15-21 days) than breast-fed children (62-76 days) and the peak concentrations were greater in formula-fed children (median = 25.0 mg/l) compared with breast-fed children (median = 22.0 mg/l). Mixed-fed infants had peak levels similar to breast-fed infants (median 19.5 mg/l) but the peak occurred at a period between the other feeding groups (31-47 days) possibly reflecting the influence of the initial feeding with breast milk. The proportion of children with detectable IgM was consistently higher in the first 12 weeks of life in formula-fed infants than the other feeding groups. These results are consistent with an antigenic challenge provided by formula either directly or through altered bacterial gut flora. The IgG levels were lower in the breast-fed children compared to the other feeding groups in the first week of life and as the levels fell at similar rates in all three groups, remained lower for the first month of life. One explanation is that factors in breast milk enhance membrane closure which is reflected in less IgG transudation. The ontogeny patterns suggest an earlier stimulation of mucosal immune system in formula-fed children concurrent with a slower maturation of the mucosal membrane. This may condition altered antigen absorption at a critical stage of immune development and predispose to any of several clinical problems such as gut and respiratory infections or the development of an atopic state.

Nutritional Status

In a cross-sectioned study of children aged from birth to 5 years in Goroka, Papua New Guinea the affect of nutritional status on ontogeny of the mucosal IgA response was examined (unpublished observations). Generally children <80% weight for age had a lower total IgA and specific IgA antibody response to *E. coli* and non-typable *Haemophilus influenzae* a common respiratory pathogen in Papua New Guinea. Preliminary analysis of the data indicates that the specific IgA antibody response is more adversely affected that the total level of salivary IgA.

Environmental Exposure

IgA levels increase with age but two environmental changes have a dramatic effect - birth and commencement of school. As has already been described[15], both events are followed by a significant increase in IgA levels which then decline. Geographical differences have also been observed. Children from Papua New Guinea have 2-3 times higher levels of salivary IgA compared with age matched Australian children (unpublished results).

MARKERS OF MUCOSAL IMMUNOCOMPETENCE

A number of potential markers of a deficit in mucosal immunocompetence have been identified; transitory IgA deficiency, the failure to develop specific IgA-antibody responses, enhanced mucosal permeability, the presence of IgD and the presence of monomeric IgA.

Evidence of enhanced mucosal permeability, transitory IgA deficiency and failure to develop IgA-specific antibody responses have already been discussed.

IgD, a marker of immaturity of the immune system, was detected in the saliva of 48% of infants studied in the first 4 weeks of life[16]. IgD was only occasionally detected after 26 weeks of age. The concentrations when detected ranged from 0.1 - 10.0 mg/l. IgD was detected in a significantly higher proportion of samples with no detectable IgA (38%) than when IgA was detected (14%) ($p<0.005$). There was no correlation between IgD and either IgG or albumin.

Monomeric IgA was detected in the saliva of 28% of infants assayed during the first year of life[17]. There were no samples with concurrent monomeric and dimeric IgA. Proteolytic degradation of dimeric IgA was excluded. The molecular form of IgA in saliva samples from 6 infants studied longitudinally indicated a conversion from monomeric to dimeric IgA after varying periods of time. Monomeric IgA was not detected in any saliva sample collected from infants over 1 year of age. SC and J chain were detected in all samples.

It is anticipated that in follow up studies it will be possible to correlate deficits of mucosal immunocompetence identified in the first years of life with the subsequent development of atopy, asthma, and recurrent respiratory infections.

CONCLUSIONS

1. The ontogeny pattern of the mucosal IgA response in normal healthy children is described and appears to reflect a polyclonal response to environmental stimuli which is under tight regulatory control. Patterns of IgA and IgM ontogeny are similar. The ontogeny pattern for specific antibodies is different from the total IgA response.

2. A number of factors have been identified which modify the pattern of IgA ontogeny, namely: an IgM response, mucosal permeability, feeding practices, nutritional status, and environmental exposure.

3. A number of potential markers of mucosal immunocompetence have been identified. These are: transitory IgA deficiency, presence of IgD, presence of monomeric IgA, failure to develop specific IgA antibody responses, and enhanced mucosal permeability.

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ARE CYTOKINES IN HUMAN MILK?

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INTRODUCTION

Breastfeeding is known to protect the recipient infant against common infectious diseases of the alimentary tract[1] and respiratory system[1]. The mechanisms of this protection have been attributed mainly to a system of direct-acting antimicrobial agents that appear to have evolved to act at mucosal surfaces[1], and which do not provoke inflammation[2]. Recent evidence suggests that the protection afforded by breastfeeding may also involve agents that stimulate the maturation of the immune system of the recipient. The evidence is as follows.

EPIDEMIOLOGIC STUDIES

Some epidemiologic studies suggest that breast-fed infants are at less risk for developing certain chronic diseases that have an immunologic basis. In a recent study the the USA[3], breast-fed infants had a lower risk of developing type I diabetes mellitus. A retrospective Canadian study revealed that the risk for developing a chronic inflammatory disease of the small intestine, Crohn's disease, was also less in children breast-fed in infancy[4]. Finally, a survey in the USA of childhood deaths due to cancer suggests that the likelihood of developing lymphomas is less among individuals who had been breast-fed[5].

EFFECTS OF BREASTFEEDING ON SERUM CONCENTRATIONS OF DEFENSE FACTORS IN THE INFANT

Two investigations indicate that serum levels of certain host resistance factors are significantly higher in breast-fed than non-breast-fed infants, and that the differences are not explained by the amounts of those agents in human milk. In the first study, breast-fed and non-breast-fed infants in Japan were prospectively studied for their response to infections with respiratory

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syncytial virus[6], a pneumovirus responsible for most cases of bronchiolotis in infants. Not only was the severity of the infections less in breast-fed infants, but serum levels of interferon-α were also substantially higher in the breast-fed infants following the infection[6]. Since levels of interferon-α in human milk are low, it appeared that human milk feedings primed the infant to produce more interferon-α in response to the viral infection. In the second study, serum levels of fibronectin, a broad spectrum opsonin for the reticuloendothelial system, were found to be significantly higher in breast-fed infants[7]. Although fibronectin was present in human milk collected during the time of lactation that corresponded to the age of the infant, the amounts of fibronectin that were consumed during breastfeeding did not account for the increments in the serum concentrations of fibronectin found in the breast-fed infants. Therefore, it was concluded that breastfeeding stimulated the production of that defense agent by the infant.

EFFECTS OF BREASTFEEDING UPON MUCOSAL DEFENSE AGENTS

Several studies report that the concentrations of defense agents in external secretions are higher in breast-fed infants[1]. Although some of the increment may have been due to the ingestion of the immunologic factors in human milk, evidence from two studies suggests that some of the increase was probably due to an enhancement of the production of the factors at mucosal sites[8,9]. Since lactoferrin, total IgA, and specific secretory IgA antibodies were increased in the urine as well as in the stools of infants fed human milk, it seems unlikely that the increase in those urinary factors was due to the ingestion of those proteins during breastfeeding.

IN VITRO EVIDENCE THAT HUMAN MILK IS IMMUNOSTIMULATORY

Although human milk contains agents that inhibit certain immunologic responses and thus are anti-inflammatory[2], certain studies indicate that human milk also contains immunostimulating agents. Within the past few years, some evidence has been reported that human milk aids in stimulating the proliferation of and immunoglobulin synthesis by LPS-primed splenic murine B cells[10]. Recently, human milk lactoferrin was reported to enhance the proliferation of peripheral blood mononuclear leukocytes stimulated with anti-CD3[11]. It was not entirely clear, however, whether the activity could have been due to other milk proteins in the lactoferrin preparation. In addition to those reports, low concentrations of unfractionated human colostrum have been found to stimulate T cell growth *in vitro*[11].

One part of the evidence regarding the immunostimulating properties of human milk was developed from a study of the motility of the leukocytes in human milk. When human milk macrophages were tested in collagen gel systems, their rate of random movement was much greater than that of their counterparts in human blood, the monocytes[12]. The genesis of this enhancement was not found to be due to a systemic activation of blood monocytes, but to stimulating agents in human milk[13]. When human blood monocytes were incubated in the fluid phase of human milk or in its whey

protein fraction, the motility of these cells increased remarkably[13]. The activity was abolished by trypsin, and the activities were found to reside in three distinct molecular weight peaks (50 kDa, 25 kDa, and 15-20 kDa). Thus, the question was raised whether there were a number of chemokinetic agents in human milk, or whether one agent existed in multiple forms.

PRIMARY EVIDENCE FOR CYTOKINES IN HUMAN MILK

Biological Activity

In 1987, Soder *et al.* reported that human milk had interleukin-1 (IL-1) activity[14]. No immunochemical evidence was presented to confirm that the biological activity was due to that interleukin. In the previously described investigation of the nature of the chemokinetic agents for monocytes in human milk, studies were undertaken to determine whether that biological activity was due to IL-1[13]. The chemokinetic activity in human milk was not inhibited by the addition of polyclonal antibodies to recombinant human IL-1, but it was discovered that the activity was reduced substantially by polyclonal antibodies to a second cytokine, recombinant human tumor necrosis factor-α (TNF-α)[13]. In a subsequent investigation, it was found that recombinant human TNF-α was chemokinetic for human monocytes[15]. Thus, it appeared that the chemokinetic agents in human milk were either TNF-α, molecules that were antigenically similar to that cytokine, or factors that induce the production and/or release of TNF-α from the target mononuclear leukocytes.

Limited studies were performed to determine if more classical biological activities ascribed to TNF-α were present in human milk[13]. In a microcytotoxicity assay, human milk, whey proteins were found to lyse a TNF-α-sensitive murine cell line (L-929). Furthermore, some of that microcytotoxicity was blocked by incubating whey proteins with polyclonal antibodies to human recombinant TNF-α.

Immunochemical measurements. Because of the foregoing biological evidence for TNF-α in human milk, immunological studies were initiated to determine if TNF-α was present in human milk[16]. Because of interfering substances in human milk, the immunodot method was not found to be a reliable method for detecting TNF-α in human milk. TNF-α was found in human milk by an enzyme-linked immunosorbent assay, but since human milk also inhibited the detection of TNF-α in this assay, the method underestimated the quantity of TNF-α in human milk. Two commercial radioimmunoassays for human TNF-α, which proved to be reliable, revealed that the mean ± standard deviation of TNF-α in human milk collected during the first 2-3 days of lactation was 620 ± 185 pg/ml. Based upon the total daily secretion of milk during that period, we estimated that an infant that age consumes about 70 ng/day of TNF-α.

CODA

Recent epidemiologic, clinical, and *in vitro* evidence suggests that breastfeeding stimulates the maturation of the immunologic system in the recipient infant and that part of the immuno-stimulation may be due to

cytokines, such as TNF-α in human milk. There are important questions that emanate from these findings.

1. Is TNF-α present in human milk throughout lactation?

2. What are the molecular forms of TNF-α in human milk? Recent evidence from our laboratory suggests that the monomeric and transmembrane (26kDa)[17] types of TNF-α are in human milk.

3. What is the spectrum of biological activities of TNF-α in human milk? It may seem incongruous that TNF-α is present in human milk, since TNF-α is one of the principal instigators of inflammation[18]. It is possible, however, that the phlogistic activities of TNF-α in human milk are not expressed because of the particular molecular forms of the cytokine (for example the transmembrane form) or the manner in which they are packaged in human milk. Non-inflammatory functions of TNF-α in human milk such as the ability to upregulate the expression of secretory component on epithelial cells or of class I and II major histocompatibility molecules on macrophages or to enhance the maturation of monocytes or T lymphocytes may be spared. In that regard, T lymphocytes as well as macrophages in human milk display phenotypic features that are in keeping with activation (unpublished data).

4. What is the fate of ingested TNF-α from human milk? Since TNF-α is susceptible to proteolytic enzymes, it may be argued that the cytokine would be digested before it could exert any effects upon the recipient. That may not, however, be the case. 1) The cytokine encounters a large mucosal surface area of the alimentary tract before the stomach, where no proteolysis occurs. 2) The production of pepsin, the only gastric proteolytic enzyme, is delayed for the first week of life. 3) A considerable amount of anti-proteases are normally found in human milk. Thus, it is likely that TNF-α from human milk will be active in the infant.

5. Are there cytokines in human milk other than TNF-α? Because of the known inter-relationships between TNF-α and other cytokines[19-22], we predict that other cytokines will be discovered in human milk. The production of one of the principal candidates, IL-6, is stimulated by TNF-α and that interleukin in turn modulates the production of TNF-α by stimulated monocytes. Our preliminary, unpublished immunochemical studies suggest that IL-6 is in human milk. Further work will be required to develop that evidence and to search for other cytokines in human milk.

Finally, once the molecular biology of the cytokine system in human milk is better understood, studies of *in vivo* effects of those cytokines upon the recipient infant should be begun.

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THE DEVELOPING GASTROINTESTINAL TRACT AND MILK-BORNE EPIDERMAL GROWTH FACTOR

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INTRODUCTION

In adult mammals, epidermal growth factor (EGF) is produced mainly in the submandibular and Brunner's glands[1-3]. The production of EGF in suckling mammals is considered to be very low. Popliker *et al.*[4] reported absence of EGF mRNA in suckling mice; others described only very small amounts of EGF to be present in submandibular glands of suckling rodents[1,5-7]. EGF is known to play an important regulatory role in the mammals; many studies suggest that EGF is a trophic factor for various regions of the developing gastrointestinal tract[8-17] and liver[18,19].

The presence of considerable amounts of EGF in milk of various species led to speculation that milk may be a major source of EGF for the suckling mammal[20]. Several years ago, we demonstrated that ^{125}I-labeled mouse EGF (mEGF) administered orogastrically to suckling rats "survives" in the gastrointestinal lumen and is delivered to peripheral organs in an immunoreactive and receptor-binding form[21]. In this presentation, we shall discuss our recent studies further characterizing the precessing of EGF in the gastrointestinal tract of suckling rat and of the human neonate.

STUDIES IN SUCKLING RATS

Difference in Absorption and Processing of ^{125}-I mEGF in Jejunum and Ileum of Suckling Rats

These studies were designed to test the hypothesis that handling of EGF is different in the jejunum than in the ileum of suckling rats. Rao *et al.*[22] demonstrated (Fig. 1) a high capacity of the small intestine to absorb ^{125}I-mEGF (doses between 10 and 1000ng/rat); absorption rate from ileum was about 3 times higher than that from the jejunum. Similarly, a higher absorption of ^{125}I-rat EGF from the ileum than from the jejunum was demonstrated[23]. It is noteworthy, that immunoreactive ^{125}I-mEGF appeared

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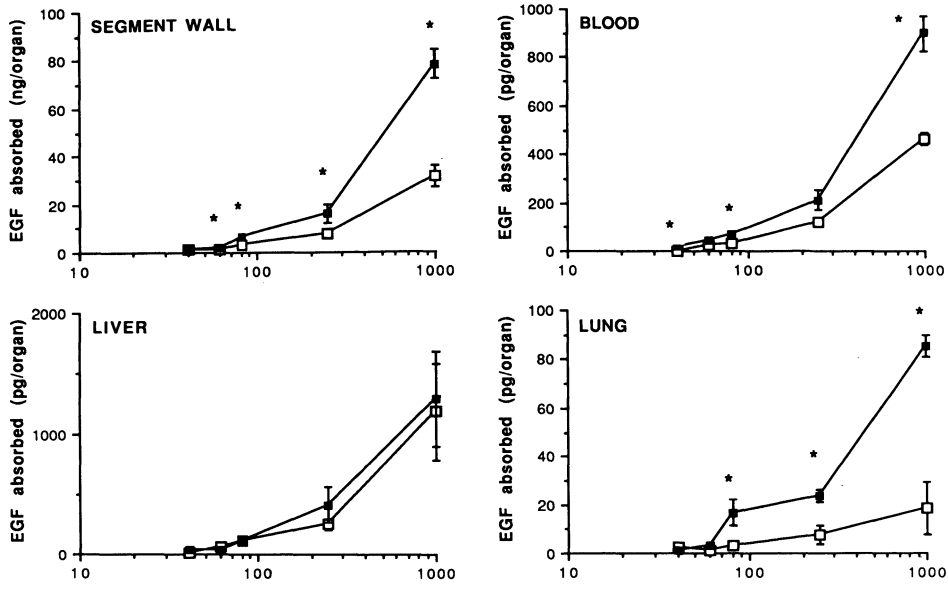

Figure 1. Dose-dependent absorption of ^{125}I-mouse EGF from isolated jejunum (open squares) and ileum (full squares) of suckling rats. Horizontal axis: ng/rat of mEGF administered in the isolated loop. Vertical axis: ir ^{125}I-mEGF per organ (units of EGF: segmental wall: ng; other organs: pg). N/group = 2-11. * = significantly different from corresponding values in the jejunum. Data taken from reference no. 22.

in lung in higher amounts, when administered into the ileum than into the jejunum[22].

Reverse phrase-HPLC analysis of extracts of jejunal and ileal luminal contents and walls showed conversion of stock ^{125}I-mEGF into several peaks with no change in immunoreactivity, but differing in receptor binding capability (unknown peak 40% binding as compared to stock mEGF; ^{125}I-des$^{49-53}$ mEGF, 43%; ^{125}I-des$^{48-53}$ mEGF, 14%; intact ^{125}I mEGF, 96% and ^{125}I-des^{53} mEGF, 157%). Whereas in the lumen of jejunum all fragments - except ^{125}I-des$^{49-53}$ mEGF - were detected, in the lumen of ileum and wall of both jejunum and ileum only ^{125}I-des$^{49-53}$ mEGF was found[22].

Studies Using Determination of Non-Labeled ("Cold") Authentic Rat EGF

In previous experiments we used ^{125}I-mEGF; however, to measure the native rat EGF we developed a species-specific radioimmunoassay (RIA)[24]. In the first studies, we determined the immunoreactive E6F (ir E6F) EGF levels in the intestinal mucosa of suckling and adult rats, fed and fasted for 18 hours.

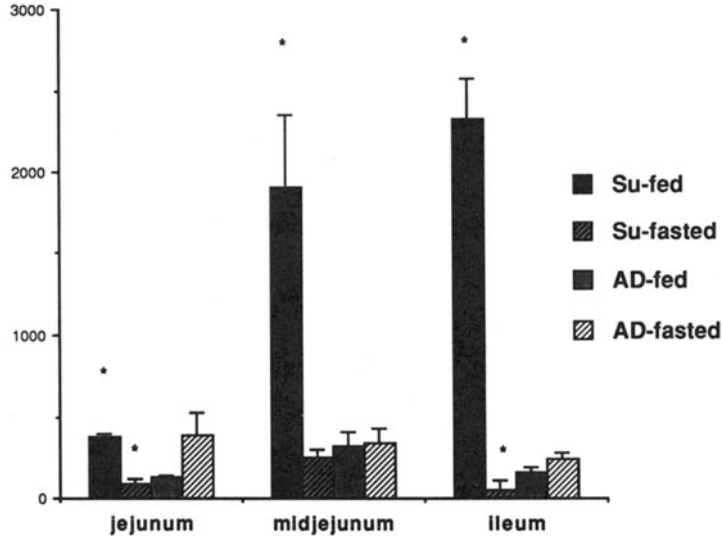

Figure 2. ir rat EGF content in the mucosa of small intestine of suckling and adult rat; effect of age and fasting. Vertical axis: pg of irEGF per mg of protein, horizontal axis: pg of EGF per mg of tissue protein; mean ± SEM. N/group: sucklings 7, adults 6. Jejunoileum was divided into three equally long segments. *Statistically significant (ANOVA followed by Fisher PLSD test) from corresponding values of adults. Data taken from reference no. 28. Su = suckling, AD = adult.

Data summarized in Fig. 2 led to three major conclusions: 1) EGF content in the fed suckling rats exceeds that of fed adult rats; 2) Due to the considerable jejunoileal gradient present only in suckling rats (ileum/jejunum = about 7), the values in the distal segment exceed in suckling rats those determined in adult rats approximately 15 times. Ileum has a special role in EGF absorption as shown by morphological techniques[25]; furthermore, studies from our laboratory show higher absorption of EGF from ileum than jejunum (see above[22,23]); and 3) Whereas in adults fasting led to no decrease of EGF content, in sucklings the decrease was considerable (the fasting values were, in the various segments, about 25%, 13%, and 2%, respectively, of the corresponding values in fed animals).

It is noteworthy that extended fasting in adult rats (48 hours) led to an increase of EGF concentration in their intestinal mucosa (data not shown). Since these experiments strongly suggested the dependency of the EGF content in the small intestinal mucosa of suckling rats, on milk intake we compared the EGF in rat milk with that present in the gastrointestinal tract of suckling rats. Determined by species-specific RIA, milk of rats lactating for 10-14 days contained EGF equivalents 35.4 ± 14.6 ng/ml (mean ± SEM, N=7). For further analysis, fresh rat milk was extracted using antibodies to submandibular rat

EGF (sm-r-EGF) covalently linked to polyacrylamide beads. Three distinct immunoreactive forms of EGF (forms A,B,C) were detected (Fig. 3); these competed with ^{125}I-r-EGF for binding to the EGF receptor (human forskin fibroblast HFF10) and stimulated DNA synthesis in growth arrested fibroblasts. Two of the forms (A,B) were converted by tryptic digestion to sm-r-EGF species (form C) as verified by RIA and migration rates in a nondenaturing polyacrylamide gel. Digestion of form C by carboxypeptidase B led to an appearance of form D (des-arginyl EGF) (Fig. 4); this form exhibited also EGF receptor binding and stimulated DNA synthesis in growth-arrested fibroblasts. In the stomach luminal content of fed suckling rats (taken directly from the mother) only the larger form (B) was found (Fig. 4). The luminal content and mucosa of the small intestine contained in all segments only the form D comigrating with des-arginyl EGF (Fig. 3).

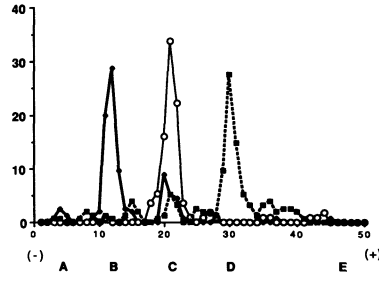

Figure 3. Polyacrylamide gel electrophoresis of milk extracts before (native) and after enzymatic degradation. Vertical axis: percent of the total immunoreactive material recovered from the gel per slice. Horizontal axis: # of slice. Direction of migration is from left to right. Heavy line = milk (#1), thin line = milk digested with trypsin (#2), dotted line = #2 digested with carboxypeptidase B. A and B: migration rates of larger forms. C: migration rate of sm-r-EGF (SG-E) D: migration rate of des-arginyl EGF (dE) E: location of the dye front. Data from from reference no. 31.

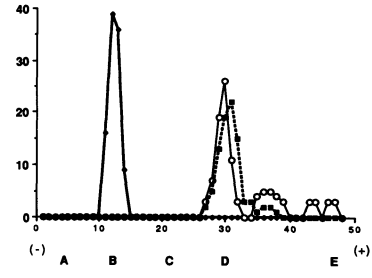

Figure 4. Polyacrylamide gel electrophoresis of extracts of luminal content of stomach (heavy line), midjujunal luminal content (thin line) and midjujunal mucosa (dotted line). Similar results as with the midjejunum were obtained with preparations of jujunum and ileum. Same arrangements as in Fig. 3. Data taken from reference no. 32.

Littermates of these suckling rats were fasted for 8 hours and then re-fed by allowing them to suckle an other (foster) rat mother (deprived of sucklings for 4 hr) for periods between 60 and 240 min (since similar results were obtained, these values were combined). Two other groups of 8 hr fasting suckling rats were fed with 0.5 ml of freshly collected rat milk or 0.5ml of rat milk substitute (RMS, see reference 16). Results of quantitative determinations of EGF by RIA are summarized in Fig. 5; 8-hr fasting led to a considerable decrease of EGF content in the entire gastrointestinal tract. Re-feeding by "re-suckling" the mother increased the EGF content to control values (rats were kept with the mother). Whereas short term re-feeding (45 min with 0.5ml fresh rat milk) increased the EGF content in the lumen of the entire gastrointestinal tract, only the mucosa of the proximal jejunum exhibited an increase (data not shown). Feeding RMS was without effect; this finding is in agreement with our previous results showing very low levels of irEGF in the small intestinal lumen and mucosa of suckling rats fed 3 days by RMS[26].

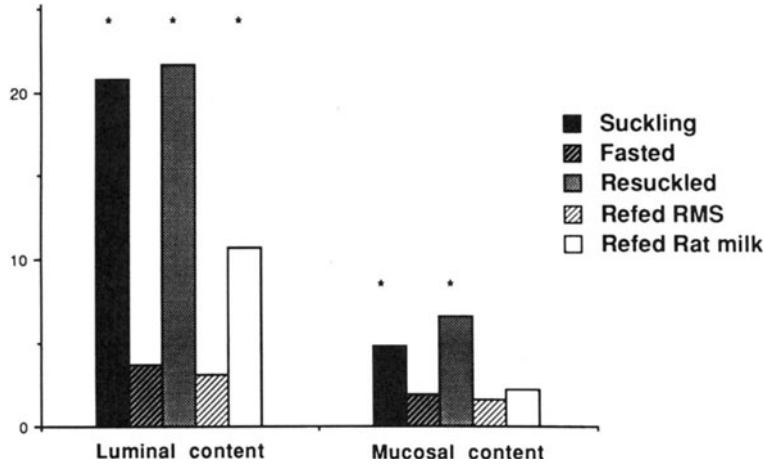

Figure 5. ir RAT-EGF content of the gastrointestinal tract of suckling (12-day-old) rats. Vertical axis: ng irEGF/per stomach and small intestine. Mean values are given, (N/group = 4 to 7). *Denote statistically significant differences from values of fed rats. Data taken from reference no. 32.

In all treatment groups similar electrophoretic patterns were seen as in control fed suckling rats; exception was the content of stomach lumen in the 8-hr fasting group. In this case, the form B represented 40% and newly appearing authentic r-EGF (form C) and des-arginyl (form D) other 40% and 20% of the total immunoreactive material, respectively. We want to stress, that in the intestinal luminal and mucosal content of suckling rats irEGF was found always in the form comigrating with the des-arginyl EGF (form D).

STUDIES IN HUMAN INFANTS

It is obvious that the extent of studies on digestion/absorption of milk-borne EGF in the human are restricted for many reasons. We approached this question with the following assumption. Resistance to gastrointestinal proteolytic degradation is necessary if ingested EGF is to function within and beyond the gastrointestinal tract. It is known, that these processes are active to a lesser extent in sucklings than in adults[27]. The "survival" of EGF in the gastrointestinal tract of the human neonate was evaluated in experiments *in vitro*.

Stomach fluid was used as the enzyme source; it was aspirated from pre-term infants one hr after feeding pre-term formula or fortified human milk[28]. This time period was chosen, because previous studies have shown maximum gastric proteolytic activity at this time[29]. ^{125}I-human recombinant EGF was incubated at several pH (1.8, 3.2, 5.8). Minimal loss of trichloracetic acid-precipitable activity occurred, in contrast to the substantial hydrolysis of iodinated casein which occurred under the same conditions. Chromatography of reaction mixtures on Sephadex G-25 showed a single major peak of radioactivity which coeluted with stock EGF. EGF also retained >75% of its ability to bind to anti-EGF affinity columns and placental membrane EGF receptors after incubation with gastric fluid. These data together with similar results obtained with gastric fluid of suckling and weanling rats[30] suggest substantial gastric survival of ingested EGF in a potential active form in infants.

CONCLUSIONS

^{125}I-mEGF absorption capacity in suckling rats is very high (ileum/jejunum = 3); higher ileum absorption ^{125}I-mEGF capacity is accompanied by correspondingly higher ir^{125}I-mEGF uptake by the lung. The content of irEGF in the gastrointestinal tract of suckling rats is influenced by intake of milk containing EGF. Milk-borne EGF is processed in the small intestine of suckling rats into forms with preserved biological activity. The variation of the EGF forms found in the milk and the gastrointestinal tract may also be influenced by differences in the rate of their intestinal absorption. *In vitro* experiments using the gastric juice indicate "survival" of biologically active EGF in the gastrointestinal tract of human neonates. Together with studies demonstrating the effect of orogastrically administered EGF to suckling rats and rabbits (for review see reference 20), these data strongly support the role of milk-borne EGF for the neonate.

ACKNOWLEDGEMENT

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GROWTH FACTOR SIGNAL TRANSDUCTION IN HUMAN INTESTINAL CELLS

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INTRODUCTION

One of the basic biological problems in the area of developmental gastroenterology is to understand the factors and mechanisms controlling the intestinal epithelial cell growth and differentiation. Enterocytes are the type of cells undergoing a rapid turnover during development and mature life. Cells in the crypt are rapidly dividing and less differentiated, whereas cells at the villus tip are terminally differentiated. Furthermore, there is a differential gene expression in both crypt cells and villus cells between the neonatal and adult intestine. Increasing evidence indicates that the control of enterocyte proliferation and differentiation appears to be mediated not only by endocrine or paracrine but also by autocrine mechanisms[1,2]. Table 1 lists several growth factors that may potentially target at the human intestinal cells. No matter how the production of growth factors are regulated, they must interact with their specific cell-surface receptors. Ligand-receptor interaction on the cell surface is then translated into activation of intracellular signaling pathways, triggering a sequence of events that eventually leads to cell division or differentiation.

The best characterized signal transduction by the growth factor receptor is the one used by EGF (epidermal growth factor). Currently, two potential signal transduction pathways are proposed for EGF[3,4]. One pathway is mediated via the activation of a tyrosine-specific protein kinase activity that is intrinsic to the receptor molecule. The other pathway is through the activation of phospholipase C (PLC) that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to produce two second messenger molecules, inositol trisphosphate (IP_3) and diacylglcyerol (DG); the former stimulates calcium mobilization from endoplasmic reticulum, while the latter activates protein kinase C. Recently, several substrates of the EGF receptor tyrosine kinase have been identified[3,4]. They include phospholipase C-γ1 (PLC-γ1), phosphatidylinositol 3-kinase (PI-3 kinase), *ras* GTPase-activating protein (GAP), microtubule-associated protein kinase (MAP kinase), *raf* kinase, *c-erb B-2*, and lipocortin I (calpactin I). It is worthy of note that these target proteins

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are all either components of second messenger pathways, cytoskeleton-associated proteins, proto-oncogene products or factors that regulate the activity of proto-oncogene products. The best characterized substrate for the EGF receptor kinase is PLC-γ1, one of a family of PLC isozymes. Most recently, an increased catalytic activity of PLC-γ1 by tyrosine phosphorylation has been demonstrated, suggesting that the EGF-stimulated formation of IP_3 and DG may result in part from catalytic activation of PLC-γ1 through the action of receptor tyrosine kinase[5].

Table 1. Potential Growth Factors for Human Intestinal Cells

Epidermal growth factor (EGF)
Platelet-derived growth factor (PDGF)
Insulin
Insulin-like growth factor I (IGF-I)
Insulin-like growth factor II (IGF-II)
Transforming growth factor α (TGF-α)
Transforming growth factor β (TGF-β)
Interlukin-2 (IL-2)

EGF is the major growth factor in human breast milk[6,7]. It has trophic effects on the intestine of the developing animals[8]. A stimulatory effect of EGF on DNA synthesis has also been noted in a rat small intestinal epithelial cell line (IEC-6)[9]. However, in the organ culture of human fetal small intestine, EGF inhibits DNA synthesis but increases lactase activity, suggesting that EGF may play a role in promoting enterocyte differentiation in human intestinal cells[10]. Membrane receptors for EGF are present in the gastrointestinal tract of both rodent species[11,12] and humans[13]. However, few studies have examined EGF signal transduction in human intestinal epithelial cell lines. Accordingly, we have begun to investigate EGF binding and receptor tyrosine kinase in the human intestinal epithelial cell (Caco-2). The results show that this cell line expresses EGF receptors with high and low affinity binding sites and the EGF stimulus is mediated via tyrosine kinase activation.

MATERIALS AND METHODS

Cell Culture

The human colon carcinoma cell line (Caco-2) was purchased from the American Type Culture Collection (ATCC, Rockville, MD) and routinely maintained in DMEM supplemented with 20% fetal bovine serum (FBS) at 37°C in humidified 5% CO_2/95% air atmosphere[14].

Receptor Binding Assay

Caco-2 cells were plated at 4.25×10^5 cells/cm^2 on 24-well plates in DMEM plus 20% FBS at day 0. The medium was changed to serum-free DMEM on day 2, and EGF binding was done on day 6. EGF binding[15] was

performed at 22°C for 3 hr in 0.1 nM [^{125}I]-EGF in HBSS plus 0.2% BSA with the absence or presence of increased concentrations of non-radioactive EGF. Scatchard analysis for the binding affinity, estimated by the apparent dissociation constant (K_d), and the receptor number or maximal binding (B_{max}) were calculated as previously described[16].

Western Blot Analysis of Tyrosine Phosphoproteins

After changing to a serum-free medium overnight, subconfluent 100 mm plates of Caco-2 cells were treated with and without EGF (200 ng/ml) at 37°C for 30 min. The cells were washed with cold PBS and lysed in 0.5 ml of a lysis buffer (20 mM Tris, pH 8.0; 137 mM NaCl; 10% glycerol; 1% NP-40) containing 1 mM PMSF, 0.15 U/ml aprotinin, and 1 mM sodium vanadate at 4°C for 20 min. Protein was separated by SDS-PAGE under reducing conditions on a 7.5% gel. Immunoblotting with a mouse anti-phosphotyrosine monoclonal IgG2b$_k$ antibody was performed according to the protocol provided by the supplier (Upstate Biotechnology, Inc., Lake Placid, NY).

RESULTS AND DISCUSSION

Scatchard analysis of [^{125}I]-EGF binding kinetics (Table 2) indicates that Caco-2 cells expressed a high affinity (K_{d1} = 0.08 nM) and low affinity (K_{d2} = 24 nM) receptor site, with the high affinity site comprising less than 3-5% of the total receptor population (6-7 x 10^4 receptors/cell). The receptor number is comparable to those reported for human fibroblasts[17]. The presence of two classes of EGF binding sites on the cell surface of human fetal small intestine and colon have been previously reported by Pothier and Menard[13]. Our studies also show that human milk down-regulates EGF receptors in Caco-2 cell due to the presence of EGF in breast milk[18]. This regulatory mechanism may in part contribute to the decline of EGF receptor binding after birth as noted in the rat small intestine[11,12].

Furthermore, other studies show that EGF receptors are present in both apical and basolateral membrane of Caco-2 cells[19], similar to the Madin-Darby canine kidney (MDCK) cells[20]. The presence of EGF receptors on both sides of the polarized intestinal epithelial cells would allow EGF to target enterocytes

Table 2. EGF Receptor Binding Kinetics in the Human Intestinal Cell Line (Caco-2)

Binding sites	K_d (nM)	B_{max} (receptors/cell)
High affinity	0.08	1,500
Low affinity	24.00	65,000

K_d = dissociation constant; B_{max} = maximal binding

via different routes. For example, the milk derived EGF may bind to the microvillus membrane receptor, while other endogenous derived EGF may bind to either the apical or basolateral receptors depending on where EGF is produced.

To determine if EGF binding activated receptor kinase activity in Caco-2 cells, tyrosine phosphoproteins of cell lysates were detected using a monoclonal anti-phosphotyrosine antibody by immunoblotting. The result (Fig. 1) shows that EGF rapidly stimulated tyrosine phosphorylation of a 170 kDa protein, known to be the EGF receptor. However, in the non-stimulated Caco-2 cells, the 170 kDa phosphotyrosyl protein was also detectable. In addition, there are many other detectable tyrosine phosphoproteins in Caco-2 cells even without EGF stimulus. The basal level of receptor tyrosine kinase activity might be in part mediated via an autocrine mechanism. It is well known that many human colon cancer cell lines[21,22] including Caco-2 cells[23] secreted TGF-α/EGF-like molecules into the serum-free medium, and that TGF-α binds to the EGF receptor to produce a mitogenic stimulus[24].

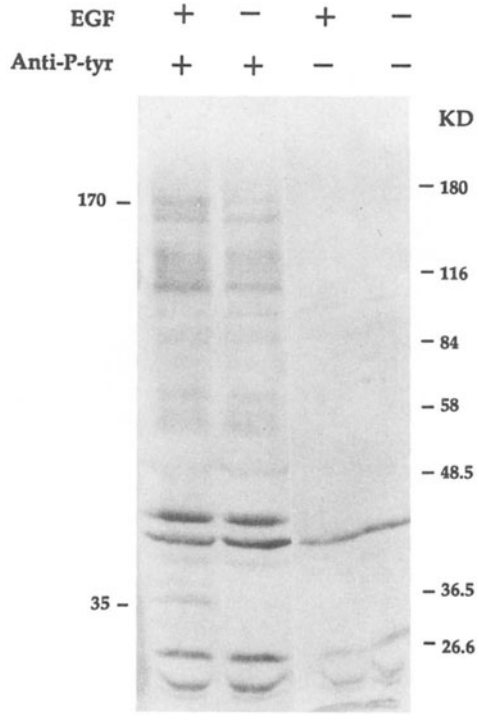

Figure 1. Immunoblotting analysis of tyrosine phosphoproteins in EGF-stimulated and non-stimulated Caco-2 cells with a monoclonal anti-phosphotyrosine antibody (Anti-P-tyr).

Fig. 1 also shows that EGF stimulated the tyrosine phosphorylation of a 35 kDa protein. Based on the molecular mass, this 35 kDa protein is likely calpactin I (also known as lipocortin I). There are several lines of indirect

evidence to support this possibility. First, the 35 kDa protein, lipocortin I, is a good substrate for EGF receptor kinase *in vitro*[25]. Secondly, it has been proposed that the cytoskeleton-associated protein, calpactin I, might be one of the predominant protein tyrosine kinase substrates in rat intestine[26]. Calpactin associates with membrane phospholipids in the presence of calcium. Previously, it has been suggested that phosphorylation of the 35 kDa calpactin might be associated with the formation of endocytic vesicles[27]. However, the physiological function of this 35 kDa protein in receptor endocytosis and/or mitogenic signaling remains to be determined.

CONCLUSIONS

This study indicates that the human intestinal epithelial cell line (Caco-2) expresses EGF receptors with high- and low-affinity binding sites. As in other cell types, the EGF stimulus is mediated via the activation of a receptor tyrosine kinase, as evidenced by the autophosphorylation of a 170 kDa receptor. In addition, a 35 kDa protein, likely calpactin I, appears to be one of predominant receptor kinase substrates in the human enterocyte. These data suggest that Caco-2 cells can be a useful *in vitro* system to help elucidate signal transduction pathways during human intestinal cell response to growth factors.

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ROLE OF IL-6 IN HUMAN ANTIGEN-SPECIFIC AND POLYCLONAL IgA RESPONSES

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INTRODUCTION

It is now well established that several cytokines are involved in B cell activation, proliferation, and differentiation. From earlier studies, it became clear that some of these cytokines acted later in B cell responses and contributed to terminal differentiation and immunoglobulin synthesis[1]. A prominent cytokine among these was a factor originally termed B cell-differentiation factor, which could be distinguished from B cell-stimulating factor-1 (BSF-1), or IL-4, and was subsequently termed BSF-2[2,3]. When BSF-2 was purified and subsequently cloned[3-5], it was shown to be identical to other cloned proteins, e.g., interferon beta (IFN-β)[6], the 26kDa protein[7], and hybridoma growth factor[8-10], and was renamed IL-6.

IL-6 is produced by B and T cells, macrophages, fibroblasts, endothelial cells, and perhaps other cell types and in turn induces a large number of biologic responses[11]. This cytokine induces acute phase proteins and is involved in various types of inflammation[11]. In addition, IL-6 induces: T cells to produce IL-2 and express IL-2 receptor, maturation into functional cytotoxic T cells, and hemopoiesis of bone marrow stem cells (reviewed in 11, 12).

In cells of the B lineage, IL-6 is mainly involved in their terminal differentiation. Thus, IL-6 induces mitogen-stimulated or EBV-transformed B cells to secrete immunoglobulin[3,13]. Further, IL-6 can augment IgM, IgG and IgA synthesis in human peripheral blood mononuclear cell (PBMC) and tonsillar cell cultures stimulated with pokeweed mitogen (PWM)[14]. Clearly, IL-6 is of key importance in B cell terminal differentiation, since incubation of these PWM-stimulated cells with recombinant human IL-6 (rhIL-6) in the presence of anti-IL-6 antibody completely blocked Ig synthesis of all isotypes[14]. Although IL-6 induces terminal B cell differentiation in various *in vitro* systems, it is not yet clear whether IL-6 alone is sufficient to induce plasma cell formation. In this review, we will provide evidence that rhIL-6 alone can serve this function in both antigen-specific and polyclonal B cell cultures.

Immunology of Milk and the Neonate
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In this study, we examined whether rhIL-6 induced the terminal differentiation of antigen (Ag)-activated human B cells[15]. To overcome the problem of low precursor frequency of these cells in peripheral blood, approved vaccines were used to induce a substantial enrichment of Ag-activated B cells in vivo. Two types of vaccines were used in this study, the T dependent protein diphtheria toxoid (DT) (Wyeth, Marietta, PA) and the T independent 23 valent pneumococcal polysaccharide (PPS) (Pnu-Imune 23, Lederle, Pearl River, NY)[15].

Ag-activated B cells were generated by systemic immunization of volunteers with PPS or DT vaccines. No specific antibody-producing spot-forming cells (SFC) were detectable before immunization, and *in vitro* stimulation of preimmune B cells with rhIL-6 failed to induce specific antibody (Ab) secretion. Seven to nine days after immunization with either PPS or DT, Ag-specific SFC were present in the circulation. When PBMC from subjects immunized 7 days earlier with DT were stimulated with rhIL-6 in vitro for 7 days, an increase of the frequency of Ag-specific SFC was observed at 20 Units/ml of rhIL-6. When the B cell-enriched fraction was cultured in the presence of rhIL-6, the increase of SFC was similar to the unfractionated PBMC (Table 1). The amount of anti-DT Ab secreted into the supernatant paralleled the numbers of SFC.

Table 1. Numbers of Anti-DT SFC and Anti-DT Antibodies in B Cell Cultures Supplemented with rhIL-6

IL-6 added to B cell cultures (/ml)	Anti-DT SFC/ 10^6 cells [a]	IgG anti-DT Ab (ELISA units/ml)[b]
None	113±7[c]	0.071±0.004
20 Units	300±23	0.120±0.001
40 Units	273±37	0.115±0.004
80 Units	340±12	0.130±0.004

[a] Numbers of anti-DT SFC were determined by ELISPOT on nitrocellulose plates coated with 1μg/well of DT. Wells were developed with a biotinylated isotype-specific anti-human Ig antibody.

[b] ELISA units (EU) based upon a reference serum arbitrarily assigned 100 EU of IgG anti-DT/ml.

[c] Arithmetic means ± SEM.

When B cell-enriched PBMC of subjects vaccinated 7 days earlier with PPS were cultured with rhIL-6, the *in vitro* secretion of PPS-specific Ab was enhanced in the same dose-dependent fashion (Table 2). Stimulation with rhIL-6 did not alter the isotype distribution of the SFC, but augmented IgA, IgG, and IgM proportionally (Table 2). Similar patterns of Ag-specific responses were also induced in splenic B cell cultures in the presence of rhIL-6 [15].

Table 2. Enhancement of IgG and IgA Anti-PPS SFC in IL-6 Supplemented B Cell Cultures[a]

IL-6 added to B cell cultures (/ml)	IgG anti-PPS SFC/10^6 cells	IgA anti-PPS SFC/10^6 cells
None	3570±550	3030±190
20 Units	4260±350	3480±225
40 Units	4890±270	4110±330

[a] Numbers of IgG and IgA anti-PPS SFC (arithmetic means ± SEM) determined by ELISPOT. Nitrocellulose wells were coated with PPS coupled to poly-L-lysine (1.5 μg/well) and developed with a biotinylated anti-γ or -α chain-specific antibodies.

To test whether the observed increase of Ag-specific SFC was indeed caused by rhIL-6, PBMC obtained from a healthy volunteer 7 days after immunization with PPS were cultured in the presence of rhIL-6 and the goat anti-IL-6 serum. The polyclonal antiserum completely inhibited the effect of rhIL-6 up to a concentration of 40 U/ml, while this inhibitory effect was partially reversed by higher doses of the cytokine[15]. Normal goat serum had no effect on *in vitro* Ag-specific Ab secretion. The same neutralizing effect was observed when anti-IL-6 serum was added to the splenic B cells and cultured in the presence of optimal concentrations of rhIL-6[15]. These results showed that administration of both T cell-dependent and -independent Ags induce circulating B cells which respond to IL-6. In this regard, it has been shown that *in vivo* immunization of humans with a variety of antigens induces the formation of large B cells in the circulation[16-18]. These cells represent activated B cells which can bind antigen and possess the potential to secrete spectific Ab.

Based on our findings, one could postulate that *in vivo* stimulation might induce IL-6 receptor (IL-6R) expression on this large B cell subset. To test this possibility, PBMC were obtained from two subjects before and after immunization with DT and stained for the pan-B cell marker CD19 and IL-6R. When $CD19^+$ cells were separated by flow cytometry according to their forward scatter into small and large cells, it was revealed that less than 3% of small B cells and 25-29% of the large B cells expressed the IL-6R[15]. The proportion of

large CD19+ cells slightly increased after immunization, although the total number of large B cells bearing the IL-6R did not change significantly[15].

When peripheral blood or splenic B cells were stimulated *in vitro* with rhIL-6, the numbers of Ag-specific SFC and the amount of secreted Ab was increased in a dose-dependent fashion. The isotype distribution was not altered by rhIL-6. Approximately 25% of the large peripheral blood B cells were IL-6R positive. It is likely that the IL-6R+ fraction includes the large B cells that were induced by immunization, because activated B cells have been shown to express that receptor[19]. RhIL-6 probably acted on IL-6R bearing large B cells by sustaining the ongoing Ab secretion and by inducing the terminal differentiation of nonsecreting cells into high-rate antibody-secreting cells. A pre-secretory phase in B cell differentiation indeed exists in mice[20]. These cells have abundant steady-state levels of Ig and J chain transcripts, but low rates of Ig secretion. Further, after stimulation of human PBMC with PWM, we found cells with a lymphoblast morphology which contained cytoplasmic Ig, but did not secrete Ig in the ELISPOT assay (unpublished data). This may be an indication that the pre-secretory phase in B cell differentiation also exists in humans. IL-6 may be one of the signals which promotes the differentiation of those cells into plasma cells.

ROLE OF IL-6 IN IgA1 AND IgA2 B CELL DIFFERENTIATION

In the murine system, IL-6 is of central importance for the induction of committed, surface IgA-positive (sIgA+) B cells to become IgA-secreting cells [21]. In those studies, B cells were obtained from murine Peyer's patches (PP), an IgA-inductive site which is enriched in sIgA+ cells, and IL-6 was shown to induce sIgA+ B cell blasts to terminally differentiate into IgA-producing plasma cells[21]. However, it remained to be determined whether IgA-committed B cells from human gut-associated lymphoreticular tissue (GALT) could also respond to IL-6 without any co-stimulants. To this end, purified B cells from human appendix were used to examine the role of human IL-6 in regulation of IgA1 and IgA2 synthesis. This population had the advantage of containing a high frequency of sIgA+ B cells[22]. In addition, the appendix has been shown to exhibit features of GALT, and this includes a dome region covered by a unique epithelium containing follicle-associated epithelial (FAE) or microfold (M) cells that function in the uptake of gut antigens for the induction of mucosal immune responses[23]. Thus, the appendix has anatomical and functional characteristics in common with the PP which have been well characterized in mice and shown to be major IgA inductive sites[24].

When B cells isolated from human appendix were incubated with rhIL-6 for 7 days, increased numbers of IgA SFC were found (Table 3). In this regard, a 10-15% increase in IgA-producing cells was already seen after 3 days in culture, while greater than 30% increases in IgA SFC were noted after 7 days of incubation[25]. Importantly, smaller increases in the number of SFC were seen for IgM and IgG isotypes. In contrast, addition of the same amount of rhIL-6 to tonsillar B cell cultures did not enhance the numbers of IgM, IgG and IgA SFC (Table 3) unless the cells were pre-stimulated with PWM, confirming results from a previous study[14]. These results show that IL-6 induces Ig synthesis in freshly isolated human appendix B cell cultures without mitogen

or antigen co-stimulation[25]. Further, the effect was predominantly restricted to the IgA isotype.

The finding that appendix B cells are responsive to rhIL-6 without any costimulation would suggest that GALT B cell subsets constitutively express IL-6R. This hypothesis was investigated by using freshly isolated appendix B cells, which were incubated with a monoclonal anti-IL-6R antibody. A high expression of IL-6R was seen on appendix B cells[25]. However, B cells isolated from PBMC, spleen or tonsils did not express IL-6R unless they were stimulated with PWM. The intensity of IL-6R expression on GALT B cells was stronger than on PWM-stimulated peripheral blood B cells. Therefore, the appendix represents the only human lymphoid tissue studied to date that contains B cells endogenously expressing IL-6R[25]. When the expression of IL-6R was examined on the different subsets of B cells according to their membrane isotype expression, two-color FACS analysis showed that $sIgA^+$ B cells expressed higher levels of IL-6R than were seen with $sIgM^+$ or $sIgG^+$ B cells[25]. This would explain the tendency of appendix B cells to differentiate into IgA-secreting cells in the presence of rhIL-6.

The observation that human appendix B cells respond to rhIL-6 without mitogen co-stimulation is a novel finding. As described in the previous studies, human B cells from PBMC, spleen, and tonsils required activation for expression of IL-6R and for induction of Ig synthesis by

Table 3. Effect of IL-6 on B Cells Isolated from Human Gut-Associated Lymphoreticular Tissue (GALT)[a]

	Number of SFC/10^6B cells[b]			
	Appendix B cells		Tonsil B cells	
Isotype	IL-6(+)	IL-6(-)	IL-6(+)	IL-6(-)
IgA	75,000 ± 10,000	30,000 ± 5,000	4,100 ± 200	4,600 ± 600
IgG	3,000 ± 900	1,900 ± 100	14,000 ± 600	16,800 ± 700
IgM	2,100 ± 200	900 ± 180	3,400 ± 400	3,100 ± 200

[a] Purified B cells from appendix and tonsils (1 x 10^6/ml) were cultured in the presence (+) or absence (-) of rhIL-6 (20 units/ml) for 7 days. Nonadherent cells were harvested and tested in the ELISPOT assay to enumerate IgM-, IgG- and IgA-SFC.

[b] Numbers are indicated as arithmetic means ± SEM.

IL-6[13,14,19,20,27]. In contrast, our studies have demonstrated that B cells freshly isolated from the human GALT express high levels of IL-6R[25]. Further, the highest intensity of IL-6R was noted on sIgA+ B cells. This would explain the predominant IgA SFC responses seen after cultivation with rhIL-6. These findings favor the notion that the appendix is an IgA-inductive site in humans, and in this regard resembles the murine PP[25,28]. Both murine PP and human appendix are enriched for sIgA+ B cells, and these subsets are responsive to IL-6 which induces them to terminally differentiate into IgA plasma cells[21,25].

Inasmuch as human IgA occurs in two subclasses, it was important to examine the effect of IL-6 for induction of IgA1 and IgA2 isotypes. Addition of rhIL-6 resulted in approximately four-fold increases in both IgA subclass SFC responses in appendix B cells (Fig. 1), while no IL-6 effect was seen in B cell cultures prepared from tonsillar B cells. The IgA2 SFC represented approximately 60-70 percent of the total IgA SFC seen with appendix B cells[25]. The increase in IgA1 and IgA2 SFC was indeed due to IL-6, since addition of anti-IL-6 antibodies to appendix B cell cultures containing rhIL-6 completely abrogated the effect of its BSF-2 activity. These results show that rhIL-6 induces GALT B cells to terminally differentiate into IgA1- and IgA2-secreting cells.

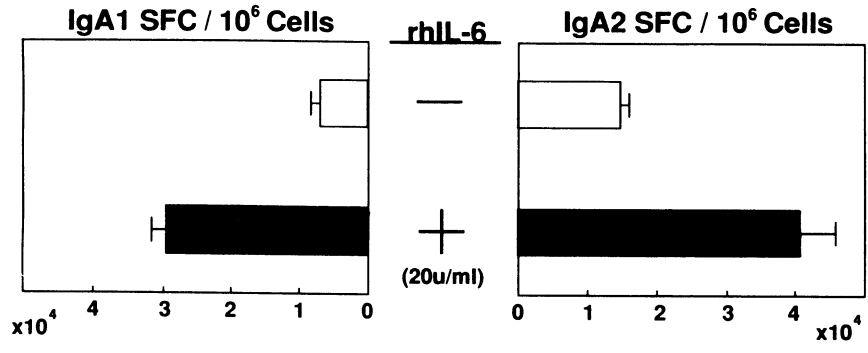

Figure 1. IL-6 support both IgA1 and IgA2 SFC responses in human appendix B cell cultures. Appendix B cells were incubated in the presence of 20 units/ml of rhIL-6, or without cytokine, and numbers of IgA1 and IgA2 SFC/10^6 viable mononuclear cells were assessed by ELISPOT assay.

When the frequency of IgA1 and IgA2 cells was compared between appendix and tonsils, two distinct patterns of IgA1 and IgA2 subclass production were noted. Appendix B cells contained higher numbers of IgA2 cells (Fig. 1), while tonsils harbored predominantly IgA1-producing cells. Our separate studies have shown that approximately equal numbers of sIgA+ B cells in both lymphoid tissues co-express IL-2R and transferrin receptors (Fujihashi, *et al.*, manuscript in preparation). An important difference was

noted between sIgA$^+$ B cells in these two tissues and induced IL-6R expression and cell size. For example, sIgA$^+$B cells in appendix were large blasts and expressed IL-6R. Furthermore, as described above, B cells isolated from appendix responded well to IL-6 without any costimulation, while tonsillar B cells did not[25]. These findings suggested that although both appendix and tonsils are organized secondary lymphoid tissues, residing IgA-committed B cells are distinct in terms of their role in the induction of IgA1 and IgA2 responses in humans.

Approximately 10% of appendix B cells were induced to differentiate into IgA-producing plasma cells by IL-6[25]. Further, approximately 50% of appendix B cells expressed surface IgA. Based on these findings, one could suggest that a relatively small number of sIgA$^+$ B cells in human appendix respond to IL-6 and terminally differentiate into IgA-secreting plasma cells. It will be important to elucidate the exact nature of sIgA$^+$ B cells which are directly responding to IL-6. To this end, it will be necessary to further separate sIgA$^+$ B cells into subsets according to the co-expression of other surface Igs (e.g., sIgM$^+$) and to their intensity of IL-6R expression (e.g., high vs low) as well as their possible occurrence in germinal centers. These types of analysis should provide the precise nature of sIgA$^+$ B cells in human GALT which are directly responsive to IL-6 and subsequently differentiate into plasma cells. We still do not know why sIgA$^+$ B cells which reside in GALT express more IL-6R when compared with sIgM$^+$ and sIgG$^+$ B cells; however, this may explain why IgM and IgG are produced less in the mucosal immune system. One could postulate that more frequent sIgM $\rightarrow$ sIgA B cell switches occur in this tissue. Along these lines, it is generally thought that $\mu \rightarrow \alpha$ isotype switches occur in PP germinal centers, since high number of surface IgA$^+$ B cells are found[29,30]. Further, cloned T cells have also been derived from human appendix and murine PP which induced both sIgM $\rightarrow$ sIgA switches and which support IgA synthesis[31-33].

CONCLUSIONS

IL-6 is a key terminal differentiation factor for Ag-induced and for polyclonally activated large B cells to become Ig-producing plasma cells. When B cells were isolated from PBMC of normal subjects that had been immunized with either T cell-dependent or -independent antigens, small but significant numbers of activated B cells were present. These large B cells expressed IL-6R and responded to exogenous IL-6 in a dose-dependent manner *in vitro* which resulted in the appearance of increased numbers of Ag-specific Ab-forming cells. Further, the secreted amount of Ab to the immunizing antigen was also enhanced by IL-6. In this process, IL-6 did not act in an isotype-specific manner since IL-6 supported both antigen-specific IgG and IgA responses. These results further support the concept that isotype-specificity of IL-6 is determined by the developmental stage and subset of B cells which are responsive to this cytokine.

In studies of polyclonal IgA synthesis, it was clearly demonstrated that the tissue source of B cells is important for the IL-6 effect. In this regard, B cells isolated from human appendix (or GALT) responded to IL-6 without any co-stimulation and led to significant increases in IgA-secreting cells. On the other hand, B cells isolated from PBMC or tonsils did not respond to IL-6

unless cells were pre-stimulated with mitogens. Further, $sIgA^+$ B cells from appendix expressed more IL-6R than those isolated from PBMC, tonsils, or spleen. The distribution of sIg^+ B cells in the appendix B cell population was $sIgA^+ > sIgG^+ > sIgM^+$, and the $sIgA^+$ B cells express higher levels of IL-6R when compared with $sIgG^+$ or $sIgM^+$ B cells. When IgA subclass responses were measured, rhIL-6 induced both IgA1 and IgA2 SFC responses; however, 60-70% of the total response was represented by the IgA2 subclass. Our studies suggest that the human appendix is an enriched source of $sIgA^+$ B cells which express high levels of IL-6R and are responsive to cytokines such as IL-6 for induction of IgA production.

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IMMUNOLOGICAL PROPERTIES AND DIFFERENTIATION POTENTIAL OF HUMAN COLOSTRAL LYMPHOCYTES OF B CELL LINEAGE

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INTRODUCTION

Human colostrum and milk contain approximately 1-5x10^6 cells/ml, identified by histochemical and immunohistochemical staining as macrophages (~40% of total cells), polymorphonuclear leukocytes (PMN), (~50%), lymphocytes (7-25%), and epithelial cells (~1%)[1-4]. Phenotypic analyses of lymphocytes revealed the presence of both B and T cells; the latter population is represented by CD4- and CD8-positive cells as well as γ/δ cells and NK cells[2,5-7]. Studies of colostral and milk lymphocytes of B cell lineage yielded controversial results with respect to their morphological properties, and their ability to respond *in vitro* to various stimuli and secrete immunoglobulins (Ig)[1,2,4,8]. The purpose of our studies was to re-evaluate the characteristics of colostral Ig-containing cells and to determine the transformability of B cells by the Epstein-Barr virus (EBV) which has been used in many studies of the differentiation potential of human B cells from various sources[9].

Ig-CONTAINING CELLS IN HUMAN COLOSTRUM

Studies of colostral B cells are hampered by difficulties encountered during the isolation of a pure population because colostral macrophages, PMN and non-cellular elements also display surface Ig[2,10,11]. In one study[12], plasma cells were identified in abundance by staining of colostral cells with Wright-Giemsa stain. However, using immunofluorescence (IF)[2], immunoelectron microscopy[13], and staining with hematological reagents we have failed to detect plasma cells in 72 samples of human colostrum[2]. Differences in the interpretation of staining may explain this discrepancy;

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colostral macrophages may have resembled plasma cells and contaminated purified colostral cell preparations.

Examination of colostral cells for antibody production by hemolytic plaque assays also provided controversial results. Although high numbers of hemolytic plaques formed by colostral elements against various bacterial antigens have been detected in all studies[10,14-16], microscopic examination of these plaques revealed the presence of individual or clustered phagocytic cells and fat-containing noncellular particles in the center[10,16]. The number of hemolytic plaques formed against a *single* antigen exceded by a great margin, expected results. Furthermore, the plaque formation was only minimally suppressed by metabolic inhibitors (Fig. 1) and the numbers of plaques did not correlate with the number of lymphoid cells represent in a given population.

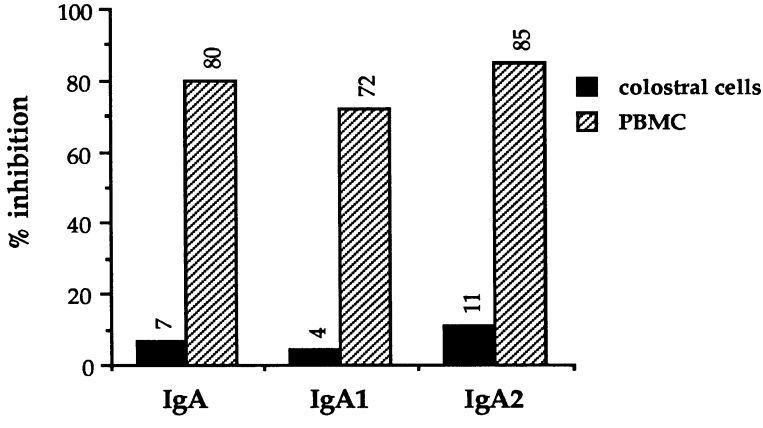

Figure 1. Inhibition of IgA spot-formation by cycloheximide. Colostral cells and peripheral blood mononuclear cells (PBMC) were cultured overnight in RPMI-1640 + 10% fetal calf serum; before analysis by ELISPOT, the cells were incubated for 4 h with cycloheximide (25 μg/ml). This treatment reduced the number of spots formed by PBMC by 72-85% while only 4-11% reduction was observed with colostral cells.

The ability to develop the same number of plaques with anti-α chain- and anti-secretory component (SC)-specific reagents[15] suggested that the elements in the plaque center contained performed secretory IgA (S-IgA) which was released during the incubation. Many studies have convincingly demonstrated that colostral phagocytic cells and noncellular particles are laden with colostral proteins, including IgA, which are released upon cell lysis[2,10,13,16-19]. Analysis of molecular properties of IgA released from lysed colostral cells revealed that these cells contained fully assembled polymeric and SC-containing S-IgA molecules[10]. This finding was in sharp contrast to the results obtained by comparative analysis of molecular forms of IgA secreted into culture supernatants or found in cell lysates of peripheral blood cells stimulated with mitogens: although polymeric IgA predominated in

culture supernatants, the intracellular IgA was found mostly in a monomeric form[10]. The mode and site of acquisition of colostral proteins by phagocytic cells remains unresolved. Although receptors for Fcα have been identified on both macrophages and PMN[20,21], the impulses that result in the internalization of S-IgA, IgM, IgG and other colostral proteins[2], have not been identified.

The total amount of intracellular IgA as related to the concentration and distribution of IgA in colostrum (free vs. cell-bound) remains controversial. It has been proposed that IgA within macrophages represents 2-10% of the total colostral and milk IgA[18,19]. When the total concentration of S-IgA in milk and colostrum (2-86 mg/ml with an average of 12.34 mg/ml in early colostrum[22,23]) are related to the total number of colostral macrophages (~$5x10^5$/ml) it appears that the reported estimates of the relative contribution of macrophages to total colostral S-IgA are unrealistically high. Using a radioimmunoassay standardized with human colostral S-IgA we have estimated that only a minute fraction of total colostral S-IgA is found within colostral cells (500ng IgA/10^6 cells)[10]. Even this amount is high when compared to the concentration of IgA measured in lysates of peripheral blood mononuclear cells cultured for 7 days in the presence of pokeweed mitogen (PWM); 15-40 ng IgA was measured per 10^6 cells. Examination of colostral phagocytic cells by immunoelectron microscopy revealed that IgA is localized within phagocytic vacuoles of highly variable sizes and densities[13,18]. Because the formation of hemolytic plaques or spots (as determined by the ELISPOT assay[24]) was not inhibitable by cycloheximide, active proteosynthesis was not required for release of intracellular Ig[24].

PROPERTIES OF COLOSTRAL B CELLS

In contrast to peripheral blood mononuclear cells, stimulation of colostral cells with various mitogens did not result in the generation of Ig-producing cells[8]. Furthermore, addition of colostral cell preparations to PWM-stimulated peripheral blood lymphocytes suppressed their responsiveness to PWM. We have shown previously[25] that colostral whey contains substances that inhibit PWM-induced differentiation of peripheral blood lymphocytes and apparently such substances were also present in colostral cell preparations. Alternatively, colostral T cells are not able to support T cell-dependent differentiation of peripheral blood B cells by PWM[8]. Furthermore, because T cell-enriched populations from peripheral blood did not augment Ig production by colostral B cells in the PWM system, we suggested that the later population is represented by B cells that are poorly responsive to PWM[8].

Our initial attempts to transform colostral B cells with EBV were unsuccessful, although B cells from corresponding peripheral blood were easily transformable[8]. Because Avital *et al.*[26] have succeeded in transforming B cells from 5 out of 8 colostral samples with EBV, we have renewed our efforts. Thus far, we have successfully transformed colostral B cells with EBV from 11 of 49 samples with an increasing rate of success. Our initial difficulties may be due to the diminished EBV transformability of B cells from Blacks (Jackson, unpublished results); almost all of our colostrum donors are Black mothers. Furthermore, we have used, in some experiments, a higher

concentration of cyclosporin. In our most recent studies, we have been able to transform B cells from 1 out of 2 colostral samples with EBV[27].

Properties of EBV-Transformed Cells.

EBV-infected cells proliferated and differentiated into Ig-producing cells, and have been kept in cultures without further subcloning for more than 100 days[27]. The cells and culture supernatants have been analyzed for the isotype (including IgA subclasses) of intracellular and secreted Ig, and for the presence of intracellular polymeric IgA linked to J chain using immunofluorescence (IF)[2], ELISPOT[24] and ELISA modified for the detection of pIgA[28]. As shown in Table 1, the colostral cells transformed with EBV secreted Ig, predominantly of IgG and IgM isotypes, as detected by ELISPOT and IF. With respect to the IgA subclasses, IgA1-positive cells were predominant, especially in long-term cultures.

Table 1. Cytoplasmic Immunofluorescence of Two Colostral Cell Cultures, Five Weeks After EBV Transformation

		Percent of cells positive for cytoplasmic:				
Culture	% Ig+ cells	IgG[a]	IgM[a]	IgA[a]	IgA1[b]	IgA2[b]
1	35	51	32	17	85	15
2	63	37	48	15	60	40

[a] % Ig^+ cells

[b] % total IgA^+ cells

When compared to parallel cultures of EBV-infected peripheral blood B cells, the total number of Ig-positive cells was lower in colostral cells. The numbers of IgA1- and especially IgA2-positive cells found in colostral cell cultures resembled, by their ratio, the distribution of IgA subclass-positive cells in the mammary gland[29] and in colostrum[30].

The quantities of IgM, IgG, IgA (including IgA1 and IgA2 and associated J chain) were measured by an ELISA[28] during 6 wks in culture (Fig. 2). Supernatants of colostral cells from different donors displayed considerable individual variances with respect to the predominance of Ig isotype. For example, after 6 wks, IgM and IgA1 were found in highest quantities in supernatant from culture #3 but not in other cultures.

Most of the IgA1- and IgA2-positive cells contained intracellular J chain (determined by IF)[2] and J chain-containing polymeric IgA1 and IgA2 were detected in culture supernatants.

In contrast to EBV-transformed peripheral blood B cells in which a three-fold increase was observed[31], the addition of IL-6 to colostral cells did not significantly influence the secretion of IgA, IgG, or IgM. However, a slight increase in the content of J chain was noted in IgA1 and IgA2 secreted by cells cultured in the presence of IL-6. Importantly, the addition of IL-6 had no effect on the isotype profiles of Ig-secreting cells from activated peripheral blood B cells[31] or EBV-transformed colostral cells cultured for up to 100 days[27].

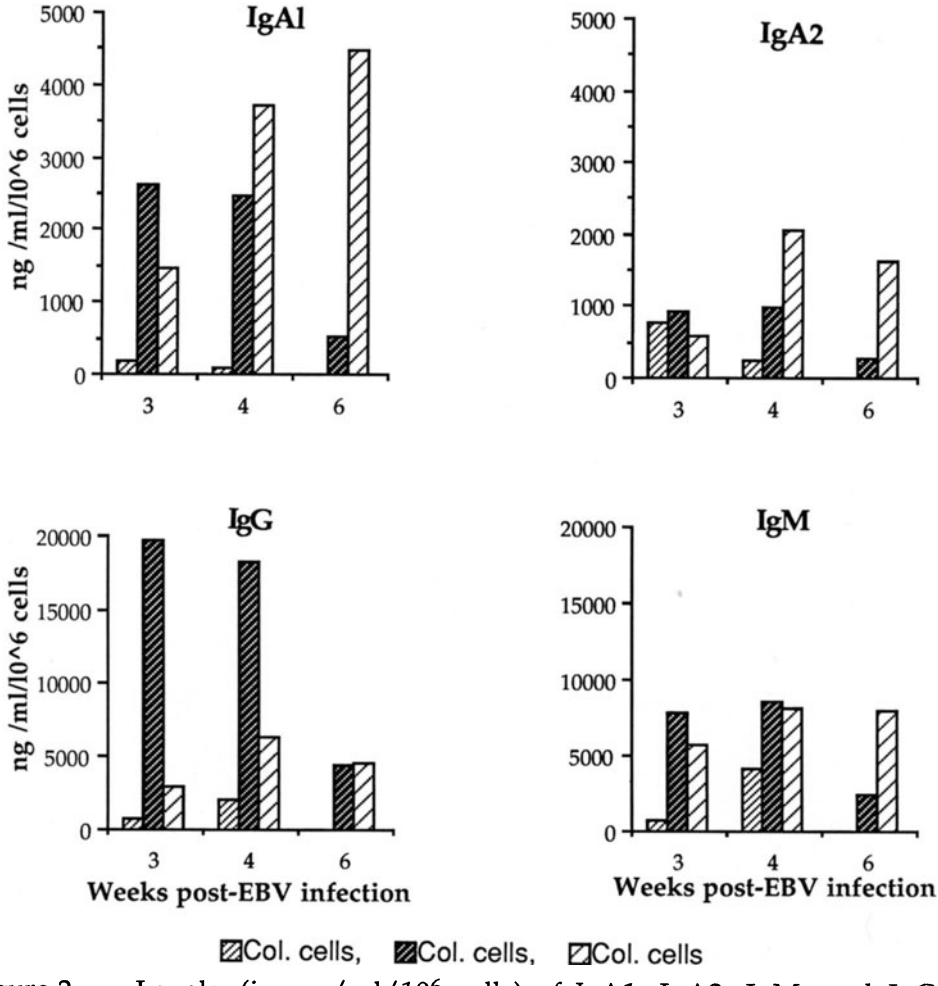

Figure 2. Levels (in ng/ml/10^6 cells) of IgA1, IgA2, IgM, and IgG in supernatants of EBV-transformed colostral cells from three donors kept in culture for 6 weeks.

Although poorly responsive or unresponsive to commonly used mitogens[8], colostral B cells from some donors can be transformed by EBV into Ig-secreting cells. Thus, our results[27] confirm and extend these of Avital *et al.*[26]. Transformed colostral B cells secreted Ig of all three major isotypes without a marked preference for IgA. Thus, it appears that the colostral B cells do not include a population of IgA plasma cell precursors pre-selected or locally expanded by the lactating mammary gland. Their origin and reasons for a decreased *in vitro* responsiveness to mitogens and IL-6 remains to be clarified.

CONCLUSIONS

1. Immunoglobulins were detected in colostral phagocytic cells and noncellular globules. Lymphoblasts and plasma cells positive for cytoplasmic Ig were not detectable. In contrast to the *active* secretion of Ig by PWM-stimulated peripheral blood lymphoblasts, the release of Ig from colostral cells was not inhibitable by cycloheximide. Together with previously reported[10] quantitation of intracellular IgA, molecular form of this IgA (SC-containing polymers), and determination of cell types that contain IgA, we conclude that a great majority of IgA associated with colostral cells is found in phagocytic cells and is not actively synthesized in high quantities by colostral cells of B lymphocyte lineage.

2. In contrast to our previous unsuccessful effort with transformation of colostral B lymphocytes with EBV[8], slight modification in culture conditions resulted in the establishment of long-term cultures from 11 out of 49 individual samples of colostral cells. IgM-, IgG-, and IgA-(including both IgA subclasses)-secreting cells were detected by the ELISPOT assay and immunofluorescence. Although the number of Ig-positive cells decreases in long-term cultures, we have maintained such cultures for several months. In contrast to cells from peripheral blood, the addition of IL-6 had no effect on the number of Ig-positive colostral cells and the isotype of secreted Ig.

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THE EFFECTS OF COLOSTRUM ON NEUTROPHIL FUNCTION: DECREASED DEFORMABILITY WITH INCREASED CYTOSKELETON-ASSOCIATED ACTIN

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INTRODUCTION

The most prominent cellular elements of human colostrum are phagocytic leukocytes, which are present in numbers ranging from 1-50 x 10^5/ml. The majority of these cells are polymorphonuclear leukocytes (neutrophils), the widely acknowledged acute inflammatory cell of the body. The presence of large numbers of neutrophils in any blood fluid other than bone marrow or blood is generally taken to imply the presence of acute inflammation, but in the newly lactating breast, the source of colostrum, *"rubor et tumor cum calore et dolore"* are not present.

The presence of acute inflammatory cells in colostrum without evidence of acute inflammation is curious and unexplained. In disease states where the function of the neutrophil as an inflammatory cell is suppressed (as in neutropenia or in inherited neutrophil dysfunction sates), altered acute inflammatory responses are the rule[1], implying a relationship between the cell's functional capabilities and the acute inflammatory response. Others have previously shown that the functional capabilities of colostral neutrophils and blood neutrophils are very different, with the colostral cells showing diminished activities[2]. We hypothesized that the colostral environment was capable of suppressing multiple facets of normal neutrophil function and that the result of this effect was acute inflammatory cells without acute inflammation. To test this hypothesis, we examined the effects of colostrum exposure on neutrophil locomotion and its associated parameters deformability and polarization.

MATERIALS AND METHODS

Human colostrum was collected and processed as previously described[3,4]. Following high speed centrifugation the cell-free aqueous fraction was collected and stored at -70°C until used. Neutrophils were purified from heparinized control donor blood as described[3,4].

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Locomotive responses of neutrophils were examined using a rectangular well, under-agarose assay[5]. Leading front distances were used to estimate locomotive responses: Hank's balanced salt solution (HBSS) was substituted for a stimulus to examine unstimulated locomotion. Chemotaxis was examined with three stimulus conditions: zymosan activated serum (AcS), 5 x 10^{-7}M f-met-leu-phe (*f*MLP) and AcS plus *f*MLP (AcS + *f*MLP). For colostrum exposure experiments, 50% aqueous colostrum was incorporated into the agarose plate.

Neutrophil deformability was assayed using a micropore filter method. A five micron pore size (13 mm size) polycarbonate filter was mounted in a modified filter holder which allowed continuous monitoring of fluid pressure on the proximal side of the filter. Purified neutrophils (2.5 x 10^6/ml) were pumped at a constant rate (1 ml/min) across the filter using a Harvard pump, and the pressure generated on the proximal side of the filter was recorded over 60 sec. Runs were performed in triplicate and averaged to produce a representative pressure x time curve for each experimental condition. The area under the pressure x time curve was taken as the measure of neutrophil deformability. Exposure conditions tested included 50% aqueous colostrum and 5 μg/ml. cytochalasin B, both at 37°C for the time periods described. Cell samples were cooled to room temperature before deformability testing.

Neutrophil polorazation was estimated visually. Purified cells were exposed to experimental conditions at 37°C for the times described, and were then fixed with 10% formalin for 30 min at 4°C. After washing, the cell suspensions were examined microscopically and 100 cells in each experimental condition were assessed as to whether they were round or polarized.

The F-actin content of colostrum-exposed neutrophils could not be assessed using the NBD-phallicidin method because of the high protein content of the aqueous colostrum. As an alternative, the neutrophil content of cytoskeleton-associated actin (CAA) was estimated[6]. Four million purified neutrophils were either kept in buffer or exposed to 50% aqueous colostrum at 37°C for the times described. The cells were then lysed (5 mM DFP, 20 mM EGTA, 20 mM imidazole, 80 mM KC1 and 2% Triton X-100), pelleted, washed, repelleted, and then solubilized for SDS-PAGE on 10% gels. After staining with coomassie blue, the gels were photographed, the negatives were densitometrically scanned, and the area under the 42 kDa actin peak was taken as the estimate of CAA for that condition. For comparisons, the unexposed, time 0 cell content was normalized to 1.0, and CAA content in other conditions was expressed as the fold increase/decrease over the unexpected, time 0 cell content.

For statistical analyses, mean $\pm$ SE values were compared by t-test, with $p<0.05$ declared as statistically significant. When natural pairing occurred over entire data sets, paired-simple t-tests were used to make comparisons.

RESULTS

Colostrum Exposure Significantly Suppresses Blood Neutrophil Locomotive Responses (Fig. 1)

Unstimulated movement in the presence of 50% aqueous colostrum was decreased to 34 $\pm$ 11% (n = 5) of unexposed control movement, while

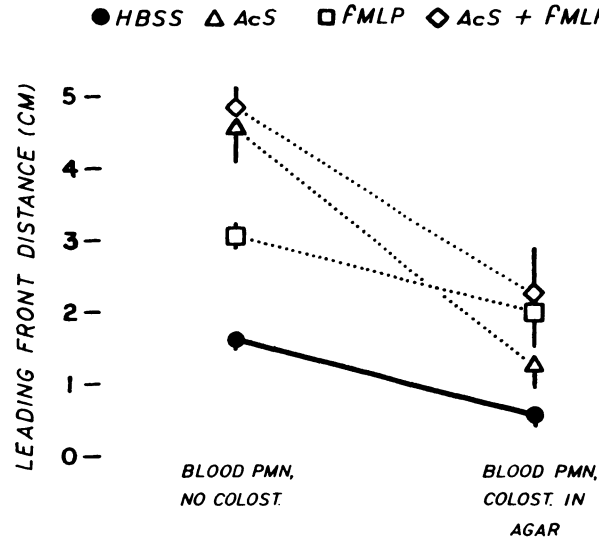

Figure 1. Blood neutrophil locomotive responses in the presence of 50% aqueous colostrum. Unstimulated locomotion (HBSS) and chemotaxis (AcS, *f*MLP, AcS + *f*MLP) are shown. Data points are mean ± SE; all colostrum effects are significant, $p < 0.05$.

chemotaxis to AcS, *f*MLP and AcS + *f*MLP were decreased to 29 ± 9% (n = 5), 59 ± 14% (n = 5) and 47 ± 12% (n = 4) of control values, respectively (all $p<0.05$).

Colostrum Exposure Significantly Decreased Blood Neutrophil Deformability (Fig. 2)

Because the ability to deform and change shape is important in neutrophil locomotion, the effects of 50% aqueous colostrum exposure on cell deformability were examined. The vertical geometry of the filtration system caused acellular buffer alone to give a pressure x time area of 95 ± 2 mm Hg sec. Neutrophils without colostrum exposure resulted in a pressure x time area of 357 ± 31 mm Hg sec. Fifteen min, 37°C exposure to aqueous colostrum increased the pressure x time area significantly (508 ± 33 mm Hg sec, $p < 0.005$ vs. unexposed cells, n = 9). This effect was present at the earliest time point examinable (5 min colostrum exposure: 645 ± 90 mm Hg sec) and could be reversed by washing the colostrum exposed cells.

Five min exposure of control neutrophils to 5 μg/ml of cytochalasin B dramatically decreased the pressure x time area observed (120 ± 8 mm Hg sec, $p<0.005$ vs control, n = 5), implying that cytoskeletal actin polymerization contributed significantly to normal cell deformability characteristics. Cytochalasin B exposure decreased the pressure x time areas of colostrum exposed neutrophils to 229 ± 30 mm Hg sec ($p<0.01$ vs. colostrum exposed cells, n = 6), but not to the levels observed with cytochalasin B exposed control cells.

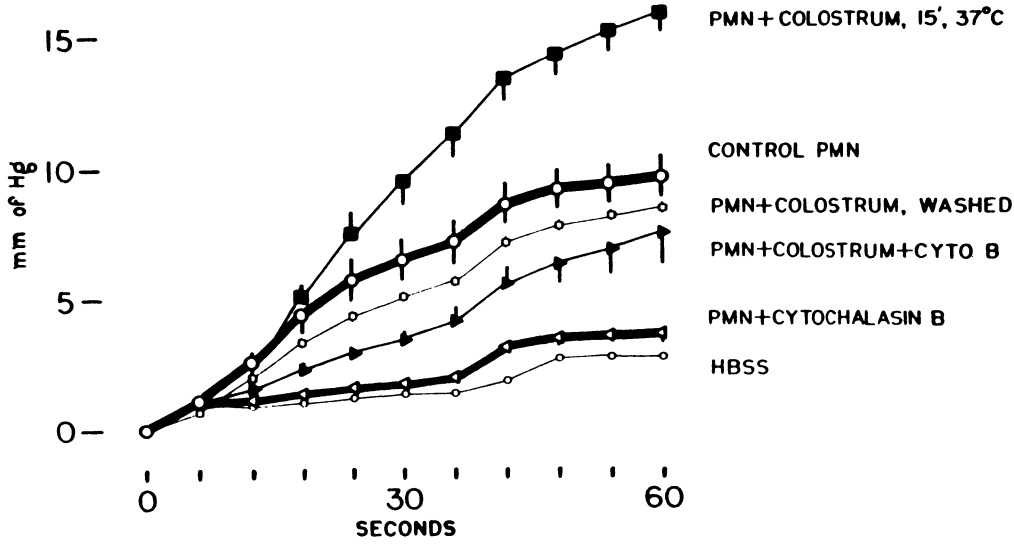

Figure 2. The effects of 50% aqueous colostrum and cytochalasin B on blood neutrophil deformability. Data points are mean ± SE. HBSS represents an acellular buffer blank.

Colostrum Exposure Significantly Increased the Percentages of Blood Neutrophils Showing Polarization (Fig. 3)

Because the decreased neutrophil deformability caused by colostrum exposure appeared to be partially related to neutrophil cytoskeletal organization, and neutrophil cytoskeletal organization is often accompanied by polarization of this cell type, the effects of colostrum exposure on neutrophil shape were examined. Freshly purified neutrophils, incubated in HBSS for 15 min at 37°C were 44 ± 7% polarized. This level of polarization was decreased to 12 ± 4% by cytochalasin B exposure, and increased to 88 ± 3% by 50% aqueous colostrum exposure. The colostrum effect was reversible, as washing decreased polarization to 28 ± 6%, and appeared to be at least partially related to cytoskeletal organization, as addition of cytochalasin B to the colostrum exposed cells decreased polarization to 41 ± 8%.

Colostrum Exposure Significantly Increased Blood Neutrophil CAA Content (Table 1)

The cytochalasin B effects observed in both the deformability and polarization assays prompted the examination of neutrophil CAA content. When various experimental exposure conditions were examined, it was clear that colostrum exposure caused very rapid increases in CAA. The largest increases were seen at the earliest time point examinable (time 0 min, i.e., the time of colostrum addition), and levels remained increased over at least 15 min of colostrum exposure. Examination of the aqueous colostrum showed no 42 kDa actin band by SDS-PAGE, making it unlikely that the increase in *cellular* CAA content was due to artifactural accumulation of *colostral* actin by the cellular cytoskeletons.

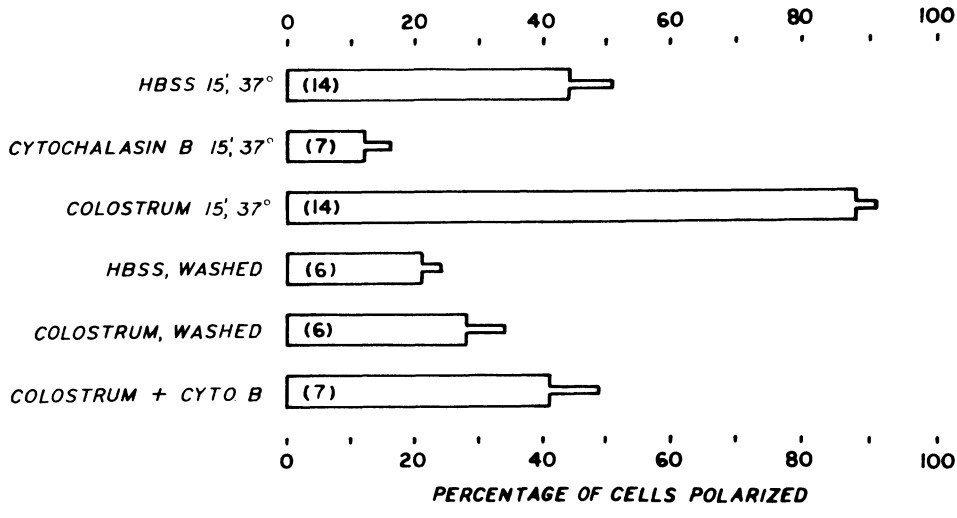

Figure 3. The effects of 50% aqueous colostrum and cytochalasin B on blood neutrophil polarization. Bars show the mean ± SE.

Table 1. The Effects of Human Colostrum Exposure on Cytoskeleton-Associated Actin Content of Neutrophils

	DURATION OF EXPOSURE#			
	0 MINUTES	5 MINUTES	10 MINUTES	15 MINUTES
NO COLOSTRUM EXPOSURE	1.0 (17)	1.0 ± 0.3 (3)	ND	4.4 ± 3.6 (3)
COLOSTRUM EXPOSED	7.0 ± 3.8* (11)	3.3 ± 0.7** (16)	3.0 ± 1.4 (10)	2.4 ± 0.2*** (17)
CYTOCHALASIN B EXPOSED	ND	0.9 ± 0.4 (6)	ND	ND
COLOSTRUM AND CYTOCHALASIN B EXPOSED	ND	1.6 ± 0.4 (6)	ND	1.8 ± 0.8 (6)

EXPRESSED AS THE X ± SE FOLD INCREASE OVER 0 MINUTES, NO COLOSTRUM EXPOSURE CELLS
* $p<0.01$ VS. 0 MINUTE, NO COLOSTRUM EXPOSURE
** $p<0.02$ VS. 0 MINUTE, NO COLOSTRUM EXPOSURE
*** $p<0.05$ VS. 0 MINUTE, NO COLOSTRUM EXPOSURE

DISCUSSION

The paradox of acute inflammatory cells in human colostrum without evidence of acute inflammation is unexplained. To determine whether functional characteristics of neutrophils important to their roles as inflammatory cells might be suppressed by the colostral environment, (thus explaining the lack of inflammation), we examined the effects of aqueous colostrum on neutrophil locomotion, polarization and deformability. These

studies showed that colostrum caused significant alteration of each of these characteristics, in directions that would be expected to adversely alter the cells ability to mediate/propagate inflammation. Mechanistically, both the polarization and deformability effects of colostrum appeared to be mediated via enhanced actin polymerization/cytoskeletal organization (based on the effects of cytochalasin B). Direct quantitation of CAA content of colostrum exposed cells confirmed this. Taken together, the data imply that for *blood* neutrophils, the colostral environment is not one that would be optimal for cellular function.

We have previously shown that human colostrum contains antioxidant activities that effectively consumes neutrophil produced H_2O_2 and can prevent neutrophil-mediated epithelial cell injury *in vitro*[3,4]. The current studies add to these observations, painting a picture that portrays the neutrophil in the colostral environment as a cell whose normal capabilities are compromised. Although the leukocytes in human milk have previously been considered to be gifts from the mother to the infant, perhaps this does not apply to the neutrophil in colostrum. We interpret our results as suggesting the opposite; the neutrophil is by its nature a potentially dangerous cell, the persona of acute inflammation. The neutrophil in colostrum appears to be altered via multiple routes, with the paradoxical effect that acute inflammatory cells are present without acute inflammation.

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PEROXIDASES IN HUMAN MILK

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INTRODUCTION

The first quantitative measurements of peroxidase activity in human milk were reported in 1963[1]. Since that time, a number of studies have been published and recently reviewed[2]. Reported peroxidase activities show great variability. However, there is general agreement on the following points: peroxidase activity is highest in human colostrum and declines to significantly lower levels in mature milk; bovine colostrum and mature milk have much higher and much less variable peroxidase activity than do their human counterparts; human milk peroxidase activity is correlated with the presence of polymorphonuclear (PMN) leukocytes.

The contribution of leukocyte myeloperoxidase (MPO) to total human milk peroxidase activity is controversial. In one study[3], the molecular exclusion chromatographic profile of human milk peroxidase was found to be similar to that of MPO derived from blood PMN leukocytes. An antibody directed against bovine lactoperoxidase (LPO) did not react with human milk peroxidase or with MPO. Another study[4], reported that the peroxidase in human milk gave a single precipitin line against an antiserum prepared against human MPO, and that anti-MPO IgG completely precipitated the peroxidase activity in human colostrum. This latter study reported that human colostral whey peroxidase, purified to homogeneity, was composed of subunits similar in size to the subunits of human MPO.

Other workers have concluded that human milk peroxidase is *not* MPO. All of the peroxidase activity in a pool of whey from human colostrum has been reported[5] to be absorbed to Phenyl Separose C1-4B and to elute with a retention time characteristic of LPO. All of the peroxidase activity in the whey was removed by an immuno-affinity column utilizing anti-bovine LPO antibodies. The ConA-Separose elution pattern of colostral peroxidase and bovine LPO antibodies absorbed the colostral peak from the ConA-Sepharose elution but did not bind human MPO. In further studies[6], an apparent 1450-fold purification of human colostral peroxidase was reported. This preparation had spectral properties and molecular size comparable to bovine LPO.

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We report here studies which show that human milk and colostrum contain variable amounts of two peroxidase enzymes: MPO, and a peroxidase with properties similar to those of human salivary peroxidase (SPO). In this study, we have used kinetic methods of analysis to distinguish between the different milk peroxidases. The conflicting results in the literature may be a consequence of real qualitative and quantitative differences in the peroxidase contents of the preparations analyzed by the different investigators and of differences in sensitivities and specificities of the methods used to characterize the enzymes.

MATERIALS AND METHODS

Early human milk (1-4 days post-partum) was collected by manual expression, refrigerated and used within 4 hours. Cells and fat were removed by two centrifugations (30-40 minutes at 31-39,000 x *g*). Peroxidase enzyme activities (Fig. 1) were measured on the supernatants as described below. The data shown in Fig. 3 were obtained from mature (1-5 months post-partum) milk. For further analysis, five samples of early milk with high activities were pooled and processed as follows. Casein was removed by adjusting the pH to 4.2 with acetic acid and centrifuging (40 minutes at 39,000 x g). Supernatant pH was adjusted to 7 with 1M NaOH, the solution was dialyzed against 0.1 M phosphate buffer, pH 7.3, concentrated by ultrafiltration (AMICON cell, YM10 membrane, and applied to a 2.5 x 15 cm column of sulfopropyl (SP) Sephadex. The column was washed thoroughly with starting buffer. In order to be certain that the column was not overloaded, these fractions were pooled and reapplied to the SP-Sephadex column. Bound material was eluted with a potassium phosphage gradient (0.1 - 1 M, pH 7.3). The wash fractions from the SP-Sephadex column were pooled, dialyzed against 2M sodium acetate, and applied to a Phenyl Sepharose C1-4B column (2.5 x 10 cm). The column was thoroughly washed with 2M sodium acetate, and adsorbed proteins eluted with a sodium acetate gradient (2.0M - 0.05M). The purpose of this step was to separate MPO from other peroxidases. In separate experiments, we have found that MPO is not eluted from the Phenyl Sepharose C1-4B column under these conditions. Fractions from the central portion of the elution peak containing peroxidase activity were pooled and used in all subsequent experiments. The gradient fraction from the initial SP-Sephadex elution was also applied to a Phenyl Sepharose column. Neither the wash nor the gradient elution fractions from this step contained any detectable peroxidase activity for Cl^- or SCN^-.

MPO, SPO, and LPO were purified and enzyme concentrations determined spectrophotometrically as previously described[7,8]. Peroxidase activities were measured with the ABTS, NBS-Cl, and NBS-SCN assays[9]. The following equation[10] was used for kinetic analyses:

$$V = \frac{[E]_0\,[H_2O_2]\,[SCN^{-1}]}{\frac{[H_2O_2]}{k_4} + \frac{[SCN^{-1}]}{k_1}\,(1 + K_i\,[SCN^-])} \qquad (1)$$

Rate constants were estimated by fitting the initial rate data to equation(1) using nonlinear regression analysis[11].

Bacterial Studies

Escherichia coli X-15 was from stock at the University of Alabama at Birmingham, *Salmonella typhimurium* his G46 was from Bruce N. Ames, University of California, Berkeley, CA. Aliquots of 3 ml of mature human milk were placed in sterile culture tubes. Various combinations of 0.1 ml each of phosphate buffered saline (PBS) solutions of LPO, KSCN, and H_2O_2 together with aliquots of bacterial suspensions were added to each tube to give the following initial concentrations: LPO, 5 μg/ml; KSCN, 3 mM; H_2O_2, and 3 mM. Tubes were placed on a rotary agitator and incubated at room temperature (21-23°C) or 4°C. Aliquots of 0.1 ml were taken at appropriate intervals, and 0.1 ml serial 10-fold dilutions in PBS were inoculated in duplicate onto standard plate count agar (Difco). Bacteria were inoculated at 1 x 10^5 to 1 x 10^9 CFU/ml. Normal contaminants of collection (1 x 10^4 to 1 x 10^7 CFU) could readily be distinguished from the inoculated *E. coli* and *S. typhimurium* by colonial morphology. Colonies were counted after incubation (18 hours) at 37°C and the results expressed as colony-forming units per ml (CFU/ml).

RESULTS AND DISCUSSION

Peroxidase Activity in Early Human Milk

We analyzed 27 milk samples from 21 different donors. The samples contained variable amounts of peroxidase enzymes. We found the following mean oxidation rates (mM donor oxidized/min): SCN^-, 3.7 (range 0.4 - 9.0); Cl^-, 0.33 (range 0.04 - 1.0). The Cl^- results corresponded to a mean MPO concentration of 6.5 μg/ml (range 0.7 - 21). Cell counts varied from non-detectable to 2.4 x 10^7 cells/ml. PMN's varied from 30 to 95% of the total cell count. Samples containing higher counts of PMN's tended to have higher MPO concentrations, but the correlation was not statistically significant. All samples contained MPO activity but the quantitative contribution of MPO to the total peroxidase activity varied widely from sample to sample. MPO activity alone did not account for the total peroxidase activity in any of the samples. In Fig. 1, the SCN^- oxidation rate is plotted against the Cl^- oxidation rate. The straight line is what would be observed with only MPO. All of the milk samples fall above the line indicating the presence of other peroxidase enzymes in addition to MPO. The mean peroxidase activity which *was not* contributed by MPO was 44% (range 17-72).

Milk Fractionation

The total recovery of the peroxidase activity applied to the SP-Sephadex column was 86% (Table 1). The distribution of the recovered peroxidase activity was 83% in the wash fractions and 17% in the gradient fractions. When a portion of the pooled wash fractions was reapplied and eluted from SP-Sephadex, 72% of the applied activity was recovered in the wash fractions and none in the gradient fractions. Wash fractions from the Phenyl Sepharose column contained no peroxidase activity. The peroxidase activity (83% of the total applied) appeared in the gradient fraction. No chloride oxidizing activity was detected in any of the fractions from this column. The

chloride oxidizing enzyme in the pooled sample showed kinetic properties identical to those of MPO. The pooled colostrum and the wash 1 fraction from the SP-Sephadex column had peroxidative activity toward both Cl^- and SCN^-. However, the Gradient 1 fraction from Phenyl Sepharose did not catalyze the oxidation of Cl^-. These procedures separated Cl^- and SCN^- peroxidative activity, but they did not completely separate peroxidase enzymes from other colostral components. The Phenyl Sepharose Gradient 1 fraction represented only a 6.6 fold purification.

The peroxidase enzyme which did not oxidize chloride had kinetic properties which closely resembled SPO. We designate this enzyme as human lactoperoxidase (HLP). Earlier results together with the data reported

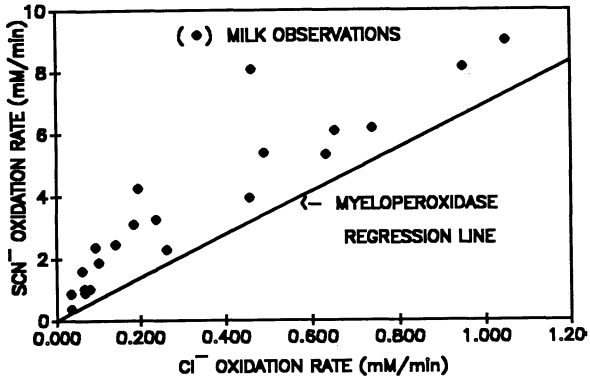

Figure 1. The SCN^- oxidation rate as a function of the Cl^- oxidation rate for human milk samples and for MPO.

Table 1. Partial Purification of Human Lactoperoxidase (HLP)

Fraction	Total activity (mM SCN^-/min oxidized)	%HLP activity	HLP/ABS 280 nm	Fold purification (%Recovery)
Pooled colostrum	5.0	2.6	---	--- (--)
Whey	3.5	26.9	0.024	1 (100)
SP Sephadex-wash 1	2.3	38.0	0.102	4.3 (86)
Phenyl Sepharose gradient 1	0.65	100	0.156	6.6 (83)

methods used in the reports of MPO in human milk[3,4], may not have been sufficiently sensitive or selective to detect HLP. The methods used by Langbakk and Flatmark[5,6] would not have detected MPO in their colostral samples because the MPO would not have appeared in the elution profile from the Phenyl Sepharose columns. They apparently did not analyze the washings from their anti-LPO affinity columns for peroxidase activity. Therefore MPO which was *not* bound to these columns would have passed through undetected prior to the step of eluting the bound LPO with glycine-HCl.

Steady State Kinetic Analyses

The data plotted in Fig. 2 show that all of the peroxidase enzymes are inhibited by high concentrations of SCN^- and that MPO is inhibited by high concentration of Cl^-. The curves for HLP and SPO have similar shapes with maximum activity near 1 mM SCN. The maxima for LPO and MPO are broader and occur near 2 mM SCN. The observations fit the predicted curves.

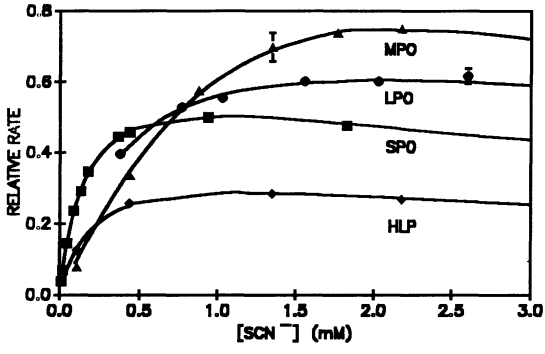

Figure 2a. Rates of peroxidation of SCN^- (pH=5.6). The Relative Rate = observed rate (μM SCN^1 oxidized/min) divided by enzyme concentration (nM) and by $[H_2O_2]$ (μM).

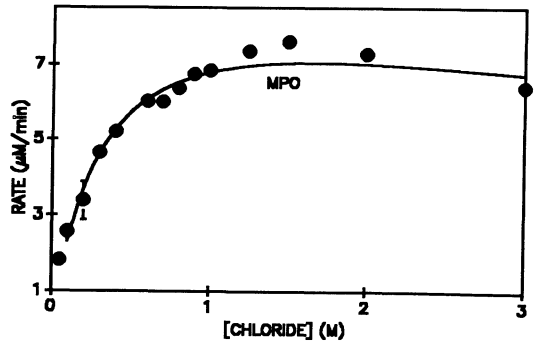

Figure 2b. Rate of peroxidation of Cl^- by human MPO (pH=7.0). Points are actual observations. Lines are predicted curves based on fitting the data to equation (1) by non-linear regression[11].

The rate constants for LPO and for SPO have been reported previously[10]. For k_1, the estimates (Table 2) were the same order of magnitude for all of the enzymes. The k_1 for MPO is approximately twice the values found for LPO and SPO as would be expected since MPO contains two heme groups per molecule. However, the MPO k_4 estimates were significantly lower than those obtained for the other peroxidases while the inhibition constant, Ki, was significantly higher for MPO. In order to obtain the values shown for HLP, the specific ABTS activity for HLP was assumed to be equal to that of SPO.

Our results show that human milk contains a peroxidase enzyme with properties similar to those of SPO. Our work is consistent with other reports[5,6]. We also found that MPO is an important contributor to the total peroxidase activity in human milk confirming earlier observations[3,4].

Table 2. Rate Constants for the Peroxidation of SCN^-, pH=5.6 and Cl^-, pH=7.0

Peroxidase	#OBS	K_1 (SD) (10^7 $M^{-1}s^{-1}$)	K_4 (SD) SCN^- (10^6 $M^{-1}s^{-1}$)	Ki (SD) (10^2 M^{-1})
LPO	22	1.46 (0.05)	3.6 (0.2)	1.10 (0.14)
HLP	12	1.55 (0.06)	8.1 (0.6)	1.61 (0.18)
MPO	17	2.04 (0.31)	0.76 (0.04)	6.54 (1.30)
SPO	24	1.09 (0.02)	6.86 (0.10)	1.50 (0.15)
			Cl^-	
MPO	24	0.059 (0.004)	0.021 (0.002)	0.11 (0.04)

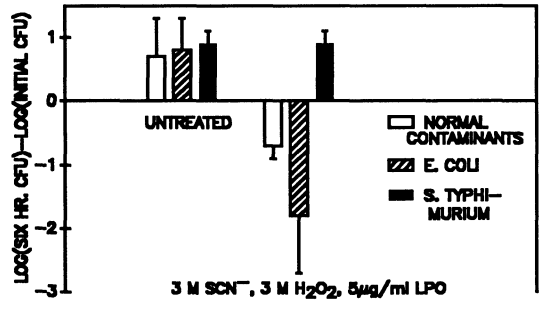

Figure 3a. Growth of bacterial contaminants of collection and of inoculated *E. coli* X-16 and *S. typhimurium* his G46 in mature human milk (21-23°C) before and after addition of indicated substances. The means of duplicate plate counts for each of 3 different samples of milk are plotted. The bars indicate 1 standard deviation.

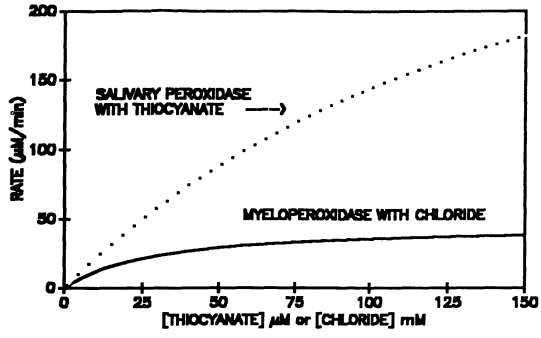

Figure 3b. Calculated rates of peroxidation of SCN^- and of Cl^-. The lines are predicted curves based on the rate constants listed in Table 2 for myeloperoxidase and on rate constants calculated at pH 7 for salivary peroxidase[10].

Antibacterial Properties of Mature Human Milk

Peroxidase activity was undetectable in the milk of seven donors collected 1 month or more post-partum. The data in Fig. 3 show that human milk will support the growth of bacteria, but when it is supplemented with bovine LPO, SCN^-, and H_2O_2 both the normal contaminants of collection and inoculated *E. coli* X-15 are killed. However, inoculated *S. typhimurium* his G46 is unaffected. Addition of SCN^- and H_2O_2 alone had no significant effect on bacterial growth. Our observations confirm that *S. typhimurium* his G46, which has a relatively impermeable cell envelope, is resistant to the lactoperoxidase system[12].

One milk donor had completed antibiotic therapy for mastitis a week before donation at 3 and 4 months post-partum. Her milk had an intrinsic peroxidase activity equivalent to 10μg/ml LPO. At the time we collected this milk, we had not developed the differential MPO/LPO assay so we are unable to report the distribution of total activity between the two peroxidases in this milk. However, this milk killed (data not shown) normal contaminants, inoculated *E. coli and* inoculated *S. typhimurium* when supplemented *only* with SCN^- and H_2O_2 to final concentrations of 3mM each. The bactericidal effects were observed at room temperature and also at 4°C. Both species of inoculated bacteria continued to grow over 48 hr in untreated samples of this milk, while the CFU for both species continuously declined during this period in the SCN^- + H_2O_2 supplemented milk. It seems likely that the peroxidase activity in this milk was due to MPO associated with elevation of leukocytes as a result of the mastitis. The killing of the *S. typhimurium* in this sample of milk as compared to the survival of these organisms in milk containing no intrinsic peroxidase activity (but with LPO added to 5 μg/ml) is difficult to explain. It is probably *not* a result generation of hypochlorite (OCl^-) by MPO-catalyzed peroxidation of chloride. The rate constants listed in Table 2 show that SCN^- would compete very effectively with Cl^- for the oxidizing equivalents of MPO Compound I and that $OSCN^-$ rather than OCl^- would be the dominant product.

CONCLUSIONS

The Biological Significance of Human Milk Peroxidase. The data reported here show that colostrum contains variable but significant levels of peroxidase activity contributed both by MPO and by human lactoperoxidase. Human milk also contains variable concentrations of thiocyanate[2]. Hydrogen peroxide may be a normal product of oxidative metabolism in the mammary gland. Thus, all of the components required to generate the antibacterial product[13] $OSCN^-$ may be present. Whether the enzyme is present as HLP or MPO, the curves in Fig. 2 and in the right panel of Fig. 3 show that $OSCN^-$ will be the major product. Thus, it seems reasonable to speculate that in early milk the peroxidase system serves an antibacterial function. It may also function to protect the mammary gland from accumulation of toxic levels of hydrogen peroxide. Such protective effects have been shown for other mammalian cells[14,15]. However, these comments apply only to colostrum and early milk, since mature milk from healthy donors contains little or no peroxidase activity.

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LACTOFERRIN BINDING TO ITS INTESTINAL RECEPTOR

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INTRODUCTION

Lactoferrin was first discovered in bovine milk in 1939[1]. It was later found in high concentration in human milk[2]. Lactoferrin is a glycoprotein with a molecular weight of 78,000 and has the capacity to bind two ferric ions concomitant with bicarbonate or carbonate ions. The primary structure[3] as well as the glycan structure[4] have been documented and the tertiary structure has been elucidated by X-ray crystallography[5]. The protein consists of two lobes, each binding one iron atom and having one glycan; because of the similarity between the two lobes, the molecule is believed to be the result of an early gene duplication[3]. Lactoferrin is sometimes referred to as lactotransferrin[3,4]. However, even if lactoferrin and transferrin have some similar physicochemical properties, they are products of different genes and their biological functions are different.

Although lactoferrin was found first in milk and is present in high concentration in the milk from several species, it is also found in plasma, tears, pancreatic fluid, bile, and urine[6,7]. Lactoferrin is also produced by neutrophils[8] and lymphocytes[9] and is known to be involved in phagocytotic killing by macrophages[10]. The concentration of lactoferrin in human milk, however, is considerably higher (1-5 mg/ml) than in other fluids, such as serum (1 μg/ml). During lactation, lactoferrin levels decrease from initially high levels in colostrum, but even after one year of lactation the concentration is usually around 1 mg/ml[11]. Maternal iron status may have some effect on the concentration of lactoferrin in milk[12]; however, this possible correlation has received limited attention. Little is known about other factors affecting milk lactoferrin levels, but nutritional status may have some influence.

PHYSIOLOGICAL SIGNIFICANCE OF LACTOFERRIN

Considering the high levels of lactoferrin in milk of several species, biological roles beyond that of a dietary protein (i.e., a source of amino acids)

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have been proposed. Early studies indicated that the iron saturation of lactoferrin in human milk is relatively low and thus a role for lactoferrin in bacteriostasis was proposed[14]. Subsequent experiments *in vitro* demonstrated that apo-lactoferrin can inhibit the growth of several iron-dependent pathogens, such as *Escherichia coli, Vibro cholerae* and *Pseudomonas aeroginosa,* and that addition of iron to the medium abolishes the bacteriostatic function of lactoferrin[15]. Although it has been demonstrated that only 1-4% of the iron-binding capacity of lactoferrin in human milk is occupied[16], there is little *in vivo* evidence for a bacteriostatic function of lactoferrin.

A role for lactoferrin in gut iron metabolism has also been proposed. The precise role of lactoferrin, however, is somewhat controversial; early work suggested that lactoferrin could inhibit iron uptake by the mucosa and therefore protects against iron overload[17]. Later, it was proposed that lactoferrin facilitates iron absorption in infants, possibly via a receptor-mediated mechanism[18]. It has also been hypothesized that lactoferrin inhibits iron absorption during early life, while iron released from digested lactoferrin becomes available for absorption during late infancy[19]. While absorption of iron from digested lactoferrin cannot be excluded as a possible pathway for iron, evidence has been gathered to support receptor-mediated iron uptake from lactoferrin (see below).

It is known that human milk stimulates the growth and proliferation of the mucosa in the small intestine, and that villous height is higher in breast-fed than in formula-fed infants. These observations were indicative of the presence of growth factors in human milk. Several such factors have now been described, among them epidermal growth factor and insulin-like growth factor[20]. Recently, studies on thymidine incorporation in rat crypt cells have demonstrated an effect of human lactoferrin in this assay system[21]. However, these studies need to be extended further and are somewhat confusing because rat milk does not contain lactoferrin[22].

PASSAGE OF LACTOFERRIN THROUGH THE GASTROINTESTINAL TRACT

In vitro digestion experiments demonstrated that the lactoferrin molecule is relatively resistant against proteolysis[23]. Pepsin shows limited capacity to cleave lactoferrin, particularly at the comparatively high pH that exists in the intestinal tract of infants; trypsin cleavage results in larger fragments of lactoferrin[24]. Consistent with these findings, Spik *et al.*[25] reported that human milk lactoferrin added to formula can be found intact in the stool of infants. Subsequently, it was shown that a significant proportion of lactoferrin in human milk survives digestion in breast-fed infants and that this proportion decreases with increasing age and degree of maturity[26]. Thus, 4-10% of milk lactoferrin escapes digestion in either intact form or as larger, associated fragments[27,28]. This amount of lactoferrin is higher than that of iron ingested during the same time period (on a molar basis) and would thus be consistent with a role for lactoferrin in iron absorption. These studies demonstrate that intact lactoferrin is present in the infant's gut and therefore has the potential to perform any of the biological functions proposed. While mucosal synthesis of lactoferrin, which was proposed to explain the presence

of lactoferrin in the urine of breast-fed premature infants[29], cannot be ruled out at this stage, the finding of intact stable isotope-labeled human milk lactoferrin in the urine of such infants[30] clearly demonstrates that milk lactoferrin can survive digestion and also pass through the mucosal barrier.

LACTOFERRIN RECEPTORS

The presence of intestinal lactoferrin receptors was first suggested by Cox *et al.*[31], who reported iron uptake by human adult duodenal biopsies incubated with radiolabled human lactoferrin. Lactoferrin receptors had previously been demonstrated on mouse peritoneal cells[32] and subsequent work has shown that human monocytes[33,34] and stimulated lymphocytes[35] also display receptors for lactoferrin. Recently, some biochemical properties of the monocyte receptor as well as the kinetics for lactoferrin uptake were described[35].

While searching for species with possible intestinal lactoferrin receptors, it is logical to focus on those species that have lactoferrin in their milk at a significant level. Such species are the human, rhesus monkey, rabbit, mouse, and pig[36]. As a consequence, these are the species in which most of this research has been performed.

Rhesus Monkey

The milk from rhesus monkeys contains lactoferrin at a relatively high level and this lactoferrin has many biochemical properties similar to human milk lactoferrin; in fact, the antibody to human lactoferrin cross-reacts with rhesus lactoferrin and *vice versa*[37]. Preliminary studies suggested the presence of lactoferrin receptors in the brush border membrane (BBM) of infant rhesus small intestine[38] and subsequent kinetic and competitive binding-studies documented that there is a specific receptor for lactoferrin[39]. This receptor recognizes human and rhesus lactoferrin, but not human transferrin or bovine lactoferrin. The dissociation constant for the lactoferrin-lactoferrin receptor complex is about 3×10^{-6}M, which is similar to that described for the macrophage[33], but lower than that for transferrin and its hepatocyte receptor, 10^{-8}M[40]. The receptor is expressed in fetal tissue and throughout adulthood, but the number of receptors per tissue weight is highest during infancy[39]. Although some experiments suggested that the glycan moiety of lactoferrin was needed for lactoferrin binding, subsequent studies have shown that the fully deglycosylated form of lactoferrin binds with an affinity similar to that of intact lactoferrin[41]. It has also been shown that lactoferrin can deliver manganese, but not zinc, to the receptor and that less saturated forms of lactoferrin bind, although the affinity is higher towards the fully iron-saturated form[42]. The receptor is also capable of binding the larger, "half-lactoferrin", fragment, but with lower affinity than that for intact lactoferrin.

Mouse

The mouse is unique in that its milk contains high concentrations of lactoferrin (~1 mg/ml) and transferrin (or "serotransferrin-like protein", ~3 mg/ml)[43]. A brush-border membrane receptor that binds mouse milk lactoferrin, but not the milk transferrin, has been described by Hu *et al.*[44]. The

affinity constant for lactoferrin was 3.5 x 10^6 M^{-1} and human and bovine lactoferrin bound with similar affinity (2.6 x 10^6 M^{-1}). The number of binding sites was 0.53 x 10^{12}/μg of membrane protein and the pH optimum for binding was 5.5. The detergent-solubilized receptor was capable of binding lactoferrin and this capacity was subsequently utilized to isolate the receptor[45]. The purified receptor had a molecular weight of 130 kDa and consisted of a single polypeptide chain with a glycan of 25 kDa. A 1:1 ratio of lactoferrin to lactoferrin receptor was suggested.

Human

The study on human duodenal biopsies by Cox *et al.*[31] demonstrated that iron can be delivered to the mucosa in a saturable manner. We have recently characterized the kinetics for iron uptake by human infant brush border membrane vesicles[41]. The binding is saturable and specific; human transferrin does not bind and, although minimal binding of bovine lactoferrin occurred, this binding was found to be nonspecific. The receptor has optimum binding at a pH of 6.5 to 7.5 and deglycosylated lactoferrin bound with a similar affinity to that of intact lactoferrin. Using affinity chromatography, the receptor was purified and characterized. It was found to have a molecular weight of 114 kDa and consisted of single ~38 kDa subunit. Each subunit contained a 4 kDa glycan. These results are somewhat similar to the data from lactoferrin receptors on human mitogen-stimulated lymphocytes[46]. These receptors have been reported to be two proteins of 100 and 110 kDa. Optimum pH for binding was between 6.5 and 7.5. A ratio of 1:2.5 for surface/intracellular receptors suggests that internalization of the lactoferrin receptor occurs in this cell type. Similar information is not yet available for human enterocytes.

Other Species

Rabbit milk contains lactoferrin, and Mazurier *et al.*[47] has shown by ligand-blotting that solubilized brush-border membranes bind human lactoferrin with a binding affinity of 1.2 x 10^6 M^{-1}. A molecular weight of ~100 kDa was reported and the receptor was found to require calcium for binding. Iron absorption studies in the rat suggested that bovine lactoferrin stimulates iron uptake in this species[48]. Although no lactoferrin receptor was found, it was demonstrated that bovine lactoferrin can deliver iron to a brush border membrane-receptor for rat milk transferrin[22]. Enhancement of iron uptake by bovine lactoferrin in suckling piglets[49] and the relatively high concentration of lactoferrin in cow milk[50] suggest the existence of a lactoferrin receptor in this species.

CONCLUSIONS

The presence of specific lactoferrin receptors has now been established in several species. Kinetics for lactoferrin binding and iron uptake have been described and the receptor has been isolated and characterized in some species. Further studies are needed to better characterize the function of lactoferrin when bound to its receptor and the ultimate fate of the molecule at the mucosal surface.

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FREE FATTY ACIDS AND MONOGLYCERIDES: ANTI-INFECTIVE AGENTS PRODUCED DURING THE DIGESTION OF MILK FAT BY THE NEWBORN

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INTRODUCTION

Milk provides immune as well as non-immune protection against disease. Indeed, the newborn of many species receives maternal antibodies through colostrum and milk. Milk provides, in addition, protection by means of an array of components with antiviral, antibacterial, and antiprotozoan action, such as lysozyme, lactoferrin, glycoproteins, cells, and lipids[1].

In this chapter, I would like to focus on the process of production of the lipid components with anti-infective properties, namely the free fatty acids and monoglycerides. Since these are products of hydrolysis of triglycerides, it is important to review the nature and structure of milk lipids as well as the process of fat digestion in the newborn. It is obvious that in order to have an optimal effect, the anti-infective products of lipolysis would have to be generated in the upper gastrointestinal tract to protect the stomach as well as the intestine. Indeed, in this chapter, I will focus specifically on the role of the stomach in the production of anti-infective lipids.

STRUCTURE AND COMPOSITION OF MILK LIPIDS

Human milk has a fat content of 3.0-4.0%; large individual variations are however possible[2]. Because of insolubility in aqueous media, the fat in milk is contained in specific structures, the milk fat globules. These consist of a triglyceride core, that contains 98% of the fat in milk, and a membrane composed of polar lipids (phospholipid and cholesterol) and proteins[2]. The milk fat globules are among the largest structural components of milk, having a diameter of 4μm in mature milk[3]. The globule membrane is provided by the plasma membrane of the mammary epithelial cells[4] and contains glycoprotein filaments whose composition is unique for each woman[5]. Furthermore, these glycoproteins are absent in the milk of other species such as the cow, goat, or rat[6]. The size of milk fat globules changes both as a function of length of lactation and length of gestation, colostrum having smaller globules (especially in milk of women who deliver prematurely) than

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mature milk[3]. The more efficient packaging of triglyceride within the globules during the progression from colostrum to mature milk results in the need for less membrane and, thus, in a decrease of milk phospholipid and cholesterol from about 1.2% to 0.4% of milk fat in colostrum and mature milk, respectively[7].

Table 1. Distribution of Fat in Human Milk

	Colostrum	Mature milk
Total fat (g/dl)	2.0 - 2.5	3.5 - 4.5
Phospholipid (% of total fat)	1.1	0.60
Cholesterol (% of total fat)	1.3	0.40
Triglyceride (% of total fat)	97.0	98 - 99
Fat globules (no/ml milk)	6×10^{10}	1.1×10^{10}
Fat globules (average diameter)	1.8 μm	4.0 μm
Surface area of fat/ml (m^2)	0.097	0.054

Data adapted from Bitman *et al.*[7] and Ruegg & Blanc[3]. The fatty acids with highest anti-infective activity[10] lauric, palmitoleic, oleic, and linoleic amount to 5-7%, 2.5-4%, 33-35%, and 13-15% of milk fat respectively[7].

The triglyceride of human milk is its most variable component, changing as a function of length of gestation, length of lactation, time of day, duration of individual feeds, as well as maternal diet[1]. Type and amount of dietary fat affect the composition of milk fat. Maternal diets low in fat and high in carbohydrate lead to *de novo* synthesis of fatty acids within the mammary gland, resulting in high concentrations of fatty acids $\leq$ C16. Therefore, although the total amount of fat remains in the normal range, the fat is more saturated. Shifts in dietary practices of populations result in changes in the fatty acid composition of human milk. For example, in the United States, the increased consumption of vegetable oil led to an increase of linoleic acid from approximately 8% in the 1960 - 1970's to 12 - 15% in the 1980's[1,7]. A similar increase in long chain polyunsaturated fatty acids such as docosahexaenoic acid (C 22:6 w3) in human milk in the U.S. can be expected as a result of the increased consumption of fish, rich in these fatty acids.

In addition to dietary effects, there seem to be intrinsic individual differences in the mechanism of milk secretion that affect the concentration of fat, some women secreting larger volumes of milk that contain lower amounts of fat[8]. Fat amount and composition can also be affected by parity; high parity (10+) is leading to markedly reduced capacity for *de novo* synthesis of fatty acids in the mammary gland[9]. The composition of milk fat and factors that affect it are listed in Tables 1 and 2.

Table 2. Factors that Affect Milk Fat Content and Composition

Variable		Change
Gestation		LC-PUFA higher in preterm colostrum, transitional milk
Lactation		Phospholipid, cholesterol higher in colostrum (preterm > term)
Parity		P10+: Lower endogenous synthesis of FA (C6-C16)
Volume		High volume: lower fat concentration
Feed		fat: fore < mid < hind milk
Diet	(High CH)	Increase in endogenous synthesis of FA (C6-C16)
	(Low cal)	Increase in palmitic acid (C16) content

FA: fatty acids, CH: carbohydrate, LC-PUFA: long chain polyunsaturated fatty acids

EVIDENCE OF ANTI-INFECTIVE ACTION OF LIPIDS

Kabara[10] has reviewed the antimicrobial effect of fatty acids, and cites observations first made by Clark in 1899 (antifungal activity of soaps) Reichenbach in 1908 (effects of fatty acids on *E. coli*) and Stock and Francis, 1943 (effects on viruses). Studies by a number of investigators have shown that fatty acids and monoglycerides have antiprotozoan[11,12] antibacterial[13] and antiviral activity, specifically against lipid-enveloped viruses[13-16]. This topic will be addressed in greater detail in Dr. Isaacs' chapter. I have listed, only briefly, the characteristics that confer this activity upon selected lipids (Table 3)[10]. As recently shown by Isaacs *et al.*[13], the formation of antiviral lipid products is not limited to milk fat but occurs also during the gastric digestion of formula fat.

Table 3. Properties that Determine Anti-infective Function of Lipids

Structure: monoglyceride, free fatty acid
Saturated FA: C12 (lauric) > C10 or C14
Unsaturated FA: C18:2 (linoleic) > C18:1 or C18:3
Isomers: cis FA active, trans FA inactive (i.e. oleic +, elaidic –)
Reactive group[a]: acid > alcohol > aldehyde > dicarboxylic acid
Ester: monoglyceride active, di- and triglyceride inactive

Adapted from Kabara[10]; [a] tested with lauric acid derivatives.

DIGESTION OF MILK FAT BY THE NEWBORN

The digestion of milk fat is initiated in the stomach through the action of lingual and gastric lipases[17,18]. These enzymes, the first secreted from lingual serous glands[19] and the second from the chief cells of the gastric mucosa[20], have an amino acid homology close to 80% and very similar characteristics (Table 4). Fat digestion is then continued in the intestine by pancreatic lipase and the milk's own digestive lipase. These two lipases have similar characteristics, chief among them are absolute dependence upon bile salts (CMC) and a pH optimum of 7.0-7.5[21].

Table 4. Characteristics for Optimal Activity of Digestive Lipase

Condition	Lingual	Gastric	Pancreatic	Milk
TG-site (Sn)	Sn 3	Sn 3	Sn 1,3	Sn 1,2,3
Product	1,2 DG (MG)	1,2 DG (MG)	2 MG	FFA
pH Optimum	2.2 - 6.0	3.5 - 6.5	6.5 - 8.0	7.0 - 8.5
Co-factors	none	none	co-lipase	none
Bile salts	no	no	> CMC	> CMC
Stability (pH 2.5 - 3.0)	yes	yes	no	yes
Protein	Stimulation	Stimulation	Inhibition	No effect
Hydrolysis of milk fat	yes	yes	no	no

TG - triglyceride; Sn - stereospecific numbering
DG - diglyceride; MG - monoglyceride
CRC - critical micellar concentration

Initial hydrolysis of fat in the stomach is essential for optimal fat digestion at all ages[22,23]. This step is, however, of significantly greater importance in the newborn because of endogenous and exogenous reasons. The first is low pancreatic lipase activity in the newborn and especially the preterm infant[24], while the second refers to the nature of the lipid digested, milk fat globules being inaccessible to both pancreatic[25], as well as milk digestive lipase[26]. Lingual and gastric lipases can, however, penetrate into the milk fat globule and initiate the hydrolysis of the core triglyceride. This step is then followed by hydrolysis of fat by both pancreatic and milk digestive lipases. Lingual and gastric lipases have specific characteristics[18], such as hydrophobicity, inability to hydrolyze the ester bonds of phospholipids and cholesterylesters, as well as stimulation of activity by dietary proteins, that reduce interfacial pressure to the range optimal for these enzymes, thus enabling them to penetrate through the milk fat globule membrane without disrupting it[27].

Quantitation of the products of lipolysis in the gastrointestinal tract of adult rats, shows that large amounts of free fatty acids are released at all levels of the GI tract, starting in the stomach (Table 5).

Table 5. Free Fatty Acids Produced During Fat Digestion in the Gastrointestinal Tract

Site	Study #1	Study #2
Stomach	9.4 ± 9.2	7.3 ± 1.2
Duodenum	35.4 ± 7.5	55.2 ± 7.4
Jejunum	35.5 ± 9.2	
		77.0 ± 3.6
Ileum	71.6 ± 11.4	

Data are mean + SEM. The animals were fasted 48 hr prior to gavage feeding of tri-^{3}H-olein fat emulsion. Free fatty acids represent ^{3}H-oleic acid released (% of total fat) one hr after gavage feeding. There were 5 animals in each study.

We have studied the hydrolysis of milk lipids in several species including the human. Although it was previously thought that hydrolysis in the stomach releases mainly short- and medium-chain fatty acids[25], recent studies show that long-chain unsaturated fatty acids are also preferentially released[27-29].

PRODUCTION OF FREE FATTY ACIDS WITH ANTI-INFECTIVE ACTIVITY DURING GASTRIC LIPOLYSIS OF MILK OR FORMULA LIPIDS

In vitro studies[27] conducted under conditions that simulate the gastric milieu, show that during the hydrolysis of human milk fat by lingual lipase there is rapid release (within 10 min) of medium-chain fatty acids (C10 and C12) followed by the release of long-chain unsaturated fatty acids (C18:1 and C18:2) within (10-30 min) (Fig. 1). It is interesting that the fatty acids with highest anti-infective activity are lauric (C12) and linoleic (C18:2). It seems, therefore, that gastric lipolysis produces high levels of antiviral and antibacterial free fatty acids from human milk. A marked increase in long-chain unsaturated fatty acids (C18:1, C18:2, and C18:3) occurs also during hydrolysis of formula triglyceride in the infant's stomach (Fig. 2)[29]. Studies on the digestion of milk fat in suckling dogs (a species similar to the human in that the milk contains digestive lipase[30], high gastric lipase activity is present at birth[31] and the milk fat is composed mainly of long-chain fatty acids[32]) show that within 60 min after gavage feeding of fresh milk as much as 27 to 35% of the fat is present as free fatty acids (Table 6). The fatty acids preferentially released were C18:1 and C18:2.

Our studies on fat digestion show, therefore, that the high level of lipolysis in the stomach as well as the type of fatty acids preferentially released are compatible with the production of anti-infective lipids in the first step of the digestion of both milk and formula fat. Furthermore, while milk digestive lipase can enhance the lipolytic process in the intestine, it is not essential for the release of anti-infective fatty acids, a process carried out efficiently by the infants' endogenous lingual and gastric lipases.

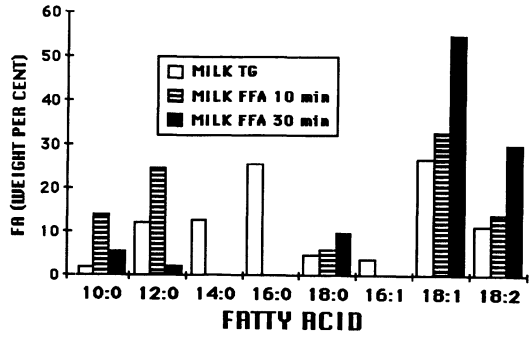

Figure 1. Hydrolysis of human milk fat by lingual lipase: *In vitro* studies (Incubation at pH 5.4). Data from Patton *et al.*[27]

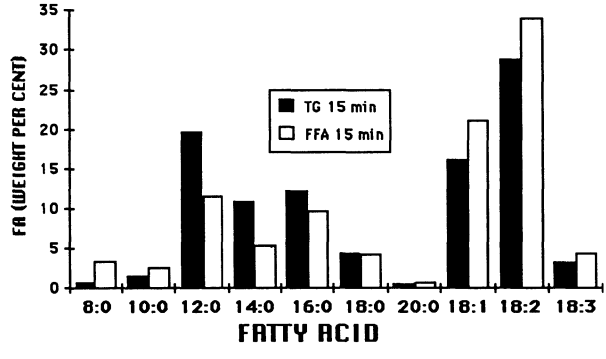

Figure 2. Hydrolysis of long-chain triglyceride formula in the stomach of premature infants. Gastric contents were aspirated for fatty acid analysis, 15 min after gavage feeding. Data from Hamosh *et al.*[29]

Furthermore, as shown in Tables 4 and 6, while lipolysis in the stomach produces measurable amounts of monoglyceride, in the intestine, monoglycerides are produced only by pancreatic lipase whereas none are formed during the action of milk digestive lipase.

The anti-infective effect of gastric lipolysis is known in a number of species. Thus, scours or calf diarrhea, an often fatal disease, can be treated by the administration of pregastric esterase[33] an enzyme similar to gastric and lingual lipases[18]. Indeed, scouring in calves is associated with rapid passage of milk through the abomasum (stomach) without sufficient gastric digestion[34]. The observation that the treatment of scouring was more effective when pregastric esterase was given to milk-fed than to milk substitute-fed calves, suggests that the products of lipolysis were the curative agent. Rabbits have a potent gastric lipase[35] leading to extensive lipolysis in the stomach[36]. In this species, it was shown that the lipids from stomach contents of sucklings were antibacterial and it was suggested that the products of lipolysis maintain the sterility of the gastrointestinal tract[37]. The gastric phase of fat digestion may have a similar function in the newborn infant[13].

Table 6. Hydrolysis of Milk Fat in the Stomach of Suckling Dogs

Age	Products of Lipolysis (mol%)		
	Free fatty acids	Diglyceride	Monoglyceride
1 wk	26.9	16.1	3.4
2 wks	35.2 ± 6.3	22.2 ± 2.4	3.9 ± 0.9
4 wks	29.8 ± 2.9	19.9 ± 1.3	3.9 ± 0.7

Data are mean ± SEM. At the three time periods studied, milk fat contained 98% triglyceride, 0.1% diglyceride and no monoglyceride or free fatty acids. Gastric contents were aspirated 60 min after gavage feeding of fresh dog milk. Data from Iverson *et al.*[32].

Hydrolysis of milk fat occurs during storage at -20°C (but not at -70°C)[38]. Thus, considerable amounts of free fatty acids (up to 20% of milk fat) could be present in stored human milk, conferring anti-infective properties to milk kept at -20°C.

CONCLUSIONS

Anti-infective agents are produced in the stomach of the newborn during the digestion of milk or formula fat. The free acids and monoglycerides produced during intragastric lipolysis have antiviral, antibacterial, and antiprotozoan activity.

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THE ROLE OF MILK-DERIVED ANTIMICROBIAL LIPIDS AS ANTIVIRAL AND ANTIBACTERIAL AGENTS

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INTRODUCTION

Milk lipids are not only nutrients but also non-immunoglobulin protective factors[1-3]. The lipids in human milk do not initially have antimicrobial activity, but become antiviral[2], antibacterial[4,5] and antiprotozoal[6] *in vitro* following storage and *in vivo* following digestion in the gastrointestinal tract of the milk-fed infant[2,5,7]. Microbial killing by milk lipids is due primarily to free fatty acids (FFAs) and monoglycerides (MGs) released from milk triglycerides by lipases and can be duplicated using purified FFAs and MGs[8,9].

Milk lipids are not unique in possessing antimicrobial activity. Human epidermis-derived skin lipids, especially FFAs, have also been found to inactivate *Staphylococcus aureus*[10,11]. The removal of skin lipids by acetone extraction permitted added bacteria to persist on the skin of volunteers whereas reapplication of the skin lipids inactivated the bacteria. The work of Coonrod[12] showed that lung surfactant from humans, dogs, rats, and guinea pigs contained FFAs that were bactericidal for pneumococci. The inhaled pneumococci were killed extracellularly in rats providing further support for the potential antimicrobial role of surfactant FFAs. Since surfactant-like particles, which are biochemically similar to lung surfactant, have recently been isolated from adult rat intestine[13], FFAs may have an antimicrobial function in the intestine which is supplemented by milk in the suckling neonate.

FFAs have also been found to have antimicrobial activity in 9 species of brown algae[14]. Both gram-positive and gram-negative bacteria were inactivated by the algal lipids. Additionally, in another study of Caribbean marine algae[15], over 70% of the lipid extracts from approximately 100 algae tested had antibacterial activity.

Lipid-dependent antimicrobial activity was thought to be absent from infant formulas. However, we have shown[7] that the lipid fraction extracted from the stomach contents of formula-fed infants inactivated herpes simplex virus-1 (HSV-1) and bacteria as readily as the lipid fraction from milk stomach

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contents. Therefore, antimicrobial lipids do not necessarily require interactions with other secretory protective factors to have a protective function.

The results presented here provide further evidence that the milk lipid fraction is made antimicrobial by the hydrolysis of milk triglycerides *in vitro* and *in vivo*.

MATERIALS AND METHODS

Human milk samples were collected under sterile conditions, 1 to 5 months postpartum, and stored at -86°C. Vesicular stomatitis virus strain Indiana and HSV-1 were grown in Vero cells and assayed as previously described[2]. Milk samples were diluted 1:5 in maintenance medium with approximately 10^5 $TCID_{50}$ of virus and incubated at 37°C for 30 min. The difference between the titer (log_{10}) of the control virus not incubated with milk and the titers of milk-virus mixtures, i.e., the reduction of virus titer, was used as a measure of antiviral activity. Human immunodeficiency virus-1 (HIV-1) studies were performed by Dr. Linda Martin, Immunology Branch, Centers for Disease Control, Atlanta, GA, as previously described[16]. Stomach contents samples were supplied by Dr. William C. Heird, Baylor College of Medicine, Houston, TX, and Dr. Sudha Kashyap, Columbia University College of Physicians and Surgeons, New York, NY, after informed consent was obtained.

RESULTS

Studies have shown an association between the appearance of lipid-dependent antiviral activity and the enzyme activity of lipoprotein lipase (LPL) but not bile salt-stimulated lipase (BSSL) in human milk[2]. Results with heat-treated milk (Table 1) also suggest that enzyme activity is required for the initiation of antiviral activity since heating the milk before storage but not after storage at 4°C prevented the appearance of antimicrobial activity. Additionally, when purified LPL was added to milk and colostrum samples (Table 2) they became antiviral in less time than in the absence of added enzyme.

Table 1. Reduction in VSV Titer (Log_{10}) by Milk Heated at 100°C Before or After Storage at 4°C

Milk sample	Not heated	Heated at 100°C for 10 min	
		After storage	Before storage
1	≥3.5	≥3.5	0
2	≥4.5	≥4.5	0

Table 2. Reduction in VSV Titer (Log_{10}) by Human Milk Stored at 23°C for 3 Days with or without LPL[a]

Milk sample	With LPL	Without LPL
Milk	≥ 3.5	0
Milk	≥ 3.5	0
Colostrum	≥ 3.5	0

[a] purified LPL was added at a concentration of 200 units/ml.

The data presented in Table 3 show that when eserine and sodium chloride, both of which inhibit BSSL and LPL, are added to human milk antimicrobial activity is not present. Rabbit serum which stimulates LPL but inhibits BSSL also prevents antimicrobial activity[17]. The addition of bile salts, which stimulate BSSL and inhibit LPL[17] (Table 4) to milk and colostrum samples produced a more rapid appearance of antiviral activity in milk following 3 days of storage. In colostrum, antiviral activity was only found when sodium cholate was present.

Antiviral and antibacterial activity was found in the stomach contents of milk-fed infants 3 h after feeding (Table 5). Viral and bacterial inactivation by the stomach contents was comparable to that found in stored human milk. When milk and stomach contents samples from milk-fed infants were tested against HIV (Table 6) 3.0-5.0 log_{10} of HIV killing was found.

Table 3. Effect of Lipase Inhibitors on the Appearance of Antiviral Activity in Human Milk

Milk sample	Stored without inhibitors	Stored in the presence of		
		1M NaCl	50mM eserine	10% rabbit serum
1	≥ 3.5*	0[a]	0	0
2	≥ 3.5	0	0	0
3	≥ 3.5	n.d.[b]	0	0

[a] reduction in VSV titer (log_{10}) by milk
[b] not done

Table 4. Reduction in Virus Titer (Log_{10}) by Human Milk Stored at 23°C with or without Sodium Cholate[a,b]

Milk sample	Days of storage	Sodium cholate +	Sodium cholate −
Milk	3	≥3.5	1.0
	9	≥3.5	≥3.5
Colostrum	4	≥3.5	0
	10	≥3.5	0

[a] VSV was the test virus

[b] sodium cholate was added at a concentration of 1 mM

DISCUSSION

The data presented here provide further evidence that the initiation of lipid-dependent antiviral activity in milk is dependent upon lipase activity. In fact, addition of purified LPL to milk before storage can reduce the time required for the appearance of antiviral activity. Results with rabbit serum suggest that a minimum concentration of LPL is required to initiate hydrolysis of triglycerides in stored human milk but that BSSL is also required to produce antiviral concentration of fatty acids and monoglycerides. Experiments with bile salts which activate BSSL and inhibit LPL showed that increased BSSL activity can reduce the time required for milk lipids to become antiviral and can increase the concentration of antiviral lipids. The experiments with lipase inhibitors, lipase activators, and the stomach contents from milk-fed infants indicate that any lipase that release fatty acids and monoglycerides from milk triglycerides, either during storage or in the gastrointestinal tract, will make the milk lipid fraction antimicrobial. This includes not only LPL and BSSL but also lingual and gastric lipases[18].

Table 5. Antiviral and Antibacterial Activity of Human Milk Stomach Contents at 3 h After Feeding[a]

Log_{10} reduction in HSV-1 titer	Log_{10} reduction in bacterial concentration *S. epidermidis*	*E. coli*	*S. enteritidis*
≥4.0	≥5.0	≥4.0	≥4.0
≥4.3	≥4.0	≥5.0	≥5.0

[a] milk samples themselves were all tested for antiviral and antibacterial activity and none was detected

Antiviral human milk and milk stomach contents inactivated HIV as readily as any other enveloped virus. Since significant levels of HIV have been found in non-cellular milk fractions[19], milk lipids released in the infants gastrointestinal tract could protect against HIV transmission by breastfeeding. Instances of HIV transmission by breastfeeding[20] most likely result from HIV that is carried intracellularly in milk lymphocytes and macrophages. This is certainly possible since maternal leukocytes fed to newborn lambs can migrate to the circulatory system[21] and bovine leukocytes penetrate the intestinal wall of newborn rabbits after feeding[22]. Additionally, cytomegalovirus, another enveloped virus pathogen, is primarily carried by polymorphonuclear leukocytes which are present in blood, human milk, and colostrum[23].

Table 6. HIV Inactivation by Antiviral Human Milk and Milk Stomach Contents

Sample	Storage	Reduction of HIV titer (log10)
1	Fresh	0
1	4 °C	5.0
2	Fresh	0
2	4 °C	5.0
3	Fresh	0
3	4 °C	3.5
4	Fresh	0
4	Stomach contents[a]	3.0

[a] stomach contents were collected 3 h after feeding

Dietary manipulation has been found to alter the composition of FFAs in human[24] and rat[25] milk. In both instances, long-chain, unsaturated FFAs were replaced by medium-chain, saturated FFAs or vice versa. However, the antimicrobial activity of FFAs and MGs is additive[5] and consequently it is the total concentration of antimicrobial lipids in milk that is critical for inactivating enveloped viruses and other pathogens. Since we have previously shown that both medium-chain, saturated and long-chain, unsaturated FFAs can inactivate enveloped viruses[8], dietary-induced changes in milk lipid composition should not decrease lipid-dependent antimicrobial activity.

Enveloped viruses have a greater susceptibility to inactivation by antimicrobial FFAs and MGs[7] than bacteria. To have a broad range of bacterial inactivation, a mixture of antimicrobial lipids is required that is comprised of FFAs and MGs varying in chain length and saturation as different bacteria are sensitive to different lipids[9]. Since FFAs and MGs are potentially easier to acquire and manipulate than other milk protective factors, it may be possible to increase the antibacterial activity in milk by adding particular lipids to banked human milk before feeding. This would

produce a "fortified" milk as has been done previously with the skim milk and cream fractions of human milk[26]. Antimicrobial lipids can provide protection against pathogens for which the neonate has not yet acquired antibodies and further studies of their antimicrobial potential are warranted.

CONCLUSIONS

Lipids appear to have an antimicrobial function at secretory surfaces including lung, skin, and intestine. Milk lipids can inactivate a number of enveloped viruses and bacteria including HIV. Lipid-dependent antimicrobial activity is initiated by the release of FFAs and MGs from milk triglycerides. Both LPL and BSSL in human milk and lipases present in the gastrointestinal tract of milk-fed infants can make the milk lipid fraction antimicrobial. FFAs and MGs which are antimicrobial can be manipulated to broaden and maximize lipid-dependent antimicrobial activity in human milk against specific pathogens.

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ANTI-ADHESIVE MOLECULES IN HUMAN MILK

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INTRODUCTION

The protection of offspring from infection is a key not only for the survival of the individual, but also of the species. A number of complementary and partially overlapping mechanisms have evolved to minimize the loss of even (the) a single defense mechanism that would leave the infant unprotected. Both innate (non-immune) and specific immune factors participate in such protection.

The term bacterial adherence denotes the binding of bacteria to mucosal surfaces. This mechanical association is a means for the organism to resist elimination by the body fluids, and to establish a population at the site where relevant receptors are expressed. Mechanisms of attachment that have been identified involve highly specific interactions[1]. The bacterial ligands, commonly called adhesins, bind to host receptors. For gram-negative bacteria, the adhesins are commonly associated with pili or fimbriae, rigid surface organelles that help bacteria reach the appropriate receptor on the complex cell surface. The fimbriae function as lectins; they show specificity for receptor epitopes provided by the oligosaccharide sequences on host glycoconjugates[2]. For gram-positive bacteria, on the other hand, the adhesins are not expressed as a surface organelle, but rather linked to cell wall components and lipoteichoic acids[3,4]. The receptor epitopes for gram-positive bacteria may consist of oligosaccharide sequences, but can also be provided by peptides, analogous to connective tissue proteins[5-7].

The functional consequences of adherence depend on the virulence of the bacterial strain and on the form of the receptor. When cell-associated, the ligand-receptor interaction facilitates colonization and tissue attack[8]. When secreted, the receptor molecule will occupy the adhesins, and competitively inhibit attachment to the corresponding cell-bound receptor[1]. Human milk is a rich source of such competing soluble receptor molecules. We have focused on one aspect of the anti-bacterial activity in human milk – the components inhibiting bacterial adherence. This review illustrates the complementary functions of the specific immunity and accessory antibacterial molecules, and

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summarizes information on mechanisms of molecules with anti-adhesive activity against *Escherichia coli, Streptococcus pneumoniae,* and *Haemophilus influenzae.*

ANTI-ADHESIVE ACTIVITY OF SECRETORY IgA

Specific Antibody Activity

The ability of specific antibodies to inhibit attachment is well established. This was first demonstrated for *Vibrio cholera* and oral streptococci[9,10]. The anti-adhesive antibodies may act in either of two ways: 1) antibodies to the receptor-binding site of the adhesin competitively inhibit receptor interaction, or 2) antibodies to bacterial surface molecules that are not directly involved in adherence may agglutinate the bacteria, and thereby reduce the number of organisms available for binding. An example of these two mechanisms is provided by the immune response to bacterial fimbriae. The majority of antibodies elicited by natural exposure to fimbriated bacteria is directed against the antigenically variable fimbrial subunit protein and does not interfere with attachment, except by specific means (??). A minor fraction of antibodies are directed against the receptor-binding lectin and when purified, such antibodies block adherence.[11-14].

In either of the above cases, the anti-adhesive activity of the antibody is attributed to the specificity of the antigen-combining site. We have recently identified an alternative mechanism of interaction between secretory IgA and *E. coli* based on lectin-carbohydrate interactions.

IgA as a Soluble Receptor for Bacterial Adhesins

The immunoglobulins are glycoproteins, with oligosaccharide sequences attached to the heavy and infrequently to the light chains. These oligosaccharides provide the basis for interactions with bacterial lectins, depending on the sequences in individual isotypes/allotypes. Type 1 fimbriae of *Enterobactericeae* are defined by the capacity to induce mannose-reversible binding to erythrocytes, polymorphonuclear leukocytes, and epithelial cells[15].

Based on the reported mannose-content of IgA[16], we analyzed the possible carbohydrate dependent interaction between *E. coli* type fimbriae and IgA[17]. The type 1-fimbriated *E. coli* agglutinated colostral IgA, with a higher titre for IgA2 than IgA1. The agglutination was strong for the strains expressing type 1 fimbriae and not for isogenic strains with similar surface antigenicity but expressing adhesins specific for the Galα1-4Galβ-disaccharide. The agglutination was inhibited by mannose. This suggested that the bacterial agglutination reaction was due to lectin-carbohydrate interactions rather than antigen recognition. This finding was confirmed using IgA2 and IgA1 myeloma proteins that lacked specific antibody activity against *E. coli.*

The oligosaccharide sequences responsible for the interaction with type 1 fimbriae were identified by carbohydrate sequence analysis of myeloma proteins. The IgA2 myeloma protein[17] that gave the highest agglutination titre with type 1-fimbriated strains was rich in high-mannose oligosaccharides. In contrast, the concentration of such oligosaccharides was low in the less active IgA1 myeloma proteins.

The functional consequences of this interaction are open to speculation. DNA sequences encoding type 1-fimbriae are constitutive among *E. coli.*

Expression of type 1 fimbriae is frequent, regardless of the virulence or clinical origin of the strain. We have proposed that the main function of the type 1 fimbriae is to enhance the colonization of the large intestine. Consistent with this, type 1 fimbriated strains bind avidly to human colonic epithelial cells in a mannose-reversible manner. It is interesting to note that milk is rich in IgA1 and IgA2, both of which interacted with the type 1 fimbriae. Furthermore, the large intestine is the site rich in IgA2-producing cells as well as the natural colonization site for type 1-fimbriated organisms. We have demonstrated that the attachment to epithelial cells, mediated by type 1 fimbriae, can be blocked by the myeloma IgA2 as well as by the colostral IgA in a mannose-specific manner. Thus, the lectin-mediated interaction thus provides a mechanism for IgA to be broadly protective regardless of the antigen-specificity of the immunoglobulin molecule (reviewed in 17,18).

NON-IMMUNOGLOBULIN MILK COMPONENTS INHIBITING BACTERIAL ATTACHMENT

Human milk drastically inhibits the attachment of *S. pneumoniae* and *H. influenzae* to human nasopharyngeal epithelial cells. It contains antibodies to numerous surface antigens on these organisms, such as the phosphoryl choline and capsular polysaccharides of *S. pneumoniae*, and the lipopolysaccharide and outer membrane proteins of *H. influenzae*. Accordingly, some of the anti-adhesive activity in milk resides in the immunoglobulin fraction. Removal of the secretory IgA antibodies by immunoabsorption reduced the inhibition effect by about 30%[19].

The remaining anti-adhesive activity in the non-immunoglobulin fraction of milk was associated with two types of molecules: free oligosaccharides and glycoproteins in the casein fraction.

Human milk is unique with regard to its content of complex carbohydrates. The free oligosaccharide fraction of milk is dominated by the lactoseries and with improved methods of isolation and characterization, more than 130 oligosaccharides, containing up to about 20 monosaccharides per molecule, have been identified[20-22].

The anti-adhesive activity against *S. pneumoniae* in the low-molecular weight fraction (<5 kDa) of milk was explained by the free oligosaccharides. In contrast there was no such effect against *H. influenzae*[23].

The anti-adhesive activity of the high-molecular weight components of milk was found in the casein fraction. Human casein drastically reduced the adherence of both *S. pneumoniae* and *H. influenzae*[23]. This effect was species-specific; bovine, goat, sheep, and buffalo milk and casein had no inhibitory activity.

The casein fraction is a complex mixture of protein in micellar form, fat and carbohydrates, with Ca^{2+} ions[24,25]. We tested the hypothesis that the anti-adhesive activity against *S. pneumoniae* was due to the carriage by glycosylated k-casein of the same oligosaccharide sequences that were found to be active in unbound form. Indeed, purified κ-casein retained the anti-adhesive activity against *S. pneumoniae* of the whole casein. The oligosaccharides released from κ-casein by sodium borohydride reduction also inhibited pneumococcal attachment. The active oligosaccharide structures remain to be identified.

The inhibitory activity against *H. influenzae* attachment showed a different pattern. Some of the inhibitory activity was retained in the κ-casein fraction, but the released oligosaccharides had no activity[23].

PROTECTIVE POTENTIAL OF ANTI-ADHESIVE MOLECULES IN MILK

The protective potential of anti-adhesive molecules has not been defined in man. Induction of anti-adhesive immunity through vaccination is common in veterinary praxis, based on the elegant studies of neonatal diarrhea in piglets infected with *E. coli* carrying the K88 adhesin[26].

Breastfeeding protects against infection by reducing the exposure to microorganisms and by the anti-microbial action of many components. To date, there are few studies that define the relative contribution of the anti-microbial and social factors to protection.

The presence of anti-adhesive molecules in human milk provides a model to examine this question. We have approached the problem in two ways:

1. Prospective study of breast-fed and non-breast fed babies.
   Collection of milk and bacteria from individual mother-child pairs.
   Correlations of anti-adhesive activities in each milk sample with clinical outcome measured as nasopharyngeal colonization and the frequency of otitis media.

   This protocol suffers from the same shortcoming as many previous studies that try to assess the protective effect of milk. The breast-fed and non-breast-fed group are bound to differ, not only in the intake of milk but also in exposure to microorganisms secondary to social differences between the breast-fed and non-breast-fed groups. Only qualitative differences related to the anti-adhesive activity of milk within the breast-fed group would support the hypothesis.

2. Intervention study.
   We propose to evaluate the protective effect of milk in weaned children with recurrent otitis media. These children would prophylactically receive breast milk with high anti-bacterial activity during six months, and the frequency of recurrent acute otitis media episodes will be compared to placebo controls. We hope that this protocol will demonstrate a protective effect of the milk, and serve as an incentive for the isolation and use of milk components in patients other than the breast-fed infants.

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THE EFFECT OF HUMAN MILK ON THE ADHERENCE OF ENTEROHEMORRHAGIC *E. COLI* TO RABBIT INTESTINAL CELLS

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INTRODUCTION

Enterohemorrhagic *Escherichia coli* (EHEC) has been recently recognized as a cause of nonbloody diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome[1]. These bacteria produce high amounts of cytotoxins and also adhere to the intestinal tract and cause mucosal damage. The nature of the intestinal receptor to EHEC is still unclear.

Bacterial adherence to epithelial surfaces has been intensively studied and recognized as an important prerequisite for infection[2,3]. Attachment to the intestinal mucosa enables bacteria to resist expulsion by the peristaltic clearing mechanism, with colonization and subsequent enteric disease. Adherence is usually mediated by binding of bacterial surface proteins, that are often called adhesins, to intestinal receptors. These receptors are usually sugar residues on cell surface glycoproteins or glycolipids. Certain carbohydrates can therefore competitively inhibit the binding of some bacteria[2,4,5].

Since human milk is rich in free oligosaccharides, glycoproteins and glycolipids[6], we examined the hypothesis that receptor analogues in human milk may interfere with the adherence of EHEC to the gut and protect against infection. Identification of such competitive, protective compounds may clarify the intestinal receptor of these bacteria.

MATERIALS AND METHODS

Bacteria

Ten *E. coli* isolates from children with diarrhea, hemorrhagic colitis or hemolytic-uremic syndrome were initially evaluated. The strains were serotyped and cytotoxin production was quantitatively determined by a radiolabeled HeLa cell assay[7]. The type of toxin produced was determined by neutralization with specific antibodies and DNA hybridization studies. For the adherence assays, bacteria were labeled by [^{3}H]thymidine.

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Human Milk and Colostrum Specimens

Pooled human milk and colostrum were obtained and the initial skimmed fraction was prepared by centrifugation to separate it from the fatty and cellular layers, as described[8]. Further processing of the milk included: preparation of fractions with defined molecular weight ranges by Amicon membranes; pretreatments with heating, trypsin, periodate, and alpha-mannosidase; preparation of oligosaccharides and protein fractions; and passing the fractions through a concanavalin A column[7,8].

Isolation of Intestinal Cells

Viable rabbit intestinal cells were obtained by treating loops of distal ileum or proximal colon with solutions containing EDTA, dithiothreitol and citrate, as described[5,9]. The viability of the intestinal cells was monitored by trypan blue exclusion.

Bacterial Adherence to Intestinal Cells

Radiolabeled bacteria (0.1 ml, $6x10^9$ bacteria/ml) were incubated with intestinal cells (0.2 ml, 10^7 cells/ml) essentially as described[9]. The reaction was terminated by adding saline and centrifugation to sediment the epithelial cells. Intestinal cells with bound bacteria were then separated from unbound bacteria by a Percoll density gradient and counted for radioactivity. Adherence was expressed as average bacteria that bound per intestinal cell. For the inhibition studies, bacteria were preincubated (30 min) with carbohydrates or human milk fractions, and the assay was then completed as described above.

Scanning Electron Microscopy

Sections of exposed intestinal mucosa were incubated with bacteria (45 min) and then washed. The tissue was then fixed, processed, and examined with a scanning electron microscope.

RESULTS

Adherence to Intestinal Cells

All 10 EHEC isolates produced high levels of cytotoxins. Of these, *E. coli* strain 43-12 showed distinctly higher binding of 36 and 32 bacteria per cell to ileal and colonic cells, respectively. Scanning electron microscopy confirmed the avid binding of this pathogen to the brush border surface of the intestine.

Further studies were conducted with this strain. To partially define the intestinal receptor that is involved, the effect of preincubating the bacteria with various carbohydrates was studied. It is found that D-mannose and alpha-methyl-mannoside caused about 50% inhibition in the binding and L-fucose about 20% inhibition. Other carbohydrates, including glucose, galactose and lactose, had no significant inhibitory activity.

Effect of Human Milk on Adherence

Preincubation of *E. coli* 43-12 with skimmed milk and colostrum caused about 40% inhibition of the binding to both ileal and colonic cells (Table 1).

When the skimmed human milk was passed through an Amicon membrane of 100 kDa, the inhibitory activity was retained in the fraction of less than 100 kDa. This fraction did not contain immunoglobulins, so that

Table 1. The Effect of Skimmed Human Milk and Colostrum on the Adherence of *E. Coli* 43-12

Fraction	Adherence (bacteria/cell)* Ileum	Colon
Control (PBS)	33 ± 7	29 ± 5
Human milk	21 ± 6	15 ± 5
Human colostrum	16 + 4	16 + 4

*Mean ± SD

the inhibition was not mediated by antibodies. Further delineation showed that the activity was in the 30 to 100 kDa fraction.

To define the protective activity better, further fractionation of the milk was performed. The oligosaccharide fraction did not show significant protective activity. Because the adherence was significantly inhibited by mannose residues, the protective protein fraction was passed through a Concanavalin A column. The non-bound fraction did not have a protective activity; the bound fraction, which was eluted by methylmannoside, retained the activity. Thus, mannosilated glycoproteins seem to be the compound that inhibited the adherence.

Additional pretreatment studies showed that boiling or treatment with trypsin did not have a significant effect on the inhibitory activity (Table 2). In contrast, treatment with periodate or alpha-mannosidase reduced the inhibition to 17% and 25% of the original activity, respectively.

Dose-response studies showed a "bell-shaped curve". The inhibitory activity was reduced by lowering the concentration of human milk fraction. At concentrations higher than the optimal, the inhibitory activity was reduced, maybe as a result of producing aggregates of bacteria.

Table 2. The Effect of Several Pretreatments on the Inhibitory Activity of Human Milk

Pretreatment	% of Original inhibition
Control	100 ± 5
Heating	97 ± 6
Trypsin	103 ± 5
Alpha-mannosidase	26 ± 9
Periodate	17 + 9

*Mean ± SD

DISCUSSION

Several studies have shown that breastfeeding can protect against enteric infections[10-12]. The protective activity of human milk is usually attributed to its high content of secretory immunoglobulins[13]. However, immune-related activity depends on exposure of the mother to the infectious microorganism and may be of limited importance in developed countries[14].

This study demonstrates another mechanism by which human milk can inhibit the attachment of enteric pathogens to the gut: receptor analogues in milk can be competitive inhibitors of such binding. In the present study, the effect of human milk on the adherence of EHEC was studied. The binding of *E. coli* 43-12, the most adherent strain, was inhibited by skimmed human milk and colostrum. The inhibition was related to mannosilated residues in the glycoprotein fraction of human milk. Previous studies have shown similar activity of human milk against other groups of diarrheagenic *E. coli*[8,14,15].

Further characterization and purification of human milk compounds which mediate the inhibitory activity are needed. It is also currently unclear if this activity differs among milk specimens of different individuals or socio-economic groups or during lactation. Also, the biologic role of this *in vitro* inhibitory activity is unknown. Experimental models, in which the receptor-like activity is separated from the antibody-mediated activity, may be helpful.

Although many questions require study, it seems that carbohydrate residues in human milk may act as receptor analogues and inhibit the binding of enteric pathogens to the gut. Characterization of these analogues will also help to clarify the natural intestinal receptors of enteric pathogens.

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IDENTIFICATION AND USE OF PROTECTIVE MONOCLONAL IgA ANTIBODIES AGAINST VIRAL AND BACTERIAL PATHOGENS

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INTRODUCTION

Research in our laboratories is focused on the roles played by intestinal epithelial cells in the secretory immune response to enteric pathogens. One of our goals is to generate and characterize specific IgA antibodies that can prevent interaction of pathogens with epithelial surfaces. It is now generally recognized that microorganisms and macromolecules that can adhere to epithelial cells are most effective in eliciting specific secretory IgA antibodies[1,2], presumably because adherent immunogens are most efficiently endocytosed and transported across the epithelium. Adherence to the M cells of follicle-associated epithelia is probably crucial in this regard, since transepithelial delivery of antigens by these cells may be a prerequisite for a mucosal immune response[3-5].

Although there is little information available concerning the molecular composition of M cell apical membranes, it is clear that membrane glycoproteins and glycolipids of the M cell surface are readily accessible to lumenal contents and that the glycocalyx on these cells is unlike the enzyme-rich glycocalyx present on enterocytes[4-6]. Certain gram-negative bacteria such as *Vibrio cholerae* adhere selectively to M cell surfaces[7], are transcytosed, and evoke a strong mucosal immune response including production of secretory IgA antibodies (S-IgA)) directed against bacterial surface antigens[8-10]. In addition, we have observed that the M cell surface is particularly accessible for binding of particles coated with cholera toxin (CT), a molecule that is highly immunogenic in the mucosal system[11].

Certain viruses also bind selectively to M cell surfaces and use the M cell transepithelial transport pathway to invade the host and spread to their target cells or distant tissue sites. For example, reovirus in the mouse intestine adheres to M cells and is transported only at this site[12]; the virus then proliferates in the mucosa before spreading to the central nervous

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system[13]. Poliovirus also adheres to human M cells, and it may be M cell transport that allows it to reach and infect its neuronal target cells[14]. We recently found that mouse mammary tumor virus (MMTV), a cancer-causing retrovirus ingested by neonates in the milk of infected mothers, also can gain access to the body by crossing the epithelium via M cell transport in mice. Finally, we have observed adherence and transport of HIV-1 by rabbit and mouse M cells. The M cell membrane components and the viral or bacterial surface molecules that mediate these interactions are unknown. Furthermore, it is not established whether any of these interactions, including bacterial colonization, viral adherence, and transepithelial transport, can be prevented by S-IgA antibodies alone in the absence of other immune protection mechanisms.

METHODS AND RESULTS

To better understand the role of S-IgA in preventing microbial adherence, uptake and disease, we have generated monoclonal IgA antibodies directed against surface components of bacterial and viral pathogens that are known to be transported by M cells. Mice were mucosally-immunized by gastric intubation or intra-luminal injection of MMTV, reovirus type 1 or *Vibrio cholerae* Ogawa 395. Hybridomas were generated by fusion of Peyer's patch cells with myeloma cells, and hybridomas secreting pathogen-specific antibodies of the IgA class were identified by ELISA[15]. The antigen specificities of the monoclonal IgA antibodies, as determined by immunoprecipitation and Western blots, indicated the major surface components of each organism that were effective immunogens in the mucosal system. These included the reovirus surface proteins sigma 3 and mu1c, the major MMTV envelope glycoprotein gp52[15], and on *Vibrio cholerae,* the serotype-specific O-linked carbohydrates of surface lipopolysaccharide (LPS)[16].

All of the IgA hybridomas generated so far produce IgA antibodies in the expected ratio of monomers, dimers, and higher polymers, and the major fraction in most cases is dimeric[15]. These antibodies are recognized by epithelial polymeric immunoglobulin receptors, as evidenced by the rapid delivery of radiolabeled S-IgA into bile following intravenous injection of radiolabeled monoclonal IgA in rats. It is important to note that the monoclonal anti-reovirus sigma 3 and anti-mu1c antibodies were non-neutralizing, i.e. they failed to prevent L cell infection *in vitro.* The anti-LPS monoclonal antibodies recognized a surface-exposed component on intact *V. cholerae* as shown by immunofluorescence and immunogold labeling, but antibody-coated live vibrios remained motile for long periods. Thus, the IgA antibodies tested for protection *in vivo* were capable of agglutinating viral and bacterial targets *in vitro,* but were non-neutralizing.

To assess the protective capacity of individual monoclonal IgA antibodies *in vivo,* we sought an assay system in which specific antibodies would be delivered onto mucosal surfaces in a continuous fashion, and would have the same distribution in the lumen as does normal, endogenous S-IgA. Hybridoma cells were therefore injected subcutaneously on the upper backs of adult BALB/c mice, and hybridoma "backpack" tumors formed at these sites over the course of 1 to 2 weeks. As the tumors grew, IgA levels in serum rose and this was accompanied by rising levels of specific IgA in the

intestinal lumen[16]. This indicated that IgA derived from the backpack tumors was delivered into bile and intestinal secretions via the normal epithelial receptor-mediated transport mechanism[17].

Adult mice are resistant to *V. cholerae* colonization and disease, but neonates are highly susceptible up to 7-8 days[18]. Therefore, newborn mice were injected subcutaneously with hybridoma cells on the day of birth, and were challenged orally with 10^7 *V. cholerae* (over 100_{LD50}) on days 6 to 8. This dose resulted in death of 100% of control mice within 40 hours. Mice with backpack tumors secreting IgA specific for Ogawa LPS were healthy after 40 hours, whereas mice with the same anti-LPS tumors were not protected when challenged with a virulent Inaba strain of a non cross-reacting serotype[16]. Control mice bearing irrelevant anti-reovirus IgA tumors or no tumors were also unprotected and died within 40 hours. Thus, secretion of specific S-IgA appears to have prevented the mucosal surface colonization that is a prerequisite to toxin production and diarrheal disease[19].

To test whether monoclonal S-IgA can prevent uptake of reovirus by M cells and infection of the Peyer's patch mucosa, adult mice bearing hybridoma tumors secreting anti-mu1c, anti-sigma 3, or irrelevant (anti-TNP) IgA antibodies were challenged with an oral dose of reovirus that is sufficient to produce a consistently high number of plaque-forming units (PFU) in the Peyer's patch mucosa after 24 hours. Mice secreting anti-sigma 3 IgA had the same level of mucosal reovirus as did control mice bearing anti-TNP IgA tumors, and therefore were not protected. Mice secreting anti-mu1c IgA, however, showed a 95% decrease in mucosal PFU. It is known that gastrointestinal proteases such as chymotrypsin partially digest the reovirus surface in the lumen, cleaving sigma 3 and causing partial processing of mu1c protein so that after intestinal passage, sigma 3 is lost but the major portion of mu1c remains[13]. Thus, it is not surprising that anti-mu1c IgA alone was able to interact locally with reovirus to prevent mucosal infection.

DISCUSSION AND CONCLUSIONS

Taken together, these studies have demonstrated that viruses and bacteria that are efficiently transported by M cells can evoke sufficient numbers of antigen-specific IgA lymphoblasts in the Peyer's patch mucosa to allow IgA hybridoma production using Peyer's patch cells alone. The antigen specificities of the IgA hybridomas thus obtained provide an indirect indicator of the microbial surface molecules that are most immunogenic in the mucosal immune system.

The ability of specific IgA antibodies to provide mucosal protection can be conveniently and directly assayed using the hybridoma "backpack" tumor method. This allows us to distinguish protective from non-protective monoclonal IgA antibodies. It also identifies microbial surface antigens and epitopes that can evoke protective antibodies, and this information could be useful in the future design of protective oral vaccines. It is noteworthy that both anti-*V. cholerae* and anti-reovirus IgA antibodies that were shown to provide protection *in vivo* were non-neutralizing in standard *in vitro* assays.

These results demonstrate that IgA antibodies alone, if delivered into secretions at sufficiently high concentration, can protect against bacterial or viral infection in the absence of other humoral and cellular immune responses. Furthermore, monoclonal IgA antibodies recognizing a single

surface epitope on the target organism are capable of providing this protection. Protective monoclonal IgA antibodies identified by these methods can potentially be produced in large quantities and used to provide passive protection to mucosal surfaces.

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PRODUCTION AND USE OF MONOCLONAL IgA ANTIBODIES COMPLEXED WITH RECOMBINANT SECRETORY COMPONENT FOR PASSIVE MUCOSAL PROTECTION

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INTRODUCTION

To respond to the constant challenge of their mucosal surfaces by pathogenic organisms, mammals have evolved non-immune protection mechanisms as well as a distinct mucosal immune system, the effector molecule of which is secretory IgA (S-IgA). Mucosal and glandular epithelial cells are able to transcytose dimeric and polymeric IgA by a specific receptor-mediated transepithelial transport mechanism. The polymeric immunoglobulin receptor (poly Ig R) is a member of the immunoglobulin supergene family[2]. The ectodomain of this integral membrane protein is composed of five domains, homologous to variable Ig domains[1,3], the first of which is responsible for binding of the polymeric IgA[4]. Upon binding of IgA at the basolateral surface of epithelial cells, the receptor-IgA complex is endocytosed and during translocation across the cell the interaction of IgA with the receptor is further stabilized by the formation of a disulfide bridge between the poly Ig R fifth domain and one constant domain of the IgA dimer heavy chain[5]. Once at the apical surface of the epithelium, the receptor is cleaved and its entire ectodomain, also called secretory component (SC) remains bound to the IgA[5]. There is some evidence that SC protects IgA dimers against proteolytic degradation[6]. S-IgA antibodies are thus produced locally by a unique collaboration between plasma cells, present in the interstitium of mucosal and glandular tissues, and the overlying epithelial cells.

Mucosal protection provided by S-IgA antibodies is thought to be mainly due to their ability to cross-link and aggregate pathogens, preventing their movement through the mucus and their access to the epithelium. Aggregates of cross-linked pathogens and S-IgA may then be evacuated by mechanical clearance, peristaltic movements in the gut or ciliated epithelium in the respiratory tract[7].

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METHODS AND RESULTS

In order to test the potential protective properties of S-IgA in the lumen of the gut, we have raised monoclonal antibodies of the IgA isotype against various pathogens that represent different models of infectious disease. Mouse mammary tumor virus (MMTV) is a model for mucosally-transmitted retroviruses; reovirus colonizes the central nervous system after entry through intestinal M cells; *Vibrio cholerae* is a model for bacteria that colonize the apical surface of the epithelium without further invasion of the epithelial cells; and *Salmonella typhimurium* invades the mucosa after destruction of epithelial cells. All these pathogens have in common a requirement for access to mucosal epithelial cells at an early stage of their pathogenesis.

Using a novel hybridoma "backpack" tumor implantation method, our group has demonstrated that a single monoclonal IgA, when secreted as S-IgA in the gut, was able to protect newborn mice against a lethal oral challenge with virulent *V. cholerae*[8]. Although the hybridoma tumor implantation method identifies protective antibodies, it is only feasible in syngeneic mice and cannot be used to deliver monoclonal antibodies prophylactically or therapeutically in other species. Passive oral administration of heterologous antibodies is feasible, however. We therefore administered 100 μg of concentrated IgA dimers by gastric intubation to newborn mice and tested their resistance to an oral challenge of *V. cholerae*. We used IgA antibodies against LPS that had been shown to be protective by the hybridoma backpack tumor method. Oral administration of IgA resulted in protection, but protection lasted only 3 hours. We hypothesized that the short duration of protection could be partly due to protease degradation, since the IgA used in these experiments lacked SC.

We therefore have designed two alternative strategies to engineer S-IgA from monoclonal IgA, using recombinant poly Ig R or SC. A full-length cDNA encoding the rabbit poly Ig R have been obtained by Mostov *et al.*[1] and by Schaerer *et al.* from our group. As one strategy, we took advantage of the fact that SC and IgA dimers can recombine spontaneously in solution. We attempted to produce free SC by transfecting SP2/0 myeloma cells with the full length cDNA, using a plasmid designed for high expression of proteins in B-cell lines[9] and tested transfectants for production and release of SC. Transfectants did express poly Ig R at their cell surfaces in a form that bound IgA dimers, as shown by FACS analysis, but cleavage of poly Ig R and release of SC-IgA complexes did not occur. Free SC was not released by these cells, in contrast to poly Ig R-transfected fibroblasts[10]. This may be because SP2/0 myeloma cells do not express the thiol protease responsible for cleavage of SC or, as they are non-polarized cells, they may lack the ability to perform some intracellular processing step that may be required for cleavage of the receptor. To circumvent this problem, we redesigned the vector such that the ectodomain of the poly Ig R is produced and released directly as a secretory protein. SC secreted by these latter transfected myeloma cells recognizes and binds IgA dimers in solution. Experiments are underway to determine the protease resistance of this S-IgA *in vitro*, and its half-life *in vivo*.

These engineered S-IgA molecules lack the covalent disulfide bonds that normally form during transport through epithelial cells. In order to produce S-IgA in which the SC and the IgA portions are covalently linked, a novel co-culture strategy was devised. In this system, MDCK cells were

transfected using a full length poly Ig R cDNA inserted in a dexamethasone-inducible vector. In transfected MDCK cells grown on Transwell filters, the itinerary of the receptor is normal, it is first directed to the basolateral surface and then transcytosed to the apical surface and cleaved, resulting in the release of large amounts of free SC in the apical medium. Monolayers of transfected MDCK cells were grown on collagen layers in which were embedded IgA-producing hybridoma cells. After hormonal stimulation, IgA produced baso-laterally bound to poly Ig R, was transcytosed and S-IgA was released apically. Disulfide bridge formation and S-IgA stabilization occurred during transcytosis in MDCK cells as shown by SDS-PAGE and Western blots.

CONCLUSIONS

Monoclonal IgA directed against a single bacterial surface epitope can protect against enteric bacterial challenge when given orally, even without secretory component.

We have shown that monoclonal IgA antibodies can be combined with recombinant secretory component produced in transfected myeloma cells to produce engineered S-IgA.

We have also demonstrated that transfected MDCK cells are able to assemble covalently bound S-IgA during transcytosis of poly Ig R-IgA complexes.

These methods can now be exploited to produce engineered S-IgA for passive protection of mucosal surfaces.

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EPITHELIAL TRANSPORT OF IgA IMMUNE COMPLEXES

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INTRODUCTION

IgA has traditionally been considered to function mainly as an immunological barrier or "antiseptic paint" along the lumenal surface of a mucous membrane, as in the respiratory or intestinal tracts[1]. In this way, IgA antibody serves to prevent foreign macromolecules or microorganisms from adhering to or penetrating the lining epithelium. In contrast, the fate of IgA immune complexes that form within the mucous membrane proper has not received much attention, either conceptually or experimentally. Such complexes could form, for example, if macromolecular antigens or fragments thereof are able to penetrate the mucosal epithelium to some extent, or if antigens derived from an infectious agent replicating within the mucous membrane combine with IgA antibodies secreted from local plasma cells. From their formation in the lamina propria of the mucous membrane, such IgA immune complexes could reach the circulation, where they could be removed by the mononuclear phagocyte system or, in certain species such as the rat but not so much in humans, by transport into the bile[2]. On the other hand, a more efficient path of removal of IgA immune complexes from the lamina propria would be direct passage across the neighboring mucosal lining cells via the same path normally used to transport free IgA into external secretions: IgA binds to the polymeric immunoglobulin receptor, secretory component, on the basolateral surface of the epithelial cell, after which the complex is endocytosed and transported through the epithelial cell into the secretions.

This report presents initial studies with a model system *in vitro* that support the feasibility of the direct route of excretion suggested above and the likelihood that it makes an important contribution to ridding the body of locally formed immune complexes.

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MATERIALS AND METHODS

The system employs purified dimeric monoclonal rat IgA antibody against the dinitrophenyl (DNP) determinant, DNP-bovine serum albumin (DNP-BSA) antigen, and polarized Madin-Darby canine kidney (MDCK) epithelial cells transfected so as to express rabbit polyimmunoglobulin receptor[3]. The monoclonal antibody (IR-1060 hybridoma) was purified from ascites provided by Dr. Hervé Bazin and the transfected MDCK cells were obtained from Dr. Keith Mostov. The transfected MDCK cells were grown as polarized monolayers on permeable nitrocellulose membranes (Fig. 1).

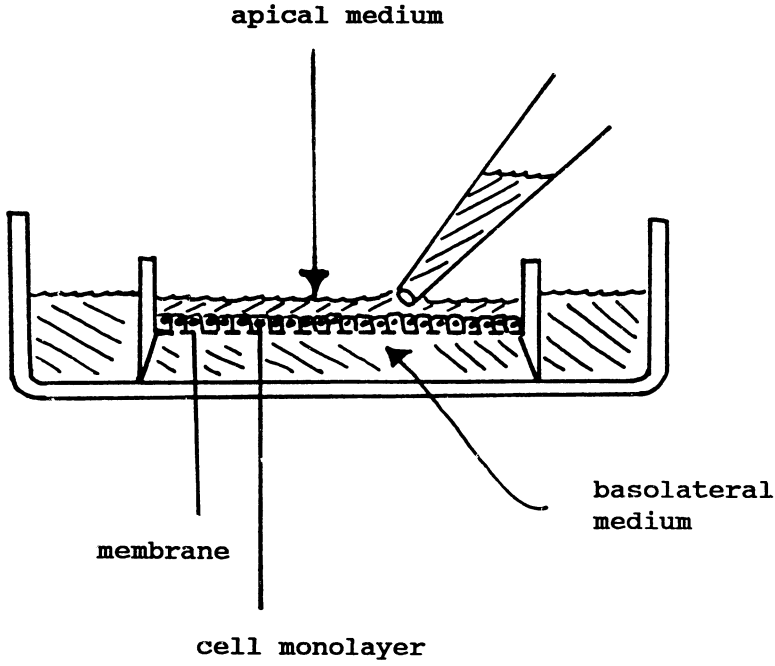

Figure 1. Diagram showing the experimental set-up. Polarized epithelial monolayers are grown on permeable nitrocellulose membranes. Immune complexes are added to the lower compartment in contact with the basolateral surface of the cells. The upper compartment if in contact with the apical cell surface. The two compartments, separated by the tight junctions between adjacent cells which prevent free diffusion, can be independently sampled.

RESULTS AND DISCUSSION

To study transcytosis, free IgA or soluble IgA immune complexes in 12-fold antigen excess (in both cases the IgA was radiolabeled with ^{125}I) were added to the compartment below the epithelial cells, thereby gaining access to the basolateral surface that bears membrane secretory component. After several hours at 0° to permit binding, the cells were washed and warmed to

37° to allow transcytosis. At intervals, medium was collected from the upper compartment (exposed to the apical cell surface) and analyzed for trichloroacetic acid (TCA)-precipitable radioactivity. In some experiments the DNP-BSA antigen was also coupled to biotin; in this way immune complexes could be precipitated with streptavidin-agarose. Figure 2 shows that both free dimeric IgA as well as immune complexes containing dimeric IgA and antigen were transported across the epithelial cells. At all time points more free dimeric IgA than immune complexes was transported, in part due to the greater accessibility of free dimer to membrane secretory component (data not shown). In contrast to the transport of dimeric IgA and immune complexes, as expected and as an additional control against nonspecific leakiness in the epithelial cell monolayer, free monomeric IgA was not transported (data not shown) because it is incapable of binding to secretory component. Interestingly, soluble immune complexes prepared with DNP/biotin-BSA and monomeric IgA antibody were not transported either (data not shown). Thus, aggregation of monomeric IgA as occurs in an immune complex is not sufficient to generate a site capable of binding to membrane secretory component. Apparently, oligomeric, J chain-containing IgA, whether free or in an immune complex, is required for binding to secretory component and subsequent transcellular transport. Since in the case of immune complexes, streptavidin was able to precipitate as much radioactive IgA from the apical medium as was TCA (data not shown), we can conclude that antigen remained bound to dimeric IgA antibody throughout cellular transport; in other words, intact immune complexes passed transcellularly from basal to apical surface and then out of the cell.

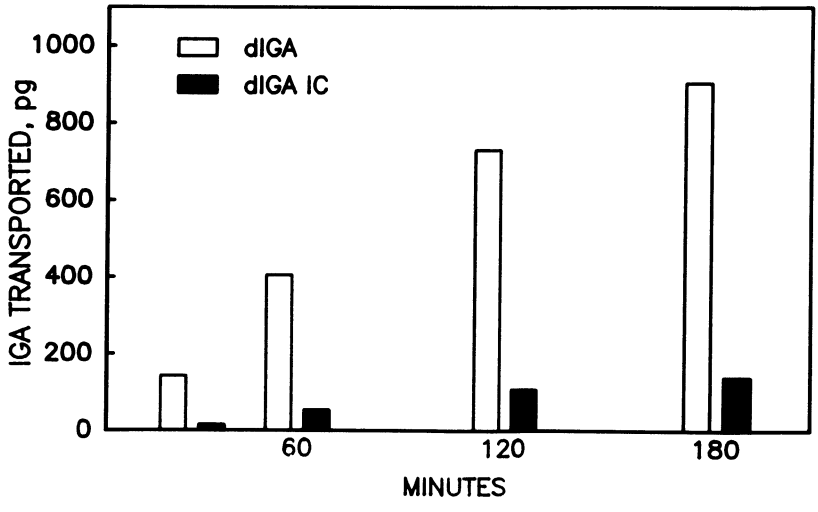

Figure 2. Transport of dimeric IgA immune complexes (or free dimeric IgA) across polarized, transfected epithelial cells. Radioactive IgA transported was quantified by gamma counting.

To study the intracellular state of endocytosed IgA immune complexes, they were added as before to the basal medium and the preparation was incubated at 0°. After washing and warming to 37° to permit endocytosis, the cells were chilled to 0°, treated with pronase to remove residual cell surface immune complexes, scraped from the nitrocellulose membrane and collected by centrifugation. The radioactivity in the cell pellets was a measure of their content of IgA immune complexes (Fig. 3). After lysis of the cells in Triton X-100 detergent, the extent of degradation of the intracellular IgA was ascertained by TCA precipitability, which was essentially complete, and by SDS-PAGE under reducing conditions followed by autoradiography. For the first 2 hours all IgA in intracellular immune complexes contained intact H and L chains. Over the next 22 hours there was evidence of partial degradation of the H chains (presumably from proteolysis at the hinge region) but no evidence of L chain degradation (data not shown). The absence of significant breakdown of intracellular IgA together with data presented earlier suggest that once endocytosed, IgA immune complexes are transported intact to the apical surface for export and are not significantly diverted to an endolysosomal compartment. In short, dimeric IgA immune complexes appear to be handled by secretory component-expressing epithelial cells in a like manner to free dimeric IgA.

Until the recent availability of secretory component-expressing epithelial cells that grow easily *in vitro* in polarized monolayers, there was no suitable means to study vectorial transport of IgA immune complexes quantitatively. In the current work, we have been able to show that such

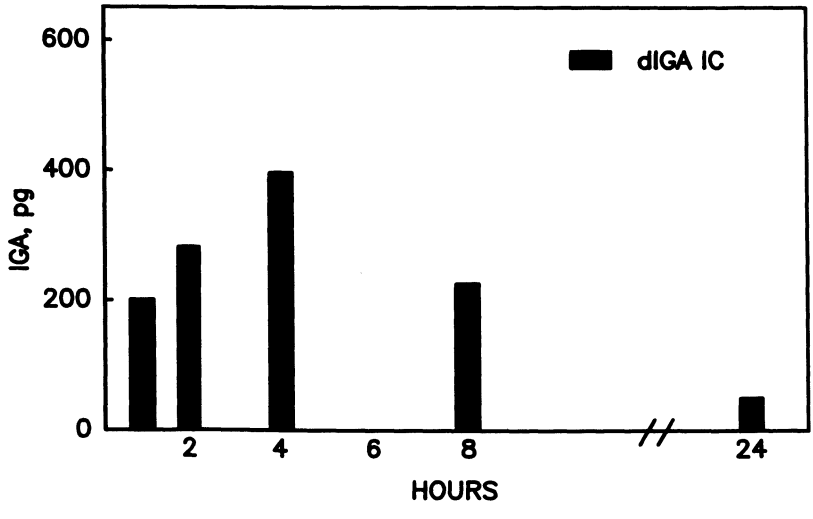

Figure 3. Endocytosis of radioactive dimeric IgA immune complexes. At the indicated intervals, epithelial cells exposed to immune complexes at the basolateral surface were treated with pronase prior to gamma counting.

transport occurs readily from basolateral to apical surface, from which export ensues, as long as the immune complexes contain oligomeric IgA. We suggest that this route, well known for transporting free dimeric IgA antibody into external secretions[1,2], could also be of major homeostatic significance for quickly ridding the body of immune complexes as they continuously form within mucous membranes. This direct route of export would limit the body's systemic exposure to free foreign molecules and immune complexes in the circulation. In so doing, the proposed route of antigen elimination may function to help prevent immune, including autoimmune, diseases.

ACKNOWLEDGEMENTS

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ASSOCIATION OF HUMAN MILK SIgA ANTIBODIES WITH MATERNAL INTESTINAL EXPOSURE TO MICROBIAL ANTIGENS

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INTRODUCTION

Human colostrum S-IgA and mature breast milk contain large quantities of secretory IgA (S-IgA)[1-3]. In general, the concentration of milk S-IgA is higher in the early postpartum period; than in the later stages of lactation[3-5]. The content of S-IgA is the result of the additive presence of a great variety of specific S-IgA antibodies that are formed locally in the mammary gland by lymphocytes that had been primed at the intestinal level by microbial and food antigens, and that migrated to the mammary gland under the influence of hormones[6-8]. In such a way, antibodies directed against *Shigella, Salmonella* and *Escherichia coli* somatic antigens, *Vibrio cholera* and *E. coli* toxins, rota- and polioviruses, and cow milk, black beans and soy beans proteins, have been detected in human colostrum and milk samples obtained in different ecosystems[1,9-14]. The ingestion of human milk containing high levels of specific antibodies has been shown to be associated with protection against diarrheal illnesses[15,16] and food allergies[17] in the breast-fed infants. Nevertheless, prospective studies have demonstrated that the presence and levels of specific S-IgA antibodies in milk do not remain constant during lactation[18,19], suggesting that its protective capacity also varies over time.

Based on observations that oral ingestion of antigens induces a temporary decrease in pre-existing, specific homologous S-IgA milk antibodies[20,21], we have suggested that intestinal infections in lactating women may induce a transient diminution in the concentration of S-IgA antibodies in milk. To explore this hypothesis, we have conducted a prospective longitudinal study among mother-infant pairs in a rural community in Guatemala. Here we present a summary of our initial findings.

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MATERIALS AND METHODS

Population

The mothers were recruited in Santa María de Jesús, a rural community 50 km from Guatemala City[22]. This traditional society is mainly formed by Maya-Cackchiquel natives among whom breastfeeding is almost universally practiced. For the purpose of this report, 15 mothers were studied. Their ages ranged from 19 to 39 years (mean = 28.3; SD = 6.1 years) at the time of delivery. They were enrolled in the program 5-7 days post-partum and we visited their homes every week. Fecal and milk samples were collected routinely every two to three weeks and, in addition, whenever an episode of diarrhea was detected among the women or their children.

Laboratory Procedures

Fecal specimens were collected in paper cans and processed as described[23]. Detection of *Shigella* spp., *Salmonella* spp., *Campylobacter jejuni, Yersinia enterocolitica, Plesiomonas shigelloides* and enteropathogenic, enterotoxigenic and adherent *E. coli*, as well as rotaviruses, *Giardia lamblia* and *Cryptosporidium* was done following standardized procedures detailed elsewhere[23]. To detect specific IgA antibodies in milk, *Shigella* spp. lipopolysaccharides were prepared by the method described by Cáceres *et al.*[24].from the homologous bacterial strains, and *Giardia* antigen was obtained from cultured trophozoites of the Portland strain[25]. All antibodies were detected by means of the enzyme-linked immunosorbent assay, as described[19], using alkaline phosphatase-labeled anti-human IgA antiserum (Tago, Inc., Burlingame, CA, USA).

RESULTS

Intestinal Infections in the Women

Five women were shown to shed *Giardia lamblia* cysts during the observation period. In three of them, the excretion of *Giardia* cysts was short-lived (1-2 weeks), one subject had positive samples taken 6 weeks apart, and one individual shed *Giardia* for at least 4 months. None of these infections were associated with diarrhea. *Shigellae* were found in 6 of the 13 women in which the *Shigellae* studies were complete (fecal culture and anti-*Shigella* antibodies). Two women had two different infections by *Shigellae*. In total, we isolated *Shigella flexneri* 2 from three cases, *Shigellae flexneri* 6 from four cases, *Shigella boydii* 4 from one and *Shigella boydii* 10 from one woman. Only one infection due to *Shigella flexneri* 2 resulted in diarrhea.

Specific IgA Antibodies in Milk

Anti-*Giardia* antibodies were detected in 10 (66.7%) of the 15 women. The antibody levels varied between 1:2 and 1:64. The women who were lacto-negative at the beginning remained negative for the duration of the follow-up. Of those who had antibodies in the milk samples taken at the start of the study, one had an increase ($\geq$ 4-fold difference), eight had a decrease ($\geq$ 4-fold difference or from positive to negative) and in one case the levels remained unchanged. In regard to anti-*Shigella* antibodies, only three (23%) women were lacto-negative. Among these three cases, we documented an increase in antibody titers in one instance; the levels did not change in the two remaining ones. Among the lacto-positive cases, in which the titers varied between 1:8

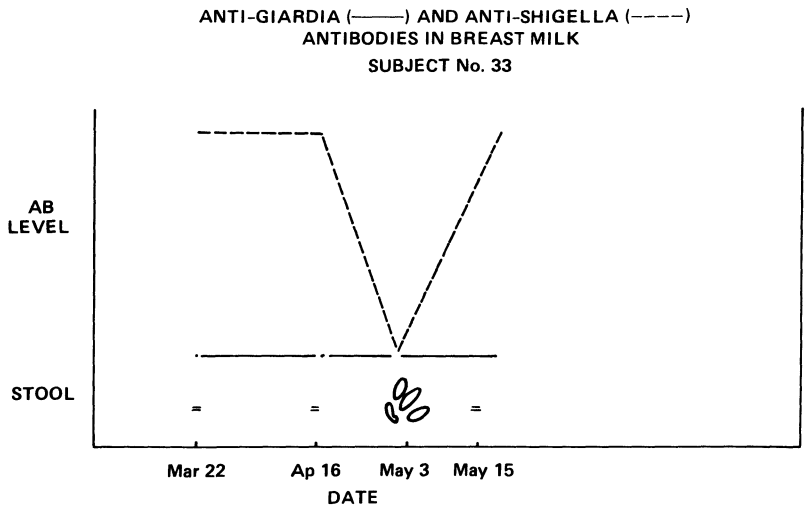

Figure 1. Association of milk antibody levels and detection of *Shigella* spp. in stool samples[a] from a woman.

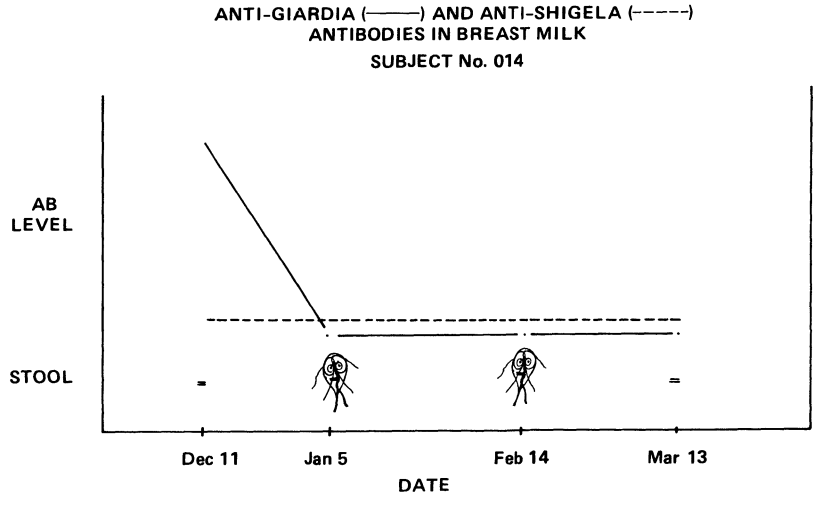

Figure 2. Association of milk antibody levels and detection of *Giardia* cysts in stool samples from a woman. Milk and stool samples were collected concomitantly on the dates shown in the horizontal axis.

and 1:256, we observed a fall in antibody levels in 7 instances (6 individuals); there were 4 women whose titers remained constant.

Association of Changes in Milk IgA Antibody Levels with Intestinal Infections

Five of the eight documented decreases in anti-*Giardia* IgA antibodies followed detection of *Giardia* cysts in the fecal samples obtained from the women. Fig. 1 depicts one case. Three falls and one increase in anti-*Giardia* antibodies were not associated with documented excretion of *Giardia* parasites. Likewise, five of the decreases in anti-*Shigella* antibodies followed infections by *Shigella* spp. (Fig. 2); lacto-conversion was seen after excretion of *Shigella*. In two cases in which infection was not documented, antibody falls were observed. In total, among 11 proven infections, a fall in antibody levels was seen in 10 cases (91%); only 5 (31%) cases of decreased titers were seen among non-infected individuals (p = 0.0047, Fisher exact test, Table 1).

Table 1. Changes in Anti-*Giardia* and Anti-*Shigella* IgA Milk Antibody Levels in Relation to Intestinal Infections in the Mother

		Decrease in antibody levels[a]		
		Present	Absent	Total
Intestinal infection documented	Yes	10[b]	1	11
	No	5	11	16
	Total	15	12	27

[a]number of individuals

[b]odds-ratio: 22; 95% CI: 1.89 - 1038.72 p=0.004 (Fisher exact test).

DISCUSSION

IgA-committed lymphocytes that are primed in the intestine migrate to the mammary gland, where they produce IgA dimers that, after being coupled with secretory component, are secreted into milk[6-8]. The ingestion by the breast-fed infant of such antibodies in high titers has been proven to be associated with protection against diarrheal disease caused by *V. cholera* and enterotoxigenic *E. coli*[15,16] and against cow milk-induced allergy[17]. These studies clearly underscore the importance of the presence and high levels of specific antibodies in human milk for breastfeeding to be protective against intestinal pathologies.

On the other hand, diarrheal illnesses are very common among children of rural areas of the developing world, where breastfeeding is practiced almost universally for prolonged periods[26,27]. These observations prompted some authors to put forward the hypothesis that underprivileged women, who commonly show nutritional deficits, have an impairment in their capacity to produce milk in adequate quantity and/or immunological quality[28]. Several studies have not been able to document deficiencies either in the volume of milk produced by underprivileged mothers or in its content of S-IgA[5,29,30]. Nevertheless, prospective longitudinal observations both in developed and developing communities have shown that the levels of

specific antibodies in milk fluctuate over time, independently of the total concentration of S-IgA and of other specific antibodies[18,19]. These changes in antibody levels do not follow specific patterns and seem to be of greater magnitude and more common among women who live in highly contaminated areas than among women of urban, more hygienic, environments. These findings, coupled with the fact that oral immunization with either live poliovirus[20] or with a vegetable protein extract[21] results in the temporary decrease of pre-existent antibodies, motivated us to suggest that intestinal infections (be they asymptomatic or symptomatic) in the lactating woman induce a drop in the content of homologous milk IgA antibodies.

The results presented here support our hypothesis. Documented infections by *Giardia lamblia* and different *Shigella* spp. in lacto-positive women resulted in decreases in antibody levels in 91% of the cases. The magnitude and duration of the observed changes varied widely among the individuals, suggesting that factors such as duration of the infections, infectious dose of the organisms, and even type of microbial agent, may play a role in determining the behavior of milk antibodies after a given infection. It is necessary to note that decreases in the levels of anti-*Giardia* and anti-*Shigella* antibodies were also observed in the absence of documented fecal excretion of these microorganisms. It is possible that our laboratory methodology to detect *Giardia* and *Shigellae* in fecal material is not 100% sensitive, especially when examining samples collected from healthy individuals. It is also very likely that other factors, different from infectious processes, influence the behavior and/or transit of IgA-commited lymphocytes that under normal conditions are programmed to migrate to the lactating mammary gland tissue.

We consider it of importance to extend these studies to a larger number of individuals and to a greater variety of diarrheagenic agents such as rotavirus, toxigenic *E. coli* and *Campylobacter*, that are among the most common pathogens associated with gastroenteritis in our populations. Of greater importance, however, is to explore the implications for the health of the breast-fed infant of these changes in milk antibody content. With the present knowledge, however, it is valid to recommend that for the protective role of breastfeeding to be expressed in its maximum capacity, efforts must be made to reduce the risk of intestinal infections in the lactating mother.

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CONCLUSIONS

Intestinal infections by *Giardia lamblia* and *Shigella* spp. in the lactating woman induce a temporary decrease in S-IgA specific homologous antibodies

found in breast milk. Other non-infectious factors may also be associated with changes in S-IgA antibody concentrations in human milk.

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SERUM AND BREAST MILK ANTIBODIES TO FOOD ANTIGENS IN AFRICAN MOTHERS AND RELATION TO THEIR DIET

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INTRODUCTION

Human milk contains secretory IgA (S-IgA) antibodies (Abs) against a number of common food antigens[1]. These Abs appear to play a role in the protection of breast-fed infants against development of infantile allergic disease. Indeed, Machtinger and Moss showed that breast milk IgA Ab levels to cow's milk are lower in mothers of allergic infants than in mothers of non-allergic infants[2]. The food Abs in human milk may also influence the infant's immune response to foreign food proteins introduced during weaning[3]. Whereas anti-bacterial IgA in breast milk reflect the mother's intestinal antigenic exposure[4], the factors that influence anti-food IgA levels in milk are still poorly understood. Results concerning the role of the mother's diet are contradictory[5,6], and modulation of the immune response by other factors at the intestinal level might be important (for instance possible adjuvant effects of intestinal infections).

In this study performed in Central Africa, in Lwiro (Zaïre), we have given cow's milk to lactating mothers suffering from marginal protein energy malnutrition, to improve their nutritional status and to increase their milk output. Concurrently, we had the opportunity to evaluate in these lactating mothers the influence of their diet on the milk and serum Ab levels against two different food antigens: 1) gliadin, which is one of the main protein of bread, consumed only sporadically and in low amount; and 2) cow's milk proteins during a milk supplementation program in a non-milk drinking population.

MATERIAL AND METHODS

Patients

Serum and breast milk from 93 lactating African mothers were collected respectively two to three months after delivery, and two months

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later. In this population, milk drinking is not a habit because of lactose-intolerance. However, during the 2 months of the study period, all these mothers ingested daily 600ml cow's milk, in the presence of a member of the medical staff to ensure compliance to the diet. A dietetic survey allowed to delineate two subgroups, consuming bread either up to once a week (maximum 3.5g of gliadin) or not at all. Protein malnutrition was only moderate at the beginning of the study as assessed by mean serum albumin levels of 33.6g/l (normal values : 35.0 - 55.0). Albumin concentration was, however, below 30.0g/l in 17% of the mothers before inclusion in the study but returned to the normal range at the end of the period of investigation.

Methods

IgA and IgG Abs against beta-lactoglobulin (BLG), bovine IgG (BIgG) and gliadin (GL) were measured by radioimmunoassays or ELISA as previously described and results were expressed in arbitrary units (A.U.) by reference to standard curves[7,8]. Milk samples were freed of fat and cells by a centrifugation at 10,000g for 30 min, and tested at two different dilutions. The amounts of Abs delivered over a 24 hours period were calculated to abolish variations in daily milk output. Molecular size of IgA Abs was analyzed in 10 samples by high-pressure liquid chromatography (HPLC)[9] and by sucrose density gradient ultracentrifugations (SDGU)[8].

RESULTS

Antibody Response to Gliadin

Anti-GL IgA were detected in the majority of both milk and serum samples, even from mothers who did not consume bread at all, or at least for 1 month before the beginning of the study (represented by crosses in Fig. 1). No other source of gliadin than bread was unravelled. The presence and titers of anti-GL IgA, both in milk and serum were not associated to the consumption of bread (Chi square test). The antigen specificity of these Abs was checked by absorption experiments; they demonstrated in some cases only, a small interference of rheumatoïd factor on the Ab titer (data not shown). In some samples, IgA Ab titers were quite elevated and values in milk and serum were correlated ($R=0.29$, $p<0.02$ for $n=69$). In contrast, the incidence of detectable serum IgG Abs was lower and no correlation was found with IgA titer.

In breast milk, anti-GL IgA were exclusively polymeric IgA (pIgA) (11s and higher molecular weights), as was total IgA; percentages of pIgA were respectively of 99% (range: 90-100%) for IgA Abs and of 100% in all samples for total IgA. Abs were revealed by both anti-alpha and anti-secretory component (SC)-radiolabeled Abs. In serum, IgA Abs were also mainly polymeric (median: 79%; range: 64-90%), whereas total IgA was mainly monomeric (median: 21% pIgA; range: 11-28%). Polymeric IgA Abs were 10.2s dimeric IgA and higher molecular weights IgA, not linked to SC.

Antibody Response to Cow's Milk Proteins

IgG Abs against BLG and BIgG were detected in most serum before the systematic introduction of cow's milk in the mother's diet, indicating a previous sensitization of the mothers to these antigens (Fig. 2 - column 1). In contrast, the incidence of IgA Abs was low both in serum and milk, as was

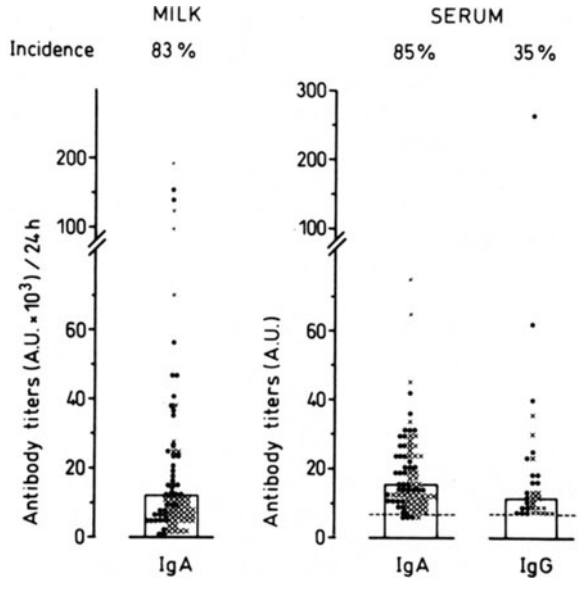

Figure 1. Anti-gliadin titers in milk and serum. Columns give the medians. Dotted lines = positivity limits. Crosses represent samples from mothers on a "gluten-free" diet.

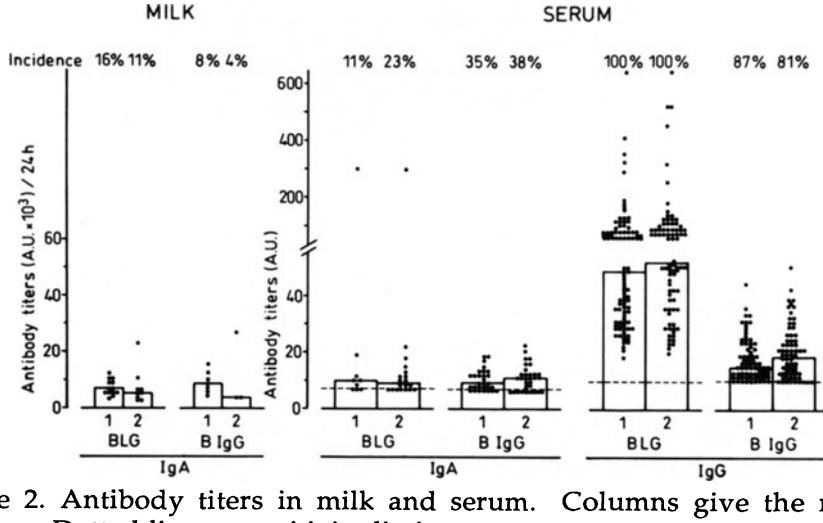

Figure 2. Antibody titers in milk and serum. Columns give the medians. Dotted lines = positivity limits.

their titer in the positive sample (Fig. 2). In this case, no correlation was found between IgA Ab titers in serum and milk.

After cow's milk introduction (600ml/day during 2 months), the number of samples with detectable Abs and the titers of IgG and IgA Abs, both in milk and serum, were not different (Fig. 2 - column 2). Ratios between the

Ab titers after and before the milk-containing diet were calculated. Histograms of the results showed, for each Ab specificity and isotype, an approximately symmetrical distribution around a mean value of 1; this suggests no modification at all of the Ab titers. This is illustrated for anti-BLG IgG in Fig. 3 showing that 84% of the ratios were comprised between 0.5 and 2.0, and 96% of them were between 0.25 and 4. This means that Ab titers before and after the cow's milk diet differed respectively from less than 1 or 2 dilutions steps of two, that could be considered as non-significant.

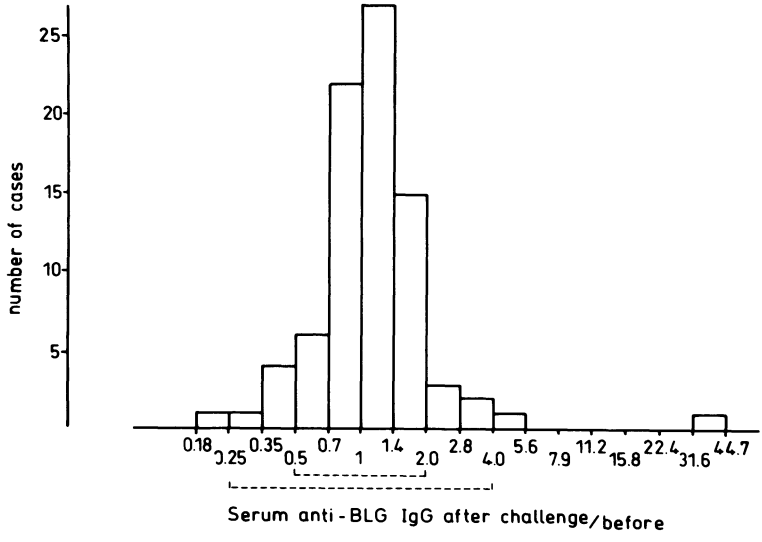

Figure 3. Histogram of the ratios (log scale) between anti-BLG IgG after and before the cow's milk diet.

DISCUSSION

We have shown that in these African mothers, both milk and serum Ab levels against two different food antigens were not influenced by the antigenic load in their diet. Anti-food Abs, essentially anti-GL IgA and anti-BLG and BIgG IgG, were detected in milk and/or serum from mothers consuming bread very sporadically, in low amount, and who were not milk drinkers. On the other hand, milk and serum Ab titers against cow's milk proteins did not change when the antigenic stimulation became considerably more important and frequent (600ml cow's milk/day during 2 months).

These results do not appear to be the consequence of possible abnormal IgA immune responses in women suffering marginally from protein malnutrition[10]. Serum and milk total IgA concentrations were within normal limits (data not shown). In addition, their aptitude to mount an IgA Ab response was apparently normal, owing to the frequent detection of significant anti-*Shigella* and anti-*Salmonella* IgA Ab levels both in milk and serum, in relation to their natural increased exposure to these pathogens (data not shown). Therefore, our data suggest that for the induction of food Abs both in milk and serum, unknown factors, other than the antigenic load must

be of greater importance. Genetic factors could be determinant as it has been suggested for serum IgE synthesis[11].

Comparisons with other data from the literature are difficult due to both epidemiological variations (degree of malnutrition, intestinal infections, pregnancy versus lactation) and differences in the antigenic exposure in term of duration and amount of antigen in the diet. In this respect, some authors suggested the existence of a relation between anti-food IgA levels in breast milk and the maternal diet[6], whereas others could not find any relation between these two parameters[2,5].

Our data could be influence by health problems in our population. The impact of malnutrition has already been evoked. However, previous studies have already documented some degree of intestinal mucosal atrophy in these populations as well as the high frequency of intestinal parasites [12,13]. Antigenic presentation at the intestinal level could be different and parasites could modify and modulate mucosal immune responses[14]. Indeed, anti-GL IgA were detected in serum at levels encountered in coeliac diseases or severe intestinal infections[8]. They were mainly polymeric even in the absence of bread consumption differing thus from patients with coeliac disease or gastroenteritis[9]. To our knowledge, this is the first example relating the presence of pIgA Abs in serum even in the absence of an acute stimulation by the corresponding antigen. The origin of this stimulation is presently unknown.

ACKNOWLEDGEMENTS

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MODULATION OF THE IMMUNE RESPONSE BY MATERNAL ANTIBODY

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INTRODUCTION

In all mouse strains studied[1,2], the primary immune response to hen egg-white lysozyme (HEL) is characterized by a predominant idiotype, IdXE (approximately 50% of population), and a predominant specificity for the 3 N-terminal amino acids of HEL (TIP), also 50% of population. The idiotype, IdXE, disappears and the TIP-specificity decreases markedly in the secondary response. Since neonatal A/J mice respond to immunization with HEL in complete Freund's adjuvant (CFA) as early as 7 days after birth and since the response is characterized by the same idiotype and specificity as the adult response, this idiotypic system provides a useful model for examining modulating effects of passively transferred maternal antibody on the immune response of the developing offspring to HEL-CFA. Our original hypothesis was that the presence of IdXE on passively transferred maternal antibody would perturb the offspring response.

METHODS

A panel of anti-HEL primary and secondary response hybridomas had been previously generated and described[1,3]. Serum-free supernatants from these hybridomas were precipitated at 50% saturation with ammonium sulfate to prepare anti-HEL mAbs with varying idiotypic (IdXE-positive or IdXE-negative) and fine specificity (TIP-dependent or TIP-independent) characteristics. All mAbs studied were IgG_1, κ and were administered intraperitoneally (IP) to the mother within 24 hours postpartum with transfer to the offspring via the colostrum and milk[4]. In additional experiments, female A/J mice were immunized IP with HEL-CFA and anti-HEL antibody was transferred to the offspring both transplacentally and via the colostrum and milk.

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Offspring were immunized IP with HEL-CFA at varying ages and compared with age-matched control litters. The immune response of the offspring was examined using ELISA techniques to determine IgM or IgG anti-HEL antibody responses. Heavy chain-specific anti-mouse IgM or IgG alkaline phosphatase conjugate was used for development with p-nitrophenyl phosphate, disodium, hexahydrate dissolved in 9.7% diethanolamine buffer at 1 mg/ml as substrate. In addition, the Elispot technique of Sedgewick and Holt[5] was adopted to examine splenic lymphocyte cultures for production of anti-HEL antibody-producing cells nine days post-immunization, the time of the peak Elispot response.

RESULTS

Induction of Suppression by Anti-HEL mAbs

Six anti-HEL mAbs were administered within 24 hrs postpartum to A/J mothers. Offspring were immunized IP with HEL-CFA at 16-20 days of age. Splenic cell suspensions were examined by Elispot assay 9 days post-immunization. The six mAbs were not equivalent in their effects. Two of the six mAbs consistently induced statistically significant suppression. One mAb gave inconsistent results and three other mAbs had no statistically significant effect. As summarized in Table 1, 2F4/2E5 (IdXE+, TIP+) and 2D1 (IdXE-TIP-) consistently suppressed the offspring response to HEL-CFA. Therefore, suppression did not correlate with IdXE, TIP-specificity or primary vs. secondary nature of the mAb. Other mAbs, 3C11 (IdXE+, TIP-), 5E11 (IdXE-, TIP-), 2C7 (IdXE-, TIP+) did not induce suppression. 2D10 (IdXE+, TIP-) gave inconsistent results. Further evidence for IdXE and TIP-specificity not being important determinants of suppression was provided by the examination of the suppressed litters for the idiotypic and fine specificity characteristics of the Elispot response. There was no change in the proportion of the IdXE component or TIP-specificity of the primary response. The induced suppression was transient and no longer present when offspring mice were immunized at 6 weeks or 2 months of age.

Transfer and Catabolism

We examined anti-HEL antibody in the sera of offspring receiving passively transferred mAb from the mother. The anti-HEL antibody present at 3 weeks of age was approximately 2.8 μg/ml. At 5 weeks of age, only a small amount of antibody remained and at 7 weeks of age, no anti-HEL antibody was detected. There was no evidence that differences in transport or catabolism of the mAbs could explain our results.

mAb Affinity/Avidity

We examined the mAbs in an antigen titration ELISA in which HEL coating concentration varied from 0.08 μg/ml to 80 μg/ml. MAb (1 μg/ml) was incubated on the plate for one hour at 37°C before addition of conjugate. Using this method, mAbs 2F4/2E5, 2D1, and 2D10 appeared of higher affinity/avidity and 3C11, 5E11, and 2C7 were of lower affinity/avidity. The three lowest affinity/avidity mAbs did not suppress in our model suggesting a possible Fc-mediated suppression. However, the inconsistent results with 2D10, a high affinity/avidity mAb make interpretation difficult.

Table 1. Suppression of Offspring Response
275 μg MAb to Mother A/J
Anti-HEL Elispots/10^6 Spleen Cells

mAb	# Control mice	# Experimental mice	#Assays	% Suppression
IdXE+, TIP+ 2F4/2E5	34	27	5	71 (± 16)[a]
IdXE-, TIP-				
5E11	13	13	2	29 (± 18)
2D1	21	17	3	74 (± 12)[a]
IdXE-, TIP+				
2C7	10	12	2	0
IdXE+, TIP-				
3C11	7	6	1	0
2D10	15	15	2	?[b]

[a] $p<.05$. In each assay, p was $<.05$.

[b] Two assays were performed. In one experiment, there was no suppression. In the other assay, suppression averaged 71% and was statistically significant ($p<.05$).

Priming with mAbs

Although only suppression was observed when 16-20 day old mice were immunized, we examined older mice to determine if there was any evidence of priming once the induced suppression had waned. For these experiments we chose the secondary response mAb, 2D1, which had consistently induced suppression in the younger mice at 16-20 days of age. We waited until offspring were 2 months of age before immunization with HEL-CFA since we knew that there was no residual antibody in the sera of the offspring at this age. Although an initial Elispot experiment suggested possible priming, priming could not be confirmed in subsequent experiments conducted under the same experimental conditions.

Since the Elispot assay only examined the peak B cell response 9 days postimmunization, we also examined total anti-HEL serum antibody in adult offspring exposed as neonates to 2D1 from the mother compared to control animals. There was no evidence for enhanced anti-HEL antibody (IgG) in the sera of experimental offspring at 9 days post immunization with HEL-CFA. In two additional experiments, mAb-exposed offspring and control 2 month old animals were studied, and total anti-HEL antibody was examined prior to immunization with HEL-CFA and at 4, 10, 15, and 22 days post HEL-CFA immunization. There was no alteration of the total anti-HEL IgG or IgM antibody response nor the kinetics of that response for experimental vs. control animals. In summary, no evidence could be found to substantiate a priming effect after exposure to 2D1 transferred from the mother A/J to the suckling.

HEL-CFA Immunized Mothers

In addition to the above described mAb experiments, we also examined offspring of mothers who had been previously immunized with HEL-CFA. We have found that high levels of serum anti-HEL antibody persist for over a year after primary immunization without the need for booster immunization. The B cell response to HEL-CFA, even in the primary response, is predominantly IgG[6].

Young (16-20 day) A/J offspring of HEL-CFA immunized A/J mothers appeared profoundly suppressed in the Elispot response to HEL-CFA immunization. In three experiments, Elispots for young offspring of immunized mothers were decreased 92% (± 7%) when studied nine days post-immunization. In additional experiments, older offspring were immunized at 8 weeks to 26 weeks of age, presumably after suppression had waned. Both Elispot assays and/or ELISA assays were performed. Elispot data are summarized in Table 2.

Table 2. Priming of Offspring Response
Immunized Mother A/J
Anti-HEL Elispots/10^6 Spleen Cells

Interval[a] mother HEL-CFA immunization	Age offspring HEL-CFA	Control Elispot' 10^6	Experimental Elispot' 10^6
10 wks	16 wks	4127 (± 4188)	5453 (± 2587)
16 wks	10 wks	3584 (± 1149)	9243[b] (± 1692)
16 wks	10 wks	10126 (± 4427)	22537[b] (± 7618)
26 wks	8 wks	7663 (± 3756)	11103 (± 2638)
27 wks	10 wks	8152 (± 2343)	6052 (± 2867)

[a] Interval from immunization of A/J mother to birth of offspring.
[b] $p<.05$ using Student's t test.

In two experiments, 10 week old offspring born to A/J mothers immunized 16 weeks prior to the birth of the offspring had an enhanced response in the Elispot assay, detecting total anti-HEL immunoglobulin (IgG plus IgM)-secreting B cells. Surprisingly, antibody was present in the sera of offspring mice at 16 weeks of age prior to HEL-CFA immunization. The offspring mice with the highest IgG antibody in pre-immune sera had an enhanced response in the Elispot assay.

In four experiments, anti-HEL IgG and IgM antibodies were examined for 8 week to 26 week old A/J offspring of A/J mothers who had been previously immunized. The interval from immunization of the mother to the birth of the offspring varied from 12 to 26 weeks. ELISA results are summarized in Table 3. Experiment #2 in Table 3 is the same as the experiment on the third line of Elispot Table 2.

In two experiments, IgM anti-HEL antibodies were significantly higher in control sera compared to experimental sera post-immunization. The pre-immunization sera contained no detectable IgM in experiments 2, 3, and 4. Experiment #1 pre-immune sera were not obtained. These results suggest a possible booster response to immunization, i.e. less IgM, in experimental animals. There was no difference between control and experimental animals for IgG anti-HEL antibody post-immunization. Pre-immune anti-HEL IgG antibody at the time of immunization in experimental and control animals were 0.57 vs. 0.04 (Exp #2), 0.05 vs. 0.00 (Exp #3), and 0.00 vs. 0.00 (Exp #4). The two groups of mice, who were not primed had no detectable IgG in pre-immune sera. It may be possible that the level of IgG antibody present in the pre-immune sera is a determinant of priming or may be evidence that priming has already occurred. Further work must be done to determine the origin of the antibody (maternal vs. offspring) in the pre-immune sera.

Table 3. IgM Anti-HEL Antibody Offspring of Immunized A/J

Interval[a] mother HEL-CFA	Age offspring HEL-CFA		Optical density 9 Days post HEL-CFA	16-21 Days post HEL-CFA
1) 12 wks	8 wks	Exp		0.04 ± 0.03
		Cont		0.22 ± 0.14[b]
2) 16 wks	10 wks	Exp	0.06 ± 0.07	
		Cont	0.38 ± 0.28[b]	
3) 21 wks	17 wks	Exp	0.11 ± 0.05	0.23 ± 0.21
		Cont	0.05 ± 0.01	0.37 ± 0.19
4) 26 wks	21 wks	Exp		0.18 ± 0.11
		Cont		0.23 ± 0.13

[a] Interval from immunization of A/J mother to birth of offspring.

[b] $p<.05$ when control and experimental groups were compared.

DISCUSSION

In our experiments with anti-HEL mAbs, we have shown that certain mAbs induced suppression of the anti-HEL response in young offspring when transferred from the mother. The induced suppression was transient and was not correlated with the IdXE-positivity or TIP-dependence of the mAb. An idiotope other than IdXE may have been involved in generating a

suppressive T cell network, but an Fc-mediated suppression could also not be excluded. Young offspring of A/J mothers immunized previously were also profoundly suppressed in Elispot response to HEL-CFA.

When older animals were examined for evidence of priming (an increased anti-HEL antibody response or altered kinetics of response), no evidence could be found when 2D1 was administered via the mother. It is possible that other mAbs with different idiotypic characteristics could prime, but it is also possible that 2D1 was administered too late in ontogeny for a priming effect. Further support for this interpretation is provided by the offspring of HEL-CFA immunized A/J mothers. Priming appeared to have occurred for some offspring born 12-16 weeks after immunization of the mother. There are at least three possible explanations for priming in the offspring of the immunized mother model, but not in the offspring of 2D1 treated mothers. 1) Since some IgG1 can cross the placenta in the mouse, offspring of immunized mothers were exposed to antibody much earlier in ontogeny. Vakil and Kearney have demonstrated that windows of susceptibility to idiotypic manipulation exist[7,8]. Administration of mAb earlier in ontogeny could resolve this issue. 2) The idiotypic characteristics of the polyclonal maternal antibody most certainly differs from mAb, 2D1. The maternal serum may contain key priming idiotypic determinants. 3) It is difficult to exclude antigen exposure *in utero* in the offspring of immunized A/J mothers. At least 10 weeks had elapsed from immunization of the mother to the birth of the offspring in an attempt to minimize this possibility. Exposure to immune complexes via the placenta could also have immunized the offspring. Further analysis of pre-immunization antibody for clonotypic analysis will help to resolve these issues. Post-immunization antibody will be further characterized for differences in idiotypy and fine specifity. Even if immunization of the offspring does occur *in utero*, these results will be of importance in selecting strategies for maternal immunization and subsequent protection of the offspring from infectious pathogens.

CONCLUSIONS

Passively transferred maternal antibody modulates the immune response to the protein antigen hen egg-white lysozyme. Both suppression and priming have been observed.

The mechanism of the observed suppression in younger animals is not clear. Priming of the older offspring may result from exposure to maternal immunoglobulin idiotypes. Future studies will determine if priming of the offspring of immunized mother A/J results in alteration of the idiotypic and fine specificity characteristics of the offspring's response to HEL.

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MATERNAL DETERMINANTS OF NEONATAL IMMUNE RESPONSE: EFFECT OF ANTI-IDIOTYPE IN THE NEONATE

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INTRODUCTION

The influence of maternal immunologic reactivity on the development of the immunologic repertoire in the neonate has been examined in a number of recent studies employing different antigens in a variety of mammalian species[1,2]. Studies carried out by Jarrett and Hall[3] in rats have shown that maternal immunization with ovalbumin (OVA) administered parenterally prior to mating is associated with significant suppression of IgE and enhancement of IgG anti-OVA response in the neonate following neonatal immunization. Significant suppression of neonatal immune response has also been observed after oral immunization of pregnant rabbits with bovine serum albumin[4]. Other studies have suggested a potential for enhanced neonatal immune response after prior maternal immunization[5]. The mechanisms underlying the maternally induced alterations in neonatal immune response remain to be determined. The relative contribution of transplacentally-acquired or breastfeeding-related maternal components in such effects is not known. The present studies were undertaken in order to characterize the nature of neonatal immune response to antigens and to evaluate the role of breastfeeding in such maternal-neonatal interactions.

MATERIAL AND METHODS

Effect of Breastfeeding on the Development of Anti-Idiotype Antibody Response to Respiratory Syncytial Virus (RSV)

BALB/c mice were mated 15 to 25 wks of age. On the 4th and 8th day after the delivery of the neonate, groups of lactating female mice and their suckling neonates which were immunized according to the following protocol. Group a and b consisted of 30 to 40 lactating mice each, which were immunized with 500 or 0.25 μg of monoclonal antibody to F glycoprotein (MAF) of RSV administered i.p. in saline. As controls, additional lactating animals were immunized with 500 μg of monoclonal antibody to G glycoprotein (MAG) in saline (group c) or saline alone (group d). In group a

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and b, five to seven suckling mice were killed without additional treatment at regular intervals for 6 to 7 weeks after birth. Other infant mice were weaned at 3 wks of age and 1 wk later challenged i.p. with a single dose of 1.5 μg of F glycoprotein of RSV in a saline suspension. Group e consisted of suckling mice whose mothers had received no RSV immunization and they were inoculated with a polyclonal Ab-2 against MAF in a dose of 50 μg/ml protein administered i.p. at 2 and 3 wks of age. These infants were subsequently challenged with F glycoprotein of RSV as described above.

Specimen collection. Specimens of serum were obtained by retro-orbital bleeding. Spleens were removed aseptically and sterile, single-cell suspensions were prepared by standard techniques. 10^7 spleen cells were cultured at 37°C in 2 ml of RPMI640 medium for 7 days and assayed for RSV antibody synthesis.

Ab-1 antibody. MAF (clone B-2-1) and MAG (clone C2) RSV antibody used in this study had been prepared in BALB/c mice in our laboratory and the characteristics were studied in detail previously[5]. The Ig isotype of MAF was found to be IgG2a and possessed complement-independent neutralizing activity of 1:512 against live RSV.

Preparation of polyclonal Ab-2 against MAF. Coupling of MAF to KLH was done according to the method of Bona *et al.*[6]. Male DBA/2 mice were immunized by whole MAF conjugated with KLH and emulsified with complete Freund's adjuvant and the serum collected was subjected to protein A column chromatography. The Ab-2 exhibited anti-Ab-1 titer of 1:16396 when tested against MAF in a cross-linked ELISA antibody assay. However, no reactivity could be detected between this Ab-2 and MAG. In competitive inhibition assay, the binding of biotin-labeled MAF to F glycoprotein was only inhibited by sera obtained from animals immunized with MAF but not with MAG. These results suggest that sera from immunized DBA/2 mice with MAF contained paratope-associated anti-idiotype which competed with antigen for the binding site on Ab-1.

Purification of F glycoprotein of RSV. The F glycoprotein derived from the long strain of RSV was prepared by affinity column chromatography, using MAF coupled Sepharose-4B columns.

Detection of anti-F glycoprotein antibody response. The development of IgG and IgM anti-F glycoprotein antibody responses were assayed by ELISA.

Ab-2 determination. The induction of Ab-2 antibody response to MAF was measured by using an Ab-1 cross-linking ELISA assay with MAF $F(ab')_2$ preparation.

Detection of neutralizing antibody. Neutralizing antibody titers against RSV were determined by a plaque reduction assay in Hep-2 cell culture monolayer in 24-well microtiter plates using 100 PFU/ml RSV long strain.

Study of Neonatal IgE Response to Ovalbumin (OVA)

Inbred female Brown-Norway (BN) rats were immunized i.p. with 1 mg OVA 14 days before and 10 days after mating. A $1x10^{10}$ volume of heat-killed *Bordetella pertussis* was used as an adjuvant in the first immunization. The other groups of female rats were sham-immunized with a saline solution containing no OVA.

Foster-feeding protocol. The mothers delivered the neonates 25-38 days after mating. The infants of immunized mothers and of sham-immunized mothers were breast-fed by their own mothers or by foster mothers (Table 2). All neonates were weaned at 3 weeks of age, and one week after weaning they

were immunized with 1 mg OVA and $1x10^{10}$ *B. pertusis* as adjuvant. Three wks later, they received a booster immunization with 1 mg OVA alone.

Passive administration of immune serum. All 4-wk-old infant rats born from and fed by sham-immunized mothers were divided into the following immunization groups. Group A infants received 0.5 ml of OVA-specific immune serum i.p.. This immune serum was collected from the BN rats immunized with OVA and contained high anti-OVA antibody titer. Two days after administration of immune serum, the infants were immunized with 1 mg OVA. Group B infants received the immune serum i.p. once a week for 4 weeks before immunization. Group C received normal rat serum before immunization with OVA. Three wks after the primary immunization, the rats in all groups received booster immunizations with OVA.

Cell co-cultures. Unprimed T cells were obtained from spleens and mesenteric lymph nodes (MLN) of the infant mice born from and fed by immunized mothers at 8 wks of age (1 wk after booster immunization) (^{b}T cells). OVA-primed T cell (^{a}T cells) and B cells (^{a}B cells) were collected from MLN obtained from male BN rats immunized with OVA. Control T cells (^{c}T cells) were obtained from male unimmunized infants of sham-immunized mothers. B cells and T cells were cultured at 37°C in RPMI 1640 medium exposed to a humidified atmosphere enriched with 5% CO_2 in the presence of OVA for 3 days. The initial medium was then removed and replaced with 2 ml fresh medium that did not contain OVA. Seven days later, the culture supernatant was harvested.

In vitro proliferative response. Purified T lymphocytes were tested with OVA or phytohemagglutinin in 96-well microplates.

Detection of anti-OVA antibody. Rat IgG, IgM, IgA, and IgE anti-OVA were assayed by ELISA. IgE anti-OVA antibody in serum was assayed by passive cutaneous anaphylaxis (PCA) in order to avoid interference in ELISA by high concentration of other isotypes.

RESULTS

Effect of Breastfeeding on the Development of Anti-Idiotype Antibody Response to RSV

After weaning at 21 days of age, no antibody activity was detected in the serum of those infants whose mothers had received 0.25 μg of MAF. However, ELISA IgG anti-F glycoprotein activity was regularly observed for up to 42 days of age and neutralizing activity persisted to 24 to 26 days of age in those infant mice of mothers who had received 500 μg of MAF postpartum. Similarly, the spleen cell culture supernatants from the infants in group b or group d exhibited no Ab-2 activity against MAF. However, Ab-2 activity was consistently observed in the spleen cells of infants of mothers who had been immunized with 500 μg of MAF (group a). The Ab-2 antibody activity appeared to reach maximum in the spleen cells collected at 4 weeks of age.

The effect of subsequent challenge immunization with purified F glycoprotein in the infants of mothers who had received MAF postpartum and in infants of control mothers who were inoculated with MAG or saline during the postpartum period are presented in Table 1. The anti-F glycoprotein antibody and plaque-neutralizing antibody to the whole virus were significantly enhanced in the group a mice whose lactating mothers had received 500 μg MAF and the response appeared to be specific against the

epitopes of F glycoprotein recognized by MAF. This is evidenced by significant inhibition of the binding of biotin-labeled MAF to the F glycoprotein by such post-immune infant sera, compared to the serum of non-immunized controls or infants whose mothers received 0.25 μg MAF or saline control postpartum.

Table 1. Development of RSV Antibody Response in Infant Mice After Active Immunization with F-gp of RSV at 4 Wks of Age

Immunization group	Anti F-gp ELISA antibody titer (Mean ± SE)*		RSV-neutralizing antibody titer (Mean + SE)[a]	Inhibition of binding of Ab-1 to F-gp (% decrease in OD)[b]
	IgG	IgM		
a	197±9	1097±102	17±1	57±1
b	114±6	206±11	5±0.3	27±2
c	126±6	229±12	5±0.2	NT
d	104±4	208±8	5±0.2	28±1
e	NT[c]	103+6	NT	NT

[a] Expressed as reciprocal of dilution
[b] Inhibition assay used a serial dilution of 1/100
[c] NT, not tested

The polyclonal Ab-2 against MAF was administered at 2 and 3 wks of age to infant mice whose mothers had received no prior immunization. These infants were subsequently challenged with F glycoprotein at 4 wks of age. A significant enhancement for IgM and IgG anti-F glycoprotein and of RSV-specific neutralizing antibody activity was observed in the Ab-2 primed animals when compared to non-primed or placebo controls.

Study of Neonatal IgE Response to OVA

Anti-OVA antibody at weaning. All mothers immunized with OVA developed predictably high anti-OVA antibody titers in IgG (1:667,000), and IgM (1:13,573) and low levels in IgE (1:18.7) when tested at delivery. No IgA anti-OVA antibody could be detected in these animals. The babies born from and fed by immunized mothers (group 1) and the babies born from sham-immunized mothers but fed by immunized mothers (group 3) exhibited significantly higher ($P<0.001$) IgG and IgM anti-OVA antibody levels at weaning than observed in infants of immunized mothers foster-fed by sham-immunized mothers (group 2) or sham-immunized controls (group 4). No infants had any detectable IgE or IgA anti-OVA antibody at the time of weaning. Thus, it appeared that high levels of IgG and possibly small quantities of IgM antibody were transferred from mother to infant via breast-feeding, but no transfer of IgE could be demonstrated (Table 2).

Table 2. Anti-OVA Antibody in Infants at Weaning and After Active Immunization with OVA at 4 Wks of Age

Group no.	Immunization status of mothers: Natural	Foster-feeding	Serum anti-OVA[a] titer at weaning (log_2 mean±SE): IgG	IgM	IgE	Serum anti-OVA titer after active immunization (log_2 mean+SE): IgG	IgM	IgE
1	Immne	Immune	18.9±14.4	9.0±5.0	<0	17.9±14.0	11.0±7.4	1.0±<0.1
2	Immune	Sham	10.0±7.0	<5	<0	19.6±16.0	13.3±10.2	7.3±4.2
3	Sham	Immune	18.9±9.2	8.6±5.7	<0	17.6±15.2	11.3±8.1	0.3±<0.1
4.	Sham	Sham	<5	<5	<0	19.9+16.2	13.3+9.8	7.3+3.4

[a] IgG and IgM were measured by ELISA; IgE was measured by PCA. Titer is expressed as log of reciprocal of dilution.

IgE anti-OVA antibody response in infants after active immunization with OVA at 4 wks of age. In the two groups of infants born from and fed by immunized mothers (group 1) or born from sham-immunized mothers but fed by immunized mothers (group 3), subsequent immunization with OVA exhibited significant suppression ($P < 0.01$) of IgE anti-OVA response, compared with the infants born from and fed by sham-immunized mothers (group 4). On the other hand, the infants born from immunized mothers but fed by sham-immunized mothers (group 2) did not demonstrate any suppression in OVA antibody response following immunization (Table 2).

Effect of passive administration of immune serum on antibody response to OVA. There were no significant differences in IgG and IgM anti-OVA responses between group A, group B, and group C. However, IgE anti-OVA response in group B infants (log mean $\pm$ SE:5.6$\pm$1.8) was significantly suppressed compared to the other two groups (7.0$\pm$4.0).

In vitro co-culture studies. When B cells from immunized infants of immunized mothers (^{b}B) were cocultured with homologous ^{b}T cells, significant IgG and IgM anti-OVA antibody synthesis was observed. However, marked suppression of IgE synthesis (with no detectable activity) was observed.

Table 3. Effect of T cells Obtained from Immunized Infants of Immunized Mothers on Anti-OVA Antibody Synthesis by OVA-Sensitized B Cells

Experiment number	Co-culture conditions (n of cells/ml)	Anti-OVA antibody titer[a] mean$\pm$SE of each experiment (%+SE)		
		IgG	IgM	IgE
1	^{a}B(2.5×10^{6})			
	^{a}T(1.5×10^{6})	9.0$\pm$6.0	3.0$\pm$<0.1	3.3$\pm$<0.1
	^{c}T(1.0×10^{6})	(100)	(100)	(100)
2	^{b}B(2.5×10^{6})			
	^{b}T(1.5×10^{6})	9.6$\pm$7.3	3.2$\pm$0.4	<0
	^{c}T(1.0×10^{6})	(170$\pm$27.9)	(110$\pm$11.0)	
3	^{a}B(2.5×10^{6})			
	^{a}T(1.5×10^{6})	10.6$\pm$8.3	4.0$\pm$0.8	1.3$\pm$<0.1
	^{b}T(1.0×10^{6})	(300$\pm$50.0	(240$\pm$17.9)	(20$\pm$2.2)
4	^{a}B(2.5x106)			
	^{b}T(1.5x106)	9.3$\pm$5.5	3.7$\pm$1.1	0.3$\pm$0.1
	^{c}T(1.0x106)	(150+14.1)	(160+11.0)	(7.5+2.2)

[a] Anti-OVA antibody titer was measured with ELISA and expressed as $\log_2$ of reciprocal of dilution.

Suppression of IgE synthesis was also observed with adult OVA-primed ^{a}B cells when co-cultured with ^{b}T cells from immunized infants of immunized mothers (Table 3).

T cell proliferative response. An *in vitro* proliferative response to PHA was clearly demonstrated in both OVA-primed (SI = 7.1±3.0, Δcpm=1193) as well as unprimed T cells (SI = 4.5±0.6, Δcpm=711). However, T cells obtained from unimmunized infants born from OVA-immunized infants (^{b}T cells) failed to exhibit any response to OVA (SI = 1.2±0.4).

DISCUSSION

The observation of particular importance reported here is that high doses of maternal antibody transferred via breastfeeding induced anti-idiotypic antibody, and subsequent immunization with the original viral protein (F glycoprotein) resulted in a specific priming effect for anti-F glycoprotein response, even in the presence of maternal antibody. It is suggested that transfer of protective idiotypes from the mother to the child could provide a dual effect to infants: priming the suckling neonate for a booster effect on subsequent exposure to antigen and at the same time, offer immediate passive protection against induced infection.

On the other hand, the passively transferred anti-OVA antibody strongly suppressed the IgE response to OVA in the neonates. Although precise mechanisms underlying the development of immunesuppression of IgE after breastfeeding by immunized mothers remain to be defined, the mixed cell culture experiments reported here suggest the induction of suppressor T cells specific for anti-OVA antibody responses. Significantly, however, such suppressor T cells did not react with OVA when tested *in vitro* in proliferative responses. These observations suggested that immunosuppressive effects mediated by breastfeeding are induced by mechanisms directed by anti-OVA antibody-specific but not by OVA antigen-specific suppressor T cells or their soluble products. It may be that the suppression of IgE is mediated also via the development of anti-OVA-specific anti-idiotypic response, though different from enhanced IgG and IgM neonatal immune response to RSV.

However, it must be emphasized that fundamental differences exist between the human and the mouse suckling infants, in regard to the uptake and systemic transport of antigen, immunoglobulin or T cell products or other macromolecules present in the suckled milk or in the infants intestinal lumen. Essentially no uptake of milk immunoglobulin has been demonstrated during breastfeeding in the human neonates, although trace amounts of milk IgA antibody may be detectable in the serum of infants under 18 h of age fed artificially, with milk containing high levels of IgA[7]. However, the bulk of neonatal IgG and all its subclasses appear to be transmitted to the fetus transplacentally in latter parts of gestation[8]. Therefore, the implications of breastfeeding-associated transfer of anti-idiotypic immunity in man must be applicable at the level of mucosa-associated lymphoid tissue and the regulation of the immune response at the mucosal surface of intestine and possibly respiratory tracts. However, this model could also be applied to the transplacental idiotype transfer and priming for subsequent neonatal immunization.

CONCLUSIONS

1. Anti-F glycoprotein of RSV monoclonal antibodies administered to lactating mice were transferred to suckling infants via the process of breastfeeding.

2. Transfer of Ab-1 RSV antibody induced anti-Ab-1 antibody after weaning.

3. Subsequent immunization with F-glycoprotein resulted in booster response.

4. Similar booster effect was seen in the infants who had received Ab-2 RSV antibody.

5. These results suggest the induction of RSV-specific anti-idiotypic antibody in the neonate via breastfeeding, secondary to maternal immunization with Ab-1 RSV antibody.

6. On the other hand, in the OVA study, transferred anti-OVA antibody via breastfeeding suppressed IgE response of the infants and the induction of the suppressor T cells for regulating anti-idiotypic production is suggested.

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IMMUNOGLOBULIN G SUBCLASSES IN HUMAN COLOSTRUM AND MILK

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INTRODUCTION

IgG, IgA, IgM, and IgD class of immunoglobulins are found in human colostrum and milk[1-3]. Although IgA comprises about 90% of human milk immunoglobulins (mean level 32 g/l), IgG levels as high as 1 g/l in colostrum and 50 mg/l in milk are found. Very little information is available regarding the distribution of IgG subclasses in human colostrum and milk. Determination of IgG subclass concentrations in human colostrum and milk may be important because they have different biological functions.

Four subclasses of human IgG (IgG1 , IgG2, IgG3, IgG4) have been identified on the basis of antigenic differences in their respective heavy chains. In normal adult serum IgG1 predominates (66%), followed by IgG2 (23%), IgG3 (7%) and IgG4 (4%)[4-5]. Recently, a large number of monoclonal antibodies to human IgG subclasses have been prepared and evaluated against a broad spectrum of immunoglobulins from different genetic pools[5]. However, their usefulness in quantitation of IgG subclass levels has not been systematically applied to human colostrum and milk. Since one study[6] using polyclonal antibody indicated local synthesis of IgG4 in the human mammary gland, it is likely that the values of IgG subclasses in the milk may differ from those of serum. Thus, the aim of the present study was to determine if the distribution of IgG subclasses in human colostrum and milk is different than that of serum and saliva.

MATERIALS AND METHODS

Collection of Colostrum, Milk, and Saliva

Serial specimens of colostrum collected from 7 women at 2 to 4 days postpartum and milk from 11 women collected between 7 to 38 weeks postpartum were obtained from Dr. M. C. Neville, Department of Physiology, University of Colorado, Denver, CO, and the Breastfeeding Association of Kopavogur Reykjavik, Iceland.

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Parotid saliva was stimulated using a sour lemon ball and collected from Stenson's duct using a modified Lashley cup[7]. This avoids contamination present in whole saliva sample. All samples were frozen at -70°C until use.

Mouse Monoclonal Antibodies

Human anti-IgG1 (HP6012), anti-IgG2 (HP6014), anti-IgG3 (HP6050), and anti-IgG4 (HP6011) were purchased from Oxoid Ltd., Basingstoke, Hampshire, England, and ICN Immunobiologicals, Lisle, IL, USA. These antibodies were characterized[5] in a solid-phase immunofluorometric assay against a panel of 62 purified human myeloma proteins of known IgG subclass, light chain types and, in some cases, known allotypes.

Quantitation of IgG Subclasses by ELISA

The quantitation of IgG subclasses was carried out by an enzyme linked immunosorbent assay (ELISA) as described by Papadea *et al.*[8]. The avidin-biotin system was used to enhance the sensitivity of ELISA.

RESULTS

IgG subclass concentrations in colostrum, milk, serum, and saliva are shown in Table 1. Subclass concentrations were highest in colostrum obtained at day 2; by day 4, they are decreased 10-fold. When concentrations were expressed as percentages of total IgG there was a different distribution of IgG1 and IgG2 in colostrum compared to those in normal adult sera. IgG1 was about 20% higher in colostrum than serum; IgG2 was 20% lower. Percentages of IgG3 and IgG4 in colostrum and serum were similar. Although the concentrations of IgG subclasses in milk were reduced by approximately 6-fold compared to colostrum values (day 4), the percentage distribution of each IgG subclass in milk was similar to that seen in colostrum. IgG subclass distribution of saliva was similar to that of serum and different from colostrum and milk.

DISCUSSION

Our results showed a significant drop in IgG subclass values in colostrum collected on day 4 in comparison to day 2 after the outset of lactation. These findings are consistent with those seen with secretory IgA (S-IgA), whole IgG and lactoferrin[9-10]. We found a lower % of IgG4 subclasses in colostrum and milk compared to that reported previously[6]. One of the reasons for this may be that the earlier investigators[6] quantitated IgG4 levels in milk and colostrum supernatants following centrifugation at 12, 100x*g* for 30 min, compared to the whole milk and colostrum used in the present study. Another difference was that the previous study[6] used polyclonal rather than monoclonal antibody to measure IgG4 levels.

Table 1. IgG Subclass Concentrations in Colostrum, Milk, Serum[a], and Saliva

	IgG Subclasses, μg /ml (mean + SE)				IgG Subclasses (%)			
	1	2	3	4	1	2	3	4
Colostrum								
day 2	2248.4 ± 531.8	162.2 ± 59.6	113.9 ± 47.0	14.7 ± 5.7	85.45 ± 3.77	8.25 ± 3.06	5.19 ± 1.32	1.14 ± 0.04
day 3	539.8 ± 123.6	38.0 ± 11.2	36.5 ± 10.1	4.7 ± 1.0	85.05 ± 2.38	7.70 ± 2.08	6.21 ± 1.18	1.08 ± 0.023
day 4	195.0 ± 83.2	12.3 ± 0.4	14.7 ± 2.5	2.4 ± .4	85.89 ± 2.58	7.00 ± 1.57	5.14 ± 1.28	1.88 ± 0.63
Milk								
days 49-266	35.72 ± 4.40	4.18 ± 0.69	1.31 ± 0.15	.516 ± .109	84.3 ± 2.12	10.9 ± 1.90	3.38 ± 0.39	1.46 ± 0.37
Serum range	3190 - 10200	1230 - 6630	160 - 1940	30 - 1330	60.3 - 71.5	19.4 - 31.0	5 - 8.4	0.7 - 4.2
Saliva	1.32 ± .4	.48 ± .14	.18 ± .04	.05 ± .01	61.01 ± 4.4	24.53 ± 4.12	9.98 ± 1.55	3.23 ± .58

[a]Data[4] expressed as 95th percentile range of IgG subclasses from serum of 172 adults.

The reason for the differences in distribution of IgG subclasses in colostrum and milk compared to saliva and serum is not clear. Further comparison of IgG subclasses between maternal serum, saliva, and colostrum should indicate whether there is a selective transfer of IgG1 from serum to colostrum, or a local synthesis of IgG1 in the mammary tissue.

IgG1 effectively fixes complement and is the major antibody response to protein antigens of viruses and bacteria[11]. Although the role of IgG1 in colostrum and milk is not known, increased levels of IgG1-specific antibodies in sera from patients with viral infections (including measles, herpes simplex, and cytomegalovirus) have been reported[12-14]. A recent study[15] also showed that in addition to S-IgA, IgG1 from milk and colostrum possessed significant antibody activity to respiratory syncytial virus, whereas the activity was absent in the IgG2, IgG3, and IgG4 subclasses. Whether IgG1 could function to protect newborns from viral infection as does S-IgA is currently under investigation.

CONCLUSIONS

The percentage of IgG1 was significantly increased and that of IgG2 was decreased in both colostrum and milk relative to the percentage distribution in healthy adult and maternal sera. Percentages of IgG3 and IgG4 colostrum, milk, and sera were similar. The IgG subclass distribution of saliva was similar to that of serum and different from milk.

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SECRETORY DEFENSES AGAINST *GIARDIA LAMBLIA*

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INTRODUCTION

Giardia lamblia is the major identified cause of waterborne enteric disease in the U.S. Nonetheless, knowledge of the biological interactions between *G. lamblia* and the host intestinal milieu is limited. We have proposed that the great variations in the clinical manifestations and duration of giardiasis are due to parasite interactions with both immune and non-immune components of this environment[1]. Investigation of these interactions *in vitro* has begun to yield valuable information on aspects of parasite biology that may determine colonization and persistence of trophozoites in the human small intestine. As summarized below, we have demonstrated powerful non-immune giardiacidal activities of normal human milk (NHM)[2] and duodenal fluid[3] and have shown that *Giardia* can utilize certain other host intestinal secretions to survive, attach, multiply, and differentiate[4-7].

We showed earlier that *G. lamblia* trophozoites are killed *in vitro* by normal human milk, independently of antibodies[2]. Several lines of evidence supported the idea that killing requires activity of the bile salt-stimulated lipase (BSL) of human milk. Killing and BSL activity[8] were inhibited by heat and by diisopropylfluorophosphate (DIFP), a specific serine esterase inhibitor[9]. Both BSL and killing activity were absent from goat's and cow's milk[8,10]. Finally, we have shown[2], and others have confirmed[10], that killing of *G. lamblia* by fresh NHM requires sodium cholate, a bile salt which activates BSL. BSL appears to function in the intestine since lipid digestion was decreased by a third in infants fed heated, versus raw breast milk[11].

BSL activity was discovered in the milk of humans and subsequently demonstrated to be present in the milk of gorillas[12], but not of rhesus monkeys, cows, or goats[9]. Accordingly, we undertook these studies to assess the appearance of this important enzyme in the milk of other higher and lower primates as a further test of the idea that BSL is responsible for the giardiacidal activity of NHM.

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MATERIALS AND METHODS

Cultivation of *G. lamblia* strain WB, collection of milk from normal humans, cows, and goats and the parasite killing assay, have all been described in detail[2]. Milk was collected by manual expression from a gorilla (*Gorilla gorilla gorilla*), three chimpanzees (*Pan troglodytes troglodytes*), a grey-cheeked mangabey (*Lophocebus alibigena*), two mandrills (*Papio sphinx*) and a mustached guenon (*Cercopithecus cephus*) at the Primatology Centre of CIRMF in Gabon. *G. lamblia* was not detected in stool specimens of these animals or of women who donated milk samples in Gabon. Milk from an orangutan (*Pongo pygmaeus*), three chimpanzees, three pygmy chimpanzees (*Pan paniscus*), two white-handed gibbons (*Hylobates lar*), three sooty mangabeys (*Cercocebus atys*), four rhesus monkeys (*Macaca mulatta*), and a squirrel monkey (*Saimiri sciureus*) were obtained from the Yerkes Regional Primate Research Center, Emory University, Atlanta, GA, under the auspices of Dr. H. McClure. Stool examinations were not performed for these animals. For collection of milk from nonhuman primates, all animals were anesthetized with ketamine. In addition, pygmy chimpanzees received droperidol. All milk samples were distributed in 0.5 to 1 ml aliquots and shipped and stored at -70°C. Antiserum against purified milk BSL[13] raised in a rabbit was the generous gift of Dr. C.-S. Wang, Oklahoma Medical Research Foundation.

Parasite Killing Assays

Milk samples were diluted 1:5 or 1:10 in growth medium without serum or bile and sonicated on ice for 20 to 30 sec to disrupt milk fat globules. Washed trophozoites (2×10^5) were mixed with sonicated milk (1% or 10% vol/vol) in the presence or absence of 3.2 mM sodium cholate, in a final volume of 0.5 ml. Controls lacked milk, bile salt, or both. After incubation for 2 hr at 37°, killing was stopped by addition of 3.5 ml of complete growth medium which contained bile and serum to arrest killing. After overnight incubation, surviving parasites were enumerated by inverted microscopy. Killed organisms were totally lysed and were, therefore, not visible[2,8]. Surviving parasites appeared normal morphologically.

ELISA Assay for BSL

Skimmed milk samples, diluted 1:2 to 1:20,000, were incubated overnight at 4°C and then for 1 hr at 37°C in ELISA plates. After washing and blocking with BSA, rabbit anti-BSL (diluted 1:100 in buffer containing 0.3% gelatin) was added and incubated for 90 min. Reactivity was detected with peroxidase-conjugated goat anti-rabbit serum (1:1000 in gelatin buffer).

BSL Activity

Milk lipase activity was assayed by release of butyric acid from tributyryn (4% vol/vol) as substrate in 0.1 M NaCl in the absence or presence of 2 mM sodium taurocholate[2].

RESULTS

Earlier studies had demonstrated BSL activity in the milk of humans[11] and gorillas[12], but not of rhesus monkeys[9]. Therefore, we tested milk from a

number of nonhuman primates for (a) ability to kill *G. lamblia* trophozoites *in vitro*, (b) BSL lipase activity, and (c) reactivity with antibody to BSL purified from human milk.

The results at both ends of the spectrum were very clear (Table 1). Milk from normal women in both San Diego and Franceville, Gabon, as well as from a gorilla, killed >96% of the parasites at a concentration of 1%. Moreover, BSL antigen was detected at milk dilutions $\geq$ 1:10,000 and lipase activity was relatively high (Table 1). At the opposite extreme, neither killing, nor enzymatic activity, nor immunologic activity was detected in milk from goat, cow, or guenon. A low level of lipase activity (with tributyryn) was detected in the latter, but it was not stimulated by taurocholate. The milk of the grey-cheeked mangabey had high killing activity and lipase activity, but the ELISA titer (1:200) was ~50-fold lower than that of the gorilla. The low ELISA titer of the mangabey milk, relative to its BSL enzymatic and killing activity, may be due to limited cross-reactivity with antibody against human BSL. The chimpanzee and mandrill milk also had ELISA titers of 1:200. Chimpanzee milk was inactive enzymatically but mandrill milk contained some BSL enzymatic activity. Neither milk killed *Giardia* (Table 1). These observations support the idea that BSL activity is responsible for killing, although other factors may also be required (see Discussion).

Table 1. Killing of *G. Lamblia* and BSL Enzymatic and Antigenic Activity

Milk donor	% Parasite killing at[a] 1%	10%	Lipase activity[b] -TC	+TC	BSL antigen[c]
Human (US)	99.4	99.9	4.75	65	>20,000
Human (Gabon)	99.2	>99.9	ND[d]	ND	>20,000
Gorilla	96.1	>99.9	2.0	17.5	10,000
Grey-cheeked mangabey	69.3	98.9	1.25	5.0	200
Mandrill	0	0	<0.25	4.2	200
Guenon	0	0	0.50	0.46	2
Chimpanzee	0	0	<0.25	<0.25	200
Goat	0	0	<0.25	<0.25	<2
Cow	0	0	<0.25	<0.25	<2

[a] Milk concentration, % vol/vol. Average of two experiments.

[b] Micro equiv. fatty acid hydrolysis/min/ml milk in the absence and presence of 2 mM sodium taurocholate (TC). Lipase and ELISA assays were carried out on milk from a single donor.

[c] Reciprocal of ELISA titer using rabbit antiserum against BSL purified from human milk.

[d] ND (not determined).

As a direct test of the idea that killing was due to BSL, samples of milk from a human, a gorilla, and a grey-cheeked mangabey were pre-treated with the specific inhibitor, DIFP[9]. In each case, DIFP-treated milk failed to kill, while 100% of parasites were killed with untreated milk (at 1% or 5%).

Milk from three other chimpanzees, three sooty mangabeys, an orangutan, two rhesus monkeys, and a squirrel monkey also failed to kill *Giardia*. Interestingly, milk from one pygmy chimpanzee and one gibbon killed all the parasites at 1%, while milk from two other pygmy chimps and a second gibbon (at 1% or 5%) did not. Killing appeared to be due to BSL since it occurred in the presence, but not in the absence of taurocholate.

DISCUSSION

BSL and Killing of *G. Lamblia* by Primate Milk

These studies have extended our earlier observation that killing of *G. lamblia* by human milk *in vitro* is dependent upon the presence of BSL. We found that milk from cows, goats, and nonhuman primates which lack BSL did not kill *Giardia*, while milk from the gorilla and grey-cheeked mangabey had both BSL and killing activity.

Early studies revealed BSL activity in milk of humans[9,11] and gorillas[12], but not of rhesus monkeys[9] or other mammals tested, including cows and goats[9,12]. Therefore, it was proposed that BSL in milk was an evolutionary newcomer[9], but the more recent finding of high BSL activity in milk from carnivorous species, such as dog, cat, bear, and seal[14] invalidated this hypothesis.

In our studies, closely related primates (chimpanzee versus gorilla and human), two species of mangabeys (grey-cheeked versus sooty mangabey), and even individual animals in the same species (pygmy chimp, gibbon) differed in killing and apparent BSL activity of their milk. Although small numbers of animals were studied, this is a further argument against the appearance of BSL in milk at a single recent point in evolution[9].

All the parasite killing we observed appeared to be due to BSL activity, since it was absolutely dependent upon the presence of taurocholate. Moreover, in each case tested, killing was ablated by pre-treatment of milk with the specific inhibitor DIFP[8] (present studies). On the other hand, it is possible that milk samples with BSL activity might not kill *G. lamblia in vitro* since killing is due to release of toxic lipolytic products such as unsaturated fatty acids[10,15]. Therefore, failure of milk with BSL to kill *in vitro* could be due to the fatty acid composition of its triglycerides, which are the substrate for this enzyme *in vitro*.

Non-specific Host Defenses and Parasite Evasion

In investigations of the mechanism of giardiacidal activity of NHM we found earlier that trophozoites are killed by certain normal products of lipolysis, such as *cis*-unsaturated fatty acids, lysolipids and monoglycerides[15]. Other studies[16,17] have shown that lingual, gastric, and pancreatic lipases act in concert with BSL to digest human milk triacylglycerol to fatty acids and glycerol. In non-breast fed individuals, pancreatic lipases also actively digest dietary lipids in the lumen of the small intestine. Therefore, we asked whether intestinal fluid from humans is also toxic to *G. lamblia*. We found that intestinal fluid from all normal subjects tested killed *G. lamblia*

trophozoites *in vitro*. As with NHM, killing was due to products of lipolysis generated during, or prior to, incubation with *Giardia*[3].

Since *G. lamblia* does infect breast-fed babies, as well as non-breast-fed individuals, we asked whether other secreted or ingested factors promote trophozoite survival. In a broader context, the upper small intestinal lumen may be regarded as a hostile environment containing high concentrations of bile, degradative enzymes and their products, and fluctuating levels of nutrients and hydrogen ions. The pH of the lower duodenum and jejunum is rather alkaline (pH 7.4 to 7.9) and the normal microbial flora in the upper small intestine is sparse ($<10^5$/ml). In earlier work, we found that *Giardia* has evolved mechanisms to use components of this unfriendly environment to promote its survival. We have shown that human small intestinal mucus promotes trophozoite attachment and growth, as well as protection from lipolytic killing[3,6,15]. Bile, a specific constituent of the small intestinal milieu, is also beneficial to the survival of *G. lamblia*. Bile salts above their critical micellar concentration (CMC) also protect trophozoites by sequestering lipolytic products in micelles[3,15]. Moreover, biliary lipids support the growth of *G. lamblia* trophozoites in a serum-free medium[4]. Bile also contributes to completion of the life cycle by stimulating encystation of *G. lamblia in vitro*[7]. Finally, the slightly alkaline pH of the small intestine (~7.5 to 7.8) promotes survival of *G. lamblia*, probably by increasing ionization of fatty acids, which prevents their interaction with the plasma membrane, and induces encystation, in concert with bile[7]. Encystation is an active means of evading host defenses since encysting parasites secrete a resistant cyst wall and also pass from the host. These findings are summarized in Fig. 1.

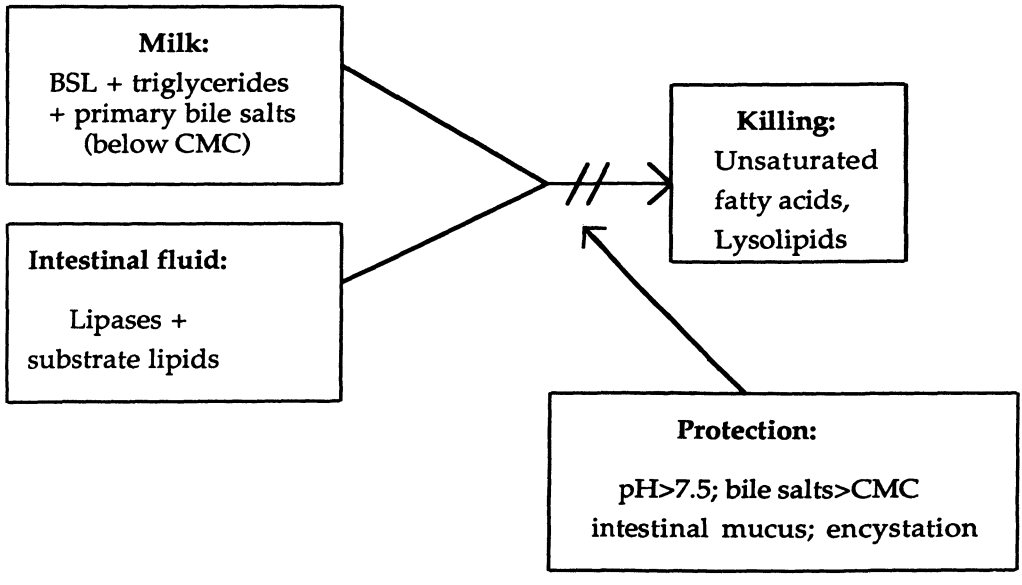

Figure 1. Non-specific secretory defenses against *Giardia* and parasite evasion.

Secretory Antibodies Against *G. lamblia*

Because of our limited understanding of the host response, we have begun to compare the isotypes and antigenic specificities of secretory antibody responses in milk with those in serum from patients with giardiasis. Earlier studies had shown that a large number of neo-antigens are expressed during exposure of *G. lamblia* to intestinal conditions[18]. While the results of Western blot analyses were complex, several observations emerged (Reiner and Gillin, manuscript in preparation). Both serum and secretory antibodies of humans tended to react with more neo-antigens than constitutive antigens, which are present in both conventionally and intestinally grown cells. Milk and sera contained IgG, IgM, and IgA antibody, which Nash *et al.*[19] described previously in samples of intestinal fluid and serum during studies of experimental human giardiasis. Further work is necessary to determine whether milk and antibody reactivity in Western blots is due to infection with *G. lamblia* or to conserved or cross-reactive antigens.

CONCLUSIONS

Manifestations of giardiasis vary from debilitating severe diarrhea, malabsorption, growth retardation, and failure of children to thrive[19] to self-limited or even asymptomatic infections[20]. Our work has suggested that this variability may be determined in part by the balance between powerful, non-immune host defenses and substances in the intestinal milieu that promote growth, attachment, and evasion of host defenses by inducing encystation. Infection may be limited by lethal lipolytic products generated in the small intestinal lumen by milk BSL[21] or intestinal fluid lipases. Other intestinal factors, may passively protect *Giardia*. The slightly alkaline pH of much of the intestinal lumen may promote ionization of fatty acids. Bile salts above their CMC may complex with fatty acids and also prevent their insertion into the *Giardia* plasma membrane. At the same time, the parasite actively evades killing by covering itself with mucus or by secreting a resistant cyst wall, under the stimulation of bile and alkaline pH. The interactions of secretory antibodies with trophozoite surface molecules could be equally important. Since little specific information about this host response is available, we have begun to elucidate isotypes and antigenic specificities of secretory and serum antibodies and to test the immunogenicity of a recombinant major surface antigen of *G. lamblia*[22].

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EPIDEMIOLOGICAL PERSPECTIVE OF BREASTFEEDING AND ACUTE RESPIRATORY ILLNESSES IN INFANTS

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Pneumonia is now the leading cause of infant deaths in many parts of the world. There is an urgent need to develop strategies to reduce serious morbidity and mortality resulting from acute respiratory infections. Protection afforded by breastfeeding against diarrheal diseases and death in infants in developing countries is accepted[1,2]. Protection against acute lower respiratory tract illness (LRI) is less convincing. Epidemiologic studies of the relationship of breastfeeding to LRI have had varying results. The purpose of this report is to explore the bases for the varying conclusions of these studies, and to develop hypotheses for enhancing the protective effects.

The variation in the results of studies of breastfeeding and infection are a consequence of the types of studies that may be performed. Ethical considerations prevent the use of randomized controlled trials. Therefore, investigations have been either observational cohort studies comparing outcomes of infants who were breast-fed with those of infants who were bottle-fed, or they have been case-control studies where the method of feeding of infants with certain infectious diseases is compared with that of matched controls.

Bauchner *et al.*[3] critically analyzed studies of breastfeeding and infections published between 1970 and 1984. They assessed the extent to which these studies met four methodological standards that relate to the scientific validity and to the generalizability of these studies (Table 1). The first standard concerned the **avoidance of detection bias**. Detection bias occurs when an outcome event is detected more readily in one group than another. For observational cohort studies, active surveillance by contacting the subject at regular intervals for illness history is important to avoid bias in the detection of illness. This is because the perception of illness may vary by method of feeding and the likelihood of hospitalization may also vary with feeding. For case-control studies, the research assistant who enrolls the control subjects should be blinded to the hypothesis of the study to avoid bias in the selection of controls.

Equally important are **adjustments for potentially confounding variables**. Unfortunately, in the United States, breastfeeding is more common among women in middle and upper socio-economic levels and

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Table 1. Methological Standards of Epidemiological Studies of the Relationship of Breastfeeding to Infections During Infancy (Bauchner *et al.*[3])

1.	Avoidance of detection bias
2.	Adjustments for potential confounding variables
3.	Definition of outcome
4.	Definition of breastfeeding

failure to breastfeed is tied to lower socio-economic status along with increased risk for infection caused by crowding, large family size, poor sanitation, and personal hygiene. Maternal smoking, family history of asthma, and daycare attendance by the infant all increase the risk for LRI. The populations under observation must be balanced for these factors or the analysis must adjust for these variables.

Finally, **breastfeeding should be defined** and the subjects must be followed carefully to determine the frequency and duration of breastfeeding. Infants may be classified into groups exclusively breast-fed, partially breast-fed, and not breast-fed. **Outcome variables** also **should be defined**; the clinical parameters of LRI and diarrhea must be described. For instance, some studies have found effects related to wheezing illnesses and not to other respiratory illnesses. Diarrhea may be more easily recognized for bottle-fed infants than for breast-fed infants. Therefore, case definition must take this into account.

Baucher *et al.*[3] found six studies - five cohort studies and one case control study that complied with at least three of the four methodological standards put forth. In addition, one case control study and three large cohort studies have been reported recently; all comply fully with the stated standards. Table 2 shows that exactly one-half of these studies showed significant protection associated with breastfeeding. The positive case control study examined the effect of breastfeeding upon hospitalization of infants with LRI caused specifically by respiratory syncytial virus; 30% of the cases had been breast-fed compared to 49% of the controls[4]. The protection held up for the more severely ill infants. Of the four cohort studies, one showed protection against acute respiratory illness (ARI) in infants of both Canada and India[5], two showed protection against ARI during the first four months of life only[6,7] and the fourth looked only at otitis media[8]. The most striking protection was observed among infants living in low socio-economic settings with older siblings and crowded living conditions.

Some of the studies that failed to show a significant protective effect of breastfeeding did have positive trends in that direction. The case control study which included infants hospitalized with any infection suggested a protective effect of breastfeeding; however, when the cases were analyzed by criteria for admission, it was found that the frequency of breastfeeding for those whose admission was mandatory was not different than controls[9]. Infants whose admission to the hospital might have been considered arbitrary

Table 2. Results of Studies of Protection of Lack of Protection Against Infection of Infants Related to Breastfeeding

Study type	Number	Protection	
		Yes	No
Case-control	2	1	1
Cohort	8	4	4
Totals	10	5	5

were significantly less likely to have been breast-fed suggesting that breastfeeding protected against admission to the hospital but not against infection. A large cohort study carried out in Denmark (a rather homogeneous population) showed no protection against respiratory or gastrointestinal infections[10]. A British study showed trends for protection of exclusively breast-fed infants that were not significant for the population under observation[11]. One study examined only gastroenteritis and breastfeeding[12]. This study suggested yet another confounding factor related to uneven recruitment by season. Most of the breast-fed infants were enrolled in the summer and early autumn so that they were at the most vulnerable age for rotavirus infection during the winter season. Most of the bottle-fed infants were enrolled later so that they may have missed the rotavirus epidemic. Few rotavirus infections were detected and this may have been the result of protection of the vulnerable group by breastfeeding, but since the comparison group was not enrolled during the comparable period no excess in that group was observed.

The only study that attempted to identify the etiology of all respiratory virus infections found no difference in the infection rates for infants who were or were not breast-fed (Table 3)[13]. During the first three months of life

Table 3. Virus Infection Rate During the First Three Months of Life by Method of Feeding (Frank *et al.*[13])

Virus type	Rate per 100 children	
	Breast-fed (N=39)	Bottle-fed (N=42)
Respiratory[a]	41	50
Other[b]	28	14

[a]Respiratory syncytial, parainfluenza, and rhinoviruses.
[b]Enteroviruses, herpesviruses, and adenoviruses.

when all were exclusively breast-fed or bottle-fed, the virus infection rate was 69 per 100 for the breast-fed infants and 64 per 100 for the bottle-fed infants. The bottle-fed infants had a slight excess of infections with the major respiratory viruses: respiratory syncytial, influenza, and parainfluenza viruses. Also, an excess of illness involving the lower respiratory tract was found among bottle-fed infants (Table 4). Particularly striking was the occurrence of eight episodes of bronchiolitis or pneumonia among 42 infants who were bottle-fed compared to only one episode among 39 breast-fed infants.

Table 4. Rate[a] of Lower Respiratory Tract Illnesses in the First Three Months of Life by Method of Feeding (Frank *et al.*[13])

Illness	Breast-fed (N=39)	Bottle-fed (N=42)
Laryngotracheobronchitis	13	7
Bronchiolitis	3	14
Pneumonia	0	5
Totals	15	26

[a]per 100 children

Infection rates in infants with RSV and parainfluenza virus type 3 were compared to maternal antibody titers specific for these viruses (Table 5). Infections rates were not lower for infants whose mothers had high titers to these viruses. This suggests that specific protection against these viruses was not transmitted via breast milk.

Table 5. Respiratory Syncytial and Parainfluenza Type 3 Virus Infection Rates[a] During the First Six Months of Life Related to Cord Antibody Titers (Frank *et al.*[13])

Antibody titers	Breast-fed	Bottle-fed
4-32	38	45
64-128	50	36
256-512	50	35

[a]per 100 children

CONCLUSIONS

The data suggest moderate protection of infants against respiratory virus infection by breastfeeding. The protection was most evident among infants less than four months of age in low income families with crowding. Although specific immune mechanisms have been suggested[14-17], the data available do not support a strong role for breast milk-mediated defenses against respiratory viruses[13]. Epidemiologic data equally support the proposition that breastfeeding during the first three months of life limits the contacts of the infant and, therefore, limits the infecting dose moderating the severity of illness (Table 6). Previous studies have shown protection in the first months of life correlated with the level of placentally transferred maternal antibody[18,19]. Boosting maternal antibody levels in both serum and breast milk could have the combined effect of increasing passive immunization and reducing the risk of the mother acquiring an infection that can be transmitted to her young infant. We have proposed that maternal immunization with vaccines against influenza and respiratory syncytial virus be tested for efficacy[20].

Table 6. Mode of Protection Afforded by Breastfeeding

1.	Specific immune mechanisms
2.	Infectious agents in feeding
	a) CMV or rubella virus in breast milk
	b) bacterial contaminants during early months of life
3.	Reduction of contacts during early months of life

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SEROTYPES OF ROTAVIRUS THAT INFECT INFANTS SYMPTOMATICALLY AND ASYMPTOMATICALLY

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INTRODUCTION

Since the initial report of rotavirus in humans in 1973[1], infection has been shown to span the age range from neonates to the elderly, with the highest rate in the group from six months to two years of age[2,3]. Rotavirus causes 10% to 50% of the diarrheal episodes that require hospitalization in this age group, and based upon community studies causes 0.2 to 0.8 episodes/child/year with 50% being asymptomatic[4,5]. The successful production of serotype-specific neutralizing monoclonal antibodies has permitted epidemiologic surveys to be conducted to examine the importance of rotavirus serotypes.

MOLECULAR VIROLOGY

Rotaviruses measure approximately 70 nm in diameter and consist of an internal core, an inner capsid, and an outer capsid[6]. The inner core contains a genome of 11 segments of double stranded RNA, each of which encodes for separate viral proteins (VP) (Table 1). The VPs can be part of the structure of the virus or they can be non-structural. The inner core is formed by VP1, VP2, and VP3; the inner capsid is formed by VP6; and VP4 and VP7 form the outer capsid. VP7 constitutes the matrix and VP4 the spikes that insert into the outer capsid as identified by cryo-electron microscopy[7].

Rotaviruses can be initially classified into groups A-F based on differences in antigenic determinants on the inner capsid protein VP6. Although groups A, B, and C have been detected in humans, group A rotaviruses are the most commonly recognized cause of viral gastroenteritis in children worldwide and are those that are detected by commercially available antigen detection assays. Group A rotaviruses can be further classified into one of two subgroups (I and II) based on non-neutralizing antigenic determinants of VP6. The majority of human group A rotaviruses are in subgroup II. Group A rotaviruses are further classified into serotypes based upon differences in neutralization epitopes located on the outer capsid

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protein VP7[7,8]. Eleven VP7 serotypes have been identified from various animal species with six of them having been isolated from humans (serotypes 1, 2, 3, 4, 8, and 9). Serotypes 1, 3, 4, and 9 belong to subgroup II and serotypes 2 and 8 belong to subgroup I. Gene sequencing data have suggested that at least nine types of VP4 exist, four of which are known to infect humans[7]. The relationship between gene types and antigenic types is unknown. In the future, a binary system of classification of rotaviruses based upon identification of both outer capsid neutralizing antigens VP7 and VP4 may be established.

The genome segments of rotaviruses can be separated by gel electrophoresis. Viruses in subgroup II display a long pattern of RNA migration while subgroup I viruses contain RNA segments that characteristically migrate with a short pattern. Gel electrophoresis is a useful tool for the study of the epidemiology of rotaviruses including comparison of strains within the same serotype, outbreak, or region.

Table 1. Structure of Rotavirus

Structure	Viral proteins
Internal core	VP1, VP2, VP3
Inner capsid	VP6
Outer capsid	VP4, VP7

ROTAVIRUS SEROTYPES

Serotypes 1-4 have a worldwide distribution, whereas the distribution of serotypes 8 and 9 is unknown. Several serotypes have been shown to co-circulate each year and to change every one to two years in areas where they have been studied. Knowledge about the distribution of the various serotypes within and outside the United States is increasing. In the U.S., the epidemiology of rotavirus gastroenteritis was investigated for two consecutive seasons from 1987-1989 in seven locations[9]. Serotype 1 was predominant in both seasons, particularly in the North and East, and serotype 3, which was second in frequency, was found most often in the South. Serotype 2 was detected uncommonly and serotypes 4, 8, and 9 were not found. This study showed a correlation between serotype and electropherotype, and identified specific RNA configurations associated with serotypes 1 and 3. In Houston, a single serotype has predominated as the cause of symptomatic and asymptomatic infection each rotavirus season in children in day care centers and in children hospitalized for diarrhea due to rotavirus[10-12]. The predominate serotype has differed every one to two years from 1979 to 1990. Symptomatic infections were not associated with a particular serotype in these studies, and overall a similar proportion of children infected with serotypes 1

and 3 had diarrhea. The serotype distribution of the day care center populations was similar to that of children hospitalized for diarrhea over the same time periods. Electrophoretic differences among rotaviruses of a same serotype were common, and more frequent among serotype 1 than serotype 3 viruses. These results indicate that serotype distribution can be heterogeneous within a city, yet highly uniform within an outbreak. In another study conducted in a rural area of the North Central United States, serotype 1 has predominated each year from 1981 through 1989[12]. Only serotype 1 has been detected in hospitalized children from many towns in this area during the eight year period of study.

The cyclic nature of rotavirus epidemics with alternating serotypes predominating at different seasons has been described in several countries[13-29] including Australia[14,21], Hong Kong[23], Japan[18,22], Argentina[25], Venezuela[16], Africa[13,17], Bangladesh[19], India[15], Guatemala[24], Mexico[28,29], Korea[26], and Thailand[20,27]. These studies have shown that frequency of serotype distribution varies within and among regions of a country and among countries (Table 2). Most of these studies have evaluated viruses identified from children hospitalized for diarrhea. Few studies have included children with asymptomatic infections for comparison and then only as groups with small numbers[11,18]. Prospective studies of children with symptomatic and asymptomatic rotavirus infection are also uncommon[11,23].

Table 2. Rotavirus Serotypes from Children with Diarrhea by Geographic Location

Study	Location	Seasons	Number serotyped	Serotype(%)
1. Gouvea *et al.*[9] 1990	7 locales in U.S.	1987-89	232	1 (78) 2 (3) 3 (19)
2. O'Ryan *et al.*[11] 1990	Houston, TX	1985-88	149	1 (64) 2 (1) 3 (35)
3. Matson *et al.*[12] 1990	Houston, TX	1979-88	488	1 (47) 2 (4) 3 (38) 4 (11)
	Ohio	1981-88	408	1 (85) 2 (3) 3 (8) 4 (4)
4. Urasawa *et al.*[13] 1987	Kenya	1982-83	13	1 (54) 2 (31) 3 (15)

5.	Birch *et al.*[14] 1988	Australia	1975-86	314	1 (51) 2 (26) 3 (19) 4 (4)
6.	Brown *et al.*[15] 1988	India	1983-85	32	1 (47) 2 (9) 3 (6) 4 (38)
7.	Flores *et al.*[16] 1988	Venezuela	1981-83	109	1 (48) 2 (16) 3 (22) 4 (14)
8.	Georges-Courbot *et al.*[17] 1988	Africa	1983-85	148	1 (70) 2 (15) 3 (13) 4 (2)
9.	Nakagomi *et al.*[18] 1988	Japan	1981-87	375	1 (47) 2 (3) 3 (3) 4 (17)
10.	Ahmed *et al.*[19] 1989	Bangladesh	1988	39	1 (38) 2 (28) 3 (3) 4 (23) mixed (8)
11.	Pongsuwanne *et al.*[20] 1989	Thailand	1983-88	58	1 (31) 2 (29) 4 (40)
12.	Unicomb *et al.*[21] 1989	Australia	1973-86	412	1 (68) 2 (9) 3 (8) 4 (15)
13.	Urasawa *et al.*[22] 1989	Japan	1986-87	411	1 (50) 2 (32) 3 (11) 4 (7)
14.	Zheng *et al.*[23] 1989	Hong Kong	not provided	19	1 (16) 2 (32) 3 (10) 4 (16) 1 and/or 4 (26)

15.	Cruz *et al.*[24] 1990	Guatemala	1987-88	37	1 (24) 2 (73) 3 (3)
16.	Gomez *et al.*[25] 1990	Argentina	1983-86	138	1 (41) 2 (19) 3 (14) 4 (26)
17.	Kim *et al.*[26] 1990	Korea	1987-89	73	1 (89) 2 (10) 3 (1)
18.	Sethabutr *et al.*[27] 1990	Thailand	1987-88	74	1 (52) 2 (47) 4 (1)
19.	Padilla-Noriega *et al.*[28] 1989	Mexico City Merida	1984-87	88	1 (43) 2 (17) 3 (15) 4 (25)
20.	Velazquez *et al.*[29] 1990	Mexico City	1987-90	44	1 (20) 2 (30) 3 (43) 4 (7)

CONCLUSIONS

Rotavirus epidemics within a community can be caused by a single serotype or may involve multiple serotypes simultaneously. The same serotype observed to infect children hospitalized with rotavirus gastroenteritis was also the most common serotype that caused symptomatic infection in the same seasons among children attending day care centers in Houston. A cyclic nature of rotavirus serotypes seems to occur on a worldwide basis. How children who live in various regions with exposure to different serotypes differ with respect to acquired protection against illness is unknown. Further studies are needed to characterize the molecular epidemiology of rotavirus antigenic types worldwide and to apply the newly developed typing reagents to the measurement of serotype specific immune responses.

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IMMUNE RESPONSE TO ROTAVIRUS VACCINES AMONG BREAST-FED AND NONBREAST-FED CHILDREN

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INTRODUCTION

Studies to determine whether breastfeeding protects children against rotavirus diarrhea have not, to date, yielded clear-cut results. Because human breast milk contains measurable and often high titers of neutralizing antibody to rotavirus, it seems likely that some protection should be conferred to breast-fed children[1,2]. Studies of rotavirus infection in the neonatal period, which is usually asymptomatic, have indicated that breast-fed infants appear to shed less virus, often mixed with antibody, and have lower rates of infection, a finding that was correlated with levels of both S-IgA and anti-trypsin activity in breast milk[3,4]. In older children, investigators failed to document any protection from breastfeeding among cohorts of children studied prospectively in Guatemala[5] and Mexico[6] or in case-control studies in the United States[7] and Bangladesh[2]; however, significant protection was observed in a case-control study conducted in Buffalo[8]. The lack of a clear protective effect has raised questions about the methods used to assess the outcome and severity of illness in these studies and has focused investigations on issues related to inoculum size and partial or complete breastfeeding that could obscure a beneficial effect if one were present.

To assess the effect of breastfeeding on rotavirus infection in a controlled, experimental setting, we have examined the immune response to rotavirus vaccines among breast-fed and nonbreast-fed children. In these trials, breastfeeding status could be determined by direct observation and by questionnaire, the time and dose of rotavirus inoculation was precisely known, and immunogenicity of the vaccine was the key outcome event measured. With the active testing of rotavirus vaccines, any possible interference of breastfeeding in the immune response to a live oral vaccine would have important implications for the administration of these vaccines in a population in which breast-feeding is common among children in their first months of life.

We began by looking for differences in the immunogenicity of rotavirus vaccines in breast-fed and nonbreast-fed infants enrolled in three

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vaccine trials conducted in Atlanta or other locations in collaboration with the CDC. Because our results showed small but insignificant trends suggesting interference by breastfeeding, we proceeded to conduct a metaanalysis similar to but larger than that of Pichichero[9] of 16 groups of infants who were administered one of three different rotavirus vaccines. Our results indicate that breastfeeding is associated with a small but significant decrease in the immunogenicity of rotavirus vaccines.

MATERIALS AND METHODS

Metaanalysis

Reports of phase 1 and phase 2 rotavirus vaccine studies published in the medical literature or available from one vaccine manufacturer (Wyeth-Ayerst Research) were reviewed for the metaanalysis. These studies included trials with several live oral vaccines: the first rotavirus vaccine (RIT-4237 bovine vaccine) prepared by Smith-Kline; rhesus rotavirus vaccine prepared by NIH or Wyeth-Ayerst Research or rhesus rotavirus reassortant vaccines, including both the monovalent serotype 1 strain (RRV-1) and the tetravalent vaccine (RRV-TV); and the bovine WC3 rotavirus vaccine strain prepared by the Wistar Institute. Studies were included in the analysis if more than 20 children were enrolled, the breastfeeding status of the children was reported, and immunogenicity of the vaccine was documented according to breast-feeding group.

Analysis

The immune response to rotavirus vaccines has been measured by various assays (e.g., neutralization activity, IgA, and IgG antibodies to rotavirus) that all reflect vaccine "take" but that are not completely comparable. For each trial, we calculated a simple odds ratio with 95% confidence intervals for whatever measure of immunogenicity was used. From this analysis, an odds ratio of less than one was consistent with the hypothesis that breastfeeding decreased the immune response to vaccine. Most studies had small numbers of participants and small trends that we suspected would not achieve statistical significance. To examine trends for the entire group of studies, we calculated and then compared the overall rates of seroconversion among breast-fed and nonbreast-fed vaccinees and tested these differences for significance by using a Fisher's exact test or a Mantel-Haenzel weighted odds ratio. We also examined the odds ratios of the individual studies to determine if these were more often less than one and tested this distribution for significance with a sign test.

RESULTS

A total of 16 groups of children studied by nine investigators met our selection criteria and were included in the analysis (Table 1). Of these, seven trials were conducted with the RIT vaccine, eight trials with the RRV vaccine or a reassortant, and one trial with the WC3 vaccine. Trials ranged in size from 21 to 222 children. In only 3 of the 16 groups, Rochester, NY[2], and Finland, was breastfeeding significantly associated with a decreased immune response to the vaccine. The doses of vaccines administered varied over a

Table 1. Review of Studies of Concomitant Breastfeeding and Different Oral Rotavirus Vaccines on Seroconversion in Infants

Rotavirus vaccine	Study report	Country/ year	Dose (PFU)	Age (months)	Seroconversion to rotavirus						
					Breastfed		Not breastfed		Odds		P
					N	%	N	%	Ratio	95% CI[a]	Value[b]
RIT 4237	Vesikari 1985	Finland, 1984	$10^{6.3}$	4-6	10/23	43	2/13	15	4.23	0.63-35.34	N.S.
			$10^{7.2}$	4-6	19/26	73	10/14	71	1.09	0.20-5.70	N.S.
			$10^{8.3}$	4-6	18/26	69	14/14	100	0.00	0.00-1.05	0.03
RIT 4237	Vesikari 1986	Finland, 1984	$10^{7.3}$	6-12	25/37	68	23/34	68	1.00	0.33-3.02	N.S.
			$10^{8.3}$	6-12	26/32	81	32/37	86	0.68	0.16-2.90	N.S.
RIT 4237	Zoppi 1986	Italy, 1984	$10^{8.1}$	4	10/15	66	6/8	75	0.67	0.06-6.28	N.S.
RIT 4237	Lanata 1989	Peru	$10^{8.3}$	2-18	34/163	21	15/59	25	0.77	0.37-1.65	N.S.
RRV MMU 18006	Davidson 1989	Maryland, New York, 1987-8	10^{3}	2-5	1/5	20	4/16	25	0.75	0.02-12.53	N.S.
			10^{5}	2-6	3/6	50	12/17	71	0.42	0.04-3.97	N.S.
RRV MMU 18006	Ho 1989	Atlanta, GA 1988[c]	10^{4}	2-3	8/15	53	48/84	57	0.86	0.25-2.94	N.S.
RRV MMU 18006	Pichichero 1990	Rochester, NY, 1986-7	10^{4}	2-4	21/40	53	33/45	73	0.40	0.15-1.09	0.05
			10^{4}	2-5	1/6	17	12/19	63	0.12	0.00-1.48	0.07
RRV-1 (NIH)			10^{4}	2-4	20/42	48	17/24	71	0.37	0.11-1.22	N.S.
RRV-TV	Ing 1990	Atlanta, GA, 1989[c]	10^{4}	2-3	2/5	40	8/39	21	2.58	0.25-24.87	N.S.
			10^{4}	2-3	4/11	36	39/80	49	0.60	0.13-2.54	N.S.
WC3	Clark 1988	Philadelphia, PA, 1985-6	$10^{7.5}$	3-12	10/15	67	25/34	74	0.72	0.16-3.26	N.S.
		Overall			212/467	45	300/537	56	0.67[d]	0.48-0.92	0.01

[a] 95% confidence interval

[b] P values by X^2 or Fisher's exact test (2-taliled). N.S. = Not significant (P>0.05).

[c] Study conducted by the Centers for Disease Control (CDC).

[d] Mantel-Haenszel weighted odds ratio for all groups.

100-fold range (RIT-$10^{6.3}$ to $10^{8.3}$; RRV-10^3 to 10^5), but the number of observations at each dose was too small to establish a dose-response effect.

Breastfeeding was associated with a small but significant decrease in the rate of seroconversion to rotavirus vaccines. Seroconversion was observed in 45% (212/467) of breast-fed children versus 56% (300/537) of those who were not breast-fed ($p<0.01$, X^2). While none of the odds ratios of the studies were significantly different from one, only 3 of the 16 studies had an odds ratio of greater than one ($p<0.05$) (Fig. 1).

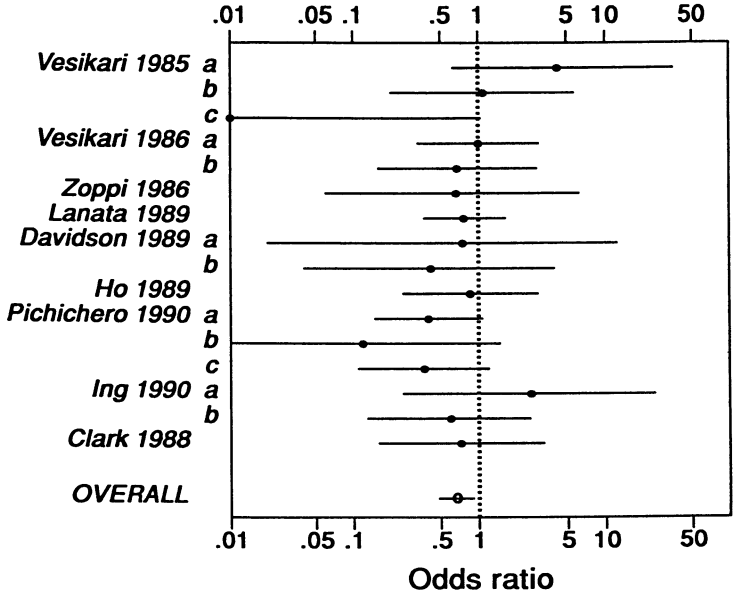

Figure 1. Odds ratios (and 95% confidence intervals) of rotavirus seroconversion among breast-fed versus nonbreast-fed infants in selected studies.

DISCUSSION

Clarification of the effect of breastfeeding on the susceptibility of children to rotavirus infection has two important clinical consequences. First, if breastfeeding decreases the incidence or severity of rotavirus diarrhea, as it does for cholera[10] or shigella[11], then breastfeeding should be encouraged and the use of oral immunoglobulins for the prevention of rotavirus diarrhea should be reassessed. Second, if breast-feeding impairs the immune response to rotavirus vaccines, then strategies for administering these vaccines to breast-fed children should be developed in future vaccine trials and programs.

In the present study, the results of the metaanalysis indicate clearly that breastfeeding decreases the immune response to a single dose of rotavirus vaccine, although this effect is relatively small. This conclusion would not have been reached with confidence using data from any of the relatively small

studies but might be expected using data from a large vaccination program. In addition, because we combined results from trials that used various vaccines and different doses and buffers, we cannot assess whether this interference would be present if a single vaccine were administered under optimal conditions as part of an immunization program. Nonetheless, our study does suggest that when rotavirus vaccine is ready for widespread use, the effect of breast-feeding on immunogenicity will have to be reassessed. Moreover, because the expected interference is small, a large scale trial will be required to determine the extent of the effect. In addition, it would be particularly interesting to know whether the titer of neutralizing activity in breast milk can be related to an alteration in immune response.

CONCLUSIONS

To assess the effect of breastfeeding on rotavirus infection in a controlled, experimental setting, we have examined the immune response to rotavirus vaccines among breast-fed and nonbreast-fed children. From a metaanalysis of the results from 16 rotavirus vaccine trials, breast-fed compared to nonbreast-fed infants were significantly less likely to develop an immune response to the vaccine, although the absolute difference was small. These results indicate that, while breastfeeding can interfere with rotavirus infection, the consequences for oral immunization with live vaccines will be marginal. Secondly, while breastfeeding might be expected to decrease a child's risk of severe rotavirus diarrhea, the lack of major interference with rotavirus infection is consistent with the ambiguous results obtained from epidemiologic field studies.

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RECENT ADVANCES IN DEVELOPMENT OF A ROTAVIRUS VACCINE FOR PREVENTION OF SEVERE DIARRHEAL ILLNESS OF INFANTS AND YOUNG CHILDREN

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INTRODUCTION

Diarrheal diseases are an important cause of morbidity in infants and young children in developed countries and a major cause of both morbidity and mortality in this same age group in developing countries[1]. The toll from diarrheal diseases in the developing countries is enormous. For example, it is estimated that in Asia, Africa, and Latin America, 3-5 billion cases of diarrhea and 5-10 million diarrhea-associated deaths occur each year[2]. In addition, diarrhea was ranked first among infectious diseases with regard to the number of episodes and the number of deaths in these areas of the world.

Rotaviruses are the single most important etiologic agents of severe diarrhea in infants and young children in both developed and developing countries. In developed countries, they are responsible for approximately 34-52% of hospitalizations for diarrhea in infants and young children[1]. The impact of rotavirus infection in this age group in the United States is substantial: 90% of this group acquires serum antibodies to rotavirus by the end of the third year of life[1]. In the United States, it is estimated that in infants and young children each year, rotavirus infections are responsible for 3 million cases of diarrhea, 82,000 hospitalizations, and 150 deaths[3-5].

Although in developing countries the rotavirus infection rate is similar to that in developed countries, (i.e. 90% of infants and young children acquire rotavirus antibody during infancy and early childhood), the consequences of such infection are quire different[1]. It is estimated that, annually, in the less than five-year age group in developing countries, rotaviruses are responsible for 110 million cases of mild diarrhea, over 9 million cases of moderately-severe diarrhea, over 8 million of severe rotavirus diarrhea, and over 870,000 deaths[6].

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Thus, there is an obvious and compelling need for a rotavirus vaccine aimed at preventing severe rotavirus diarrhea during the first two years of life when the consequences of such illness are most serious. Although oral rehydration salt solutions are effective in the treatment of diarrheal diseases, their availability, use, and implementation are a major problem in developing countries and, thus, the proven efficacy of this treatment does not diminish the need for a rotavirus vaccine.

ROTAVIRUS CHARACTERISTICS RELATED TO VACCINE DEVELOPMENT

Rotaviruses are 70nm in diameter, non-enveloped, and possess a distinct double shelled outer capsid structure[1]. The name "rotavirus" (rota = wheel) was proposed because the smooth outermost margin gives the appearance of the rim of a wheel placed on short spokes radiating from a wide hub when visualized by electron microscopy[7]. Within the inner capsid is the core which contains the virus genome comprised of 11 segments of double-stranded RNA[8]. The segmented genome readily undergoes genetic reassortment during co-infection[1,8].

Rotaviruses have 3 important antigenic specificities – group, subgroup and serotype – that are mediated by various proteins[8]. Group specificity is defined predominantly by VP6, the major structural protein located on the outer surface of the inner capsid and encoded by RNA segment 6. Most rotaviruses of epidemiological importance share a common group antigen and thus are classified together as group A rotaviruses, the only group for which vaccines are considered necessary at present[1]. The "non-group A" rotaviruses are divided into groups B, C, D, E, F, and G; only groups B and C have been detected in humans[9]. Subgroup specificity is mediated by VP6 and has been defined only for the group A rotaviruses; most group A strains belong to one of two subgroups designated I or II[1,8].

Serotype specificity has been defined by VP7, a glycoprotein that is one of the 2 major neutralization antigens located on the outer capsid and is encoded by RNA segment 7, 8, or 9. There are 7 human rotavirus VP7 serotypes, and those numbered 1-4 are of epidemiologic importance[1,8,10]. Several human rotavirus strains share VP7 serotype specificity with rotavirus strains of animals. It should be noted that rotaviruses are also an important cause of diarrheal illness in animals and that most human and animal rotaviruses share the common group A antigen[1].

The other outer capsid protein VP4 (encoded by the 4th RNA segment) also induces neutralizing antibodies, and is the hemagglutinin in certain strains[8]. It protrudes from the outer surface in the form of 60 slender spikes, each about 12nm in length[11,12]. A serotyping scheme based on VP4 specificity has recently been described[13]. Antibodies to VP4 or VP7 are each independently associated with protection against rotavirus illness[14].

APPROACHES TO ROTAVIRUS VACCINE DEVELOPMENT

Approaches to the development of a rotavirus vaccine range from growth of human or animal strains in cell culture to molecular biologic techniques. The most extensively evaluated strategy in rotavirus vaccinology

is the concept pioneered by Edward Jenner in 1798 for human smallpox vaccination in which a related, live, attenuated agent from a non-human host is used as the immunizing antigen. Several early studies were instrumental in suggesting the feasibility of a "Jennerian" approach to rotavirus vaccination, including: 1) the sharing of a common group antigen between human and animal rotavirus strains studied up to that time[7,15]; and 2) protection of animals against challenge with a human rotavirus strain following prior infection with an animal rotavirus strain[16,17].

Efficacy Trials

The "Jennerian" approach to rotavirus vaccination was first evaluated in humans with a bovine rotavirus strain. The bovine strain induced protection against clinically significant diarrhea in older infants and in young children, but in later studies in infants under 6 mo of age its success was limited[18-25].

We have pursued the "Jennerian" approach using as the vaccine strain, the simian rhesus rotavirus strain MMU18006 that is antigenically similar by neutralization to human rotaviruses of VP7 serotype 3 specificity[26-28]. This candidate vaccine grows efficiently in a semi-continuous strain of fetal rhesus diploid lung cells (DBS-FRhL-2), an acceptable vaccine cell substrate[29].

The safety and antigenicity of an orally administered live RRV vaccine was evaluated in phase 1 studies sequentially, first in adults and later in children of progressively younger age[26-28,30]. The vaccine was safe and antigenic in adults and older children. However, in certain locations, the optimal dose of 10^4 plaque forming units (PFU) induced characteristically mild, transient febrile responses in approximately one-third of 2-5 mo old vaccinees[26-28,30,31]. These reactions were considered acceptable by parents, clinicians, and investigators. Because the vaccine was also antigenic in this target population of 2-5 mo old infants, phase II double-blind efficacy trials were initiated with collaborators world-wide. As shown in the table, seven field trials involving over 1,000 infants and young children have been reported with the RRV vaccine.

The vaccine was variably effective in these trials. For example, in the Venezuela trial (#3) in which 151 vaccinees and 151 controls were studied over a one-year period, the vaccine had an efficacy rate of 64% against rotavirus diarrhea in 1-10 mo old vaccinees, whereas in the 1-4 mo old age group, the efficacy was 82%[34,35]. Results from field trials in older children in Maryland [#1] and Sweden [#2] were encouraging and, in addition, in 2-5 mo old infants in Finland [#7], the vaccine induced limited protection[31-33]. However, the RRV vaccine failed to protect against rotavirus diarrhea in trials in 2-5 mo old vaccinees in Rochester (#4), Arizona (#5), and Maryland (#6)[36-38]. The immunogenicity of the RRV vaccine was comparable in each of the locations and thus could not account for the marked difference in efficacy.

An explanation for the variable efficacy was formulated after the rotavirus strains in the various studies were serotyped. The predominant strains responsible for rotavirus diarrhea in the Venezuelan study were of VP7 serotype 3 specificity (the same serotype as the RRV vaccine strain), whereas in the trials in which the vaccine failed to induce protection, serotype 1 strains were predominant[34-38]. These observations suggested that VP7 serotype-specific immunity is required for protection against individual serotypes responsible for rotavirus diarrhea. It appeared that the RRV vaccine

did not induce appreciable heterotypic immunity in young infants not primed by previous rotavirus infection.

When heterotypic protection has been observed with rotavirus vaccines (see table), it is generally either: 1) limited in extent, or 2) involves older infants who may have been primed by natural rotavirus infection and thus may have developed broadly reactive (heterotypic) antibodies following vaccination. The inability of young infants to mount a heterotypic antibody response in comparison to adults or older children following rotavirus vaccination was recently shown by epitope blocking and neutralization assays[39]. Thus, a major factor responsible for efficacy of a rotavirus vaccine against heterotypic strains may be the priming of the infant immune system by natural rotavirus infection (subclinical or clinical) prior to immunization. Therefore, in trials involving young infants, the presence of circulating rotavirus strains that cause inapparent infections may not only affect the outcome of the trial but may confound the analysis of the mechanisms of protection.

In this regard, it is of interest that in a recently completed study in Rochester (not included in the table) in 2-5 mo old infants, the RRV vaccine induced heterotypic protection against serotype 1 rotavirus diarrhea[40]. This is in sharp contrast to the earlier trial in Rochester (#4) in which this vaccine failed to induce protection against rotavirus diarrhea associated with serotype 1[36]. This unanticipated finding is under investigation.

MODIFIED JENNERIAN APPROACH TO ROTAVIRUS VACCINATION

Because variability in protection by the RRV vaccine was likely a result of the failure of young infants without prior exposure to rotavirus to develop heterotypic antibodies to each of the four epidemiologically important serotypes following vaccination, we adopted a modified Jennerian approach. With this strategy, our goal is to develop a quadrivalent rotavirus vaccine of broader antigenic coverage that includes not only RRV (serotype 3) but also three reassortant rotaviruses. These reassortants were prepared by coinfection of cell cultures with RRV and a human rotavirus of each of 3 serotypes under selective pressure of antibody against the RRV[41,42]. Thus, RRV was the donor of 10 attenuating genes, whereas a human rotavirus of VP7 serotype 1, 2, or 4 specificity was the donor of a single gene that encodes VP7, a major outer capsid neutralization protein. Presently, human rotavirus-RRV reassortants are available for VP7 serotypes 1, 2, and 4. RRV is the serotype 3 component in the quadrivalent vaccine.

Phase I studies with individual reassortant vaccine strains of VP7 serotype 1, 2, or 4 specificity indicated that each behaved similarly to the RRV vaccine with regard to reactogenicity and antigenicity[43-46]. Two phase II studies with individual rotavirus reassortant have been completed[40,47].

In Finland, human-rhesus rotavirus reassortant vaccine strains D x RRV and DS-1 x RRV with VP7 human serotype 1 and serotype 2 specificity, respectively, were evaluated individually for safety, immunogenicity, and protective efficacy in a double-blind placebo controlled three-cell trial of 359 infants who were 2-5 mo of age[47]. After the first season of surveillance, the D x RRV vaccine induced 67% protection against the development of homotypic serotype 1 diarrhea and surprisingly the DS-1 x RRV vaccine induced the same level of protection against this heterotypic strain. The D x RRV vaccine

Table 1. Reported Phase 2 Field Trials of Rhesus Rotavirus (RRV) Vaccine

Institution (Investigator [Ref])	Number of children (age in months)	Number in each group		Efficacy vs RV diarrhea		Predominant VP7 serotype
		vaccine[a]	placebo	all	mod. severe/severe	
1. University of Maryland (*Rennels, et al.* [32])	27 (5-20)	14	10	74%	Not available	Not available
2. University of Umea (*Gothefors, et al.* [33])	104 (4-12)	53	51	48%	80%	1
3. Central University of Venezuela (*Flores, et al.* [34], *Perez-Schael, et al.* [35])	302 (1-10)	151	151	64%	85%	3
4. University of Rochester (*Christy, et al.* [36])	103 (2-4)	85	88	NIL	NIL	1
5. Johns Hopkins University (*Santosham, et al.* [37])	301[b] (2-5)	108	107	NIL	NIL	1
6. University of Maryland (*Rennels, et al.* [38])	112 (2-4)	63	49	29%	~29%	1
7. University of Tampere (*Vesikari, et al.* [31])	200 (2-5)	100	100	38%	67%	4

[a] 10^4 PFU dose of RRV except in studies 1 & 2 (10^5 PFU)

[b] RIT 4237 (10^8 PFU) administered to a 3rd group of 106 children; efficacy nil.

was not as effective in inducing protection in the second year of the study when serotype 1 and 4 strains were predominant, whereas the DS-1 x RRV vaccine continued to be protective. The heterotypic protection observed with the DS-1 x RRV vaccine was welcome but unexpected and needs further investigation by analysis of serologic responses to vaccination and natural infection as well as genetic analysis of strains recovered from ill individuals. As noted earlier, the role of priming with naturally-occurring rotavirus strains prior to vaccination may again be an important factor in explaining this heterotypic protection. The placebo group may also have been primed but because they were not vaccinated, they would likely develop an anamnestic immunologic response only after a subsequent naturally-occurring rotavirus infection.

In Rochester where the D x RRV reassortant vaccine (with VP7 serotype 1 specificity) and the RRV vaccine were evaluated individually along with a control group in 300 infants and young children 2-5 mo of age, the D x RRV vaccine induced protection against VP7 serotype 1 rotavirus diarrhea[40]. Moreover, as discussed above, unexpectedly and in contrast to the earlier Rochester trial, the RRV vaccine also induced protection against serotype 1 rotavirus diarrhea.

Encouraged by the lack of significant reactogenicity along with the demonstrated immunogenicity of individual reassortant strains, we pursued phase I studies, in Venezuela, in which RRV and the three human rotavirus-RRV reassortants were combined into a single quadrivalent vaccine containing D x RRV (VP7 serotype 1), DS-1 x RRV (VP7 serotype 2), RRV (VP7 serotype 3), and ST-3 x RRV (VP7 serotype 4). The quadrivalent vaccine was observed to be similar to the RRV parental strain or individual reassortants with regard to the nature and frequency of reactions observed in 2-5 mo old infants[48]. However, the "take rate" induced by the quadrivalent vaccine to each of the four VP7 serotypes represented in the vaccine as measured by neutralization assay, was disappointing[28,48,49].

Thus, we made efforts to adjust the dose or the number of doses, or to administer divided doses or to utilize different vaccine combinations (as with the M37 vaccine [described below]) in an attempt to achieve a "take rate" of at least 50% by neutralization assay with each of the serotypes represented in the vaccine[28,48-50]. After various trials in which reactogenicity and antigenicity were evaluated (Fig. 1), we found that by increasing the dose of each component in the quadrivalent vaccine ten-fold (i.e., from 10^4 PFU to 10^5 PFU) and by administering 2 doses of this formulation, the reactogenicity was similar to the lower dose and in addition, a "take rate" approaching or exceeding the 50% level was achieved against each of the 4 serotypes[50]. Thus, our plan at present is to begin a "catchment" study in Venezuela of 3,500 infants and young children with 3 doses of this 4×10^5 PFU quadrivalent vaccine. In this trial, only individuals who seek medical attention at a central clinic or hospital will be included in the evaluation.

Other studies with the quadrivalent vaccine are in progress or planned. In the United States, under the direction of Dr. Bruce Davidson is sponsoring a collaborative efficacy trial in 900 infants with 3 doses of the quadrivalent vaccine with the 10^4 PFU dose of each of the components in one group, 3 doses of the 10^4 PFU dose of the DxRRV reassortant vaccine alone to another group and 3 doses of a placebo in a third group. In Peru, Dr. Lanata is carrying out an efficacy trial similar to the preceding one in 650 infants. In Myanmar (Burma), Dr. Moe is beginning anefficacy trial in 550 neonates of 3 doses

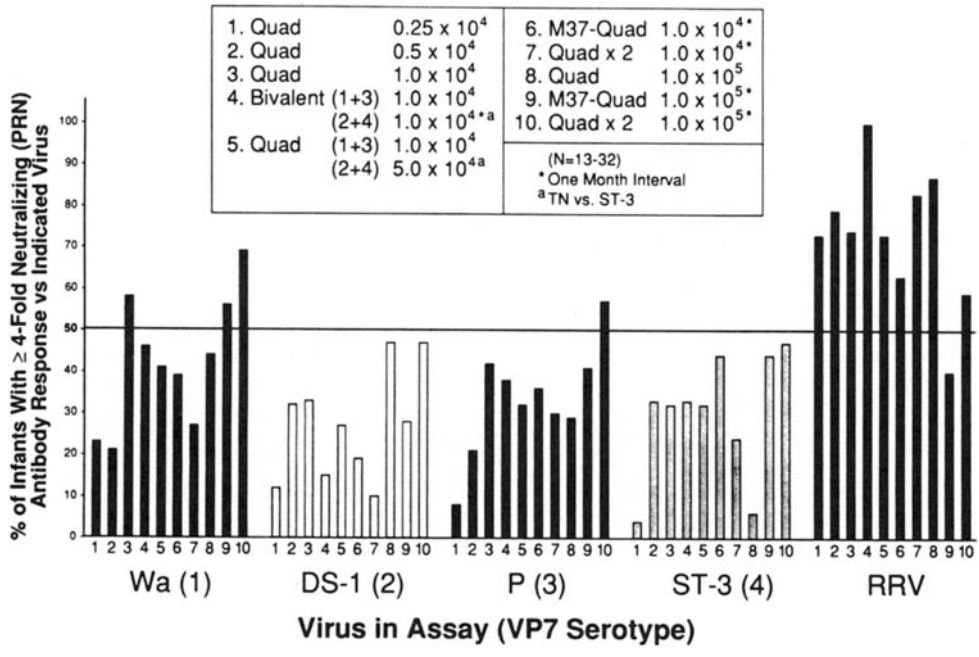

Figure 1. Progression of quadrivalent (Quad) (VP7 serotype 1,2,3,&4) rotavirus vaccine phase I studies in infants 10-20 wks of age in Venezuela. Data from 28,48-50; data for category 10 is partial (50). Note: TN = Tube neutralization assay.

of the quadrivalent vaccine with the 10^5 PFU formulation of each component or a placebo. In Brazil, Dr. Linhares is about to begin an efficacy trial in 500 infants with 1 or 3 doses of 10^4 PFU of each component of the quadrivalent vaccine or a placebo in 650 infants.

Finally, a non-Jennerian approach to rotavirus vaccination is also being evaluated. It involves the use of a neonatal VP7 serotype 1 rotavirus strain, M37, that may be naturally attenuated[28,49,51]. The impetus for evaluating this strain is based on the observation by Bishop *et al.*[52] that neonates who developed a subclinical rotavirus infection during the first 14 days of life were protected against severe rotavirus diarrhea during a 3-year follow-up period. The M37 human rotavirus strain was originally recovered from the stool of an asymptomatic neonate in Venezuela and subsequently cultivated in AGMK cells[28,49,51]. M37 has the VP7 specificity of human rotavirus serotype 1 and a unique VP4 which is shares with other neonatal strains of VP7 serotype 1, 2, 3, or 4[13,28,49,51].

Phase I studies in Baltimore, Venezuela, and Finland revealed that the vaccine was safe or acceptably reactogenic and antigenic[49,53,54]. In preliminary results of a combined phase I, phase II trial of the M37 vaccine in Finland, the 10^4 PFU dose of the M37 vaccine failed to induce protection against rotavirus diarrhea[53]. The number of infants who received the 10^5 PFU dose was too small to allow evaluation of efficacy of this higher dose. Although the M37

vaccine was antigenic as determined by IgA ELISA antibody responses, neither the 10^4 PFU nor the 10^5 PFU dose induced an acceptable rate of serum antibody responses to serotype 2, 3, or 4 rotavirus antigens. Thus, this strain may not be sufficiently broadly reactive to induce protection against all 4 serotypes.

Thus, progress is being made in the development of a rotavirus vaccine with emphasis on a vaccine that induces antibody to the VP7 of each of the four epidemiologically important serotypes. If this approach should not yield adequate protection, other avenues which take advantage of the immunogenicity of both VP7 and VP4 of human rotaviruses will be explored including: 1) the use of cold-adapted strains of human rotaviruses; 2) the development of reassortants between human rotavirus strains with different VP7 and VP4 antigenic specificities; 3) use of recombinant DNA to deliver specific rotavirus antigens; and 4) delivery of rotavirus to specific intestinal sites by microencapsulation technology[1,28,55,56].

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ROTAVIRUS SPECIFIC BREAST MILK ANTIBODY IN TWO POPULATIONS AND POSSIBLE CORRELATES OF PROTECTION

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INTRODUCTION

The group A Rotaviruses are the major cause of severe viral diarrhea worldwide, contributing to severe dehydration in approximately 1% of infants less than a year old[1]. Rotavirus is associated with approximately 1 million deaths per year[2]. Although a protective role for breastfeeding has been pursued for rotavirus infection and disease, results of epidemiologic studies have been contradictory[3-5]. Part of the problem with the interpretation of these studies has been that the association of specific protective factors in milk with protection against infection or disease has not been investigated.

With recent advances in the field of rotavirus research, more is known about potential protective antigens. The two major virion surface proteins, VP4 coded by gene 4 and VP7 coded by gene 7, 8, or 9 must be considered the prime candidates for the immunoprotective antigens of rotavirus[6-7]. Both elicit type specific neutralizing antibody. Rotavirus attachment is mediated by VP7. Internalization and thus infectivity of rotavirus is determined by VP4[8]. There are six human VP7 serotypes and only 3 basic classes of human VP4.

Using two different breastfeeding populations, one in which rotavirus infection is endemic, Caracas, Venezuela, and the other where rotavirus is seasonal, Rochester, U.S.A., we characterized the rotavirus specific antibodies present in breast milk and attempted to find antibody correlates of "protection" using vaccine trials with Rhesus rotavirus serotype 3 (RRV).

MATERIALS AND METHODS

Breast Milk Samples and Subjects

Breast milk samples were taken from U.S. mothers of 2-6 month old infants who participated in various RRV trials (RRV $1x10^4$ PFU or $1x10^5$ PFU, RRV reassortants, or placebo recipients) and from 3-8 day old and 4-10 months old Venezuelan infants who received 1.10^4 PFU RRV. These trials were used to evaluate both rotavirus-specific antibody content of breast milk and correlates of interference with vaccine "take". Breast milk samples were

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supplied by Dr. M. Pichichero, University of Rochester, NY. All breast milk samples from Caracas were supplied by Dr. Jorges Flores, NIH.

Rotavirus Specific Antibody Determinations

Breast milk samples were defatted by centrifugation. All breast milk samples were processed to contain 10 mg% total IgA as determined by an enzyme linked immunosorbent assay (ELISA)[9]. Plaque reduction neutralization assays were performed against the RRV strain and human P strain. These rotovirus strains have common VP7 antigens but do not share VP4 antigens. Serial two-fold dilutions of breast milk starting at 1:10 was mixed with an equal volume of either virus suspension containing 600 PFU/ml. After 30 minute incubation at 56°C, neutralizing activity was determined as previously described[10]. Neutralizing titers were based on the highest reciprocal dilution causing a 60% or greater reduction in plaque formation.

RRV VP4 and VP7 specific breast milk activity was determined using a modification of a competitive binding ELISA[11]. Monoclonal antibodies were supplied by Dr. Kim Green, NIH. A homotypic serotype 3 specific VP7 monoclonal antibody (MAb), 954/159, and a RRV strain specific VP4 MAb, 954/23, located in the VP8 cleavage polypeptide were used[12]. Specific activity of the monoclonals was determined against gradient purified RRV and human P outer capsid antigens. Briefly, serial diluted milk sample starting at a 1:10 dilution were incubated with the rotavirus antigen containing wells overnight at 4°C. After washing, specific MAb was added at 37°C for two hours. After washing, the reaction was developed with specific conjugate. Epitope blocking antibody (EBA) titers were determined to be the highest reciprocal milk dilution giving a $\geq$50% reduction in absorbance from the positive unblocked control wells.

RESULTS

Prevalence Rotavirus Specific Antibody in Milk

Sixty-five breast milk samples from Caracas women and 58 breast milk samples from Rochester women were available for analysis of rotavirus specific antibody. Forty-five of the Venezuelan milk samples were obtained in the first week of life. Forty-five breast milk samples from the United States were obtained from mothers of infants who were vaccinated prior to the winter rotavirus season.

As seen in Table 1, 84.6% of the Venezuelan breast milks and 50% of the U.S. breast milk samples contained detectable antibody (reciprocal titer $\geq$10) to RRV by Plaque reduction neutralization assay (PRNA). This is a statistically significant difference (Chi sqr, $p = 0.00009$). There were 13 U.S. breast milk samples obtained during rotavirus season (November to March). Eight of these samples had RRV specific PRN antibody and 12 had human P neutralizing antibody. When these "winter" breast milk samples were analyzed separately with the Venezuelan samples the differences between Venezuelan and U.S. milk neutralizing antibody to serotype 3 was no longer statistically apparent ($p = 0.2$). 36/58 (62%) of the Venezuelan breast milks and 23/55 (42%) of the U.S. breast milks had serotype 3 VP7 epitope blocking activity ($p = 0.04$). Thirty-six per cent of the Venezuelan milk samples had RRV specific epitope blocking VP4 activity but only 9% of the U.S. milks had this activity ($p = 0.003$).

Rotavirus-Specific Antibody in Breast Milk and Vaccine "Take"

In order to evaluate the effects of specific milk antibody on interference with vaccine "take", we used P and RRV PRNA results in addition to the VP4 and VP7 epitope blocking antibody levels. We designated functional VP4- and VP7-neutralizing activity for the breast milk samples: a PRNA RRV/P reciprocal antibody titer ≥ 4 indicative of predominantly VP4 specific activity and a PRNA RRV/P $\geq 1 \leq 2$ indicative of predominantly VP7 activity (i.e. neutralization shared by P and RRV).

We evaluated 20 breast fed 4-10 month old Caracas infants who received RRV 10^4 PFU. Breast milk was available from 15 infants. Eight of the 15 (53%) had evidence of predominantly VP4-neutralizing antibody; 7 had predominantly VP7 PRN activity. Breast milk reciprocal neutralizing antibody titer for RRV ranged from 10 to 160 (mean 111); P PRN reciprocal titers ranged from <10 to 640 (mean 77). There was no significant difference in the magnitude of the P or RRV PRN titers ($p = 0.5$).

Table 1. Breast Milk Rotavirus Specific Neutralizing and VP4 and VP7 Antibodies

SITE	RRV PRN	P PRN	VP4 EBA	VP7 EBA
	nos. positive/total nos. tested (T)			
Caracas	55/65 (85)	44/50 (88)	21/58 (36)	36/58 (62)
Rochester	28/56 (50)	NT	5/56 (9)	23/55 (42)
	$p = 0.00009$		$p = 0.003$	$p = 0.04$

Only 2 breast milk samples had detectable VP4-blocking antibody suggesting either that the epitope recognized by the VP4 monoclonal antibody used was not responsible for the VP4-neutralizing activity seen or that the binding affinity of the milk VP4 antibodies was poor in the ELISA. VP7 epitope-blocking antibody was present (reciprocal titer ≥ 10 in 11/15 (73%) breast milk samples with titers ranging from <10 to 160 (mean 41).

Of these 15 vaccinees, 9 (60%) had evidence of a seroresponse to vaccination. 6/11 (55%) who received breast milk containing epitope-blocking VP7 antibody had a seroresponse. We were unable to show interference by these blocking antibodies to vaccine take ($p = 0.4$, Fishers). In contrast, 6/8 (75%) infants fed with breast milk containing VP4 predominant PRN activity (RRV/P PRN titer ≥ 4) had no seroresponse. 5/7 (71%) infants breast fed with milk negative for VP4 PRN antibody but positive for VP7 PRN antibody had a seroresponse. Statistical evaluation of the lack of interference by milk VP7-neutralizing antibody and presence of interference by RRV VP4-neutralizing antibody approached significance (Fisher's $p = 0.1$).

DISCUSSION

Because the breast is an integral part of the mucosal immune system, specific antibody activity contained in breast milk is dynamic reflecting the ongoing infectious and immunologic experiences of the mother. It is not surprising, therefore, that rotavirus specific breast milk antibody activity is more prevalent in a rotavirus endemic area like Caracas than in the United States where rotavirus infection is seasonal. In fact, this difference in antibody activity disappeared when U.S. breast milk sample collected during rotavirus season were compared to Venezuelan samples. What is somewhat surprising is the fact that 50% of breast milk samples taken in the U.S. during non-rotavirus season had rotavirus-specific antibody, suggesting either that there is a low-level of rotavirus infectious activity persisting during the non-winter months, antigenic stimulation with cross-reacting antigens, or active memory.

There were differences in the type of specific antibody present in breast milk sampled from the rotavirus endemic and non-endemic areas. There was very little activity in VP4 epitope-blocking antibody in the U.S. samples compared to the Venezuelan samples. Whether this reflects decreased exposure to this epitope in the U.S., variations in the VP4 antibody specificity in the two populations, or a "broadening" of milk antibody response from continuous antigenic exposure in Venezuela is unknown. The primary breast milk antibody responses to RRV and human P rotaviruses in both populations seemed to be against VP7 antigens, rather than VP4. Whether this is true for other rotavirus serotypes is unknown at present.

Finally, VP7 specific activity in breast milk did not seem to be correlated with interference of rotavirus infectivity as measured by RRV "take." Our data suggests but does not prove that VP4 activity in breast milk is important for limiting the infectivity of rotavirus. This type of analysis needs to be expanded in additional vaccine trials and extended to studies of natural rotavirus infection.

CONCLUSIONS

Rotavirus specific antibody activity in human milk is dependent on rotavirus exposure and varies depending on geographic location. In addition, rotavirus specific breast milk activity probably does play a role in interfering with rotavirus infectivity. Using rotavirus vaccine studies as a model, it was found that VP4 specific antibody activity and not VP7 activity, was associated with lack of vaccine "take" in breastfed RRV immunized newborns.

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HUMAN MILK AND HIV INFECTION: EPIDEMIOLOGIC AND LABORATORY DATA

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INTRODUCTION

The role of breastfeeding in Human Immunodeficiency Virus (HIV) infection is unknown. Major issues include:

1) The risk of vertical HIV transmission associated with breastfeeding,
2) The effect of breastfeeding on the HIV-infected infant,
3) The effect of breastfeeding on uninfected infants of HIV-infected women,
4) The importance of breastfeeding to infants in the absence of HIV infection, and
5) The effect of lactation on HIV-infected women.

The question of whether or not and at what frequency HIV may be transmitted through breastfeeding is important for both industrialized and industrializing countries. In the United States, the number of HIV-infected women continues to increase. Approximately one third of their offspring acquire HIV infection and develop AIDS as a result of vertical transmission. At the end of 1990, 2,786 pediatric AIDS cases that met the Centers for Disease Control (CDC) case definition had been reported to the CDC. In addition, 15,493 AIDS cases had been reported among adolescent and adult women.[9] The World Health Organization has estimated that in sub-Saharan Africa, 2.5 million women and 500,000 children are HIV-infected.[6] In the United States, 84% of the reported pediatric AIDS cases are due to vertical transmission.[9] In Africa, pediatric AIDS occurs as a result of vertical transmission and blood transfusions.

Theoretically, vertical transmission could occur in any of the three stages of the relationship between mother and offspring. The evidence for intrauterine transmission is that virus has been isolated from fetal tissue.[15,26] Intrapartum transmission might occur when the baby is exposed to infected maternal blood or secretions. Postnatal transmission might occur in

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situations where infective maternal body fluids contact receptive ports of entry in the infant. Reports of virus isolation from milk, saliva, and tears have appeared in the literature. [31,34,11,10]

There is still much to be learned about the timing and determinants of vertical transmission. Barriers to answering the breastfeeding transmission question definitively include the following research feasibility problems:

1) Inability to distinguish prepartum, intrapartum, and postpartum transmission;
2) Difficulties in identifying breast-fed and artificially fed infants who are similar except for feeding mode;
3) Difficulties in distinguishing meaningful levels of exposure to virus in breast milk;
4) Sample size considerations; and
5) Ethical and political considerations.

Several case reports and two laboratory observations of HIV isolation from milk have strongly suggested that HIV can be transmitted by breast feeding. Two small studies of mother-infant pairs who seroconverted in the first 2 years postpartum add strength to this evidence. Reports from case series, surveillance cohorts, and prospective cohorts of HIV-infected women and their offspring suggest that the increment in vertical transmission of HIV infection attributable to breastfeeding is likely to be small.[12,22,26,43]

The benefits of breastfeeding under normal circumstances are well known. The incidence and duration of breastfeeding, as well as the magnitude of the benefits, vary tremendously from one country to another and within countries by culture and by social class. Recommendations regarding breastfeeding in the context of HIV infection have been developed to address the possibility of breastfeeding transmission of HIV. The differences in the recommendations reflect the different health conditions confronting the constituent populations of the health agencies. They also reflect the fact that the data from which policy can be formulated is meager. In December, 1985, the Centers for Disease Control (CDC) issued the following recommendation for the United States: "HTLV-III/LAV-infected women should be advised against breastfeeding to avoid postnatal transmission to a child who may not yet be infected."[5] In 1987, in a Special Programme Statement on AIDS, the World Health Organization (WHO) recommended "continued promotion, support and protection of breastfeeding" and, in the case of women known to be HIV-infected, that careful consideration be given to the availability of safe effective use of alternatives before advising against breastfeeding.[38] In the United Kingdom and in Australia, the recommendations regarding the feeding of infants of HIV-infected women are similar to those of the CDC. In contrast, in developing countries where safe, effective affordable use of alternative milk is not available, breastfeeding is recommended in general even for women known to be HIV positive.[21]

The potential benefits and hazards of breastfeeding in the presence of HIV infection have been postulated but not adequately studied. Human milk contains immunologic, and nutritional properties which have documented activity against HIV and other viruses.[20,37,40] There is a hypothetical possibility that these antiviral properties may play a role in limiting transmission through breastfeeding. They may also benefit HIV-infected infants by improving their nutritional status, modulating their immune

response to opportunistic infections, or perhaps altering the progress of their immune deficiency. Alternatively, the usual benefits of breastfeeding may be inadequate against HIV, particularly if the mother is ill. These potential benefits to infected infants would be especially important in settings where breastfeeding is the norm and HIV status is unknown for most women, a situation which exists in several African countries where HIV is a major problem. These hypotheses remain unexamined. Other questions regarding the immunologic and nutritional quality of the milk of HIV-infected women and the health effects of lactation on women in different stages of HIV disease might also be addressed.

EVIDENCE FOR HUMAN MILK TRANSMISSION OF HIV

Case Reports and Prospective Studies

Epidemiologic evidence for human milk transmission has come primarily from 2 types of studies:

1) Retrospective examinations of individual breast-fed children whose mothers were apparently infected postpartum, usually through contaminated blood transfusions. These children came to attention either because maternal recipients of contaminated blood were traced or because the children presented to clinical attention with HIV disease, and a plausible source of postpartum maternal infection was discovered; and

2) Prospective studies of mother-infant pairs who seroconverted postpartum. There are 3 different studies which provide a systematic examination of women who were documented to be seronegative at delivery and seropositive during the first 2 postpartum years.[8,13,34]

Eight reported children correspond to the first category (3 in Australia,[41,4,42] 2 in Rwanda,[16] 1 in Zaire,[8] 1 in France,[36] and 1 in the USA[30]). Six of them are breast-fed infants of mothers presumed infected through postpartum transfusions. The 7th and 8th cases are a Zairean child who was wet nursed by an HIV-infected relative and an Australian child of an IV drug user who seroconverted while breastfeeding.

In April, 1985, Ziegler and his colleagues in Sydney, Australia reported the case of a breast-fed child whose mother had had a postpartum transfusion.[41] During the child's second year of life, the mother and child were found to be HIV-seropositive. Other family members remained seronegative. The mother was assumed to be seronegative prior to her transfusion; a blood donor was HIV-seropositive.

In Australia there have been 8 breast-fed infants of women who presumably became infected by postpartum transfusions.[42] Breastfeeding durations were from two weeks to three mo and most mothers developed lymphadenopathy. Two infants became infected and 6 are presumed uninfected.

In September, 1985, Stewart and colleagues reported from Australia that three breast-fed children of HIV-infected mothers did not become infected.[29] This report provides an interesting contrast to the Ziegler report. The difference in timing of breastfeeding with respect to the onset of the

mothers' infection may be important. These women were infected by artificial insemination one year prior to becoming pregnant whereas the first woman, if she became infected from her transfusion, began breastfeeding immediately after acquiring HIV, a period of HIV viremia when the risk of transmission may be higher.

The mother of an Australian child proposed as a breastfeeding transmission case[4] came to clinical attention when she was 11 mo postpartum because of weight loss and lymphadenopathy. She reported illicit intravenous drug use for 8 yr and a needle sharing event 9 mo postpartum which was temporally associated with an illness consistent with acute HIV infection. She was seronegative at 9 mo and seropositive at 11 mo. Her child was breast-fed for 11 mo, was seropositive, and had a cervical lymph node biopsy positive for HIV. The breast milk was not cultured but electron microscopy revealed the presence of intracellular and extracellular virus, and the milk was noted to be hypercellular with a marked increase in lymphocytes and histiocytes.

Two cases have been reported in Zaire.[8] One child was born to a healthy 28 yr old woman of unknown HIV status who died during the cesarean section delivery of the child. The child was breast-fed by an HIV-seropositive aunt with AIDS-Related Complex (ARC) and was seropositive at 1 yr of age with a history of recurrent fever, diarrhea, cough, and failure to thrive. He received injections but had no history of transfusion or scarification. The father was HIV-negative.

The other case report described 3 Zairean children whose HIV-seronegative mothers became infected following blood transfusions.[8] One mother received a contaminated transfusion during a neurosurgical procedure 11 mo postpartum. Her child was breast-fed for 7 more mo and seroconverted between 3 and 4 mo after the procedure. The child had received no injections, transfusions, or scarifications after the operation. The other 2 mothers received contaminated transfusions during cesarean sections and seroconverted within 6 mo. Their infants were seronegative at 6 and 9 mo of age while breastfeeding almost exclusively.

In Rwanda and France, 3 mothers and their infants became HIV seropositive following postpartum maternal transfusions.[16,36] In all 3 cases, the infants were breast-fed, a blood donor was HIV-seropositive, and spouses and other children were HIV-seronegative. The 2 Rwandan mothers were assumed, but not documented, to be HIV-seronegative prior to their transfusions, and one of the seropositive infants was reported to have had 12 IM injections during infancy. In Rwanda, injections are a potential source of infection as it is very unlikely that sterile injection equipment is used in most instances. The French woman, on the other hand, had a documented seroconversion. A frozen blood sample from her fourth mo of pregnancy was negative for HIV antibodies, suggesting that she became infected after that time and probably at the time of her postpartum transfusion.

In 1991, Stiehm and Vink[30] reported another possible transmission of HIV infection through breastfeeding following a maternal postpartum transfusion. The child was breast-fed for 8 mo and did well until age 4 yr when she developed diarrhea and anemia and was transfused with blood from an HIV-seronegative donor. She was found to be HIV-seropositive. Her father remained seronegative, but her mother and younger brother were also seropositive, consistent with the mother becoming infected as a result of

her postpartum transfusion. One of the mother's plasma donors was seropositive, and no other known risk behaviors were noted.

Two studies of women in Zambia and Rwanda found that several breastfeeding mother-infant pairs seroconverted over the first 2 postpartum years. In 1989, Hira and colleagues[13] reported that 16 of 641 Zambian women who were HIV-seronegative at labor seroconverted during the first year after delivery. Two of 8 breast-fed children seroconverted at 20 and 22 mo of age. The investigators believed there were no risk factors other than breastfeeding and that the women became infected through sexual intercourse. They also followed 30 breast-fed children whose mothers were seronegative at labor but whose fathers were seropositive at the time of labor. Three women seroconverted at 8, 10, and 12 mo after delivery. The child of the woman who seroconverted at 12 mo was p24 positive at 15 mo of age.

In a prospective study of 212 mother-infant pairs, HIV-seronegative at delivery in Kigali, Rwanda, 16 women seroconverted over a mean of 17 mo of follow-up. Postnatal seroconversion also occurred in 9 of their infants, 5 in the first 3 postpartum mo and 4 in mo 4 to 21. The authors concluded that 4 of the 5 infants who seroconverted in the first 3 postpartum mo had possibly acquired HIV postnatally, and that 4 infants who seroconverted between postpartum mo 4 and 21 had proven postnatally acquired infections. One infant was PCR-positive at birth and judged to be infected prior to the postnatal period. Supporting the possibility of intrapartum of late *in utero* transmission to the infants who were early seroconverters was the fact that 2 of their mothers had reversed OKT4/OKT8 ratios at 15 days postpartum.

There are numerous observations of the offspring of women known to be seropositive during pregnancy. However, comparing transmission proportions among these studies in an attempt to find the increment possibly attributable to breastfeeding is not generally useful because the populations and study designs are frequently quite different and serious bias is possible. Transmission proportions even within studies have varied from one year to the next by amounts which may end up being the amount attributable to breastfeeding without further systematic study.

SUMMARY OF THE EPIDEMIOLOGIC EVIDENCE FOR HUMAN MILK TRANSMISSION

The infected, breast-fed infants reported here include those of mothers believed or documented to be seronegative at delivery who later seroconverted presumably following a contaminated blood transfusion or sexual intercourse with an infected man. In several postpartum transfusion cases a seropositive blood donor was identified, husbands and other children remained seronegative, and known factors and behaviors thought to increase risk for infection were not present. Those cases involving documented seroconversions[8,28,13,34] are particularly compelling in support of the breastfeeding transmission hypothesis. Further, they tend to support the hypothesis that transmission is more likely to occur when the mother has an incident infection and is viremic.

When one accepts the hypothesis that breastfeeding can transmit HIV, the important transmission questions concern frequency and determinants. Limited data from case series and cohort studies suggest that most breast-fed infants of infected women do not become infected.[12,22,31] In only 1 study did

most (5/6) breast-fed babies develop HIV infection.[3] A postpartum maternal transfusion while breastfeeding has not ensured transmission. There are at least 4 such cases (2 in Australia and 2 in Zaire)[8,42]. Transmission proportions in the case series and cohort studies of women already infected during or prior to pregnancy in Australia,[42] Haiti,[12] Zaire,[8] and the European Collaborative Study[22,43] have been approximately the same as in the United States where women did not breastfeed. As noted above, however, these proportions are changing over time and may not be comparable figures depending on the population and the study design.

The timing of acquiring infection relative to the timing of seroconversion is usually unknown and creates problems for categorizing vertical transmission as prenatal, intrapartum, or postpartum. A rigorous analysis would have to make note of the fact that there is not exhaustive evidence that mothers reported here were not infected before the birth of their infants since in most cases women's HIV status was not available. In the 5 cases[8,28,13,34] where prior serology was available, we have to interpret the data with the knowledge that the development of antibodies is often undetected for long periods after infection has occurred.[23] The evidence provided by Van de Perre[34] regarding PCR results, maternal T-cell subset ratios, and timing of seroconversion with respect to delivery, supports a model of variable time to seroconversion and peak transmission efficiency throwing into doubt the timing of perinatal infection in these cases of early seroconversion.

The mechanism of acquiring HIV infection through breastfeeding has not been studied. Transmission might occur via skin or mucosal lacerations secondary to oral candidiasis or instrumentation during delivery. Or HIV present in milk might cross the gastrointestinal mucosa barrier under specific conditions. Nipple cracking and bleeding has been suggested as a possible source of transmission associated with breastfeeding. Maternal or infant lacerations, in isolation or in combination might increase risk of transmission during breastfeeding.

It has been pointed out that there are other hypothetical routes of infection transmission, such as oral exchange of infective body fluids or introduction of virus through uncornified newborn skin, although there are no data to support such hypotheses.[7]

Laboratory Observations

Viral isolation. In 1985, Thiry *et al.*[32] in Belgium reported the successful isolation of HIV in 5 samples of milk from 3 HIV-infected women with sick infants. In 1986, Vogt *et al.*[35] in Boston reported isolating HIV from the colostrum of an HIV-seropositive woman in a study designed to examine cervical secretions for HIV. In 1988, electron microscopic examination of the milk of an HIV-infected Australian woman with an HIV-infected, breast-fed child revealed the presence of an intracellular and extracellular virus, and the milk was noted to be hypercellular with a marked increase in lymphocytes and histiocytes.[4]

In 1991, Ruff *et al.* reported that 22/31 (71%) of breast milk specimens from HIV-1 seropositive women were positive for HIV DNA by polymerase chain reaction (PCR) technique. 17/25 colostrum samples were HIV-positive by PCR, and 7/13 mature milk samples were PCR-positive. Samples which were p24 antigen-positive were more likely to be PCR positive than those which were p24-negative.[25]

Anti-HIV antibodies. In 1988, Van de Perre *et al.*[33] reported the detection, using western blot techniques, of IgG, IgM, and IgA antibodies to HIV in the milk of 4 HIV-seropositive women but not in the milk of 2 HIV-seronegative women. In 1989, Ruff, *et al.*[24] reported that 11 of 12 HIV-seropositive women had HIV antibodies in their milk and none of the 7 seronegative women tested had HIV antibodies in her milk. In 1990, Yolken *et al.*[40] examined the milk of 10 HIV-seropositive women from Haiti and Malawi and detected HIV IgG antibody and smaller amounts of IgA antibody directed against envelope protein in all specimens but no HIV antibodies in the milk of seronegative women. There was no detectable virus in the milk using PCR. In 1990, Belec *et al.*[2] reported finding IgG, IgM, and IgA to an array of HIV core and envelope proteins in the milk of 15 HIV-seropositive women and only to p25 and p18 in the milk of 4 HIV-seronegative women. In 1990, McDougal, *et al.*[18] used a monoclonal based ELISA to examine 9 serial colostrum and milk specimens from 3 seropositive North American women in the first few postpartum days and found IgA in titers from <1:10 (negative) to 1:160 and IgG in titers of from <1:10 (negative) to >1:1280. The milk from a seropositive woman who relactated 2 yr after discontinuing breastfeeding had an IgA titer of 1:20 and an IgG titer of >1:1280. No HIV antibodies were detected in control milk from 2 seronegative women.

SUMMARY OF THE LABORATORY DATA ON HIV AND HUMAN MILK

The mere presence of HIV and anti-HIV antibodies has been documented. These results are provocative, but their meaning and usefulness is unclear. Presence of HIV in milk does not necessarily imply infectivity, and presence of HIV antibodies does not necessarily imply protection. These findings must be systematically and statistically associated with clinical outcomes. The small numbers of milk samples from unsystematically selected women have not revealed enough variance in the presence or amounts of antibody to suggest the possibility of a meaningful correlation between clinical outcomes and quantitative antibody measures. Confirmation of these findings will be interesting only if the confirmatory antibody and culture studies expand the scope of laboratory investigations to include specification and quantitation of various antibody isotypes, correlation of these results with immunologic entities in blood and other secretions and also with maternal stage of disease and infant outcome. The women who donate milk specimens must be selected in a systematic manner, and serial specimens would be desirable.

The work of Welsh and May, Isaacs, Yolken, and Newberg should be expanded to explore the extent to which and the mechanisms through which human milk is capable of inactivating HIV. Basic studies examining the log kill of HIV, both free and intracellular virus, introduced into expressed human milk and gastric aspirate following breastfeeding could produce valuable data about the general capability of milk to inhibit ingested HIV. Examining the immunologic profile and the HIV status of serial human milk specimens from HIV-infected women whose peripheral blood immunologic status is available for correlation with the milk data, as well as with the clinical data on the women and their infants, might produce valuable information regarding infectivity of the breast milk of HIV-infected women. Until the technical capability to diagnose HIV in the first wk of life is

achieved, allowing investigators to distinguish which infants are infected at birth, this approach would probably produce the most meaningful results.

Laboratory reports of the presence of whole HIV virus, HIV virus particles, or anti-HIV antibodies in human milk are important preliminary steps in efforts to learn more about the transmissibility of HIV infection via breastfeeding. To proceed further toward meaningful results which could be useful in clinical practice and public health policy, considerable basic information must first be generated. The substantial work performed over the past 10-15 yr on milk from women without HIV infection can serve as a guide for similar studies to determine the quantity and quality of milk produced by women with HIV infection at various stages of disease. The nutritional and immunologic properties of the milk of HIV-infected women may differ significantly from those of uninfected women, particularly when they in advanced stages of HIV disease. In order to accomplish this work, it would be useful for human milk investigators, who usually lack HIV biohazard protection in their laboratories, to collaborate with HIV investigators, who usually have little familiarity with laboratory methods to study milk. Collaboration between these 2 groups in systematic studies examining milk as well as blood and other secretions such as cervical secretions and saliva would be likely to be fruitful.

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CHARACTERIZATION OF A HUMAN MILK FACTOR THAT INHIBITS BINDING OF HIV GP120 TO ITS CD4 RECEPTOR

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INTRODUCTION

Infants born to mothers infected with human immunodeficiency virus (HIV) are at high risk for HIV infection, with reported rates of transmission of between 20 and 50%[1-3]. Breast milk has been suggested as one of the agents of transmission, based on reports of isolation of HIV from breast milk[4], and on the seroconversion of infants breastfeeding on the milk of mothers who had recently seroconverted[5,6]. This has resulted in the medical dilemma that breastfeeding has been questioned in populations in which HIV is a major health concern, despite the knowledge that it is often these very populations in which breastfeeding has its greatest effect in reducing non-HIV associated infant morbidity and mortality[7,8].

Although human milk can be the agent of vertical transmission of other viruses, such as cytomegalovirus[9] and HTLV-1[10,11], breast milk is also known to contain a number of immunoglobulin and non-immunoglobulin factors which can inhibit the pathogenesis of microbial agents[12-15].

We studied a factor in human milk that might protect against the human immunodeficiency virus. The binding of the viral envelope glycoprotein, gp120, to the host cell receptor, CD4, is thought to be necessary for the entry of HIV into most target cells, and is thus the critical first step in the infection of most cells by HIV. The ability of milk samples to inhibit the binding of CD4 with HIV membrane fragments, with gp120, or with OKT4A, a monoclonal antibody to CD4 which binds at or near the site of HIV attachment, was measured by use of a solid phase assay system.

Pooled milk was fractionated and the fractions containing inhibitory activity were identified by use of the solid phase assay. Milk fractions with high inhibitory activity were subjected to subfractionation, and chemical and enzymatic treatment; changes in their inhibitory activity were monitored to provide information on the nature of the inhibitory factor.

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METHODS

Samples

Milk samples from healthy mothers in the Boston area represented a broad spectrum of human milk with regard to stage of lactation, age of the mother, and parity. The Baltimore samples were obtained from healthy mothers shortly after they delivered at the Johns Hopkins Hospital. Milk samples were obtained within 1 week postpartum from HIV-seropositive and HIV-seronegative mothers residing in Port-au-Prince, Haiti. All milk samples were stored at -70°C until testing was performed. Unpasteurized bovine milk samples were obtained from cows from Maryland and Massachusetts. The bovine milk samples were immediately frozen and tested without additional processing. Serum samples were obtained from HIV seropositive and HIV-seronegative individuals residing in the Baltimore, Maryland area.

Solid Phase Assay

Purified recombinant CD4 was immobilized onto the wells of a polyvinyl microtiter plate. The sample of milk or its components was incubated with recombinant gp120 and applied to the wells. After the wells were washed, the amount of gp120 adhering was quantitated by reaction with monoclonal antibody to gp120 followed by peroxidase-linked goat anti-mouse IgG and then o-phenylenediamine. The resulting color development is proportional to the amount of gp120 that binds to the plate and was measured at 405 nm.

Several monoclonal antibodies to CD4 are available which bind to or near the loop region which is the binding site for gp120. When antibodies OKT4A, OKT4B, OKT4D, OKT4E, and MT151 were substituted for the gp120 in the binding assay, milk inhibited their binding to CD4 to the same extent that it inhibited the binding of gp120 to CD4. As OKT4A is commercially available, it was substituted for gp120 in the assay, and positive results using the OKT4A assay were routinely confirmed with the gp120-binding assay. Therefore, the ability to block binding to the loop region of CD4, regardless of the specific ligand used to measure the blocking, will be referred to as CD4-blocking activity or CD4 -inhibitory activity.

Preparation of Milk Fractions

Oligosaccharide fractions were prepared from pooled human milk by removing the cream by centrifugation, precipitating the macromolecules in cold 50% acetone, separating lactose from the oligosaccharides on charcoal, and separating acidic from neutral oligosaccharides by ion exchange, as described elsewhere[16].

Lipid fractions were prepared by Folch partition followed by normal phase, reversed phase, and ion-exchange chromatography, as previously described[17].

Protein fractions were prepared from pooled milk by first removing the cream by centrifugation; the resulting skimmed milk was passed through a 0.45 μm filter, the filtrate and retentate were independently passed through a Concanavalin A affinity column, and each was separated into the flow-through, the fraction released from the column by 1M NaCl, and the fraction released by 1 M methyl-mannoside and methyl-glucoside in 1 M NaCl. The protein fraction with the highest activity was passed through a Sepadex G-200

SF molecular-sieving column, and the several resulting peaks were tested for blocking activity.

The crude milk protein fraction was also separated by preparative isoelectric focusing in a Rotofor (BioRad, Richmond, CA) apparatus. The protein fraction was made 1% with ampholytes (pH 3-10) and resolved at 16 watts for 4 hours. This resulted in 20 fractions resolved on the basis of their isoelectric points. These fractions were tested for CD4-blocking activity.

Enzymatic Treatments

Aliquots of human milk protein were dissolved in buffer with Gentamicin sulfate and incubated with enzyme for 50 hours at 37°C. The enzymes tested were neuraminidase from *Vibrio cholerae* (0.05 U/mL, Hoechst), sulfatase from *Patella vulgata* (limpet) at 0.5 mg (5 U/mL), *Aerobacter aerogenes* (1 U/mL) sulfatase, and sulfatase from abalone entrails (10 U/mL). For each enzymatic digestion, a blank sample with only enzyme and buffer was used as a control, as well as sample plus buffer without enzyme.

Chemical Treatments

Aliquots of human milk protein were subjected to 80°C distilled deionized water for 3 hours, methanol at ambient temperature for 24 hours, or reductive alkylation with 450 μg dithiothreitol at 37°C for 4 hours, followed by 5 mg iodoacetamide at 37°C for 2 hours, followed by dialysis and lyophilization.

RESULTS

Figure 1 shows the CD4-blocking activity of the 34 milk samples tested, expressed as the titer of material at which 50% inhibition occurs in the solid phase assay. All human milks tested displayed CD4-blocking activity, with a mean titer of 1:1600, and no significant differences were noted among the milks from Massachusetts, Maryland, and Haiti, and no differences in activity were apparent between the milks from HIV-seropositive mothers and HIV-seronegative mothers in Haiti. In contrast, no blocking activity greater than 1:20 was noted in any of the 23 sera obtained from HIV-seropositive and HIV-seronegative individuals.

Figure 2 displays the level of inhibitory activity in serial samples of a Massachusetts mother whose lactation was in the mature phase. Despite variations in total protein content (broken line), inhibitory activity was consistently present over an 80-day period, with titers ranging from 1:700 to 1:3000. These data suggest that the inhibitory activity may be due to some constituative component of human milk, and the remainder of our efforts constituted an attempt to characterize this factor.

Figure 3 demonstrates that human milk contains the inhibitory activity, but bovine milk does not. When tested at the concentration at which they are found in human milk, none of the five major lipid classes nor the total, neutral or acidic oligosaccharide fractions demonstrate appreciable inhibitory activity. However, both the cream and the skim fractions of human milk contain as much activity as the original milk, causing us to hypothesize that the activity might be found in the protein fraction. Furthermore, finding that dextran sulfate, but not other dextrans,

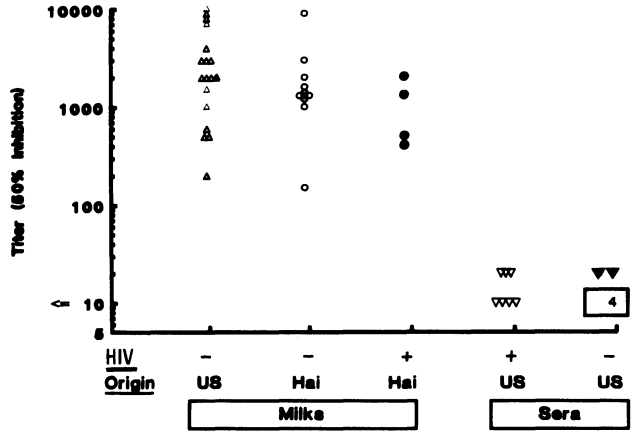

Figure 1. CD4 blocking activity of human milk and serum. Milk samples from the United States (US) were obtained in Boston and Baltimore from HIV seronegative mothers. Milk samples from Haiti (Hai) were obtained from seronegative mothers (–) and seropositive mothers (+). Sera from HIV seropositive (US+) and seronegative (US–) mothers were obtained in Baltimore. The CD4 blocking activity is found in milk samples regardless of HIV status, whereas it is not found in serum.

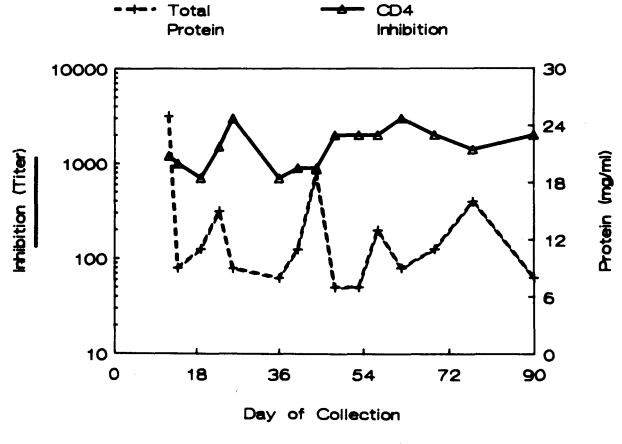

Figure 2. CD4 blocking activity of serial samples of human milk. A mother in the Boston area with well established lactation provided serial milk samples. the ability of human milk to block CD4 binding appears to be consistent and stable over the mature phase of lactation.

also inhibits binding to CD4, led us to investigate whether the active factor might be a sulfated constituent of the protein fraction.

To test this hypothesis, a skimmed milk preparation was prepared from pooled milk, and it was clarified through a 0.45 μm filter. The proteins from both the filtrate and the material retained by the filter were separated from any low-molecular-weight contaminants by diafiltration through a 10 kDa

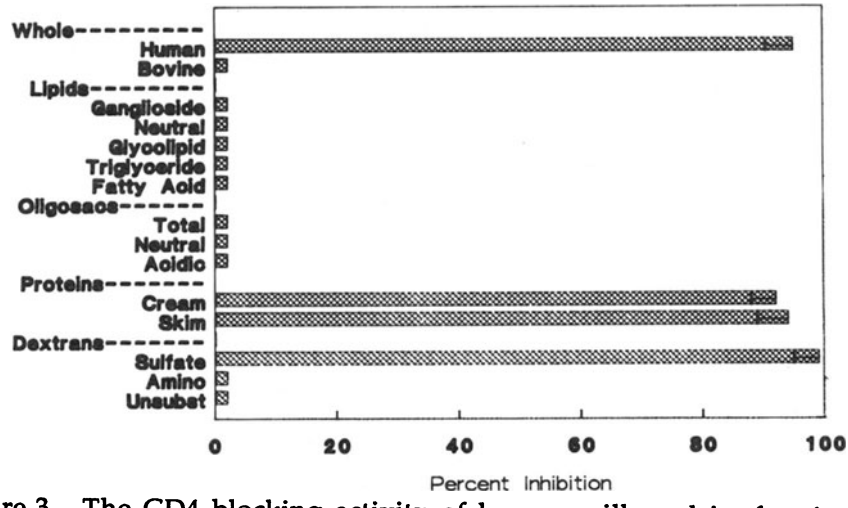

Figure 3. The CD4 blocking activity of human milk and its fractions. The oligosaccharide and lipid fractions of human milk did not display any CD4 blocking activity; however, activity was found in both the cream and skim fraction, indicating that the activity might reside in a macromolecule that distributes across both compartments. Bovine milk did not display blocking activity. Dextran sulfate, but not other dextrans, had blocking activity, suggesting the possibility that active macromolecule might be sulfated.

cut-off ultrafilter. Both of these fractions retained significant activity (Fig. 4), indicating that the active factor, when in its native state, might be part of a large aggregate. These fractions were then passed through a Concanavalin A lectin affinity column, which is known to bind to mannosylated glycoproteins. The activity was found to remain in the flow-through buffer fractions, indicating that the active factor is not mannosylated. The most active of these fractions was passed through a Sephadex G-200 SF molecular sizing column (Fig. 5). The activity was found in the void volume, indicating a molecular weight greater than 250 kDa for the CD4-inhibitory molecule in its native state.

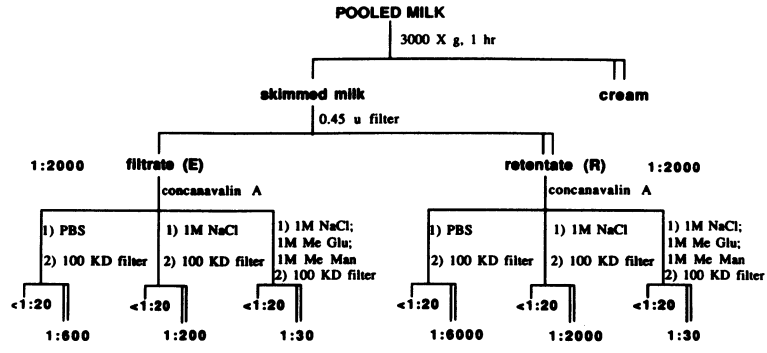

Figure 4. Fractionation of the macromolecular portion of human milk based on filtration and affinity to Concanavalin A. The activity is partially retained by a 0.45 μ filter, indicating its association with an aggregate, and the activity does not specifically bind to Concanavalin A, indicating a lack of mannose in the active factor.

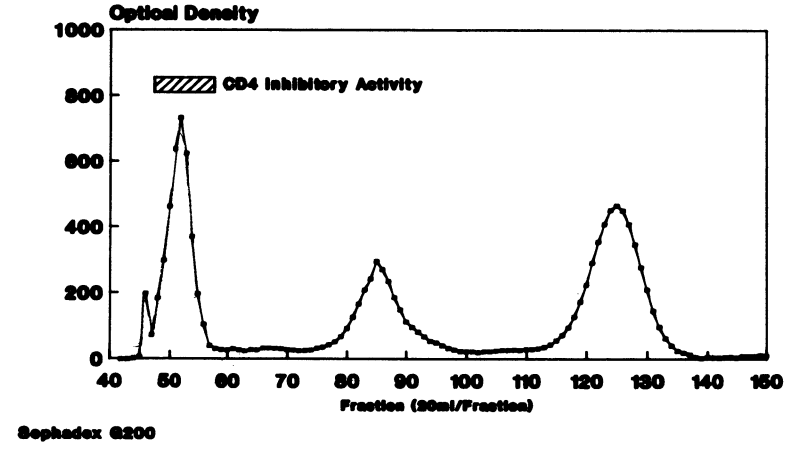

Figure 5. Fractionation of the macromolecular portion of human milk based on size. The CD4 blocking activity is found in the void volume of a Sephadex G-200 SF column eluate indicating a size of greater than 250 kDa for the native CD4 blocking material.

Aliquots of the active fraction were subjected to several enzymatic and chemical treatments to investigate the chemical properties of the inhibitory molecule's active component. Incubation with the sulfatase from *A. aerogenes* resulted in a significant reduction in inhibitory activity (Fig. 6), although other sulfatases were less effective, supporting the concept that the active molecule may be sulfated. Neuraminidase did not affect the activity of the inhibitory molecule, indicating that sialic acid is not part of its active site. The loss of activity by exposure to methanol or by alkylation, but stability to boiling water, are consistent with the properties of some membrane-associated proteins.

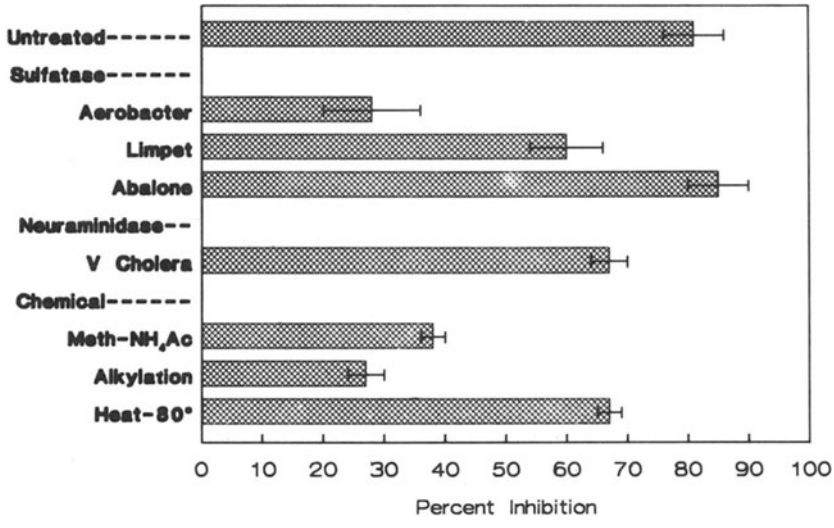

Figure 6. Changes in CD4 blocking activity due to enzymatic and chemical treatment. The activity is diminished by incubation with Aerobacter aerogenes sulfatase, by treatment with methanol, and by reductive alkylation.

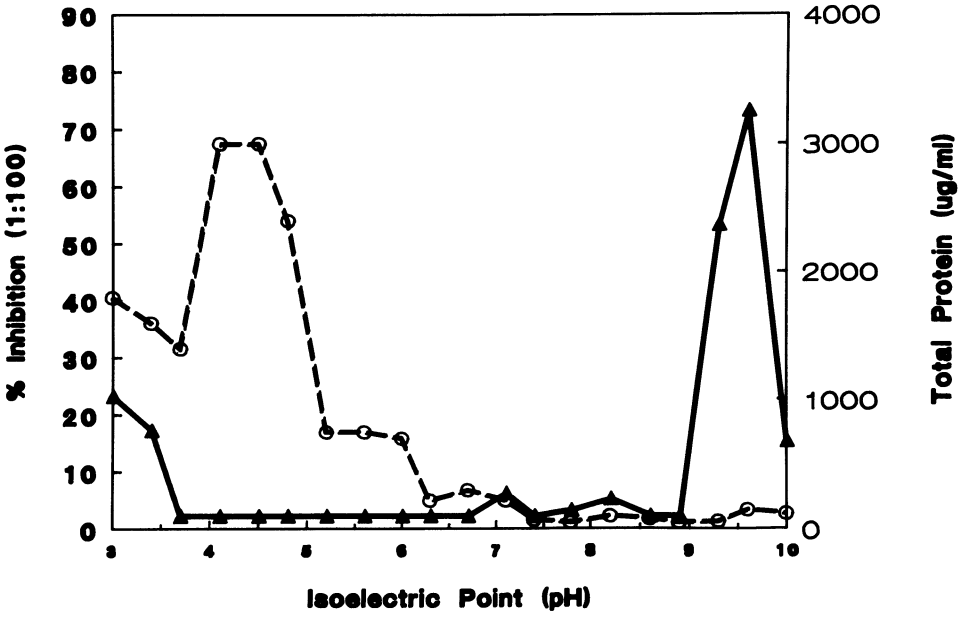

Figure 7. Preparative isoelectric focussing of the human milk macromolecular fraction. The activity is found mainly in the fractions with isoelectric points of approximately 9.3-9.6. The protective macromolecule is enriched 300-fold by isoelectric focussing.

The total protein fraction from a new sample of pooled human milk was separated by preparative isoelectric focusing on a Rotofor apparatus. Most of the CD4-inhibitory activity was found in the fractions that correspond to an isoelectric point of approximately 9.3-9.6 (Fig. 7). As these fractions were low in total protein, this represents a 300-fold enrichment of activity. In view of this high isoelectric point, and in conjunction with the high molecular weight and lack of mannose seen previously, it is likely that the CD4-inhibitory molecule is not an immunoglobulin. Also, none of the milk samples or milk fractions contained detectable levels of CD4 as measured by a solid phase immunoassay with a detection limit of 1 ng/mL. Thus, the inhibitory activity in milk cannot be accounted for by the presence of soluble CD4.

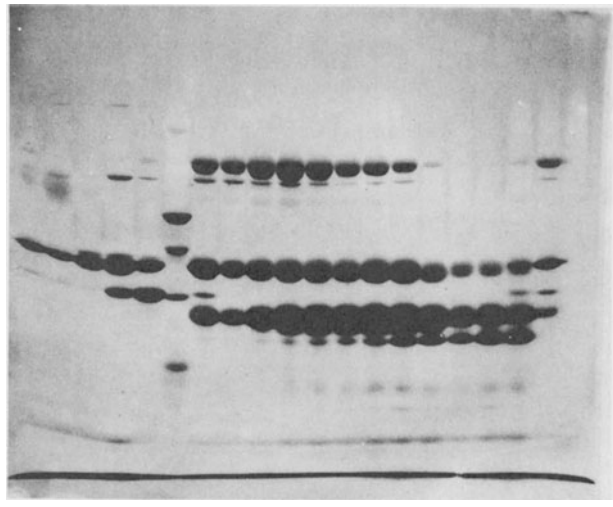

Figure 8. Polyacrylamide gel electrophoresis of the isoelectric focussing subfractions of human milk macromolecules. The active fractions, found in lanes 18 and 19 from the left, display four bands (the top four) that one enriched or specific to these lanes; the uppermost 3 of these bands are glycosylated, as indicted by Periodic Acid Schiff reagent (PAS). The band at the very top of the gel stains most heavily by PAS, consistent with the behavior of milk mucins or glycosaminoglycans.

The above protein fractions from the Rotofor apparatus were separated by polyacrylamide gel electrophoresis using a 4 to 12% gradient (Fig. 8). Those fractions which displayed high CD4-inhibitory activity (lanes 18 and 19 from the left) contained four characteristic bands revealed by silver stain. The gel was also stained for carbohydrate by PAS reagent, showing that the top three of these bands were glycosylated. The band at the top of the gel stained faintly with silver stain, but was much more pronounced with PAS staining, indicating the presence of a very large glycosylated material. Lane 6 contains molecular weight markers of 200, 116, 97, 66, and 45 kDa.

DISCUSSION

The data indicate that human milk strongly inhibits the interaction of CD4 with HIV envelope protein and with monoclonal antibodies which recognize epitopes involved in HIV binding. The activity was highly prevalent in milks obtained from geographically and ethnically diverse populations. That the activity was not found in bovine and some other milks suggests that the inhibitory activity is due to a specific interaction with human CD4 molecules rather than a non-specific effect of milk on solid phase binding. We could not detect CD4 antigen directly in the milk specimens, indicating that the blocking effect was not due to the presence of soluble CD4 in human milk.

The activity of human milk was largely confined to the macromolecular fraction. Chromatographic analyses indicated that the active material has negatively charged regions, but has an isoelectric point in the 9.3-9.6 range. Enzymatic analysis indicated resistance to trypsin, but sensitivity to sulfatase from *A. aerogenes* and, to a lesser extent, to limpet sulfatase. These findings indicate that the active material may be a sulfated macromolecule. This finding is of interest in light of the recognized inhibitory activity of dextran sulfate and other sulfated glycoproteins on the binding of HIV to CD4 and on cellular replication of HIV virions[18].

A striking property of the active milk protein is its high isoelectric point, a property which contrasts with the acidic nature of lactalbumin, lactoglobulin, and other milk proteins[19]. This high isoelectric point, the stability of the protein to heat denaturation in water, its sensitivity to methanol denaturation at ambient temperatures, and its tendency to aggregate and be retained in a 0.45 μm filter are characteristics consistent with those of molecules containing regions that resemble hydrophobic proteolipid[20-22]. The active material in human milk may thus prove to be a sulfated membrane glycoprotein or proteoglycan which is capable of binding to CD4 and preventing the attachment of HIV envelope glycoprotein. The material could also be a glycosaminoglycan that associates with a membrane protein. The purification and isolation of this milk constituent and the elucidation of its mechanism of action should be the subject of additional investigations.

We found inhibitory activity in human milks but not in human sera. A similar activity has been described in human saliva[23]. This distribution of inhibitory activities may be related to the epidemiology of HIV infection, in which a high rate of HIV transmission is linked to parenteral exposure, in contrast to the relatively low rate by fecal-oral contact.

We used a solid phase assay for human milk analysis, rather than a tissue culture neutralization assay, because milk is toxic for many cultured cells, and because of difficulties encountered in reproducing neutralization assays with milk components. The biological relevance of our system is supported by the fact that dextran sulfate and other glyco-polymers with known anti-HIV activity also inhibit the binding of CD4 to OKT4A and related monoclonal antibodies. In addition, the active milk fraction inhibited the binding of purified HIV envelope protein gp120 to CD4 suggesting the possiblity of a universal inhibitory effect against the strains of HIV-1 and HIV-2[24]. The effect of the milk CD4-inhibitory activity on the replication of strains of HIV should be the subject of various additional investigations.

Numerous studies have indicated a protective effect of breastfeeding on infant morbidity and mortality, especially in children living in less developed countries[8,25-28]. However, the role of breast feeding in the management of children born to HIV-infected mothers remains controversial in light of anecdotal reports of apparent postnatal HIV transmission from mothers to breast-fed children and of the rare isolation of HIV from human milk[3-6]. Our data indicate that human milk, but not bovine milk, contains a macromolecule which may inhibit the binding of HIV to CD4 receptor molecules; thus, human milk is unlikely to be an efficient medium for the vertical transmission of AIDS. Our findings support the continued study of the role of breastfeeding in the health of HIV-infected children, so that the risks and benefits of breastfeeding in populations at risk for HIV can be evaluated.

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BREAST MILK TRANSMISSION OF CYTOMEGALOVIRUS (CMV) INFECTION

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Human CMV is highly species-specific, and humans are believed to be its only reservoir[1]. Seroepidemiologic surveys have found CMV infection in every population that has been tested including remote Indian tribes in the Amazon basin who lacked evidence of past measles or influenza infections[2,3]. CMV infection is endemic and without seasonal variation. Climate does not affect the prevalence of the infection, and there are no known vectors in the natural transmission cycle. The prevalence of CMV infection increases with age, but according to geographic, ethnic, and socioeconomic factors. The pattern of acquisition of this infection varies widely among populations[4]. CMV is acquired earlier in life in developing countries and among the lower socioeconomic segments in developed countries. Differences between populations can be particularly striking during childhood, with rates of seropositivity in four to six year old children varying from less than 10 percent in Great Britain and certain populations in the United States, to nearly 100 percent in Africa and the South Pacific[4]. Presumably, these significant differences are the reflection of factors that account for increased exposure to CMV such as crowding, breastfeeding, sexual practices, and certain child rearing practices. Transmission occurs by direct or indirect person to person contact. Sources of virus include urine, oropharygeal secretions, cervical and vaginal secretions, semen, milk, tears, and blood[5,6]. CMV is not very contagious; the spread of infection requires close or intimate contact with infected secretions. Restriction enzyme analysis of CMV DNA has been used to demonstrate person-to-person spread of virus in situations where close contact occurs such as breastfeeding, sex partners, toddlers in day care centers, and parents or caretakers of infected toddlers[6]. Under special circumstances fomites may also play a role as CMV has been shown to retain infectivity for hours on plastic surfaces and CMV has been isolated from randomly selected toys and surfaces in day care centers[7,8].

CMV is a member of the herpesvirus family and like other members can cause primary and latent infections, the latter of which can reactivate. In addition, because of an enormous diversity in genetic strains, CMV can also

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cause reinfections[6]. Given the property of latency, reactivation and reinfection along with the numerous sources of virus, it is not surprising that CMV has a number of different routes of transmission. These include vertical transmission resulting in congenital infections, perinatal transmission via infected genital secretions, and through infected breast milk, respiratory spread through intimate contact, venereal transmission and transmission via infected blood products. From a public health standpoint, the congenital and perinatally acquired infections represent important routes of transmission[6]. In the Unites States, congenital infections occur in 0.2 - 2.4 percent of all live births averaging about 1 percent. The perinatal infections are even more prevalent on a worldwide basis. Using virus isolations as a means of diagnosis, perinatal infections have been found in 7 - 60 percent of infants by six months of age in various populations throughout the world[9]. In areas of high prevalence (30 - 60 percent) infection is associated primarily with universal breastfeeding practices rather than crowding or poverty.

Diosi, *et al.* were the first to report the isolation of CMV from human milk[10]. The virus was found in 1/49 milk specimens collected from parturient women. Hayes *et al.* subsequently reported the isolation of CMV from 14 milk specimens collected from 63 (27 percent) of seropositive women[11]. In addition, that group found that virolactia was more common in samples collected more than one week postpartum (50 percent) than in those collected during the first week (11 percent). About ten years ago, our group at Alabama did a large scale cross-sectional study to better understand the relationship between the excretion of CMV in breast milk and transmission to the infant[9]. Two hundred seventy-eight women and their offspring were included in this study. Thirty-eight of the 278 women were found to excrete CMV at least once in the colostrum or milk for a rate of 13 percent. CMV was found more often in milk (25/70 specimens; 36 percent) than in colostrum (20/244 specimens; 8 percent). This difference was highly significant with a P value of <0.001. To define whether CMV could be transmitted by breast milk, the infants of 28 women shedding CMV only in breast milk were studied prospectively; 19 of them were breast-fed and nine were exclusively bottle-fed. None of the infants had been infected *in utero* or received blood transfusions. Whereas none of the nine bottle-fed infants became infected, 11 of the 19 infants (58 percent) fed infected breast milk acquired CMV infection. These infants became viruric between four weeks and four months of age, except for one who became viruric at nine months -- three months after his mother had her first CMV positive milk specimen. In all infected infants, infection was established in the presence of considerable levels of maternally derived neutralizing antibody (geometric mean titers [GMT] = 48; range, 8-256). There was no significant demographic differences to explain the difference in the transmission rates between the two groups of women.

In these studies, virus infection in various sites of the mother was related to perinatal acquisition of infection by the infant. Association between maternal excretion of CMV from various sites and the subsequent acquisition of infection by the infant (from birth to six months of age) is summarized in Table 1. When fed infected breast milk or exposed to CMV in the genital tract during delivery, the rate of infection in young infants was high and nearly the same – 58 percent in those fed infected breast milk and 57 percent in those exposed to CMV during delivery. Lesser transmission rates were noted when viral excretion occurred in the maternal genital tract in the first or second trimester of pregnancy. Notably, perinatal infection was not associated with

isolated maternal excretion of CMV into the urine or the saliva. Only one of the 136 infants born to women who did not excrete CMV became viruric at four months of age; this baby was amongst the 11 who were breast-fed. Unfortunately, in this particular case, only two specimens of milk were studied at two and 91 days postpartum; both were reported negative for CMV.

Table 1. Association Between Maternal Excretion of CMV from Various Sites and Subsequent Infection of the Infant

Only site of maternal excretion	No. of infants infection/ No. exposed[a]
Breast milk	
Breast-fed infant	11/19 (58)
Bottle-fed infant	0/9 (0)
Cervix	
3rd trimester & postpartum	8/14 (57)
3rd trimester	18/68 (26)
1st & 2nd trimester	1/8 (12)
Urine[b]	0/11
Saliva[c]	0/15
Nonexcreting women	
Bottle-fed infant	0/125
Breast-fed infant	1/11 (9)

[a] Figures in parentheses denote percentages
[b] Late 3rd trimester
[c] Excretion 1 day postpartum

In this particular study, specific IgA antibodies (titer ≥ 4) were found in 11 of the 35 (31 percent) milk specimens examined. Antibody was detected in 10/22 (45 percent) of the infected samples and only 1/13 (7 percent) of the uninfected specimens. Thus, the lack of infectivity was significantly related to the presence of IgA antibody in milk ($P = 0.027$). To investigate whether the lack of IgA antibody in the infected samples could be explained by the formation of immune complexes between CMV antigens and antibody, infectivity titers were assessed by means of a plaque assay in ten CMV-infected milk specimens before and after incubation with anti-human immunoglobulins and C3. Immune complex formation could not be incriminated by these approaches. Significant reduction of infectivity was detected in only one specimen after incubation with anti-C3 but not with anti-immunoglobulins. This study rather clearly established the fact that CMV could be transmitted to the young infant via infected breast milk as well as infected genital secretions, as was previously shown.

In order to establish the importance of the routes of transmission for perinatally acquired infection, a prospective study was undertaken by our group at Alabama. The study included 58 mother-baby pairs who attended the clinic for at least two months[12]. Vaginal secretions, urine, saliva, and breast

milk from the mother and urine and saliva the baby were obtained within five days of delivery and when possible at 3, 6, 14, 24, 36, and 52 weeks after birth. Samples of blood for immunologic studies were collected from the mother at enrollment, 6, 24, and 52 weeks postnatally. Intrauterine CMV infection was excluded by failure to isolate CMV from the urine and saliva collected within five days of birth. Postnatal infection of the infant was defined by the initiation of viral excretion after three weeks postnatally. Of the 58 mothers, 17 (29 percent) were seronegative, and 41 (71 percent) were seropositive for CMV antibody at enrollment. These two groups did not differ in demographic characteristics or in length of follow-up or lactation. None of the susceptible women seroconverted during the course of the study. In the seropositive group, the geometric mean titer of serum ACF antibody was 60 and for neutralizing antibody it was 108.

None of the seronegative women shed CMV from any site at any time during the study. Among the 41 seropositive women, CMV was shed in the breast milk in 13 women (32 percent), into the vagina in four (10 percent), into the urine in three (7 percent), and into saliva in one (2 percent). Four women shed virus into more than one site (two into milk and urine; one into milk, vagina, and saliva; and one into vagina and urine). In all cases, viral shedding was intermittent irrespective of the site involved. Even though frequency of sampling was less, CMV excretion occurred significantly more often into breast milk than into other sites. CMV was shed into milk most frequently between two and 12 weeks postpartum. During this period 18/50 milk samples collected from 13/31 mothers (42 percent) contained virus. In contrast, only 2/40 women (5 percent) shed virus into colostrum and 1/10 (10 percent) into milk after 12 weeks. There were no significant demographic differences between mothers who excreted virus and those who did not.

None of the infants born to seronegative mothers became infected by CMV during the course of the studies. In marked contrast, 12 of the infants or 30 percent born to the 41 seropositive mothers became infected between birth and eight months of age (nine between birth and 4 months, and two between 2 and 6 months, and one between 5 and 8 months). Infants who breast-fed for longer than one month became infected more often ($P = 0.015$). Infection of the infants occurred significantly more often ($P = 0.007$) when the infants were fed by the mothers who shed virus into milk (9/13, 69 percent) than when infants were breast-fed by mothers who shed CMV from other sites (0/3), or by mothers in whom there was no demonstrable viral excretion (3/25, 12 percent). Clearly, this study indicated that transmission of virus via breast milk is very likely the most important source for perinatally acquired infections by the infant. Natal transmission is second to breast milk transmission as a cause for perinatal infection.

As noted in Table 2, among 40 milk specimens collected from 14 women who were consistently seronegative for anticomplementary immunofluorescence and neutralizing antibodies, only four samples obtained from two women contained substances that neutralized CMV with titers ranging from 4 to 16. Although neutralizing substance was detected more often in milk collected from seropositive women, it was demonstrated in less than 50 percent (11/24) of the cases, and then only intermittently and infrequently in 15/77 samples (19 percent). In a few instances in which milk samples were obtained at frequent intervals, the neutralizing substance apparently persisted for a brief period (days) in contrast to the persistent systemic antibody response. In addition, the titers of milk neutralizing

substance were low, ranging from 4 to 64 (GMT 11), especially when compared with paired serum neutralizing antibody titers which ranged from 8 to 256 (GMT 108). There was not correlation between the intermittent appearance of neutralizing substance in milk and maternal viral excretion or acquisition of infection by the infant, Table 2. Likewise, there was no correlation between the presence of virus and the presence or amount of neutralizing substance in individual milk samples. Neutralizing substance was found in 3/21 (14 percent) of virus positive samples and 12/56 (23 percent) of virus-negative samples.

No CMV immunofluorescent (IF) antibodies were detected in 18 samples of milk collected from eight seronegative women (Table 2). IF antibodies were detected in milk obtained from seropositive mothers significantly more often than neutralizing substance (9/11 women [$P = 0.4$] and 15/30 samples [$P < 0.01$]). IgA, IgG, and IgM antibodies were detected in decreasing order. These IF antibodies were detected intermittently, could not be correlated with maternal viral excretion, acquisition of infection by the infant, or the presence or absence of virus in individual milk samples. Even though IF antibodies and neutralizing substances were not concordant among individual milk samples, they were found more frequently in association with neutralizing substance, 9/11 samples, than in the absence of neutralizing ability, 13/29 samples ($P = 0.035$).

Table 2. Relation of Milk Cytomegalovirus (CMV)-Neutralizing Substance and Immunofluorescence (IF) Antibodies to Maternal Viral Excretion and Acquisition of Infection by Infant

Maternal status	Neutralizing substance	IgM	IgG	IgA	Any IF antibodies
Seronegative	2/14	0/8	0/8	0/8	0/8
Seropositive	11/24	2/11	4/11	6/11	9/11[a]
CMV milk excretion	4/12	1/6	2/6	2/6	4/6
No CMV milk excretion	7/12	1/5	2/5	4/5	5/5
Infants infected	5/11	1/5	2/5	2/5	4/5
Infants uninfected	6/13	2/6	2/6	4/6	5/6

[a] Versus neutralizing substance ($P = 0.04$, Fisher exact test).

These studies indicate that consumption of infected breast milk is the most common source for perinatally acquired CMV infection in breast-feeding societies. Apparently, infection of breast milk occurs most often as a result of reactivation of latent maternal virus rather than primary infection. The exact location of the latently infected cells in the seropositive women is unclear, i.e. breast cells versus white blood cells. Neither systemic nor local maternal immunity can completely control reactivation of CMV in the breast

and likely not in other sites. Indeed, transmission of infection to the suckling infant occurs in the face of maternal immunity, at least systemic immunity. Though placentally acquired maternal antibody does not protect the infant from infection, it apparently ameliorates the virulence of the infection as it does with transfusion acquired CMV infection in young infants. Rarely perinatally infected infants develop an obstructive form of CMV pneumonia in the first six months of life, but in the vast majority of cases the infection remains subclinical, albeit chronic in nature. Rather than being detrimental then, infection by breast milk could actually be useful by stimulating natural immunity in female infants and conceivably protecting against virulent congenital infection during the child bearing years. Contrarily, feeding infected breast milk from donors other than the mother could prove to be problematic, especially in small, seronegative premature infants. CMV can survive in stored breast milk for a number of days, depending on how the milk is treated[13]. Another area of concern is the transmission of CMV to HIV-infected or other immunologically defective infants. All of the above problems are in need of resolution.

Using a variety of *in vitro* techniques, human milk has been shown to possess many elements that would provide both specific and non-specific antiviral or antimicrobial activity. Even though CMV replicates in the breast, it is apparently a poor immunogen for stimulating local antibody production, in contrast to its ability to stimulate prompt systemic antibody production. Substances capable of neutralizing CMV in milk are found only sporadically and then in small amounts when compared with serum, IgA, IgG, and IgM antibodies directed against CMV are also sporadically found in milk with IgA predominating. These antibodies must have functions, as yet unknown, other than neutralization of virus, as they appear more often than neutralizing substances. The presence of neutralizing substances, IF antibodies, or their combination cannot prevent reactivation of CMV in the breast or its transmission to the suckling infants via milk.

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ANTIBODY RESPONSES TO CYTOMEGALOVIRUS IN SERUM AND MILK OF NEWLY DELIVERED MOTHERS

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INTRODUCTION

The present study was conducted to study IgG and IgA antibody responses to cytomegalovirus (CMV) in serum and milk. Another more practical objective was to find out whether or not an analysis of a milk sample could be used instead of the classical testing of a serum sample to ascertain a latent CMV infection. As a comparison, a virus with a different mode of transmission and strategy of survival was studied. For this purpose adenovirus was chosen.

MATERIAL AND METHODS

One hundred ninety-nine mothers delivered at the Department of Obstetrics at University Hospital of Umeå participated in the study. Their median age was 28 years (range 17-40 years). A blood sample was obtained from all and a colostrum sample from 144 mothers. From 15 of them, mature milk was collected. Milk samples were centrifuged at 3000 rpm for 30 min and the fat layer and cell pellet were discarded. Milk supernatants and sera were stored frozen at -20°C until analysed.

Antigens

Each vial of CMV antigen and control antigen (SBL, Stockholm, Sweden) was diluted in 2 ml extraction buffer and sonicated with two 30 sec pulses on ice. The working dilution was 1/80 as titrated against a positive reference serum.

The adenovirus antigen originated from two local strains serotypes 3 and 5. The viruses were propagated on A 549 cells in serum-free medium in the laboratory. After extraction with 0.25% Triton X 10, freeze-thawing, sonication, and clarification at 3000 rpm for 15 min, the supernatants of the two antigens were titrated against positive reference sera. The two antigens

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were pooled and used in 1/100 dilution. A control antigen of non-infected cells was produced in a similar manner.

ELISA Procedure

In every run of the test fresh-coated plates were used. Polystyren microplates (NUNC, Århus, Denmark) were sensitized with appropriate concentrations of virus and control antigens for 2 hours at 37°C. After coating, the plates were saturated with 0.5% bovine serum albumin. Samples and controls were analyzed in the following dilutions: IgG in serum 1/100, IgG in milk undiluted; IgA in serum 1/50, and IgA in milk 1/100. Samples were incubated overnight at 4°C. Conjugates were incubated for 1 h at 37°C. The IgG conjugate labeled with alkaline phosphatase was diluted 1/500 (Orion, Helsinki, Finland). The IgA conjugate labeled with peroxidase was diluted 1/750 (Sigma, St. Louis, USA). The IgG substrate was developed for 30 min at 37°C and the IgA for 10 min at room temperature. The plates were read immediately in a Titertek spectrophotometer at 405 and 492 nm, respectively. A significant antibody response was defined by a difference of absorbance values between virus and control antigen rows by $\geq$0.10 for IgG and $\geq$0.20 for IgA.

RESULTS

IgG Antibodies to CMV and Adenovirus

As is shown in Table 1, there was a close correlation between sero- and milk-positivity to CMV; 71.4% vs 70.8%, respectively. The corresponding figures for adenovirus were 95.9% vs 74%. Table 2 shows the coefficient of correlation between absorbance values in serum and colostrum samples. As can be seen, there was the same high positive correlation of IgG levels for both viruses ($r = 0.79$, $p<0.001$). CMV IgG levels were unchanged in mature milks at 1, 2 and 3 months as compared to colostrum (data not shown).

IgA Antibodies to CMV and Adenovirus

As is shown in Table 1, there was an inverse pattern of IgA in colostrum and serum samples of the two viruses. CMV IgA was four times more prevalent in colostrum (50.3%) than in serum (11.7%). Prevalence of adenovirus-specific IgA in colostrum (43.3%) was half of that in serum (90.3%). In mature milk samples the CMV IgA levels were significantly lower than in colostrum; at 1 month ($p<0.001$), 2 months ($p=0.004$), and 3 months ($p=0.001$) (data not shown).

Relation Between Presence/Absence of CMV IgG in Colostrum and Serum and Relation Between CMV IgA in Colostrum and CMV IgG in Serum

The concordance for presence/absence of CMV IgG in colostrum samples and sera was 96.5% (Table 3). Four of the five discrepant samples had absorbance values in the range of 0.07 to 0.09; i.e., just below the cut-off. The concordance for CMV IgG in sera and CMV IgA in colostrum samples was low; 59%. Fourteen sera negative for IgG and IgA and IgG in colostrum, were positive for IgA in colostrum (Table 3).

Table 1. Sero- and Lacto-positivity of CMV and Adenovirus IgG and IgA Antibodies

	IgG		IgA	
	CMV	Adeno	CMV	Adeno
Sero-positivity	71.4%	95.9%	11.7%	90.3%
Lacto-positivity	70.8%	74.0%	50.3%	43.3%

Table 2. Coefficients of Correlation Between Absorbance Values of Serum IgG and Colostrum IgG, and Between Serum IgG and Colostrum IgA Against CMV and Adenovirus

	IgG	IgA
CMV	r = 0.79 (p<0.001)	r = 0.21 (p<0.05)
Adenovirus	r = 0.79 (å<0.001)	r = 0.50 (p<0.001)

Table 3. Relation Between Presence/Absence of IgG in Colostrum and Serum and Relation Between CMV IgA in Colostrum and CMV IgG in Serum

Serum IgG	Colostrum IgG +	Colostrum IgG −
+	100	3*
−	2*	39

Concordance: 96.5%
*Abs-values: 0.07-0.09-0.00
**Abs-values: 0.07-0.09

Serum IgG	Colostrum IgA +	Colostrum IgA −
+	58	44
−	14*	27

Concordance: 59%
*Re-analysis at 1/25 dilution yielded 2 further sera positive; Abs-values 0.22 and 0.25. The remaining had Abs-values $\leq$0.01.

DISCUSSION

CMV gives, as do the other members of the herpesviruses, a persistent infection. In general, the virus is dormant and of trivial importance for the infected individual. Transmission occurs by close physical contact. Most individuals are infected during the first years of life. At birth 0.5-1.0% newborns excrete virus in urine and at 1-4 years of life 40% are seropositive[1]. This indicates that pregnancy and lactation are associated with reactivation of the virus[2,3]. Accordingly, one study showed that as many as 70% of seropositive mothers excreted infectious virus in their milk[4]. Apparently, feeding with breast milk represents a major way of transmission of the virus. The infection is subclinical and most probably modified by transplacentally and milk acquired antibodies. Feeding with fresh, not heat-treated, milk may therefore be a risk for an infant born to a seronegative mother. In such circumstances a knowledge of the immune status of the milk donor is needed. In practical terms an analysis of the milk sample is most convenient. This study shows that an analysis of CMV IgG in undiluted defatted milk is reliable for that purpose.

The high concordance between serum and colostrum IgG regarding both presence and absence of antibodies and levels of antibodies indicates that milk IgG is derived from serum. The serum antibody levels were at least 100 times higher than the milk levels, which is an expected concentration gradient between serum and secretions.

When prevalences of CMV and adenovirus IgA antibodies were compared, a clear difference was noted for the two viruses. For CMV there was a four-fold higher prevalence of antibodies in colostrum than in serum (50.3% vs 11.7%), compared to adenovirus to which antibodies were twice more often present in serum than colostrum (90.3% vs 43.3%). The difference may be explained by the different biology of the two viruses. In healthy individuals CMV remains dormant, which is indicated by the absence of specific IgA[5]. This is in contrast to herpes simplex virus which is frequently reactivated and consequently IgA has been reported to occur in 50% of blood donor sera[6]. The higher prevalence of CMV IgA in milk than in serum indicates that reactivation occurs locally in the mammary gland. A systemic reactivation in unlikely, as indicated by the low prevalence of IgA in sera and lack of clinical signs of infection in pregnant women. On the other hand, the high prevalence of IgA to adenovirus in serum indicates frequent reinfections. The presence of adenovirus IgA in milk may be a manifestation of a migration of lymphocytes from the gut to the mammary gland, because this virus replicates in the gut. CMV does not replicate in the gut, and hence milk CMV IgA is most probably not synthetized by migrating lymphocytes.

The established mode to determine a persistent infection with CMV is by testing for IgG antibodies in serum. In this study, we found that 14 mothers who were both IgG and IgA seronegative to CMV, had a specific IgA response in milk. One can question whether or not the IgA response was specific, or if the serum analysis was of a low sensitivity. To address the last question, the 14 sera were reanalyzed in a four-fold lower serum dilution (i.e., 1/25). Two of the samples were undoubtedly positive at 1/25 dilution. Obviously, some of them were discordant due to low levels of IgG in serum not detected at the 1/100 dilution. It is unlikely that non-specific reactions could explain the discordant result, since a control antigen was used for every sample tested. A probable explanation would be that the virus had been dormant in the

mammary gland without being replicated for a very long period. Therefore, a systemic immune response had not been maintained. A recent study using the polymerse chain reaction (PCR), to amplify viral DNA showed that a substantial proportion of seronegative blood donors had CMV DNA in their blood mononuclear cells[7]. This finding indicates that seronegativity to CMV may not exclude the possibility of a latent infection. The PCR study indirectly supports the finding of CMV-specific IgA antibodies in milk as being a reality in seronegative subjects.

A rather unexpected result was the stability of IgG antibody levels in mature milk compared to the significant decrease of IgA in mature milk. The decrease is most likely explained by a dilution effect of greater volumes of mature milk on the locally produced IgA. It is also additional evidence for the passive transfusion of IgG from serum to milk. The IgG leaks from serum to milk along a concentration gradient, that is not influenced by the volume of the milk.

CONCLUSIONS

The present study indicates that:

1) CMV is reactivated locally in the mammary gland of the lactating mother,
2) CMV IgG as tested in undiluted milk is as reliable as a serum test for determination of immune status to CMV,
3) CMV IgG levels remain unchanged in mature milk, whereas CMV IgA levels decrease significantly, and
4) lack of CMV IgG in serum may not exclude a dormant infection of CMV in the mammary gland.

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PROTECTION OF NEONATAL MICE FROM FATAL REOVIRUS INFECTION BY IMMUNE SERUM AND GUT DERIVED LYMPHOCYTES

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INTRODUCTION

Murine reovirus infection has been used as a model to define the mechanisms of immunity to enteric viruses. Previous studies have shown that both cellular[1-5] and humoral[4,6-8] immune responses develop in reovirus-infected mice. These specific immune responses, and particularly mucosal immune responses, probably play a pivotal role in containing and resolving enteric reovirus infection.

Passive immunity to reovirus-induced meningoencephalitis has been characterized by several investigators. A report by Gaulton *et al.*[9], described maternal transfer of immunity to serotype 3-induced meningoencephalitis. The investigators found that female mice immunized with either reovirus serotype 3/Dearing (3/D) or type 3/D-specific anti-idiotope antibody protected their neonatal offspring against the development of neurologic abnormalities and death following intramuscular (i.m.) infection of their neonates with type 3/D reovirus. Studies have also shown that polyclonal rabbit anti-reovirus antiserum as well as murine anti-reovirus monoclonal antibodies specific for the virus attachment polypeptide are capable of protecting neonatal mice from serotype 3/D-induced meningoencephalitis following intracerebral (i.c.) or i.m. inoculation[10].

We have used a murine isolate of serotype 3 reovirus (designated Type 3 clone 9 or T3c9)[11,12] to study the roles of various lymphocyte populations in protection against disease. Following oral inoculation of neonatal mice, T3c9 replicates in the small intestine and disseminates to the central nervous system[12,13]. The infection results in a highly lethal meningoencephalitis in non-immune neonates. A previous study[13] has demonstrated that monoclonal antibodies directed against the serotype-specific σ1 polypeptide can protect neonatal mice from systemic disease caused by oral (p.o.) inoculation of T3c9 reovirus. We have reported that immunity can be

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passively transferred from immune mothers to pups following maternal immunization with either reovirus 3/D or type 1/Lang (1/L). Protection can be imparted either transplacentally or during suckling[14]. Furthermore, serum from adult mice that were immunized with either reovirus type 1/L or type 3/D can protect infected neonates from reovirus-induced meningoencephalitis.

One of the first lines of defense against enteric virus infections is the epithelium of the small intestine. Since reovirus invades the host through the gastrointestinal (GI) tract, we determined the capacity of passively transferred gut-associated lymphoid tissue (GALT) to protect neonates from lethal infection following peroral challenge with T3c9. We report here that reovirus-immune intraepithelial lymphocytes (IELs) and Peyer's patch lymphocytes (PPLs) can protect neonates from lethal infection. Analyses of virus titers in several tissues following infection indicate that in mice that received IELs, early infection was established and then cleared before neurologic signs of infection developed. These results suggest that mucosal humoral and cellular immune responses that are induced as a result of enteric priming with virus have the capacity to contain or limit lethal reovirus infection.

MATERIALS AND METHODS

Viruses

Reoviruses 1/L, 3/D, and T3c9[11,12] were obtained from B. N. Fields (Harvard Medical School, Boston, MA). Viral stocks were grown in L-cells and purified as previously described elsewhere[4,14] on CsCl gradients. A third passage stock of CsCl gradient purified reovirus was used for all immunizations and infections.

Mice

Adult BALB/cByJ mice were obtained from Jackson Laboratories (Bar Harbor, ME) and housed and bred in the animal facility in the Department of Biology at the University of Pennsylvania.

Immunization

Adult mice were immunized with 3×10^7 plaque forming units (pfu) of reovirus by the intraduodenal route (i.d.) 1 week before cell transfer as previously described[4].

Lymphocyte Preparation

IELs were isolated using a modified method from Dillon and MacDonald[15]. Briefly, PP and mesentery were removed from isolated intestines. The intestines were split longitudinally then cut into 2-4 cm pieces, and placed in Ca^{2+}, Mg^{2+} free Hank's balanced salt solution (HBSS). IELs were eluted with two 15 min incubations at 37°C with stirring in HBSS supplemented with 0.1mM EDTA followed by 4-5 15 min incubations in HBSS supplemented with 5% horse serum. The eluted cells were passed through glass wool columns and lymphocytes were separated from epithelial cells by discontinuous Percoll density gradient centrifugation. PPLs were isolated from disassociated Peyer's patches by mechanical disruption.

Cell Transfer and Infection

One day old mice received 10^6 lymphocytes intraperitoneally. Cells were injected in a total volume of 50µl intraperitoneally using a 26 gauge needle. The following day the neonatal mice were orally infected with 3×10^6 pfu of T3c9. Virus was suspended in 35µl of borate-buffered saline containing gelatin, pH 7.4 (gel saline) and neonates were inoculated using a piece of PE10 tubing attached to a 30 gauge needle and 1 ml syringe.

Tissue Handling and Preparation

At various times after infection, neonatal mice were sacrificed by decapitation and livers, small intestines, and brains were removed for virus titration. Virus titers were determined using a plaque assay as previously described[16] and protein determinations were performed using a micro-Lowry method. Virus titers are expressed as $\log_{10}$ pfu/mg protein. Brains from 22 day old (20 days post infection) mice were prepared for pathologic examination by fixing whole brains in Bouin's solution for 3 h and cutting coronal sections. Tissues were processed and stained with hematoxylin and eosin at the pathology department of the Hospital of the University of Pennsylvania. Pathologic changes in brains were assessed by evaluating sections in a blinded manner.

RESULTS

Infection of Neonatal Mice with Reovirus Type 3 Clone 9

Neonatal mice that were infected perorally with 3×10^6 pfu of reovirus T3c9 developed a highly lethal meningoencephalitis. Previous experiments indicated that the peroral LD_{50} for this virus was approximately 10^4 pfu. Thus, the challenge dose was approximately 300 times the LD_{50}. In these experiments, 3×10^6 pfu routinely killed 75-100% of infected control mice.

Most non-immune neonates developed detectable virus titers in brain tissue by day 4 and high virus titers were found in all brains from non-immune pups by day 7 (mean titer $\log_{10} = 4.9 \pm 0.3$ /mg protein). The mean time to death was approximately 10 days. Pathologic changes in the brains of survivors at day 20 included foci of necrosis and neuronal cell dropout that were found throughout the cortex, hippocampus and medulla. Surrounding neurons exhibited oval or spherical hyaline inclusions. There was moderate to severe perivascular cuffing of capillaries by mononuclear cells and occasional polymorphonuclear leukocytes. The leptomeninges were severely infiltrated with similar inflammatory cells. These findings are consistent with previous reports of reovirus-induced meningoencephalitis following i.c., i.m., or p.o. inoculation[9,13,17,18].

Passive Protection Against Fatal Reovirus Infection

Groups of female mice were immunized with two oral inoculations of 1×10^7 pfu of 1/L reovirus or 3/D reovirus given two weeks apart. Two weeks after the second immunization, reovirus immune female mice were mated with non-immune male mice. The resulting pups were orally infected with T3c9 two days after birth. Although 10/13 control animals died, all neonates from immune dams survived the infection (Table 1).

Table 1. Passive Transfer of Protection Against Lethal Infection

Maternal immune status[a]	# Infected[b]	#Survived	% Survival
Non-immune	13	3	23
Type 1/L	18	18	100
Type 3/D	17	17	100

[a] females were given two oral inoculations of 3×10^7 pfu of reovirus one week apart. Two weeks after the second inoculation, the mice were mated with non-immune male mice.

[b] two day old neonatal mice were orally inoculated with 3×10^6 pfu of T3c9.

Foster-Nursing

From the previous experiments it was not clear if the protection was mediated by passive transfer of antibody *in utero* or in milk that the neonates obtained by nursing on immune mothers. Experiments were performed to determine whether one or both mechanisms were responsible for protection. Female mice were immunized with two p.o. inoculations of type 3/D and then mated. The offspring from this group were delivered by cesarean section to ensure that newborns received no milk from immune mothers. The cesarean delivered pups were placed in cages with lactating non-immune female mice. Two days later the mice were infected with virus. All the infected mice (n=4) survived and displayed no evidence of meningoencephalitis by pathologic examination of brains at day 20. The converse experiment was also performed. After natural delivery, pups (n=10) from non-immune females were caged with lactating serotype 3-immune females and infected two days later. These pups also survived and no evidence of pathology was found at day 20 (Table 2).

Protection Against Fatal Reovirus Infection by Immune Serum

One to two day old mice were given 30 µl of type 1/L-immune, type 3/D-immune or non-immune serum intraperitoneally. The neonates were orally inoculated with 3×10^6 pfu of T3c9 one day later. Both type 1/L-immune and type 3/D-immune serum protected against lethal infection. Non-immune serum had no detectable protective activity. No pathologic changes were observed in brain sections from randomly selected survivors 20 days after infection, indicating that immune serum protected against reovirus-induced meningoencephalitis.

Protection Mediated by Transferred IELs and PPLs

One to two day old mice were given intraperitoneal injections of 10^6 immune or non-immune IELs and then were infected with T3c9 24 h later. The results in Table 3 are compiled from three separate experiments and demonstrate

Table 2. Protection in Foster-Nursed Neonates

Mothers/neonates	% Survival	% with Pathology
Normal/normal	33%	100%
3/D-Immune[a]/normal	100%	0%
Normal/3/D-immune[b]	100%	0%

[a] females were immunized with two p.o. inoculations of 1 x 10^7 pfu of reovirus type 3/D one week apart. Mice were mated two weeks after the second immunization.

[b] neonates were derived by cesarean section to preclude the possibility that milk was obtained by nursing following birth.

Table 3. Passive Transfer of IELs Protects Against Lethal Infection[a]

Cells[b]	# Infected[c]	# Dead (%)	# Pathology
No cells	20	15 (75)	4/4
Immune IELs	21	3 (15)	10/18
Non-immune IELs	9	5 (55)	4/4

[a] results are from 3 separate experiments.

[b] immune IELs were derived from donor mice that were given one i.d. inoculation of 3 x 10^7 pfu of reovirus 1 - 2 weeks before cell transfer. Non-immune IELs were derived from non-immune adult mice.

[c] one to two day old neonates received either non-cells (No cells) or an i.p. injection of 10^6 IELs. Twenty four hours later the neonates were orally inoculated with 3 x 10^6 pfu of T3c9.

that IELs derived from reovirus-immune mice could protect neonates against death when the cells were given prior to infection. Non-immune IELs appeared to mediate slight, and variable protection. In addition to IELs, immune PPLs could protect neonates against the lethal effects of infection (data not shown). The protective cells were serotype cross-reactive because both IELs and PPLs from serotype 1/L-immune mice protected against lethal serotype 3 disease.

Virus Titers in Protected Mice

To determine the site of virus containment, virus titers in small intestines, livers, and brains of infected mice were quantitated at 4, 7, and 10 days after infection in mice that received immune or non-immune IELs. At 4 and 7 days after infection, significant virus titers were found in all tissues tested. By day 10 however, virus titers were significantly diminished in all tissues tested, particularly in the brains of infected mice that received reovirus-immune IELs (data not shown). Thus, systemic viral clearance is associated with survival in mice that received reovirus-immune IELs.

DISCUSSION

Mucosal immunity may play a pivotal role in mediating protection against pathogens that normally gain entry to the host through the GI tract. Because the normal portal of entry for reovirus is the gastrointestinal tract, infection by the oral route allows an in depth characterization of mucosal immune mechanisms. The results in this report demonstrate that humoral and cellular components of mucosal immunity can protect reovirus-infected neonates against a lethal outcome.

It was determined that the immune response induced in adult mice following immunization with active reovirus can confer protection to offspring. This passively transferred protection against fatal infection is serotype cross-reactive. Reovirus type 1-immune females could confer immunity to pups challenged with reovirus type 3 virus[14]. As noted previously[14] our related findings concerning intestinal reovirus infection are similar to those reported by Offit and Clark[19,20] using a rotavirus model of infection. However, in contrast to our findings, peroral rotavirus immunization of female mice induced an immune response which, by passive transfer via nursing, failed to protect their neonates from infection with heterotypic rotavirus. In their model, diarrhea resulting from destruction of absorptive epithelial cells indicated virus replication in the gastrointestinal tract. In our studies, immunization with heterotypic virus protected against the development of CNS disease but not virus replication in the small intestine.

Because mice that were born to dams previously immunized p.o. with reovirus type 3 manifested no detectable virus in any tissue tested[14], it is possible that a specific secretory IgA response to the homotypic virus resulting from p.o. immunization of dams provides the most potent protection. Foster-nursing experiments in which milk from immune dams could protect pups born to non-immune dams further suggest that secretory factors such as IgA antibodies may be sufficient to protect against death and/or the

development of severe pathology. This interpretation is consistent with work from our laboratory[4] that intraduodenal application of reovirus in adult mice markedly increased the frequency of IgA memory precursor cells in Peyer's patches. It may be possible to determine the protective effects of IgA anti-reovirus antibodies by treating non-immune dams with monoclonal IgA anti-reovirus antibodies and determining if these antibodies appear in milk and protect nursing neonates against infection. Preliminary results indicate that non-neutralizing IgA monoclonal antibodies raised against type 1/L virus fail to protect against death, but further studies are in progress. In addition to IgA antibodies, it is possible that non-IgA immunoglobulin isotypes were transferred to neonates during suckling and absorbed before gut closure.

Transplacental transfer of antibody from immune dams to neonates is also protective as indicated in the results of the foster-nursing experiments. Pups delivered by cesarean section from a reovirus type 3-immune mouse did not develop meningoencephalitis following infection when nursed by a non-immune, lactating dam. Thus, transplacental antibody, as well as factors obtained during suckling, can protect neonates against disease.

Virgin *et al.* [10] and Tyler *et al.*[13] previously showed that parenterally transferred hyperimmune rabbit immunoglobulin and mouse monoclonal antibodies specific for the neutralizing epitope on the σ1 polypeptide could protect against serotype 3/D-induced meningoencephalitis. Our finding that passive transfer of immune serum could protect against disease provides further evidence that virus-specific antibody produced in response to reovirus infection can protect against meningoencephalitis. Further work needs to be done to identify the determinant(s) recognized by the murine immune system that results in the development of an immune response that is protective against CNS disease.

It has recently been reported that IELs derived from the mucosal epithelium of rotavirus-immune mice can partially protect mice bearing the severe combine immunodeficiency defect (SCID) that are chronically infected with rotavirus[21]. Our cell transfer experiments demonstrate that both IELs and PPLs are highly efficacious at preventing meningoencephalitis. Cell depletion studies indicate that the phenotype of protective IELs is CD8+, Thy-1+ (Cuff *et al.*, data not shown).

Oral inoculation of adult germfree mice with reovirus results in a marked increase in the percentage of Thy-1+, TCR2+ (α/β form of the T-cell receptor for antigen) cells in the intestinal epithelium of primed mice. IELs contain populations of precursor cytotoxic T-cells (pCTL) which lyse virus infected targets *in vitro* in an MHC-restricted, virus-specific manner ([5] and Cuff *et al.*, submitted). It is likely that these virus-specific pCTL are responsible for the protection mediated by transferred IELs. Interestingly, viral clearance occurs systemically in mice that receive immune IELs suggesting that they are capable of systemic re-circulation and can reach the brain.

In addition to T-cell-mediated protection by IELs, adoptively transferred PPLs also protect against infection. The phenotype of protective PP lymphocytes is CD8-, Thy-1- (Cuff *et al.*, data not shown). This observation, and the finding that immune serum is protective, is consistent with the conclusion that the protective elements in PPLs are members of the B-cell lineage and probably mediate protection by antibody production.

CONCLUSIONS

Protection against reovirus-induced meningoencephalitis can be passively transferred either transplacentally or by suckling and protection is serotype cross-reactive because dams immunized with reovirus type 1/L protected neonates infected with T3c9. Most significantly, it appears that T-cells as well as B-cells derived from the intestine are capable of mediating virus-specific immune responses as a result of enteric priming and these cells can contribute to the resolution of disease. It may be important to consider these findings in developing strategies of immunization against enteric pathogens such as cholera and rotavirus, or pathogens such as HIV that enter the host through mucosal surfaces.

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PASSIVE IMMUNE PROTECTION FROM DIARRHEA CAUSED BY ROTAVIRUS OR *E. COLI*: AN ANIMAL MODEL TO DEMONSTRATE AND QUANTITATE EFFICACY

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INTRODUCTION

Several studies have described the use of orally administered passive antibodies from heterologous species origin to control enteric disease caused by bacteria[1-4], viruses[5-13], and protozoa[14]. Both the therapeutic and prophylactic properties of passive antibodies have been evaluated. The therapeutic efficacy has been confined to suppressed pathogen shedding with limited effects reported on the acute clinical disease[2,12]. The reported lack of therapeutic effect on the course of the *Escherichia coli* and rotavirus diarrhea episodes may be attributed to the self-limiting nature of these diarrheas in healthy patients. Passive antibody is effective in treating *Cryptosporidium* infection[14] and may also be effective in treating chronic diarrhea in immunocompromised patients.

There is substantial evidence supporting the use of passive antibodies from heterologous species to prevent enteric disease in both animals and man[1,3-11,13]. In particular, the use of orally administered antibodies from cows immunized with the appropriate pathogens from heterologous species to prevent specific diarrheal disease is well established[3,4,7,11,13,14].

In the United States, human rotaviruses (HRV) are the most frequent pathogens identified in children hospitalized with acute diarrhea, particularly during the winter[15]. Likewise, enterotoxigenic *E. coli* (ETEC) is the most frequent cause of diarrhea in travelers to developing countries[16-18] and is a leading cause of diarrhea in children in these countries[24]. Thus, both agents have world-wide epidemiological significance and both are candidates for novel approaches to control the diseases they induce.

The studies reported here: 1) describe an animal model to evaluate bovine passive antibodies to prevent diarrhea caused by HRV and ETEC, and 2) investigate the mechanisms by which orally administered antibodies prevent disease. Both pathogens were studied in a gnotobiotic piglet model. Gnotobiotic piglets were chosen because: 1) piglet gastric physiology is similar to man, 2) significant trans-placental transfer of antibody from the sow to the

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piglet does not occur, 3) there is no competition between experimental pathogens and the "normal gut flora" possibly masking the effects of the passive antibody, and 4) the piglet is susceptible to infection by HRV serotypes 1 and 3 allowing for the testing of human pathogen-specific antibody systems.

The specific objectives of the present studies were to: 1) establish quantitative relationships between antibody dose and protection from diarrhea caused by both HRV and ETEC, and 2) establish the concept of comprehensive protection based on the mechanism of antibody-mediated blocking of shared virulence factors in the ETEC system.

MATERIALS AND METHODS

Gnotobiotic Piglets

Gnotobiotic piglets were derived and maintained as described earlier[19]. Antibody preparations were fed to individually housed piglets at varying doses mixed with Similac with Iron® ready-to-feed infant formula. The feeding/challenge schedule for the ETEC experiments involved an initial feeding of 120 ml antibody/Similac solution (Ab), at 6 h post-derivation (PD), followed by 30 ml Ab feedings every 3 h beginning at 24 h PD. Following ETEC challenge (at 46.5 h PD) piglets were fed Ab every 3 h for an additional 10 feedings. The piglets were necropsied 30 h after infection.

The feeding/challenge schedules for the HRV experiments were modified as follows: The piglets were fed Similac with Iron without antibody supplement for the first 48 h PD in three 80 ml feedings per day. On the third day and thereafter, the piglets were fed Similac with antibody supplement three times daily, 80 ml per feeding. Piglets were challenged with virus midway between the first and second antibody feedings on day four. Antibody feedings continued for the duration of the experiment, three times per day, 80 ml per feeding.

Pathogens

Two ETEC strains were used for the challenge studies: RB 09:K103, 987P, LT-ST+ and FW 0141:K101, 987P, LT-ST+. The strain used for cow immunizations was RB 09:K103, 987P, LT-ST+. The piglets were challenged orally with 1.0 ml of an 8 h Brain Heart Infusion culture, diluted to a challenge dose of 10^4-10^5 viable ETEC.

Three HRV serotypes were used for the rotavirus challenge studies: HRV serotype 1 (Wa) derived from infant stool, was initially obtained from R.G. Wyatt[20], Laboratory of Infectious Diseases, Bethesda, MD, and maintained by *in vivo* passage in gnotobiotic pigs (Saif, unpublished). Piglets were challenged with 2.0 ml of a suspension of infected piglet intestinal contents, diluted to contain $5x10^5$ infectious virus units.

HRV serotype 2 (S2) was obtained through J. Hughes (Columbus, Ohio Children's Hospital) from T. Urasawa[21] Sapporo, Japan. This virus was passaged in MA 104 cells grown in MEM-E containing 10% fetal calf serum. All attempts to cause diarrheal disease in piglets using this virus failed. confirming earlier work indicating that specific strains of HRV serotype 2 did not appear to be pathogenic in pigs[22]. Because of the lack of pathogenicity no protection studies were conducted with HRV serotype 2. S2 virus was used as a component of the trivalent cow immunogen, however.

HRV serotype 3 (M) was obtained from R. G. Wyatt as an infected infant stool. The virus was passaged in gnotobiotic piglets to obtain infectious challenge pools (Saif, unpublished). Piglets were challenged with 2.0 ml of a suspension of infected piglet intestinal contents diluted to contain approximately 10^6 infectious virus units. The SA-11 strain of rotavirus was obtained from J. Hughes, Columbus, Ohio, Children's Hospital, as passage eight of the isolate originally obtained from H. Malherbe[23]. This strain was grown in MA 104 cells and was used to provide the rotavirus serotype 3 specificity for the cow immunogen and for *in vitro* VN assays.

Antibody Preparations

Bovine antibodies to porcine ETEC (RB 09:K103,987P, LT-ST+) were prepared using a whole bacterin immunogen (approximately $3x10^{10}$ washed cells per immunizing dose treated with 0.4% formaldehyde). The bacterin was suspended in phosphate buffered saline (PBS) and mixed 1:1 with incomplete Freund's adjuvant. Three pregnant Holstein dairy cows were immunized intramuscularily 7 days prior to the beginning of the cow's dry period. Seven days after the dry period had started the cows were given a booster immunization by intramammary infusion[25]. Upon calving, the first three milkings of colostrum were collected, defatted by centrifugation, and processed to a whey fraction by precipitating the casein using rennet. The whey samples were pH neutralized, clarified, and pooled. An immunoglobulin concentrate was prepared by precipitating the pooled whey using ammonium sulfate at 40% saturation. The immunoglobulin was resolubalized, dialyzed against PBS, adjusted to 150 mg/ml IgG1, dispensed into tubes, and sterilized by exposing the tubes to 0.4 Mrad of gamma radiation. "Control" material for the ETEC experiments was prepared in the same way using colostrum from two unimmunized cows. The antibody titers of the immune and control materials were measured with a passive hemagglutination assay which used sheep red blood cells that had been formalin-sensitized with an antigen extract made from the immunogen which contained substantial amounts of 987p pilus antigen. The antibody titers were 8.15×10^5 HA units/g IgG1 for the immune concentrate and 5.1×10^4 for the control material. The immune concentrate was polyclonal and multispecific. A detailed analysis of its antigenic specificity was not conducted, but it is clear that the material contained significant pilus-specific antibodies and *did not* contain significant anti-enterotoxin.

Bovine antibodies to HRV were prepared by immunizing groups of Holstein dairy cows with an immunogen containing purified, inactivated HRV, serotypes 1(Wa), 2(S2), and 3(SA-11). The viruses were grown using MA 104 cells, purified by centrifugation on sucrose/cesium chloride gradients and inactivated. The antigen was emulsified with incomplete Freund's adjuvant and administered to the cows using the method described above or by intramuscular injections for cows first immunized in early lactation. Antibody concentrates were prepared from colostrum as outlined above or from bulk mid-lactation milk samples using a proprietary process. The antibody concentrate derived from this process contained 85% total protein which was 30-35 IgG1. To control microbial contaminants test materials were either sterilized by gamma irradiation or an antibiotic mixture (100 μg/ml gentamicin, 300 μg/ml vancomycin, and 20 μg/ml fungizone) was added. The antibiotic mixture had no effect on HRV pathogenicity. Anti-HRV antibody potencies of the materials were measured using an infected cell reduction -

immunoperoxidase (ICRIP) microtiter assay. Average antibody-specific activities (antibody titer units per g IgG1 x 10^{-6}) for each HRV serotype for the immune materials were: Wa = 51 $\pm$ 13.6, S2 = 23 $\pm$ 10.8, and SA-11 = 216 $\pm$ 41. Average antibody specific activities for the control materials were: Wa = 5.0 $\pm$ 2.1, S2 = 1.8 $\pm$ 0.8, and SA-11 = 13.2 $\pm$ 2.4.

Indicators of Efficacy

The following criteria were used to evaluate the disease response to ETEC challenge: Diarrhea - time of onset, duration from onset to necropsy at 30 h post challenge, and severity (0 = no diarrhea, 1 = scant loose runny stools, 4 = profuse diarrhea with copious pale feces, consistency of water); *E. coli* per g of ileum - ileal segments were collected, weighed, ground, serially diluted ten fold, and colonies enumerated using Endo Agar plates; Liver cultures - the number of colonies from a loop plunged into the liver at necropsy and streaked on a blood agar plate; Histopathology - microscopic identification of *E. coli* adhering to segments of duodenum, jejunum or ileum.

The following criteria were used in the rotavirus studies: Diarrhea - time of onset, duration from onset post-challenge to 6 days post-challenge, and severity (mild or profuse); Virus shedding - daily rectal swabs were cultured to determine the presence or absence of virus by immunofluorescence-infected-cell-focus (CCIF) assay for HRV-Wa and an infected cell focus assay by stained using either an immunoperoxidase or an immunofluorescence staining system for HRV-M challenge studies. Seroconversion - serum samples were collected on the day of infection, and at the termination of the experiment (21 to 30 days post-challenge). Virus neutralizing antibodies contained in serum were quantitated to assess the immune response to rotavirus challenge (plaque reduction VN method for HRV-Wa studies, ICRIP VN method for HRV-M).

RESULTS

ETEC Passive Protection

Infective dose. An appropriate infectious dose of the challenge ETEC for these studies was identified in a preliminary experiment. Groups of piglets were challenged with $5x10^3$, $4x10^6$, or $4x10^9$ viable organisms. All piglets developed profuse diarrhea, the onset occurring later with lower challenge doses (onset = 22, 12, and 6 h post challenge respectively). A target infectious dose of 10^4 - 10^5 viable bacteria was chosen and resulted in reproducible kinetics of diarrhea onset with a safety margin to insure infection. The challenge dose for each protection experiment was confirmed by a plate count of the challenge inoculum immediately after use.

Antibody efficacy. Results of the ETEC passive protection studies are in Table 1. Feeding groups were: buffer (PBS plus Similac), Nonimmune Immunoglobulin fed at levels of 6.0, 2.4, 1.2, and 0.24 g IgG1 per day, and Immune Immunoglobulin fed at the same levels.

Data are presented showing the average number of hours the piglets displayed diarrhea followed by the severity scores. Using the 10^4-10^5 challenge dose, the onset of diarrhea was usually at 16-22 h post-infection (PI). Therefore, the maximum duration of diarrhea at necropsy (30 h PI) was 14-8 h. Data show that both challenge ETEC caused severe diarrhea in the piglets.

Table 1. Protection from Diarrhea Following *E. Coli* Challenge

Antibody	IgG1 g/day	Diarrhea[a] Homolo	Diarrhea[a] Hetero	Log *E. coli*/g Ileum Homolo	Log *E. coli*/g Ileum Hetero	Histopathology[b] Homolo	Histopathology[b] Hetero	Liver Culture[c] Homolo	Liver Culture[c] Hetero
Buffer	0	9/4	NT	0.63	NT	I	NT	3	NT
Nonimmune	6.0	NT	11/4	NT	5.28	I	I	NT	0
Nonimmune	2.4	11/4	14/4	6.54	8.41	I	I	<1	H
Nonimmune	1.2	10/4	14/4	9.23	8.41	I	I	1	1
Nonimmune	0.24	NT	14/4	NT	9.18	NT	I	NT	14
Immune	6.0	NT	ND	NT	3.93	NT	I	NT	0
Immune	2.4	ND	ND	5.28	4.86	0	0	0	<1
Immune	1.2	7/1	11/2	5.36	5.23	0	I[d]	0	<1
Immune	0.24	6/4	4/3	6.20	4.34	0	0	<1	8

[a] average duration (h)/severity score

[b] I = detected in Ileum, (no *E. coli* detected in duodenum or jejunum)

[c] average number of colonies/loop plunged into liver at necropsy, H = heavy colonization

[d] only one piglet which had been challenged with $5x10^5$ *E. coli* showed colonization

ND = no diarrhea, NT = not tested

Challenge ETEC strains: Homolo (immunogen) = RB 09:K103, 987p, LT-St+, Hetero = FW 0141:K101, 987P, LT-ST+

This diarrhea was prevented completely by feeding the anti-ETEC antibody at or above 2.4 g IgG1 per day. Protection was achieved without regard to the homologous or heterologous nature of the challenge organism. Some protection was observed at feeding levels of 1.2 and 0.24 g IgG1 per day. None of the piglets fed 1.2 g IgG1 per day developed severe diarrhea and some of them did not develop diarrhea. However, the protection seen at this level was variable, with some piglets developing a mild diarrhea that lasted 1-14 h. At this dose, no significant differences were seen between piglets in the homologous and heterologous challenge groups. Feeding at the 0.24 g level or below was not protective. Most of the piglets in this group developed severe diarrhea. Thus, the minimum protective antibody dose in this model was between 1.2 and 2.4 g of IgG1 from the ETEC immunized cows (1-2 million HA antibody units) fed per day.

This estimate was supported by the magnitude of *E. coli* colonization of the ileum, histopathological identification of *E. coli* in the piglets' intestine, and systemic colonization as reflected by bacteria present in the liver at necropsy (Table 1). Heavy colonization was seen in the piglets fed control material (all three assays). In contrast, the antibody feeding significantly limited, but did not eliminate, ETEC colonization. Interestingly, the antibody inhibited colonization even at doses which did not prevent diarrhea.

Rotavirus Passive Protection

Disease indicators/kinetics. The kinetics of HRV shedding and diarrhea in HRV-1 infected gnotobiotic piglets fed unsupplemented diet are shown in Fig. 1. An almost identical pattern was observed in HRV-3 challenged piglets. The highest incidence of virus shedding was observed for 1 to 7 days PI. Mild diarrhea usually appeared by day 2 PI and commonly lasted until day 12 PI. Profuse diarrhea was usually established by day 3 and lasted at least until day 7 PI. For group comparisons, analysis of virus shedding and diarrhea was restricted to days 1-6 PI.

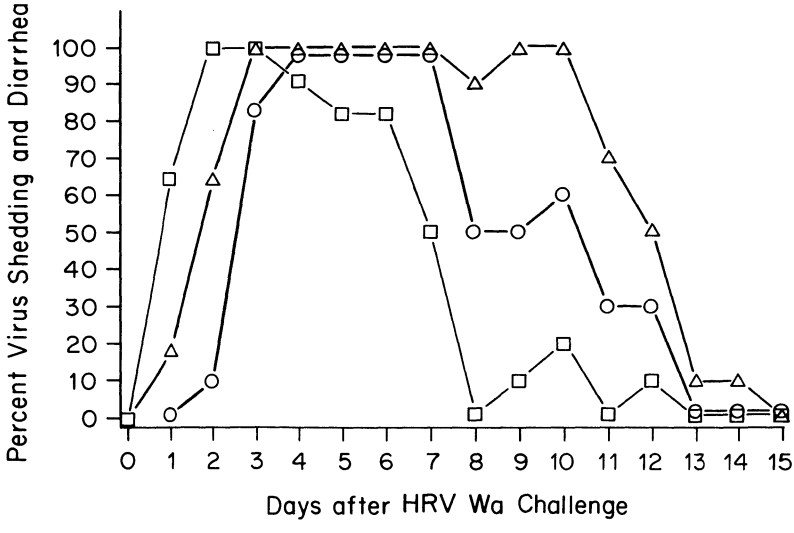

Figure 1. Virus shedding and diarrhea in HRV-challenged piglets (n=17) fed control diet. ❑—❑ HRV in feces, Δ--Δ mild + profuse diarrhea, o—o profuse diarrhea.

Antibody efficacy. Results of the passive protection studies for HRV serotype 1 are shown in Fig. 2. Ten feeding levels of immune immunoglobulin are reported along with two feeding levels of control immunoglobulin and a no-immunoglobulin Similac control. The figure shows the average percent days with diarrhea (mild or profuse), and the percent of days fecal virus shedding, as a function of total virus neutralizing (VN) antibody units and IgG1 (ing) fed per day. A clear dose-dependent relationship was observed between the dose of VN antibody fed and protection from diarrhea and inhibition of fecal virus shedding.

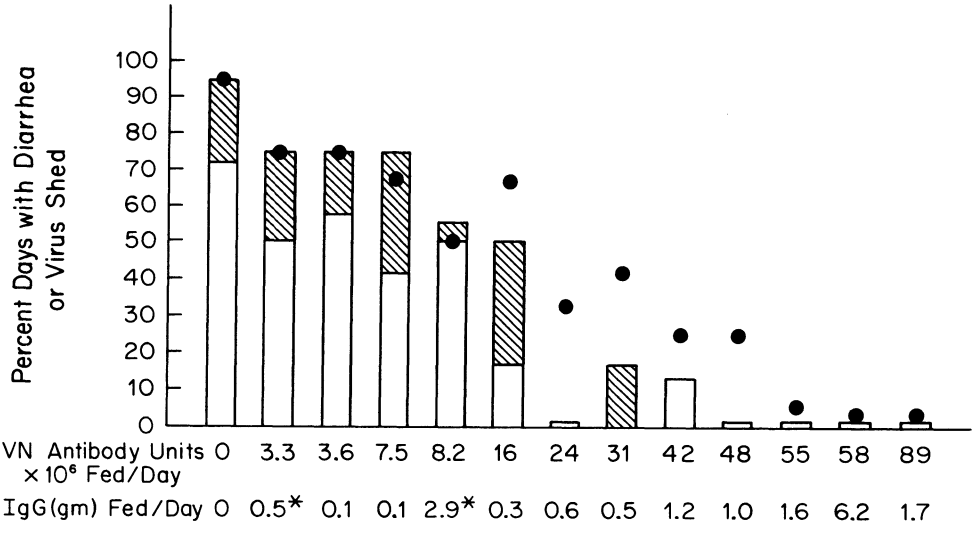

Figure 2. Antibody dose - diarrhea/virus shedding response for HRV serotype 1 (Wa)-challenged piglets. ☐ mild diarrhea, ▨ profuse diarrhea, ● virus shedding, * nonimmune.

Protection results for HRV serotype 3 are shown in Fig. 3. Six immune and one control immunoglobulin and one no-immunoglobulin Similac feeding groups are presented. As with the serotype 1 protection data, these experiments shown a clear antibody dose-dependent protection from mild and profuse diarrhea and a dramatic reduction of infectious virus shed in feces.

One potentially negative consequence of providing effective passive immune protection would be an inhibition of the recipients own immune response to pathogenic challenge. To measure this effect, serum samples collected from the piglets at the end of the feeding trial were tested for HRV-neutralizing antibody activity by plaque reduction VN. The relationship between the amount of antibody fed and the serum neutralizing antibody titer for the serotype 1 challenge series is shown in Fig. 4. Seroconversion in all of

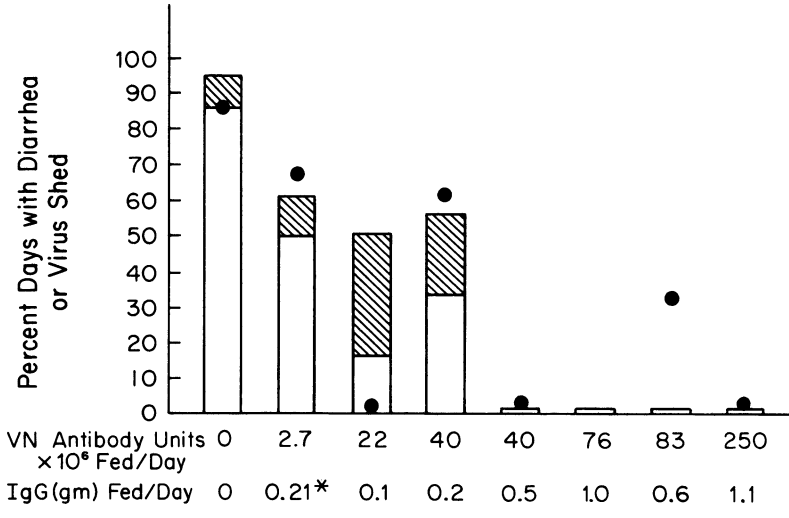

Figure 3. Antibody dose - diarrhea/virus shedding response for HRV serotype 3(M)-challenged piglets. ☐ mild diarrhea, ▨ profuse diarrhea, ● virus shedding, * nonimmune.

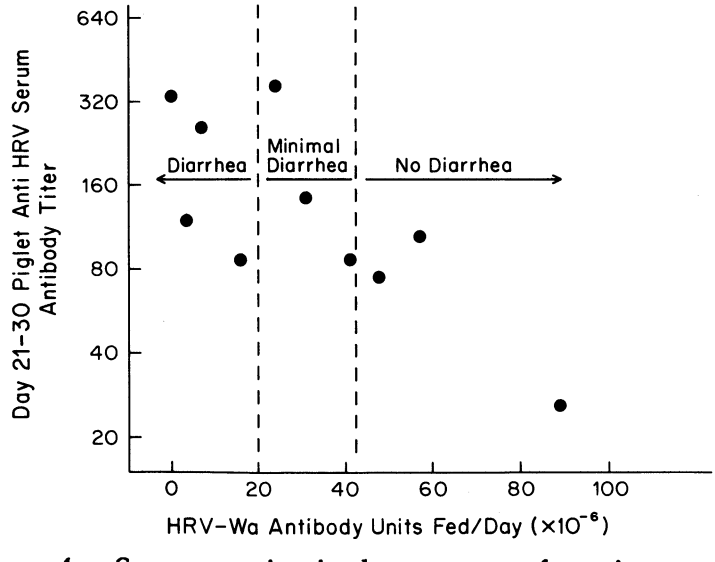

Figure 4. Seroconversion in the presence of passive protection.

the antibody treated piglets was observed. While there are insufficient data to clearly define small differences between control-fed and antibody-fed piglets, the present data indicate that no significant adverse effect on seroconversion occurred at the moderate antibody feeding levels required for protection from diarrhea.

DISCUSSION

There is a continuing interest in the use of heterologous species orally administered passive antibodies to prevent enteric disease. Several commercial products are available for animals and a number of clinical trials in humans have been reported. While vaccine approaches might provide an easier and possibly more cost-effective means to prevent enteric diseases, safe and effective vaccines are not available for all important enteric pathogens. Passive antibodies have a number of attractive features which seem to ensure continued interest in their development: high potential for safety, specificity, immediate effectiveness, easily controlled dose, and efficacy in immunocompromised patients. One of the concerns in clinical testing of passive antibodies is the time and expense involved in conducting trials to evaluate prophylactic effects. Further, clinical populations with high incidences of natural attack are uncommon for many enteric pathogens.

Data presented here describe an animal model which was useful in establishing and understanding the utility of bovine antibodies to ETEC or HRV in preventing diarrheal disease caused by these pathogens. Using this model, we documented the protective capacity of orally administered bovine antibodies at both the clinical level (no diarrhea) and the microbiological level (limited colonization, reduced viral shedding) and established relationships between antibody dose and efficacy. This is an important indicator of the practicality of a prophylactic strategy for a particular antibody preparation and can also be important in assessing improvements in product potency or formulation. The model was also used to address safety concerns regarding the passive/active immunization issue (with HRV) by demonstrating the ability to seroconvert in the presence of protection from symptomatic disease conferred by the passive antibody. These data confirm, in a heterologous antibody system, previous studies by Bridger and Brown who used delayed rechallenge of piglets with porcine rotavirus to demonstrate passive/active enteric immunity[7]. Finally, we have used the model to better understand the mechanism of enteric antibody action. Specifically, in the ETEC example, we were able to demonstrate protection based on blocking pilus-mediated attachment. Antibodies to toxins did not play a significant role in the protection since both the immunogen (homologous) strain and the heterologous challenge strain were specifically selected to be heat-stable toxin-positive and heat-labile toxin-negative. Since heat-stable toxin is a very weak immunogen, we were assured that the main mode of efficacy would be through anti-attachment factor antibodies. While the data are incomplete, our experiments clearly showed extended serotype protection based on antibodies to shared attachment factor antigens.

CONCLUSIONS

1. A quantitative relationship between oral anti-pathogen antibody dose and protection from diarrhea caused by either rotavirus or *E. coli* was established using the gnotobiotic piglet model.

2. The minimum protective dose of the bovine anti-*E. coli* antibodies studied was 2.0 to 2.5 g IgG fed/day.

3. Protection from heterologous *E. coli* challenge based on bovine antibodies to a shared virulence factor antigen was established and the minimum protective antibody dose was the same as for the homologous challenge.

4. Minimum protective doses in piglets for bovine anti-human rotavirus antibodies were established: Serotype 1 (Wa) = 40 x 10^6 VN Units fed/day, Serotype 3 (M) = 75 x 10^6 VN Units fed/day.

5. Seroconversion following the rotavirus challenge was not prevented by the levels of passive needed for protection from diarrhea.

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THE ANTIBODY RESPONSE IN INFANTS AFTER COLONIZATION OF THE INTESTINE WITH *E. COLI* O83. ARTIFICIAL COLONIZATION USED AS A PREVENTION AGAINST NOSOCOMIAL INFECTIONS

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INTRODUCTION

Starting at birth, the infant is exposed to a multitude of microbes including potential pathogens. Within the first few days, *Escherichia coli* appears in the stool, the strains originating from the mother and/or from the environment[1]. The infant can usually cope with these microorganisms with the support of transplacentally transferred IgG antibodies and breast milk that contains various defense factors including secretory IgA (S-IgA) antibodies against various *E. coli* antigens[2]. An *E. coli* strain O83 was used to efficiently colonize the colon of neonates and was found to remain for months[3]. In this study, two groups of infants were observed: healthy full term infants colonized with *E. coli* O83 (Group A), in which antibody responses against the O83 antigen in serum, saliva, and stool were monitored in order to determine whether the common mucosal immune system was triggered; and high risk infants in an intensive care unit, (Group B), in which the effect of artificial colonization of the intestine on the occurrence of nosocomial infections, presence of bacterial pathogens, and infant mortality were studied.

MATERIALS AND METHODS

Group A

In 9 breast-fed and 8 formula-fed infants, artificial colonization of the intestine was started during the first six days of their life by oral administration of a suspension prepared from a live 24-hour old culture of the serotype *E. coli* O83:K24:H31 containing 5×10^8 organisms in 1 ml. One ml of the suspension was given to each child diluted in 10 ml of tea, 3 times a week for four successive weeks. Five breast-fed and six formula-fed infants were followed as controls. The infants participated in the study with the written informed consent of their mothers and the study was approved by the Ministry of Health.

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The presence of *E. coli* O83 was detected by agglutination with a specific rabbit antiserum. Samples of blood, stool, and unstimulated saliva were taken before colonization and during the 2-3rd week, 4-7th week, 8-11th week, and 12-15th week after colonization. The same schedule of sampling was followed in the controls.

Antibodies of the IgA, IgG, and IgM isotypes against the O83 antigen were measured by enzyme-linked immunosorbent assay (ELISA)[4]. The level of antibody activity was expressed as a percentage of references prepared from samples of serum, stool, and saliva from adults colonized with the *E. coli* O83. The specificity of the ELISA assay was determined by inhibition studies. Ninety-seven percent of the binding activity of the hyperimmune serum was inhibited by incubation with an *E. coli* O83 sonicate. If *E. coli* O86 sonicate was used, only 14% of the antibody activity was inhibited. In adults colonized with *E. coli* O83, the inhibition of serum IgA antibody activity by *E. coli* O83 and *E. coli* O86 sonicates were significantly different. Coefficients of intraassay and interassay variation were 6.9% and 13.5%, respectively.

Group B

A group of 230 infants was colonized after admission to an intensive care unit during two 6 month periods for 4 successive weeks in the manner described for group A. Study parameters were: 1) the number of infections; 2) the presence of pathogenic microflora in samples taken from stool and elsewhere, detected by routine bacteriological examination before, during, and after colonization; 3) infant mortality; and 4) the necessity of administering antibiotics. Identical parameters were investigated in a group of 204 control infants during two 5 month periods.

Statistical evaluation included analysis of variance, chi-square test, and Fisher's test.

RESULTS

Group A

The strain *E. coli* O83 was detected in stools of all colonized infants starting on the 2nd day after oral administration and persisted throughout the study. No mothers were found to be colonized with *E. coli* O83.

Antibody response against *E. coli* O83. IgA antibodies against *E. coli* O83 in saliva increased after colonization in breast-fed, as well as in formula-fed infants, reaching higher levels between the 2nd and 11th weeks in comparison with the formula-fed controls ($p<0.01$) and in the interval 4-7 weeks compared to breast-fed controls ($p<0.01$). The IgM levels were higher in the colonized groups than in both control groups between 2-8 weeks after colonization ($p<0.01$) (Fig. 1). IgA antibodies against *E. coli* O83 were found in breast-fed infants' stools whether they had been colonized or not; presumably these antibodies originated from the mothers' milk. In the formula-fed infants, the IgA antibodies to *E. coli* O83 increased from the 2nd week after colonization and were significantly higher than in formula-fed controls until the 7th week after colonization ($p<0.05$). The level of IgM anti-O83 in stool did not differ between colonized and control infants (Fig. 2). In the serum, no increase of IgA, IgM, and IgG antibodies against the O83 antigen could be detected by the ELISA method.

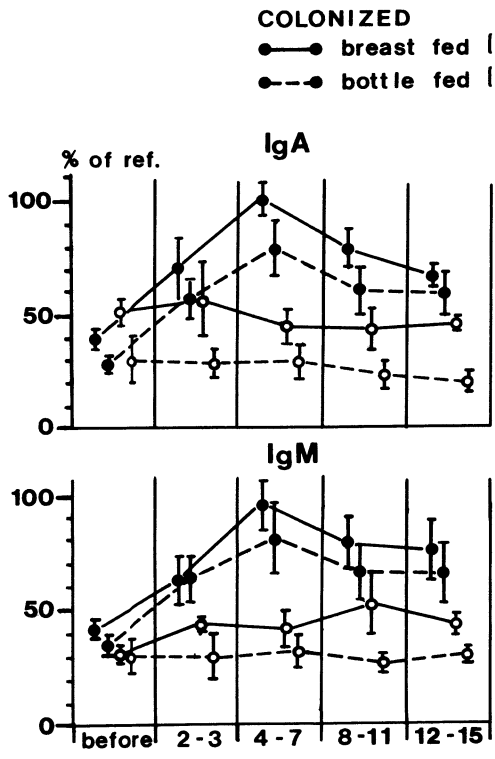

Figure 1. Levels of IgA and IgM *E. coli* O83 antibodies in saliva. Results of ELISA expressed as a percentage of reference in means ± SEM. The IgA levels were significantly higher in both colonized groups compared to bottle-fed controls in the intervals of 2-3, 4-7, and 8-11 wks ($p<0.01$) and in the interval 4-7 wks after colonization compared to breast-fed controls. The IgM levels were significantly higher in colonized groups than in both control groups in intervals of 2-3 and 4-7 wks after colonization ($p<0.01$).

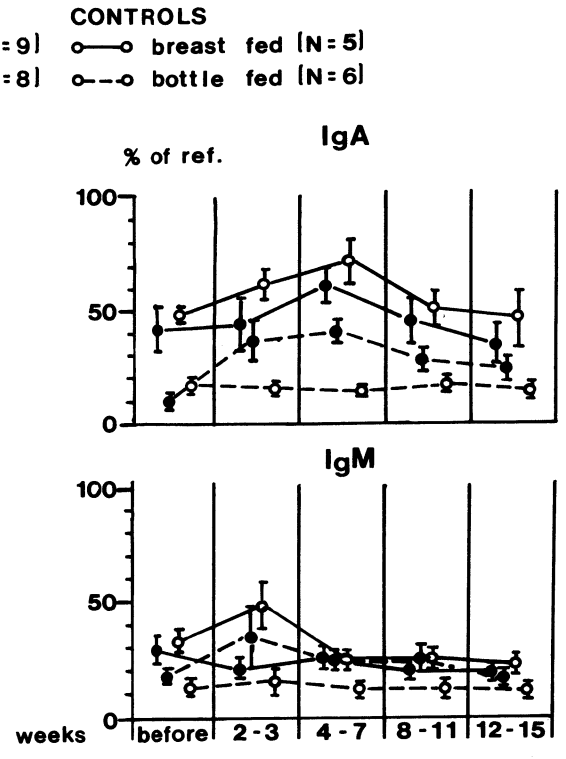

Figure 2. Levels of *E. coli* O83 IgA and IgM antibodies in stools. Results of ELISA are expressed as a percentage of reference in means ± SEM. The IgA O83 antibodies were significantly higher in breast-fed colonized and control groups than in formula-fed controls in all intervals ($p<0.05$). In formula-fed colonized infants the IgA O83 antibodies were significantly higher than in formula-fed controls in intervals of 2-3, and 4-7 wks after colonization ($p<0.05$).

Group B

E. coli O83 was detected in stools of 87% of colonized infants. Overall infections (septicemia, meningitis, pneumonia, viral, and candida infections) occurred in 20% of colonized infants and in 37% of controls. Infections caused the death of 2 infants from the colonized group compared to 21 deaths in the control group (Table 1).

Table 1. Incidence of Infection Compared Between Colonized and Control Groups of Infants

Group	Colonized (n=230)	Controls (n=204)
Number of sick infants	45 (19.56%)	75 (36.76%)
Number of infections	65	112
Contribution of specific infections to the total number of infections (in %)		
Septicemia	32.3	47.3
Candida infections	18.5	10.7
Bronchopneumonia	15.4	13.4
Viral infections	7.7	6.2
Urinary tract infections	6.1	7.1
Gastrointestinal infections	6.5	4.5
Meningitis	4.6	5.3
Other infections	9.2	5.3
Number of deaths:		
Group	Colonized	Controls
Birth weight (g)		
<999		4
1000-1499	1	2
1500-1999		3
2000-2499	1	8
2500-2999		1
Total	2 (0.86%)	27 (13.23%)
Caused by infection	2 (0.86)	21 (10.29%)

Bacterial pathogens were detected in 44% of infants before colonization and in 60% of controls. After colonization, pathogens have disappeared in 85% infants of the colonized group but only in 25% of control infants treated conventionally. Antibiotics had to be used in 27% of colonized infants and in 46% of controls. These differences are statistically significant ($p<0.01$) (Table 2).

DISCUSSION

Bacterial colonization of the intestine or oral administration of killed bacteria has been tested for the prevention against enteric infections and as a means of stimulation of the local immune system in animals[5], in human adults[6], and in some cases also in infants[7]. The characteristics of the *E. coli* O83 strain have been described in our previous work. Oral administration of the O83 strain evoked an increase in total stool S-IgA as well as a specific serum antibody response in infants[8].

Table 2. Presence and Decrease of Bacterial Pathogens Detected after Admission and During Hospitalization

Groups of infants	Colonized (n=230)		Controls (n=204)	
		%		%
Pathogens detected after admission	100	44	124	60
in stool only	14	14	17	14
elsewhere[a]	71	71	76	61
in stool and elsewhere	15	15	31	25
Pathogens detected during hospitalization	90		117	
in stool only	29	32	10	9
elsewhere	33	37	65	56
in stool and elsewhere	28	31	42	36
Pathogens detected after admission and/or during hospitalization	143		171	
Absence of pathogens	122	86	43	25
in stool only	28	20	18	10
elsewhere	63	44	19	11
in stool and elsewhere	31	22	6	4

[a] elsewhere = nose, throat, spinal fluid, blood, urine, others

The definition of the antigenic structure of the strain as O83:K24:H31 has recently been completed (Orskov, personal communication). In *E. coli* strains, isolated from infants with septicemia, meningitis, or urinary tract infections, the antigens K1, K2, K3, K12, and K13 are detected in most cases[9,10]. The strain *E. coli* O83:K24:H31 used in our work has never been isolated from infants with meningitis or septicemia, and has caused no complications in our previous studies of colonized newborns[11].

Results of this study showed significant increases of specific IgA and IgM responses in saliva after oral colonization of the gut. These findings are in agreement with the concept of the common mucosal immune system. Mellander *et al.*[12] reported an S-IgA response in saliva against *E. coli* O antigens at birth. It is suggested that these salivary antibodies in newborns may result from priming *in utero* via maternal anti-idiotypic antibodies[13].

The *E. coli* O83 IgA antibodies were higher in stool in both breast-fed groups, and presumably originated from maternal milk. Significant production of *E. coli* O83 IgA antibodies in the intestine after colonization could, however, be shown in the formula-fed group 2-7 weeks after colonization. Colonization with *E. coli* O83 might, therefore, protect formula-fed infants by inducting early antibody production. In addition, the possibility of cross-reactions between *E. coli* strains can also be considered.

Presumably, the best protection for young infants against infection is achieved by breastfeeding[14]. Nonetheless, some infants at high risk such as those born prematurely, are often deprived of fresh breast milk. The protective effect of intentional colonization with *E. coli* O83 has been demonstrated clinically in an intensive care unit. The presence of *E. coli* O83 in the intestine displaced pathogens in the gut. Additionally, pathogens were removed from other body locations. This effect could be explained by non-specific stimulation of the immune system.

CONCLUSIONS

Oral colonization of infants with the non-pathogenic *E. coli* O83 strain stimulated antibody production in stool and saliva. Long-term presence of *E. coli* O83 in the intestine possibly protects formula-fed infants by an early induction of S-IgA antibodies, partially compensating for the lack of S-IgA from maternal milk.

In high risk infants, preventive colonization of the intestine significantly decreased the number of infections, the infants' mortality, the presence of pathogens in the intestine and other body locations, and reduced the need for antibiotics.

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ANTIBODIES TO *STREPTOCOCCI PNEUMONIAE* IN SERA AND SECRETIONS OF MOTHERS AND THEIR INFANTS

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INTRODUCTION

Human milk normally contains antibodies against a variety of common pathogens, and there is epidemiologic evidence that breast-fed infants are less susceptible to certain gastrointestinal infections.[1] The role of breastfeeding in protection against respiratory bacteria is less clear and is undoubtedly complicated by the practical and social factors that determine the exposure of infants to organisms such as the pneumococcus.[2] The immunological mechanism of protection is assumed to be the presence of type-specific antibodies; however, there are no quantitative data on antibodies to pneumococci in human milk. In this paper we describe a prospective study of antibody levels to four common pneumococcal serotypes in mothers' milk and in the sera and saliva of their infants during the first year of life. The pneumococcal types selected were types 6, 14, 19, and 23, which are the most common types associated with carriage and infection in early childhood.[3] Because some of these capsular antigens bear structural similarities to oligosaccharides found in normal human secretions, we also studied the inhibitory effect of milk and saliva on the binding of type-specific antibodies to the pneumococcal polysaccharides.

MATERIALS AND METHODS

Patient Population and Study Design

Thirty-seven mothers and their infants in a suburban private practice in Birmingham, Alabama were followed from birth. Patients were middle-class, healthy, and without known immunologic disease. Twenty-three infants were breast-fed for 3 to 12 months; 14 infants who were exclusively bottle-fed were included in studies of antibody in serum and saliva. Cultures for pneumococcal colonization were obtained from mothers and infants at each office visit; cotton-tipped swabs from high in the oropharynx were plated directly upon a selective sheep blood agar containing 5 mg/l gentamicin sulfate (Schering Pharmaceuticals, Kenilworth, NJ), processed as previously described, and the pneumococci serotyped with Danish antisera.[4]

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Serum samples were obtained by finger-prick. Whole unstimulated saliva was collected with cotton gauze pledgets, put into a plastic syringe barrel, and the saliva squeezed out into a collecting tube. Mother's milk was obtained by expression with a manual breast pump. Milk samples were centrifuged to remove cells and lipids, and the aqueous portion was used for testing. All samples were stored frozen at -20° C until used.

Antibody Assays

The assay was an ELISA designed to detect total specific antibody using an anti-F(ab')$_2$ secondary antibody conjugated to horseradish peroxidase (Southern Biotechnology Assoc., Birmingham, AL). Pneumococcal polysaccharides of types 6A, 14, 19F, and 23F (Lederle Laboratories, Pearl River, NY) were treated with nitrous acid to destroy any contaminating C-polysaccharide: 100 µl of 2 mg/ml aqueous solution of polysaccharide was added to 100 µl of 1 M HNO_2 and 50 µl of 0.1 M acetic acid, pH 5.5, allowed to react at room temperature for 10 minutes, then neutralized with 12 µl 1 N NaOH with 4 µl of 0.1% phenolphthalein. Polysaccharides were then coupled to poly-lysine, as previously described[5], and dispensed in 200 µl volumes in microwell strips (Immulon II or IV, Dynatech, Chantilly, VA). Antigen-coated microwells were tested in the ELISA with mouse monoclonal anti-phosphocholine antibodies (Dr. David Briles, University of Alabama at Birmingham) to confirm the elimination of C-polysaccharide determinants. Samples were tested at three or more dilutions in phosphate buffered saline containing 0.05% Brij-35 (Aldrich Chemical Co., Atlanta, GA) and 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO), incubated in the wells for 3 hours at room temperature. Secondary antibody was incubated to 2 hours, washed out, and colour developed with 2 mg/ml ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), Sigma) in 0.01% H_2O_2. Antibody levels were estimated against a standard curve, as described elsewhere.[6]

Inhibition of Antibody Binding by Secretions

To determine if oligosaccharide moieties cross-reacting with pneumococcal polysaccharides occurred in human milk or saliva, an "inhibition ELISA" was employed, as described in studies of group B streptococcus type II.[7] This was done as above except that the antibody (pooled human IgG) was incubated for 1.5 hours in the presence of varying concentrations of milk, saliva, lactose or sucrose. For these experiments milk (aqueous portion) and saliva were heated to 100°C for 10 minutes, then centrifuged to remove antibodies and other denatured proteins.

RESULTS

Antibody levels in infant sera were highest shortly after birth, declining to low levels by about three months of age, as shown in Fig. 1. Antibody levels were similar for the four types tested, averaging about 3 µg/ml over the study period; during this time, some infants developed antibody in response to known colonization or infection. Levels in saliva were very low (0.01-0.20 µg/ml) or not detectable. Of 34 infants from whom saliva samples were available eight had detectable antibody to type 6A, five to type 14, one to type 19F, and two to type 23F.

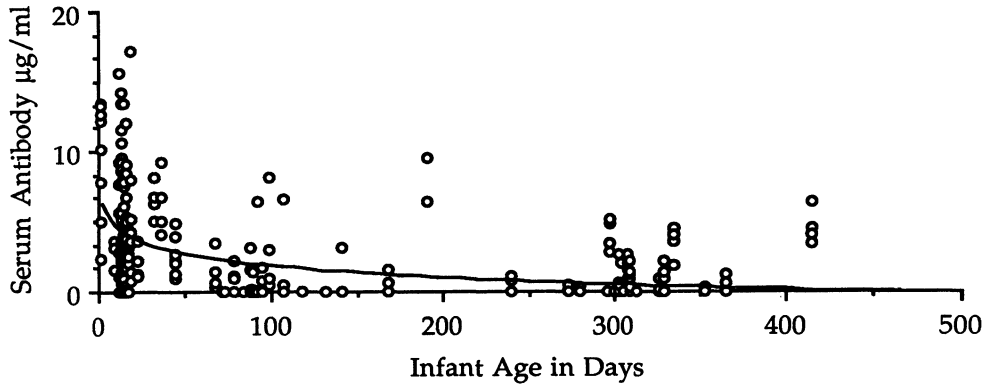

Figure 1. Infant serum antibody to pneumococcus types 6A, 14,19F, and 23F.

Milk antibody levels remained fairly constant over the course of lactation, as shown in Fig. 2. However, breast feeding trailed off after three or four months, and only a few mothers continued to nurse through the first year. This left the breast feeding population too small to make any assertions regarding the relation between antibody level and pneumococcal carriage or infection. Antibody levels in the mothers' milk and saliva were considerably higher than in infants and are shown in Table 1. Most milk samples had some detectable antibody, averaging a bit over 0.5 µg/ml, and ranging up to about 2 µg/ml. About two-thirds of mothers also had antibody in their saliva, with levels about half that of the respective milk levels, except for type 19F which was unexpectedly low.

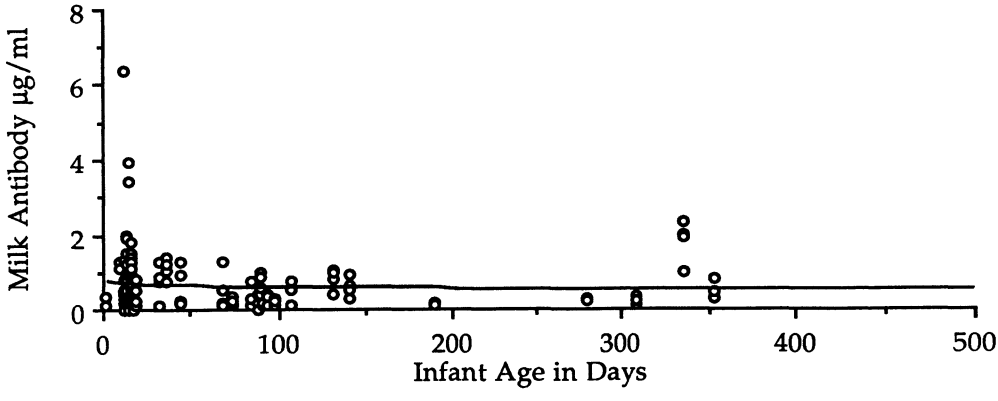

Figure 2. Milk antibody to pneumococcus types 6A, 14, 19F, and 23F.

The inhibition of antibody binding by constituents of normal human secretions was studied using the inhibition ELISA. We began with pneumococcus type 14, because this antigen has an important galactose determinant, and milk contains lactose and other galactosyl oligosaccharides. We selected a concentration of pooled human IgG that bound in the middle of the ELISA curve. Keeping the concentration of antibody constant, we added increasing concentrations of milk from two different donors (heated to remove antibodies), to act as an inhibitor in the liquid phase. As shown in Fig. 3A, the more milk added, the less IgG bound to the pneumococcus type 14 antigen on the microwells, and the lower the absorbance after incubating with the peroxidase-labeled second antibody. The milk inhibited about 40% of the

Table 1. Pneumococcal Antibody (μg/ml) in Milk and Saliva of 37 Mothers

	#Samples	Type 6A	Type 14	Type 19F	Type 23F
Milk	72	0.62 ± 0.53	0.57 ± 0.53	0.67 ± 0.47	0.44 ± 0.45
[Range]		[0-1.94]	[0-1.95]	[0-2.30]	[0-2.00]
Saliva	67	0.32 ± 1.20	0.32 ± 1.26	0.06 ± 0.11	0.17 ± 0.60
(Pts+/Pts tested)		(23/34)	(24/34)	(20/33)	(23/33)

IgG binding to the solid phase. Since mature human milk contains about 7% (200 mM) lactose, lactose was substituted for milk, and an unrelated disaccharide, sucrose, was used as the control (Fig. 3B). The inhibition by lactose was equal to that of milk. Inhibition by saliva (not shown) yielded curves identical to those with milk, using equal volumes of heat-treated saliva. Saliva does not contain free oligosaccharides but does have galactosyl determinants on mucin side chains.

The inhibition of antibodies to pneumococcus type 23F was examined in a similar manner. As shown in Fig. 3C, there was little inhibition by milk or lactose, but moderate (30%) inhibition by saliva. The inhibition pattern of type 6A (not shown) was similar to that of type 23F, and type 19F (not shown) was minimal with either milk or saliva.

Figures 3A-3C. Using the inhibition ELISA described in the text, pooled human IgG at a constant concentration was incubated in microwells with increasing concentrations of milk or saliva (heated to remove antibodies) or with lactose or sucrose at concentrations equivalent to the normal lactose content of milk. Figures 3A and 3B show inhibition of antibodies to pneumococcus type 14 polysaccharide by milk, saliva, and lactose; figure 3C reveals inhibition of antibodies to type 23F by saliva but not milk or lactose.

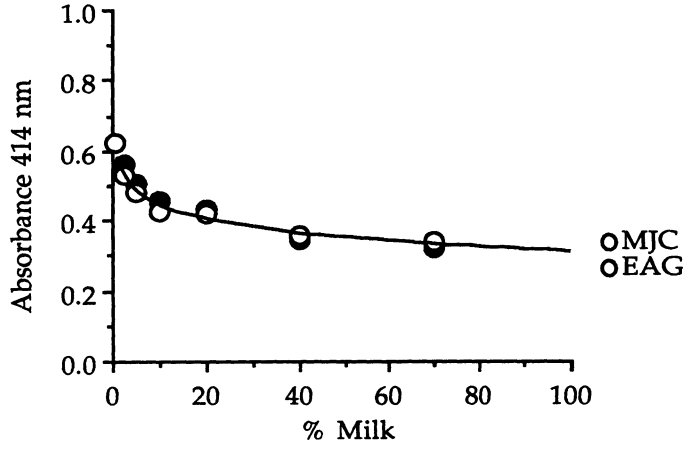

Figure 3A. Inhibition of IgG binding to pneumococcus type 14 by lactose.

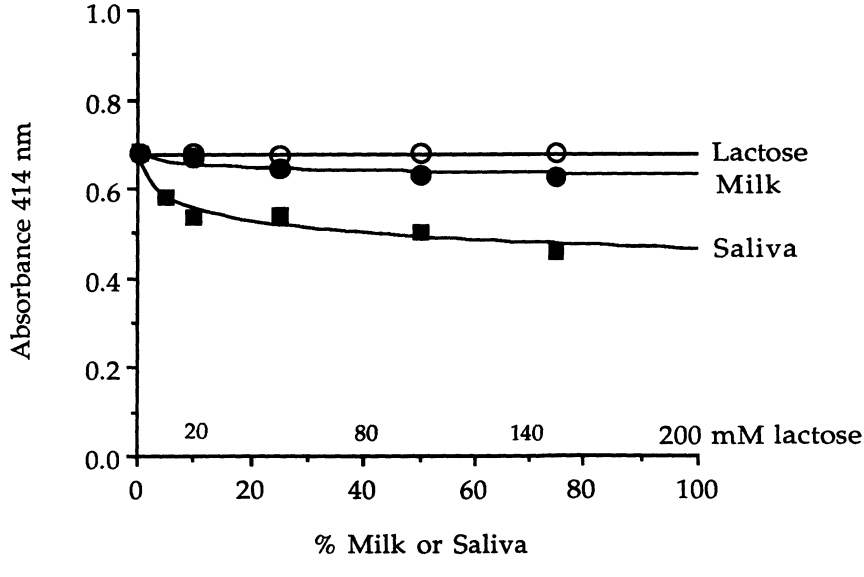

Figure 3B. Inhibition of IgG binding to pneumococcus type 14 by lactose.

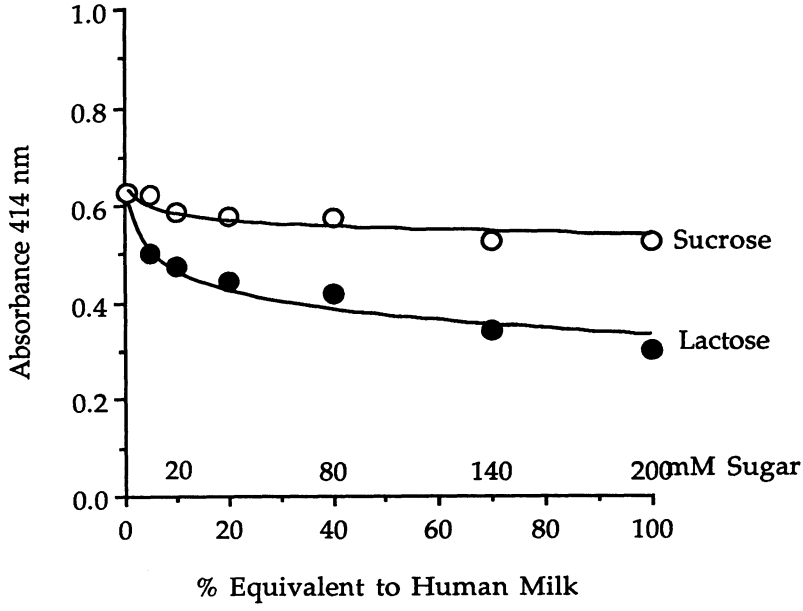

Figure 3C. Inhibition of IgG binding to pneumococcus type 23F.

DISCUSSION

Breastfeeding has long been thought to reduce the risk of infection during infancy. Since milk antibodies are predominantly secretory IgA and do not normally participate in complement activation or phagocytosis, their mechanisms of protection are thought to be agglutination and prevention of attachment of organisms to mucosal surfaces.[8] Although antibodies to specific respiratory pathogens have been detected in human milk[9, 10], there is no direct evidence that immunological mechanisms, rather than social and environmental factors, actually account for any reduction in morbidity from respiratory infections.[2] Nevertheless, other important roles for milk antibodies have been proposed, including the modulation of immune responses.[10] We have also noted that antibodies to the galactosyl determinant of group B streptococcus type II cross-react, and indeed bind to, components of normal human milk and saliva[7]; this may provide a mechanism whereby antibodies could bind specifically both to the bacteria and to the mucin side chains, thus fixing the organisms to the mucus layer and preventing invasion. Although infants rarely had antibodies to pneumococcal antigens in their saliva, their mothers did. Their mothers' milk contained type-specific antibody at levels of about 0.5 μg/ml; this may provide a considerable amount of total antibody when one considers that an infant may consume a liter or more of milk per day. The levels of antibody we observed, about 0.5 μg/ml, were similar to levels observed to *Haemophilus influenzae* type b.[9]

Quantitative antibody levels to other respiratory bacteria are not currently available.

The pneumococcal capsular polysaccharides are quite varied, and many bear structural similarities to food substances and host components.[11, 12] The pneumococcal type 14 antigen, for example, is similar to the backbone of the ABH blood group substance and is apparently identical to the desialylated group B streptococcus type III polysaccharide.[13] It was for this reason that we suspected that antibodies to the type 14 antigen might cross-react with salivary mucins, milk oligosaccharides, or lactose. The type 6 antigen was of interest because it shares some determinants with *H. influenzae* type b and with *Escherichia coli* K100.[14] Type 19 has determinants in common with *Enterobacter* species and amylopectins. Rhamnose determinants of type 23 are thought to be responsible for cross-reactions with the streptococcus group B antigen and with gum arabic, an ubiquitous food additive.

Apart from their theoretical role in defense against disease, milk antibodies to antigens with significant structural similarities to food substances may be important in the modulation of the infant's immune response.[10] Young infants do not respond well to parenteral immunization with bacterial polysaccharides, perhaps in part because they are confronted early in life with an enormous array of dietary and bacterial polysaccharides via the alimentary tract. There is some evidence that oral tolerance to polysaccharides may occur in both man and animals.[15, 16] Milk antibodies may bind to pneumococcal or dietary polysaccharides and limit entry of these antigens into the infant's gut-associated lymphoid tissue or otherwise alter their immunologic processing. A poorly controlled immune response to dietary proteins is thought to contribute to childhood eczema and allergy, as discussed elsewhere in this symposium. What part milk antibodies to polysaccharides may play in protection or in the maturation of the infant's immune system remains to be determined.

CONCLUSIONS

In a prospective study of mothers and their infants we found that human milk contains antibodies to pneumococcal polysaccharides of types 6, 14, 19, and 23. Levels were highest early in lactation and were fairly constant thereafter, averaging about 0.5 μg/mL of milk, as measured by ELISA. Infants' serum antibody levels were highest at birth and declined progressively with age. Some pneumococcal polysaccharides appear to have epitopes that cross-react with oligosaccharides and other components of human milk and saliva. The role of milk antibodies in defense against pneumococcal disease remains to be defined.

ACKNOWLEDGEMENTS

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THE POTENTIAL IMPACT OF GROUP B STREPTOCOCCAL ANTIBODIES IN BREAST MILK

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INTRODUCTION

Group B streptococcal (GBS) infection continues to cause significant morbidity and mortality in neonates despite advances in early diagnosis and treatment[1-4]. While a great deal is known about this organism, and the pathology it causes, its precies route and mechanism of entry into its host is still not clear. In suckling rats[5,6] and neonatal rhesus monkeys[7], oral administration of GBS has resulted in systemic disease. It seems likely that the gastrointestinal tract may also be a source of GBS infection in the human neonate via swallowed amniotic fluid[2] or contaminated breast milk[8-12]. Enteral administration of GBS specific antibody via breast milk[13] or formula[5], in animals, appears to decrease GBS disease. Prior to the conduct of studies assessing a similar impact in humans, we sought to determine the potential of human breast milk (colostrum) to provide the neonate with GBS type-specific antibody and factors effecting these levels in breast milk and neonatal stools.

METHODS

Source of Samples

Forty-six consecutive women whose pregnancies were full term, uncomplicated, and gave consent provided samples of maternal sera at 3 days postpartum, maternal milk at 3 days postpartum, post-transitional neonatal stools at 3 days postpartum, and neonatal cord sera.

Processing of Samples

All milk samples were cultured for group B streptococcus. Heavy cellular components and fat were separated by centrifugation at 5000 x *g* and discarded. Fresh stools were weighed and lyophilized for storage. Prior to analysis, the stools were ground into a powder and resuspended to the original wet weight with normal saline then spun at 20,000 x *g* and supernatant removed for analysis. All samples were stored at -70°C until

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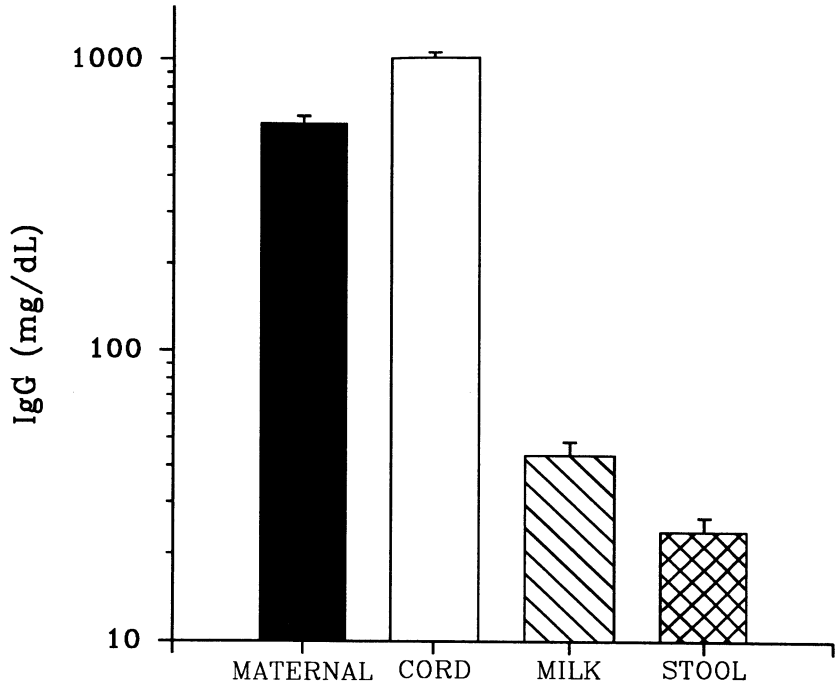

Figure 1. IgG (mg/dL) levels (mean ± SEM) of maternal sera (black), cord sera (white), maternal milk (hatched), and neonatal stool (cross hatched) n = 45.

analysis. GBS was cultured from only 1 milk sample, and this patient was excluded from analysis.

Immunoglobulin Determination

Total IgG was determined by radial immunodiffusion[14].

GBS Specific IgG Determination

GBS type-specific IgG was measured using an enzyme-linked immunoassay[15].

Statistical Analysis

Linear and multilinear regression analysis was used to determine the relationship of paired data.

RESULTS

The 45 women who participated in this study had the following characteristics (mean ± SEM): age 26.9 (1.1) yrs, gravidity 2.4 (0.2), Caucasian 36 (80%), first time breastfeeding 25 (56%). Their neonates had the following characteristics: gestation 39.9 (0.2) wks, birthweight 3451 (66) grams, male sex 27 (60%), Apgar[1] 8.2 (0.1), Apgar[5] 9.0 (0.4), SGA 1 (2%), LGA 7 (16%).

Figure 1 compares the mean (± SEM) total IgG in mg/dL for the different sources of samples: maternal sera, cord sera, maternal milk, and

neonatal stool. These values are comparable to previously reported values. Milk samples contained <10% the IgG of maternal sera.

Figure 2 compares the geometric mean (± SEM) titer of GBS type-specific IgG for serotypes Ia, II, and III from maternal sera, cord sera, maternal milk, and neonatal stool. Milk samples contained approximately 10% of the GBS type-specific IgG of maternal sera.

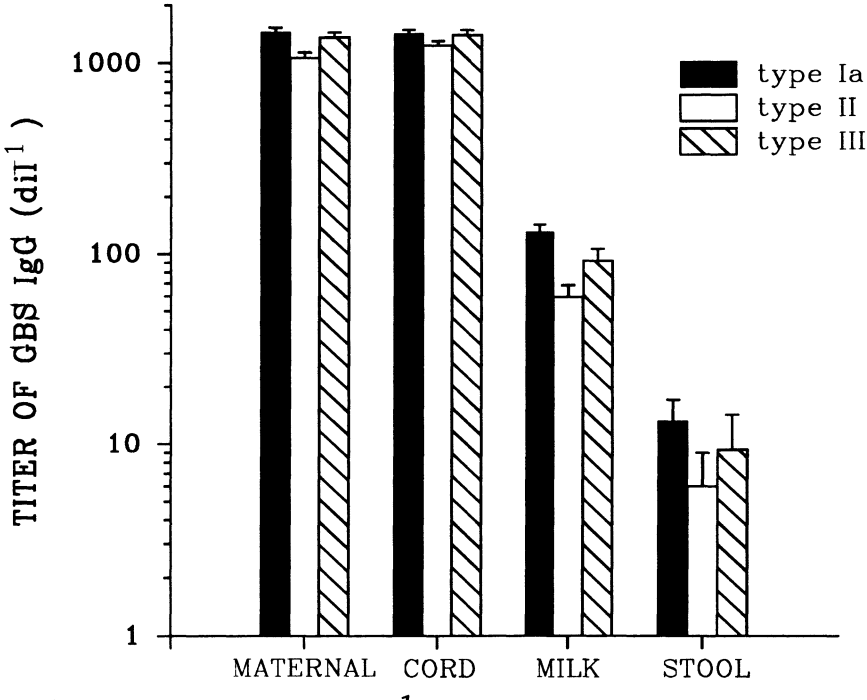

Figure 2. GBS type-specific IgG (dil^{-1}) (geometric mean ± SEM) of maternal sera and milk, cord sera and stool for GBS serotypes.

Figure 3 compares maternal sera versus paired cord sera for total IgG. Linear regression analysis found that only 25% of the variability of cord sera IgG could be attributed to the maternal sera.

Figure 4 compares GBS type-specific IgG in maternal sera versus paired cord sera. Maternal sera GBS type-specific IgG was a much better predictor of cord sera GBS type-specific IgG accounting for as much as 50% of the variability, as demonstrated here by serotype Ia.

Table 1 compares the effect maternal sera have on the concentration of total IgG and GBS type-specific IgG in maternal milk. The effect of cord blood on stool and maternal milk on stool. In each instance, organism-specific antibody is more likely to influence specific antibody than total IgG. Multiple linear regression of the effect of cord sera and maternal milk on stool suggests this effect is somewhat additive (R = 0.68).

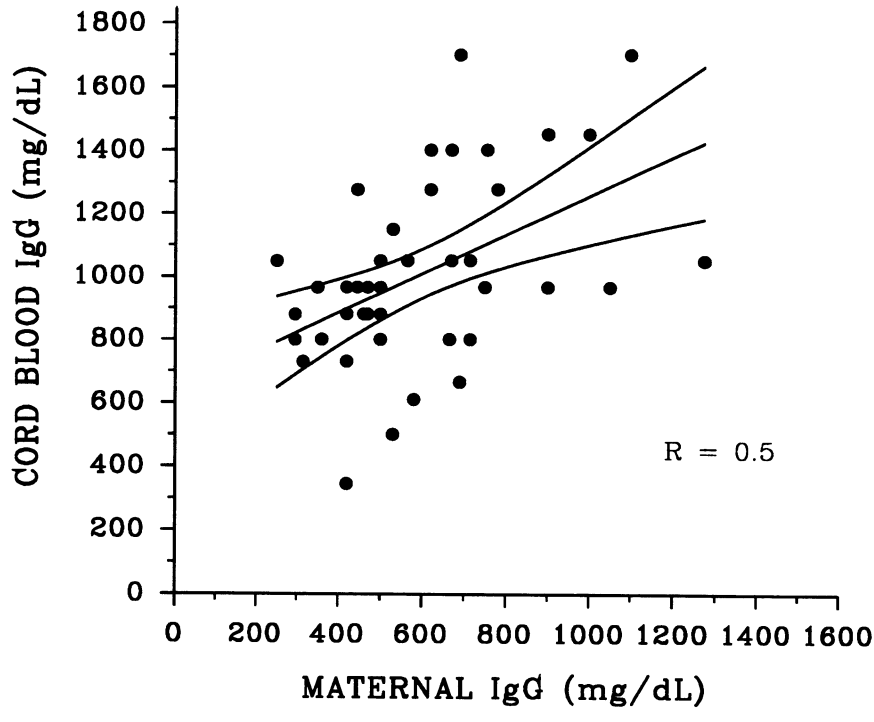

Figure 3. IgG (mg/dL) (mean ± SEM) of maternal sera vs cord sera, n = 45.

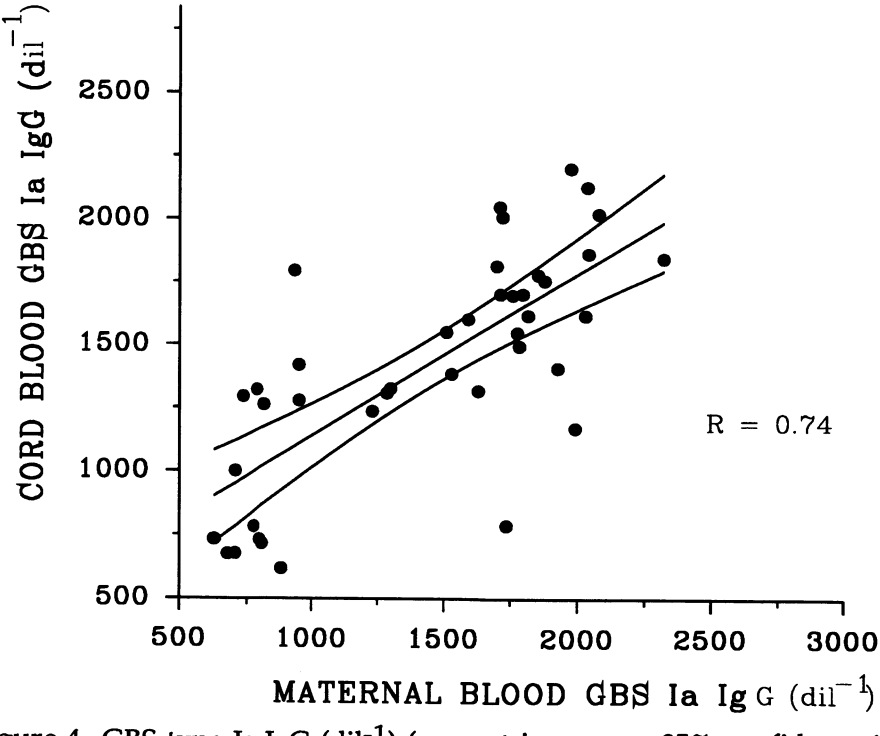

Figure 4. GBS type Ia IgG (dil^{-1}) (geometric mean ± 95% confidence interval) of maternal sera vs cord sera, n = 45.

Table 1. Comparison of Paired Secretions for Total IgG, GBS Type-Specific IgG by Regression Analysis*

Secretions compared	Total IgG	GBS type-specific IgG
Maternal vs cord sera	0.50	0.69
Maternal sera vs milk	0.28	0.48
Cord sera vs stool	0.07	0.44
Maternal milk vs stool	0.05	0.41

*indicates R values

DISCUSSION

Despite continued advances in early diagnosis and treatment, neonatal bacterial infections continue to cause thousands of infant deaths annually. Of all infant deaths related to infection, neonatal (birth to 28 days) infectious related deaths account for 17%[1]. GBS is the most common cause of early-onset neonatal bacterial infection and as such, is the model most commonly used to study newborn sepsis.

GBS infections can be divided into early-onset - usually occurring <3 days of age, and late-onset - occurring >7 days of age. GBS serotypes from infants with early-onset disease reflects maternal colonization and are one-third each: types I, II, and III; late-onset disease is due to predominantly type III[2]. Current estimates suggest 12,000 neonates will develop GBS infection this year with 70% occurring as early-onset[3]. Early-onset disease usually takes one of three forms: sepsis without a focus (48%), sepsis with pneumonia (40%), or sepsis with meningitis (12%); 85% of late-onset infections present as meningitis[3]. Even though mortality rates have improved for both early-onset (<20%) and late-onset (<10%), a significant number of survivors have permanent neurological or physical sequelae[4]. Early-onset infections are probably acquired in-utero or during birth. Late-onset infections appear to be acquired from the postnatal surroundings (including human carriers, and contaminated materials and equipment). While hematologic, enteral, and tracheal routes of entry have been suggested, the precise route or mechanism of entry are still debated.

In suckling rats, the oral administration of GBS has resulted in sepsis, meningitis, and pneumonia[5,6]. These systemic infections decreased with age, increased with dose, varied with strain, and were effected by cold stress and asphyxia[6]. In neonatal rhesus monkeys, gastric colonization resulted in sepsis, meningitis, and pneumonia. In this study, three neonates received direct *tracheal* instillation of GBS via an endotracheal tube and none of the infants developed bacteremia or died. Thirteen other neonates received direct *gastric* instillation of GBS via an orogastric tube. Six (46%) developed bacteremia and 5 with bacteremia died[7]. Thus, in suckling rats and neonatal rhesus monkeys, GBS systemic disease follows enteral inoculation and appears to occur via mucosal invasion. While no

one knows precisely, it seems likely that the gastrointestinal tract is also a site of this organism's entry into the human neonate[2].

In rare instances, breast milk may be the source of GBS leading to infection in a neonate. GBS are known to colonize about 3.5% of breast milk collected[8]. This appears to be independent of the method used[9,10] and may result in systemic infection in the nursing infant[11,12].

Enteral administration of GBS-specific antibody via breast milk or formula may decrease GBS disease. Recently, we found that rats suckling breast milk with GBS type-specific antibody had improved survival following oral colonization with GBS. This improved survival in rat pups appeared to be related to both their increased serum GBS type-specific antibody and their decreased GBS enteral colonization[13]. Others have observed that rats suckling human intravenous immunoglobulin with GBS type-specific IgG had decreased enteral colonization and bacteremia following oral GBS infection[5]. These observations suggest that GBS type-specific IgG may have an effect on mucosal or systemic immunity.

The importance of bacterial specific IgG antibodies in early neonatal feedings is yet unclear. The existence of these bacterial IgG antibodies in human milk has been known for some time[16], as has their ability to survive passage through the gastrointestinal tract[17] and retain function[18]. Though several authors have suggested that passively acquired IgG may diffuse into the intestinal lumen[16,17], its absorption is not clearly documented[19,20]. Organism-specific IgG, however, may provide protection from adherence and penetration of pathogenic organisms such as GBS and *Escherichia coli* similar to its actions in human urinary tract epithelial cells[21].

CONCLUSIONS

We conclude that in term, uncomplicated pregnancies, significant amounts of GBS type-specific IgG is present in colostrum. The amount of organism-specific antibody in colostrum is dependent upon maternal sera, and the amount of organism-specific antibody in neonatal stools is dependent upon both neonatal sera and maternal milk. We speculate that GBS type-specific antibody present in maternal colostrum may play a role in local or systemic immunity against GBS infection.

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IgA PROTEASES OF *HAEMOPHILUS INFLUENZAE* DIVIDING IN HUMAN MILK ARE INHIBITED BY IgA ANTIBODY IN THE MILK

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INTRODUCTION

IgA proteases[1-4] are extracellular enzymes produced by bacteria specifically pathogenic for human beings. These enzymes cleave both serum and secretory human IgA immunoglobulins (S-IgA), and thus may be virulence factors, or may contribute to colonization of mucosal surfaces by these bacteria. All clinical isolates of the important childhood pathogens *Haemophilus influenzae, Streptococcus pneumoniae,* and *Neisseria meningitidis* are IgA protease-positive, and the non-pathogenic members of these genera are enzyme-negative. IgA proteases are produced by all strains, those harmlessly colonizing the upper respiratory mucosa of children, and those isolated from infected sites e.g., the cerebrospinal fluid in meningitis. The role of these enzymes in disease has been difficult to verify because of their high specificity for human IgA as substrate, so animal models are inappropriate, and human tissues *in vitro*[5] cannot precisely reproduce *in vivo* conditions to examine this question.

The relationship between IgA proteases and human S-IgA is complex. First, these enzymes are microbial antigens which give rise to S-IgA antibodies, which, in human milk inhibit their proteolytic activity[6]. Thus S-IgA is both substrate and inhibitor. Second, secretion of the mature IgA protease from Gram-negative species is dependent on IgA protease activity itself. In *H. influenzae,* the organism used in this report, final release of the enzyme (about 110kDa) from the bacterial cell is by autoproteolysis of a much larger precursor of approximately 169kDa[2,7,8,11]. Because this autoproteolysis takes place in secretions containing anti-enzyme antibodies, release of the enzyme from the bacterial cell could be blocked. Third, of the two IgA subclasses in human secretions, IgA1 and IgA2, IgA1 is the sole substrate for nearly all of the known IgA proteases. However, the subclass which carries the enzyme-inhibiting antibody has not been defined. Finally, the protease-derived Fab and Fc fragments of S-IgA1 retain many biological properties; for

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example, S-IgA Fab binds to surface antigens of infective microorganisms, as recently documented with oral streptococcal pathogens[9]. Such fragment binding does not necessarily benefit the host, and may favor bacterial colonization or infection by blocking access of other immune elements to the bacterial membrane, or by changing the interaction of fragment-bearing bacteria with host tissues.

To explore these complex relationships we have established conditions for the growth of *H. influenzae* in S-IgA-rich human milk whey as the sole nutrient. Our findings are that this microorganism proliferates in human milk whey, that cells produce IgA protease during such growth, that all IgA protease activity is inhibited, and the inhibition is attributable to secretory IgA antibodies in the normal milk samples examined.

METHODS

Bacteria

H. influenzae strain Rd, a non-encapsulated derivative of a serotype d strain[10], produces type 1 IgA1 protease which it secretes into the extracellular environment.

Assay of IgA proteases

Qualitative assays for the enzyme was by identification of cleavage products of human ^{125}I-IgA1 substrate. Fragments were resolved by SDS-polyacrylamide gel electrophoresis, after which they were autoradiographed, as shown in Figure 3 (see below).

Quantitative assay was done by methods detailed in an earlier report[6]. Enzyme unknowns were incubated at 37°C with equal volumes of a substrate consisting of trace amounts of radio-iodinated human serum monoclonal IgA1 protein in 2 mg\ml unlabeled IgA. The IgA digestion products were resolved on 10% SDS-polyacrylamide gel electrophoresis and the amount of substrate cleaved was determined by the relative concentrations of intact, uncleaved IgA and its fragments measured by gamma counting of cut membranes. All assays incorporated a positive enzyme control consisting of IgA protease from strain Rd.

Bacterial Culture

H. influenzae was cultured in brain heart infusion (BHI) medium supplemented with 10 μg/ml β-nicotinamide adenine dinucleotide (NAD) and hemin, both from Sigma Corporation (St. Louis, MO). Enumeration of colony forming units (cfu) was done by subculture of dilutions on chocolate agar plates.

Human milk cultures of *H. influenzae* were conducted as follows: Donors were randomly chosen from healthy nursing mothers who were 3-6 days post-partum, were not taking antibiotics or other drugs, and who provided informed consent for our studies. Aliquots of 10-50 ml milk were collected and refrigerated in sterile glass bottles; within 24 hrs these milk samples were centrifuged at 9,500 rpm for 20 min. The clear whey, free of cells, was withdrawn by needle from beneath the lipid layer and was preserved by freezing at -70°C until thawed for use. Donor samples were arbitrarily numbered 1-4.

Starter cultures of bacteria for milk inoculation were grown in BHI supplemented with NAD and hemin. Thirty microliters of stationary phase cells following overnight culture were diluted with 9.0 ml fresh medium, and 30 μg of this dilution were inoculated into 1.0 ml aliquots of filter-sterilized milk whey pre-warmed to 37°C. Milk was sterilized just before use by passage through an 0.45 μm filter (Millipore Corp, Bedford, MA). No additional NAD or hemin was added to milk before inoculation. Cultures were in room air in screw-capped 50 ml polypropylene centrifuge tubes (Corning Glass Works, Corning, NY) which stood vertically, slowly rotating in a horizontal axis in a water bath at 37°C. To quantitate growth 30 μm samples were removed immediately (zero time) and at various time intervals, serially diluted with sterile normal saline, and plated onto chocolate agar. Plates were incubated at 37°C in 5% CO_2 in air. Growth was expressed as absolute cfu at each time point during incubation. For culture in dilutions of milk whey, sterile normal saline was the diluent.

RESULTS and DISCUSSION

All of four randomly selected milk whey samples supported the growth of *H. influenzae* strain Rd. Growth curves of bacteria expressed as cfu over a seven hour culture period in all four samples, and in BHI broth, are shown in Fig. 1. When contrasted to the relatively smooth growth curves in BHI broth, the cultures in milk whey were somewhat non-linear. This was attributable to cell aggregation, as seen by phase microscopy at various times during the culture period. The increases in cfu number also reached a plateau at about five hours in most milk cultures, again due to aggregation.

In experiments not shown, *Haemophilus influenzae* Group b also grew in human milk, indicating that growth in milk was not restricted to non-typable strains such as eg. Rd.

Haemophilus proliferation in human milk whey did not require supplementation with any growth factors including NAD and hemin, both of which are an absolute requirement for haemophilus growth in BHI. Control experiments showed that there was insufficient carryover of NAD and hemin from diluted starter cultures to explain the sufficiency of milk in this regard. Addition of one or both supplements to milk did not alter the growth curves obtained. In experiments not shown, milk whey conferred on unsupplemented BHI broth the ability to support cell growth without addition of either NAD or hemin, suggesting that milk contains these substances, or their equivalents.

IgA protease enzyme protein was synthesized by the cells proliferating in human milk whey, but the activity of the secreted enzyme was fully blocked. Fig. 2 shows the *Haemophilus influenzae* IgA protease activity as a function of cfu in BHI and in human milk sample #3. During a culture period of 7 h there was no detectable IgA protease activity in milk culture. Cultures maintained as long as 48 h also had no activity, although cfu number did not increase beyond 7-8 h. The absence of IgA protease activity in milk was not due to a faulty synthesis or secretion of the enzyme protein from cells, because Western blot analysis using rabbit anti-IgA protease detected the enzyme protein in such cultures Milk whey used as first antibody in Western blots also detected the enzyme protein. (data not shown).

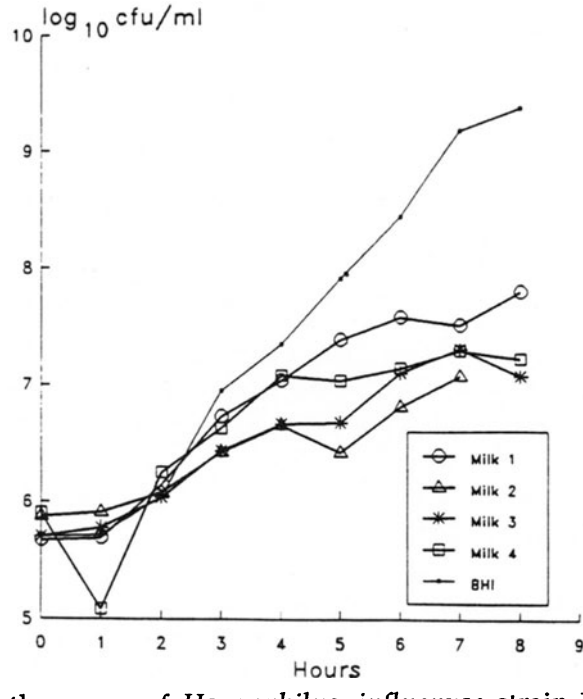

Figure 1. Growth curves of *Haemophilus influenzae* strain Rd in brain heart infusion broth (BHI) and milk whey #1-4 of four women.

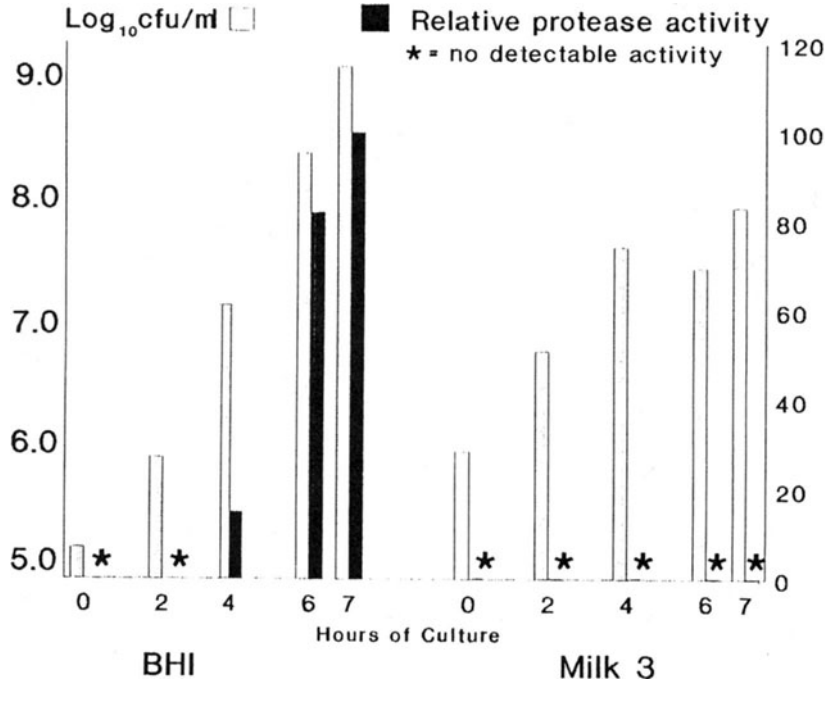

Figure 2. IgA protease activity as a function of cfu (per ml) at various times during culture of *H. influenzae* strain Rd in BHI, human milk whey sample #3. There was no enzyme activity detected in milk culture despite cfu reaching levels of 10^8/ml.

Cultures in milk whey diluted with normal saline yielded higher numbers of cfu of strain Rd. The 7 h value reached a maximum of approximately 10^9/ml in milk sample #3 diluted in the range 1:20-1:80 (v/v) (Fig. 3). Dilutions beyond 1:80 (data not shown) showed progressively less cfu, attributed to insufficient nutrient or inadequate buffering capacity of the medium. As with whole milk whey, growth in diluted milk did not require NAD or hemin. These data indicate that normal human milk has a concentration-dependent suppressive effect on haemophilus proliferation, although the increased number of cfu with dilution could in part be due to lesser degree of bacterial aggregation. Several factors in human milk are known to limit bacterial growth[12-14], but we have not yet defined what factor(s) specifically reduce strain Rd growth in milk whey. In addition to higher growth in diluted milk, haemophilus IgA protease activity was also found in the dilutions of 1:20 or higher (Fig. 3, top). This shows that the capacity of milk to inhibit IgA protease is also concentration-dependent, a result consistent with an antibody-based mechanism, as proposed earlier[6].

From these experiments we conclude that human milk whey is a fully sufficient medium for the proliferation of *H. influenzae*, providing an opportunity to examine in greater detail the relationship of IgA protease secreted from the cell with native IgA in human milk. Inhibition of IgA protease by milk is an antibody mechanism, as discussed earlier[6]. Whey lacks phagocytic cells or high levels of membrane-bound lipid found in natural milk, so the influence of these on haemophilus growth under these conditions are not yet known, but there is evidence that Haemophilus can become resistant to host defense mechanisms during growth in natural media[15].

In earlier studies using IgA protease added directly to milk samples it had been shown that whey was a potent inhibitor of the IgA proteases of several pathogens. This inhibition was immunologic, mediated by S-IgA antibody with specificity for the enzyme protein, and not a function of IgA as a competitive substrate. The present work extends these findings, showing that IgA protease is synthesized by *H. influenzae* cells allowed to proliferate in human milk. Further, unless milk whey has first been diluted for use as culture medium the enzyme is again fully inhibited. The IgA subclass inhibiting the enzyme in milk (IgA1 or IgA2, or both) has not been clearly established. Although Western blot analysis indicates that most of the protease is in the extracellular medium, we are now conducting experiments to determine if these antibodies can block, in part, the bacterial secretion of the enzyme by interrupting the autocatalytic component of processing.

These data raise the possibility that anti-IgA protease antibodies in milk provide a generalized biological defense mechanism, because they are directed against enzymes of bacteria known to colonize the upper respiratory mucosa of infants[16]. Inhibition of IgA proteases at mucosal surfaces could spare S-IgA1 of both maternal and child origin, such IgA having a broad anti-microbial specificity which extends beyond the antigens of the protease-producing microorganisms. While these considerations do not address the role of IgA2 antibodies at mucosal surfaces, in normal human subjects the relative numbers of lymphocytes producing IgA1 antibodies are much higher than IgA2 in the upper respiratory tract[17]. It is also important to emphasize that milk may be unable to inhibit the IgA proteases of all bacteria colonizing the oral cavity and upper respiratory mucosa. For example, the enzyme of

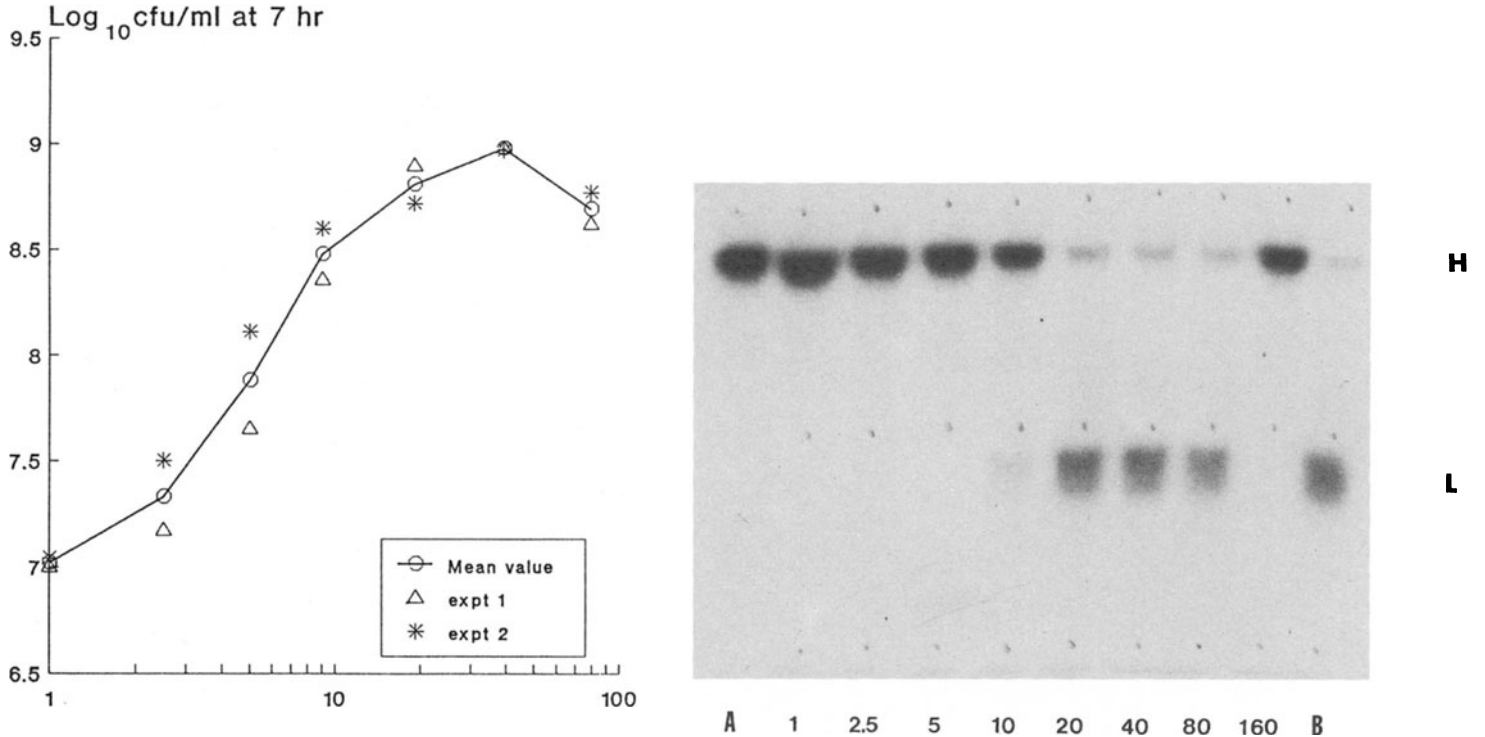

Figure 3. Cultures of *H. influenzae* strain Rd in various dilutions of human milk whey. Left panel: cfu per ml at each dilution at 7h, plotted as mean of the two experiments. Right panel: IgA protease activity using ^{125}I-IgA1 substrate (see Methods). Substrate was incubated with completed cultures and autoradiographed to reveal products. Lane numbers are the dilutions (see left panel); lane A–substrate control (no enzyme); B–enzyme control showing complete IgA cleavage; H–intact IgA1 heavy chain; L–Fd portion of H chain in the Fab digestion fragment. Protease activity was found only in milk dilutions of 1:20 or higher.

streptococcal species e.g. *Streptococcus sanguis* has already been found resistant to antibody-mediated inhibition.

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BOVINE LACTOGENIC IMMUNITY AGAINST PEDIATRIC ENTEROPATHOGENS

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Diarrheal diseases affect over 1.5 billion and kill more than 4 million children less than 5 years of age each year in the lesser developed countries (LDCs) of the world[1]. Surveys of 60 LDCs revealed an average incidence of about 4 bouts of diarrheal disease/child/year[1] whereas some countries, Peru, for example[2], have an incidence of nearly 10 bouts annually in children under 5 years of age. If these children survive, they fail to thrive unless they are properly nourished and treated during their illness.

While it is widely accepted that breast-feeding is an effective means of preventing diarrheal disease in infants[3-7], a substantial proportion of infants and children in Third World countries are still not breast-fed[8]. Breastfeeding provides the infant with maternal antibodies as well as a variety of protective factors, as yet not well-defined[9-11], in mothers' milk. Studies in our laboratory[12-14] have extended numerous reports[9-11,15] which show that mothers' milk has antimicrobial properties *in vitro*. However, they also show that, as these potentially protective components vary with individual mothers as well as with the genus, species, and strain of the etiologic agents, the milk of an individual mother cannot be expected to protect her child against all potential infectious threats. Breastfeeding may also protect indirectly, by avoidance of environmental contamination through dirty containers, water, and food. Unfortunately, despite rigorous campaigns by WHO, UNICEF, and other health-concerned organizations, a large proportion of Third World children are not breast-fed and, if they are, not for sufficiently long[8]. If this is so, why do we not further consider the potential alternative of providing formulae which are protective as well as nutritious?

That orally administered antibody is protective was demonstrated by Paul Ehrlich[16] in mice nearly a century ago. A number of attempts have since been made to evaluate the **therapeutic** effects of perorally administered preformed antibody. Mietens *et al.*[17] fed a hyperimmune bovine milk immunoglobulin concentrate (MIC) (containing about 40% Ig) including antibodies to 14 serologically different strains of enteropathogenic *Escherichia coli* (EPEC) to 60 infants (10 days to 18 months) suffering from *E. coli* diarrhea. No therapeutic effect was demonstrated although the period of excretion of homologous serotypes was reduced. Similarly, MIC containing antibodies to 4

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different serotypes of human rotavirus failed to affect the course of rotavirus diarrhea in infants[18,19]. Recently, McClead *et al.*[20] orally administered purified bovine Ig containing anti-cholera toxin to cholera patients. Again, no therapeutic effect was evident. Thus, several studies have shown that **orally administered antibody has little, if any, effect on already established disease.** An exception, which should be noted, is the therapeutic efficacy of orally administered hyperimmune bovine colostrum in treatment of chronic diarrhea caused by *Cryptosporidium* in immunologically compromised patients (i.e., patients with hypogammaglobulinemia[21,22], patients on immunosuppressive chemotherapy[22], and AIDS patients[22,23]). The bovine anti-oocyst antibodies are probably interrupting the organism's life cycle by preventing oocyst re-attachment.

On the other hand, the **prophylactic efficacy of passive orally administered antibody has been documented in numerous studies** in experimental animal models as well as in humans. Snodgrass *et al.*[24] showed conclusively that lambs fed human IgG containing antibody to rotavirus from 24-72 hr after birth and challenged with human rotavirus at 30 hours were protected from diarrhea. This treatment did not prevent excretion of the virus or development of antibodies to it. In our laboratory[25], feeding purified hyperimmune colostral bovine IgG1 containing antibodies against either cholera or *E. coli* heat-labile enterotoxin or *Vibrio cholerae* outer membranes protected infant rabbits from diarrhea following intraintestinal challenge with virulent cholera vibrios. There is also evidence for the prophylactic efficacy of orally administered antibody in children and adults against two of the most prevalent pediatric diarrheagenic pathogens, rotavirus and *E. coli*, as summarized in Table 1.

Human milk or colostrum, administered to infants, protected against EPEC according to the studies of Svirsky-Gross[26], Tassovatz and Kotsitch[27], and Larguia *et al.*[28]. In 1980, Narayanan *et al.*[29] claimed to have obtained significant protection against a variety of infections in 32 high-risk, low-birth-weight infants by partially feeding human breast milk (breast milk by day and formula at night) compared with 38 infants receiving formula alone. Barnes *et al.*[30] orally administered commercial pooled human serum gammaglobulin (at a dose of 640 mg in 4 ml, four times a day for the first week of life) to a group of 75 low-birth-weight infants in a nursery where rotavirus was known to be endemic. Twenty-five of these babies excreted rotavirus. Of these, 6 of 11 babies given placebo developed rotavirus diarrhea, whereas, only 1 of 14 babies fed gammaglobulin were so affected. Of note also in this study, none of the 39 low-birth-weight babies given gammaglobulin developed necrotizing enterocolitis (NEC), while 3 of the 34 given placebo had NEC. A recent study by Eibl *et al.*[31] showed that feeding 600 mg of pooled human serum Cohn fraction II (IgA-IgG) to low-birth-weight infants/day in 3-4 doses as a feeding supplement was completely protective against NEC. There were no cases in the 88 infants receiving Ig compared with 6 in the control group of 91.

A number of studies (see Table 1) have examined the therapeutic/protective effects of feeding hyperimmune bovine immunoglobulin. The newly parturient cow secretes into her colostrum enormous quantities of immunoglobulin, predominantly IgG1[35], the counterpart of secretory IgA in human. Like human IgA[36,37], bovine IgG1 is relatively protease-resistant and immunologic reactivity is retained after passage through the gut[38,39]. Ebina *et al.*[32] fed 20 ml of hyperimmune bovine anti-rotavirus colostrum daily to 6 infants in an orphanage. Seven control

infants received 20 ml of commercial milk. After 1 month, 6/7 control infants had developed diarrhea, while 5/6 colostrum-fed infants were diarrhea-free. Recently, Davidson *et al.*[34] demonstrated that infants fed resuspended dried bovine colostrum containing antibody against rotavirus were protected from nosocomial rotavirus diarrhea. In that study, 9/65 control children, but none of the 55 colostrum-fed infants developed diarrhea. While it is not altogether clear that antibody, rather than other colostrum components was responsible[41], clearly, their colostrum preparations protected against rotavirus diarrhea.

Table 1. Prophylactic Efficacy of Passive Oral Immunization with Human or Bovine Colostrum or Milk

Immunoglobulin source	Protection vs.	Reference (year)
Human		
Milk	*E. coli* 0111:B4	26 (1958)
Milk	*E. coli* 0111:B4	27 (1961)
Colostrum	EPEC[a]	28 (1977)
Milk[b]	Varied pathogens	29 (1980)
Serum Ig pool	Rotavirus	30 (1982)
Serum Ig pool	NEC[c]	31 (1988)
Bovine		
Colostrum[d]	Rotavirus	32 (1985)
MIC[d,e]	EPEC	33 (1988)
Colostrum[d]	Rotavirus	34 (1989)

[a] Enteropathogenic *E. coli.*
[b] Partial milk feed: milk by day and formula by night.
[c] Necrotizing enterocolitis.
[d] Hyperimmune.
[e] Milk immunoglobulin concentrate.

Tacket *et al.*[33] have shown definitively that oral administration of a milk immunoglobulin concentrate prepared from cows hyperimmune to EPEC, cholera toxin, and heat-labile *E. coli* toxin completely protected adult American volunteers against challenge with 10^9 colony-forming units of enterotoxigenic *E. coli* H10407. None of the ten volunteers fed MIC developed diarrhea whereas 9/10 receiving control MIC did.

If we then accept that hyperimmune bovine immunoglobulin can be protective, can it then be a practical option to be used on a broad scale to protect children in developing countries? No direct information is available to answer this question. Some assumptions can be made, however, based particularly on the data from the study in American volunteers[33] (see Table 2). Protection was attained against 10^9 viable enterotoxigenic *E. coli* by feeding 3.55 g MIC three times daily for 7 days. The MIC used contained 40%

immunoglobulin which was a mixture of antibodies, of which many may be assumed to be irrelevant. If we assume, e.g., that 10% of the antibodies were effective, then the dose of effective immunoglobulin per volunteer per day was of the order of 400 mg. We may also assume that this dose was excessive in view of the fact that it resulted in 100% protection against an unnaturally high challenge. Can we assume that 10-fold less, i.e., 40 mg per day, would be sufficient? And, can we also assume that an infant or young child would require only one fourth of the dose? Although this remains to be shown by appropriately controlled experimental studies, they seem to us to be potentially acceptable assumptions. Inasmuch as a kilogram or more of immunoglobulin can be harvested from the colostrum of a newly parturient cow, based on the demonstration of Tacket *et al.*[33], a single cow could provide at least 250 daily doses for adults and perhaps 1,000 doses for children. These assumptions are related to using colostrum from newly parturient cows. Is it possible that there could be sufficient antibody present in mature milk from immunized cows to be protective? Inasmuch as a liter of milk contains between 100 and 400 mg of IgG1, the answer would depend on the protective dose, which still remains to be defined. If it is feasible, it is equally feasible that herds of cows could potentially be immunized with multiple antigens to provide milk which could be protective against a variety of pathogens.

Inasmuch as so many infants in this world are not being breast-fed, we suggest that the prophylactic effects of passive oral immunization with preformed bovine antibody should be evaluated in controlled studies in Third

Table 2. The "Bottom Line Question": What is the Minimal Effective Dose of Bovine IgG1?

	Answer[a]		
Fact [or asumption]		Dose (g) /day	Protection (%)
1.	M.I.C.[b]	10.7	100[c]
2.	IgG1 (M.I.C.=40% Ig)	4.3	100
[3.	IgG1 (if 10% of IgG1 is relevant)	0.43	100]
[3a.	IgG1 (if 1% of IgG1 is relevant	0.043	?]
[4.	Protective dose/child	0.1	100]
[4a.	Protective dose/child	0.01	?]
5.	Mature milk (0.1-0.4 g IgG1/l)[41-43]	0.1-0.4	?
6.	Protective dose against natural challenge	?	?

[a] Based upon the volunteer challenge study of Tacket *et al.*[33]

[b] Milk Immunoglobulin Concentrate = 40% IgG1.

[c] Against 100% effective challenge, i.e., 1.2×10^9 EPEC.

World pediatric populations. Should the results of such initial studies be encouraging, further studies should then be performed to determine the minimal effective dose, for that will have a significant bearing on the question of whether passive oral immunization can be cost-effective. We think it should be technologically feasible to overcome the problem of sanitary delivery of such protective formulae and, should that be the case, such formulae could be an important life-saving interim measure until the health and welfare of the next generation is effectively made a primary commitment of the Third World countries concerned.

That the pattern of infant morbidity and mortality can be altered by vigorous government intervention through immunization, education, sanitation, and delivery of health care has been demonstrated dramatically. The pioneering studies of Dr. Leonardo Mata[3,7] provided the blue-print for the social and public health reforms which, when implemented by Dr. Oscar Arias and his government, reversed the tide of infant mortality in Costa Rica[44,45]. The under-five mortality rate (U5MR) mortality rate in Costa Rica is now under 30/1000 live births[46]. Could protective infant feeding formulae reduce it further?

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MILK SECRETORY IgA RELATED TO *SHIGELLA* VIRULENCE ANTIGENS

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INTRODUCTION

It has long been suggested that breastfeeding has a protective effect against shigellosis both in terms of decreasing the frequency of disease and lessening the severity in those infants who acquired infection. Antibodies have been demonstrated in milk directed toward the lipopolysaccharide (LPS) antigens of *Shigella*. However, the four species include a large number of antigenically distinct LPS types (*S. dysenteriae* [serogroup A] twelve unrelated LPS types, *S. flexneri* [serogroup B] six serotypes, *S. boydii* [serogroup C] eighteen immunologically unrelated LPS types, and *S. sonnei* [serogroup D] one LPS type). For human milk to provide protection against shigellosis on the basis of antibodies against LPS, a very large repertoire of serotype specific antibodies might be required. Although the LPS antigens of *Shigella* are not generally closely related, these organisms do share a common virulence mechanism. Virulence in *Shigellae* is a complex phenomenon depending on both chromosomal and plasmid coded loci working in concert to produce multiple virulence enhancing factors (Fig. 1). The most important of these factors, those responsible for invasion of mammalian cells, are encoded on a 30-37 kb segment of the large plasmid that is similar in all *Shigellae*. This plasmid encodes for production of a group of immunodominant proteins, the invasion plasmid antigens A (*ipaA*), B (*ipaB*), C (*ipaC*), and D (*ipaD*), that are genetically and immunologically identical or nearly identical in all of these organisms. Because these *ipa* proteins represent the shared molecular basis for *Shigella* virulence, we sought to demonstrate whether antibodies to these determinants might be present in human milk. We compared milk samples from two populations of women with differing *Shigella* exposure risk: one with a high frequency of shigellosis (Mexican women) and the other with a lower frequency (US women).

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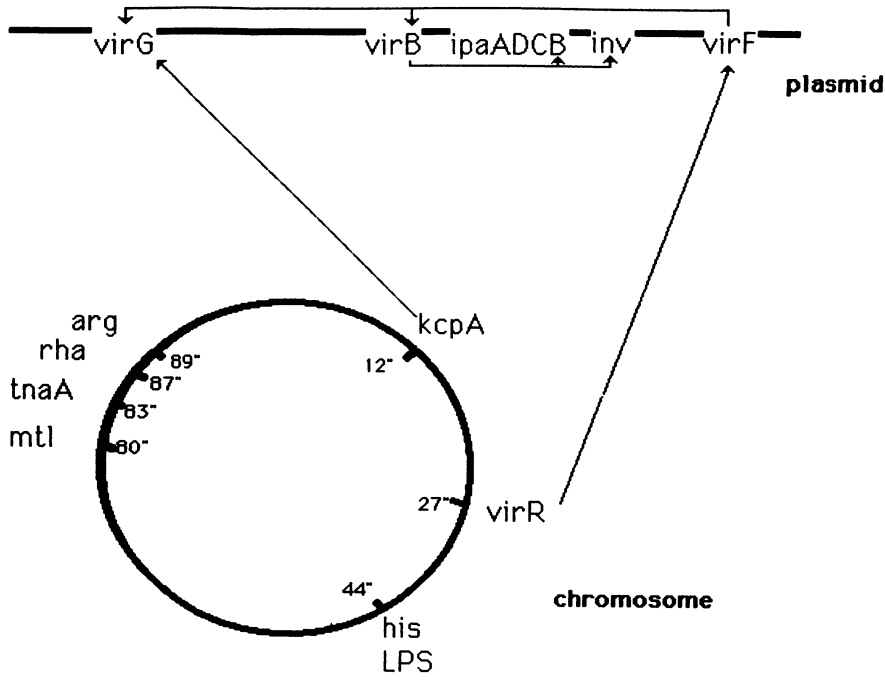

Figure 1. Map of chromosomal and plasmid loci which determine *Shigella* virulence. Although the plasmid is circular, it is shown in a linear representation in this schematic to focus on the part of the plasmid which encodes the virulence associated proteins. The structural genes for the major virulence proteins (*ipaA, ipaB, ipaC, ipaD*) are controlled by the *virB* locus whose function is in turn controlled by *virF*. The *inv* region is required for translocation of the *ipa* proteins to the cell surface. The *virG* locus is responsible for synthesis of a protein involved in intracellular spread of organisms. The *virG* locus is controlled both by a plasmid locus (*virF*) and a chromosomal locus (*kcpA*). The chromosomal locus *virR* is a temperature-regulated repressor which allows plasmid genes to be expressed when the organisms are grown at 37°C. The chromosomal *his* locus is linked to the genes controlling synthesis of O repeat units on LPS. The *mtl-arg* region is responsible for fluid accumulation and enhanced inflammation in gut infection models.

MATERIALS AND METHODS

Milk was obtained by an Egnell pump from fifty-two women living in Houston, Texas and fifty-four women living in Mexico City, DF. Both colostrum and mature milk samples were collected and stored frozen at -20°C until assay.

Absorption to remove antibodies to non-*ipa* proteins of each milk aqueous phase (lipid layer removed after low speed centrifugation) was performed as previously described[1]. Western blot analysis of the absorbed

milks was performed using water extracts[2] from *S. flexneri* serotype 5 strains M90T (fully virulent) and M90T A2 (lacking the plasmid virulence loci) as antigens. Specific IgA-*ipa* reactions were detected with horseradish peroxidase-conjugated goat anti-human IgA (Organon Teknika, Melvern, PA) followed by staining with 0.06% 4-chloro-1-naphthol with 0.01% H_2O_2.

ELISA determinations of S-IgA titers for virulence plasmid associated antigens were measured using water extracts of M90T and M90T A2 diluted with 0.1M sodium carbonate buffer at pH 9.6 for overnight coating at 4C of 96 well plates (Immulon 1, Dynatech; Alexandria, VA). After blocking with 3% nonfat dry milk in phosphate buffered saline for one hour and washing twice with PBS 0.05% Tween 80 (PBS-T), aqueous milk samples preabsorbed with M90T A2 as described above, were serially diluted, incubated for 1hr at 37°C, washed five times with PBS-T, washed twice with PBS and specific IgA detected with peroxidase-conjugated goat anti-human S-IgA (Cappel, Westchester, PA) with ortho-phenylenediamine dihydrochloride as the color indicator. The titer was taken as the last well having a difference in OD of (M90T-M90TA2) greater than three standard deviations above a carbonate buffer background[3].

RESULTS

Immunoblots with strains M90T and M90TA2 side by side demonstrated that the absorption scheme had left little antibody to M90T A2. Antigens whose molecular weights were consistent with the major immunogenic virulence plasmid coded proteins (*ipaA*- 78 kD, *ipaB*- 57 kD, *ipaC*- 43 kD, and *ipaD*- 39 kD) were recognized by immunoblot in all twenty one milk samples from Mexican women and in 40% of milk samples from twenty-five Houston women. Milk samples which detected these major immunodominant antigens also detected antigens whose molecular weights were not consistent with the *ipa* proteins.

ELISA for antibody to virulence plasmid coded antigens demonstrated that all colostrum samples from Mexican women (n=20) had detectable titers ($\geq$1.5) while only 52% of colostrum samples from Houston women (n=23) had detectable antibody levels. Positive titers were noted in 93% of samples from Mexican women (n=31) taken $\geq$ 8 days into lactation and 46% of samples from Houston women (n=26). Serially sampled women in general showed stable titers (within one tube dilution) after the first two weeks of lactation. These differences between frequency of antibodies in the Mexican and Houston colostrum and milk samples were highly significant ($P<0.001$).

DISCUSSION

The putative protective effect of human milk feeding in prevention of shigellosis has not been defined. Potentially important factors include the consumption of a less contaminated diet during breastfeeding, specific immune factors including S-IgA directed toward LPS or virulence plasmid associated antigens, milk leukocytes, alteration of stool pH by breastfeeding with overgrowth of organisms that compete with *Shigella* for the colonic ecologic niche, and non specific factors in milk such as lactoferrin, lysozyme,

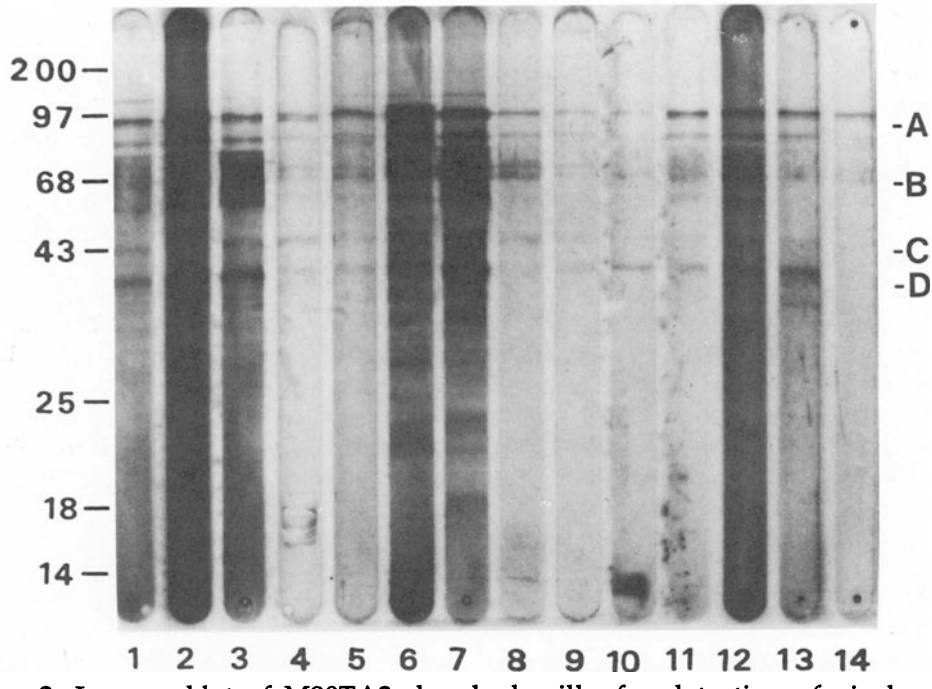

Figure 2. Immunoblot of M90TA2-absorbed milks for detection of virulence plasmid-specific antibodies. Antigens of a water extract of M90T have been separated by SDS-PAGE, transferred to nitrocellulose, and individual milk samples incubated in a miniblot apparatus. The location of the major immunodominant virulence plasmid proteins (*ipaA, ipaB, ipaC, ipaD*) is shown. Most samples have antibodies both to the *ipa* proteins and to undefined antigens.

oligosaccharides, fatty acids, etc. The significance of antibodies to the *Shigella* virulence plasmid antigens in the colostrum and milk of women from Mexico, an area with a high frequency of shigellosis, is that these antibodies may provide a means for protecting against all serotypes of *Shigella*.

Of more interest is the demonstration of virulence plasmid-related antibodies in the colostrum and milk of women living in Houston, and area with a lower frequency of *Shigella* infection. In this environment, some women may have never experienced an episode of shigellosis. It is extremely unlikely that so many women living in this environment would have had recent *Shigella* infection. *Shigella* although briefly excreted after clinical illness, is rarely carried chronically. Antibodies that recognize *Shigella* virulence plasmid associated antigens are very unlikely to be related to cross-reactive antigens of other bacteria. Studies of DNA of twenty-two different enteric genera (1240 different strains) have shown no homology to the genes encoding the *ipa* proteins[4-6]. Stool samples of children evaluated for the presence of the genes coding the *Shigella* virulence plasmid proteins have demonstrated that the genes are detectable only when *Shigella* or the closely related invasive *E. coli* are cultured[7]. Studies comparing the frequency of

milk antibodies to the *ipa* proteins and *Shigella* LPS demonstrate that the antibodies are associated (Hyani K. *et al.* manuscript in preparation). Thus, the finding of antibodies to the virulence plasmid coded antigens appears to be specific and to reflect prior infection with *Shigella*. That such antibodies are present in a population unlikely to have recent experience with *Shigella*, suggests that *Shigella*-specific, gut-derived lymphocytes, stimulated many years before, have been recruited to the breast prior to the onset of lactation.

CONCLUSIONS

1. Human milk commonly contains antibodies to the major virulence antigens shared by all *Shigellae*.

2. The levels of these antibodies in milk do not change significantly during lactation either in a high (Mexico) or low *Shigella* risk population (US).

3. The presence of antibodies to *Shigella* virulence plasmid-coded antigens in the milk of women from an area where *Shigella* infection is not common suggests that *Shigella*-specific, IgA-secreting cells which have been programmed in the distant past are recruited to the breast during pregnancy or lactation.

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CORTISONE STRENGTHENS THE INTESTINAL MUCOSAL BARRIER IN A RODENT NECROTIZING ENTEROCOLITIS MODEL

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INTRODUCTION

Necrotizing enterocolitis (NEC) is the most common acquired gastrointestinal emergency in the neonate[1]. The disease is an enigmatic one with no identifiable risk factor except for prematurity. The pathogenesis of the disease appears to be multifactorial with bacterial proliferation, intestinal ischemia, and enteral alimentation all appearing to have a role, either individually or in concert, in any specific patient[2]. It has been suggested that the breakdown of the gastrointestinal mucosal barrier, which is already immature secondary to the prematurity, may very well be the common pathway through which the different factors manifest themselves, allowing for the systemic penetration of toxins, bacteria, and antigens and the development of NEC[3]. There is no specific treatment for this disease, but based on this hypothesis on its etiology precocious maturation of the intestinal barrier might ameliorate or prevent this life-threatening disease. The aim of this study, therefore, was to re-establish a model of NEC in the rat and to see if the lesion could be prevented with the prenatal administration of corticosteroids, a known maturational agent. We then went on to study the intestinal mucosal barrier in this model that may explain the beneficial effects of the corticosteroids.

MATERIALS AND METHODS

The NEC Rat Model

Pregnant Sprague Dawley rats, 18 days' gestation were treated with either cortisone acetate, 20mg/100g body weight, a dose previously noted to mature surface characteristics of the neonatal rat[4], or normal saline. Four to five pups from each mother were taken immediately after birth, before nursing had been initiated, and were placed in a 36°C incubator. Using the model of Barlow which takes into account the multifactorial pathogenesis of

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NEC[5] the pups received an artificial formula by orogastric feedings and daily ischemic insult with hypoxia and cold stress[5,6]. Their urogenital areas were stroked gently every 4 h to stimulate voiding and defecation. The pups were weighed at the same time daily. The effects on the pups after four days of such treatment was assessed by looking for weight loss, pathologic changes, and mortality. The intestinal mucosal barrier was then examined by studying three aspects: 1) the *in vivo* intestinal uptake of a fed antigen, ovalbumin, was evaluated as a monitor of the ability of the intestine to take up macromolecules; 2) the bacteria found in close association with the small intestine was measured; and 3) the translocation of these bacteria to the liver was evaluated.

Bacterial Colonization and Translocation to Liver

The small intestines of some of the animals killed on day 4 were assessed for associated aerobic bacteria. The small intestine from the pylorus to the cecum was removed under sterile conditions. The lumen was flushed with 6 ml of sterile PBS to remove non-adherent organisms. The wet weight of each tissue was recorded. Each intestine was homogenized separately under sterile conditions and ten-fold dilutions were prepared in sterile PBS. Each dilution from 10^1 to 10^6 was plated in duplicate on horse blood agar plates which were incubated at 37°C for 24 h. The colonies were then counted and expressed as colony forming units (CFU)/g wet tissue. To assess whether translocation of bacteria from the gastrointestinal tract to the liver occurred, the liver was removed under sterile conditions and homogenized in sterile PBS and serial dilutions were plated on horse blood agar. Plates were examined for colonies at 24 h.

Ovalbumin Uptake Studies

To study the uptake of an antigen from the intestine into the systemic circulation, ovalbumin (Sigma Chem. Co., St. Louis, MO), 2 mg/g of body weight, was fed to the pups by orogastric intubation at 2 days of age with the silicone rubber tubing used for the formula feeding. Blood was obtained by cardiac puncture 1 h later and sera were kept at 4°C for determination of ovalbumin content by an ELISA using horseradish peroxidase-conjugated rabbit anti-ovalbumin antibody as the detector.

Statistics

The logit-binomial model was used to estimate the probability of all yes or no phenomena such as dying, pathologic lesions, and translocation. For the continuous outcomes of ovalbumin concentration, bacterial colonization and weight change, a linear model estimated the difference between the two groups.

RESULTS

Mortality (Table 1)

Two of the 29 pups whose mothers were treated with cortisone died by the end of the experiment, the fourth day of life. However, in the group of animals subjected to the NEC protocol but not pre-treated with cortisone, 12 pups out of a total of 28 died by day 4. A log likelihood ratio test indicated that this difference is significant with $p<.01$.

Weight Change (Table 1)

Over the 4 days studied, this rodent NEC model resulted in a net weight loss to all of the pups relative to their birth weight, but this weight loss was minimized by the prenatal administration of cortisone with a mean individual weight loss of 0.08 g after treatment compared to a 0.75 g loss, demonstrating that the pretreatment resulted in a better clinical outcome.

Table 1. Morbidity and Mortality in 4 Day Old Rat Pups Subjected to NEC

	NS[a]	CA[b]	p
Mortality (%)	39.3	6.9	<.01
Weight Change[c] (g)	-0.75±0.16	-0.09±0.14	.0009
Pathology			
Gaseous distention(%)	75	25	<.01
Hemorrhagic lesion(%)	50	0	<.01

[a]rat pups born to normal saline (NS)-treated mothers, n=28
[b]rat pups born to cortisone acetate (CA)-treated mothers, n=29
[c]mean ± SEM

Pathology

The gross findings in the intestine were primarily gaseous distention of the intestinal loops along with hemorrhagic lesions distributed in patches through the distal small bowel, analogous to the findings in human neonatal NEC. Gaseous distension was seen in 75% of the pups in the control group as compared to 25% of the cortisone group, with a p-value less than <.01 (Table 1). *None* of the rats born of the mothers treated with cortisone showed hemorrhagic patchy lesions *whereas 50%* of the pups from untreated mothers had this finding (Table 1).

Microscopically, interstitial and intraluminal hemorrhage were noted and epithelial damage with villous shortening was seen in the ileum of the group which did not receive the cortisone (Fig. 1A), whereas the architecture was maintained in the cortisone treated pups (Fig. 1B).

Bacterial Colonization

The total number of bacterial colonies was significantly less in the steroid-treated group (Table 2), suggesting that the steroid treatment rendered the pups less susceptible to the association of organisms with their intestinal microenvironment. Changes of surface glycoconjugates secondary to the corticosteroid treatment[4] are potentially the cause of this finding.

Bacterial Translocation

The levels of bacteria populating the gastrointestinal tract as well as the strength of the barrier affects the incidence of translocation of these bacteria to the mesenteric lymph nodes, and to the liver, spleen, and blood[7], resulting in

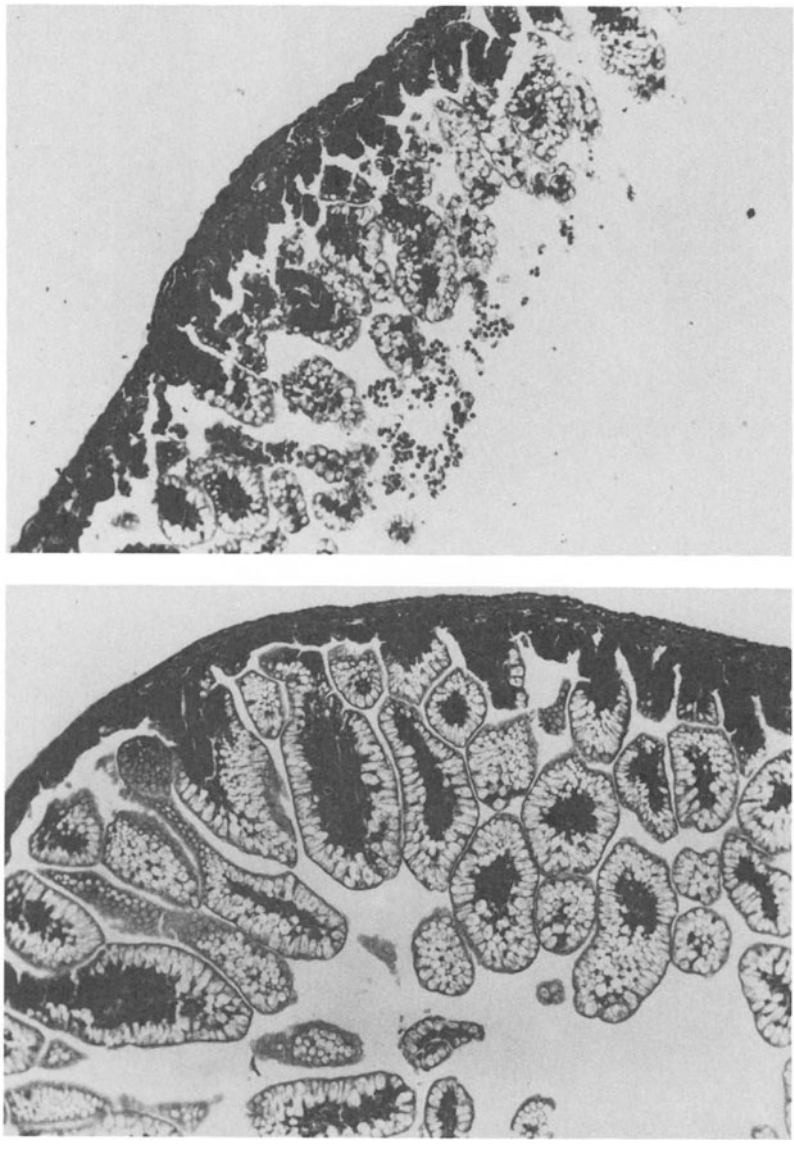

Figure 1. Samples of distal ileum of 4-day old rat pups subjected to NEC protocol (H&E; original magnification x 150).

A. Mothers received normal saline prenatally. Blunting of villi together with desquamation of epithelium, intramural hemorrhage, and loss of red blood cells into the intestinal lumen are noted in a representative section.

B. Cortisone was administered to the mother prenatally. Villus length is preserved and the normally vacuolated epithelium is not altered in a representative section.

systemic illness. We therefore, assessed for the presence of bacteria in the liver, as a measure of systemic penetration of the bacteria. We found bacteria *much less* frequently in the livers of the cortisone animals than in animals whose mothers had not been treated with steroids (Table 2), confirming the relatively healthy status of the steroid-treated pups.

Ovalbumin Uptake

Finally, the penetration of intact proteins as well appears to be related to maturation of the gastrointestinal barrier, with macromolecules being absorbed readily in the newborn pup and much less so in the adult rat[8]. The data presented here shows that the NEC model causes a pertubation of this already immature barrier as evidenced by the *extremely* high levels of serum ovalbumin after an oral feeding while the prenatal administration of cortisone prevented the influx of large amounts of the ovalbumin into the systemic circulation even in the presence of the insult known to produce the lesion of NEC (Table 2).

Table 2. The Effects of Prenatal Cortisone Acetate (CA) on the Gastrointestinal Barrier in a Rodent Model of NEC

	NS(n)	CA(n)	p
Ovalbumin	3169±830[a] ng/ml	590±111[a]	.0065
Bacterial colonization	5.94±0.20[a]	4.16±0.30[a,b]	.0001
Translocation	42.1%	10.5%	<.01

[a]mean ± S.E.
[b]$\log_{10}$ CFU/g intestine

DISCUSSION

It is well known that corticosteroids as well as thyroxine and other growth factors can induce structural and functional changes in the neonatal rat's intestine, making it approach a more mature state. In 1984, Bauer and his colleagues[9] showed that corticosteroids given to women at risk of delivering prematurely, resulted in a decreased incidence of NEC in the offspring. More recently, Halac *et al.*[10] found a significant decrease in the incidence of NEC in premature neonates whose mothers were treated prior to delivery with steroids. In addition, infants whose mothers did not receive treatment were then randomized to treatment or placebo and again there was a decreased incidence of NEC and an improved clinical outcome when NEC did occur.

The present study confirms this finding of a beneficial effect of corticosteroid treatment in an experimental rodent model of NEC. This rodent model serves as a useful adjunct to examine the mechanisms by

which corticosteroids protect against the development of NEC. Prenatal corticosteroids enhanced the intestinal mucosal barrier of the neonate to luminal antigens and decreased small intestinal bacterial colonization.

It has been suggested that bacterial colonization is partially determined by the availability of certain glycoconjugates on the mucosal surface by allowing for the binding of the bacteria with specific carbohydrate moieties. Modification of the carbohydrate moieties of the intestinal microvillus surface and of mucus glycoproteins has been described with development[11,12] and corticosteroids have been shown to precociously mature the developmental process[4] on the intestinal microvillus membrane. We speculate that the protective effect of corticosteroids in NEC is related to these types of maturational changes at the intestinal surface, which result in decreased bacterial colonization and decreased foreign antigen penetration.

CONCLUSIONS

This observation reinforces the role of bacteria in the multifactorial pathogenesis of NEC. Prenatal treatment with cortisone improved the outcome in this rodent NEC model, enhanced the intestinal mucosal barriers, and decreased bacterial colonization. The further assessment of pharmacologic manipulation with glucocorticoids, thyroid hormone, and factors in milk such as epidermal growth factor which may have a more localized effect on intestinal barrier function, should further enhance our understanding of the pathogenesis and treatment of NEC.

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THE RELEVANCE OF IMMUNOGLOBULIN IN THE PREVENTION OF NECROTIZING ENTEROCOLITIS

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INTRODUCTION

Necrotizing enterocolitis (NEC) is a severe acquired gastrointestinal disease and an important cause of morbidity and mortality among stressed low-birth-weight infants in neonatal intensive care units. Although prematurity is the primary risk factor for NEC, it is generally accepted that the pathogenesis of the disease is multifactorial, and a combination of immaturity of the host's gastrointestinal defense mechanisms, intestinal ischemia, and colonization of the gut by infectious agents leads to the development of the disease[1]. NEC develops in 2 to 7% of premature infants admitted to a neonatal intensive care unit[2]. Early abdominal signs indicative of NEC include abdominal distension, diarrhea, gastric retention, emesis, and macroscopic or occult gastrointestinal bleeding. When the disease progresses to unstable stage with vital signs that resemble sepsis, perforation of the intestine or an obstruction pattern on abdominal radiograph, patients require aggressive medical and/or surgical therapy. Histologically the disease, affecting primarily the terminal ileum and ascending colon, is characterized by coagulation necrosis of mucosa and submucosa, inflammation, ulceration, peritonitis, and intramural gas-filled cysts (i.e. pneumatosis intestinalis)[3].

The mortality rate approaches 40-50%, depending on birth weight, maturity and coexisting medical problems[4,5]. Prevention of NEC would therefore substantially contribute to a decrease in morbidity and mortality rates among premature infants. In a recent controlled, randomized clinical trial published in detail[6], we evaluated whether supplementation of feeding of low-birth-weight infants with an oral IgA-IgG preparation would reduce the incidence of NEC.

MATERIALS AND METHODS

Study Design and IgA-IgG Preparation

A detailed description of the study design has been published elsewhere[6]. In brief, a total of 434 infants with birth weights between 800 and

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2000 g, for whom breast milk from their mothers was not available, were prospectively enrolled in the study and randomly assigned to one of two groups. For the first 4 weeks of life infants in group A (N=211) received 600 mg per day of an oral IgA-IgG preparation as a supplement to their regular feeding (infant formula with or without pasteurized, pooled human milk), whereas 223 infants in group B received their regular feeding only (control group).

Two hundred thirty-four infants (123 in the IgA-IgG treatment group and 111 in the control group) were withdrawn during the first week of the study because breast milk from their mothers became available, and 21 control infants were excluded during weeks 2 to 4 of the study because of violations of the study protocol or because breast milk from their mothers became available.

The oral IgA-IgG preparation used in our study was made from human serum, Cohn fraction II, by ion-exchange chromatography (Igabulin, kindly supplied by Immuno AG, Vienna, Austria)[6]. Nine different lots contained 66-84% IgA, 15-33% IgG and <1.5% IgM. As determined using standard techniques (hemagglutination inhibition, neutralization, radioimmunoassay, indirect immunofluorescence, bacterial agglutination), the preparation contained high titers of antibodies against a multitude of infectious pathogens (bacterial toxins such as pertussis, tetanus, diphtheria; viruses such as poliovirus, coxsackievirus, rotavirus and echovirus)[6].

Examination of Fecal Immunoglobulins

Stool samples were collected over a period of 24 hours up to twice a week during the four-week study period. The feces were lyophilized, dissolved in PBS, and immunoglobulin concentrations were determined by single radial immunodiffusion (for a detailed description see Ref. 6).

Immunofluorescence Analysis of Anti-Bacterial Antibodies

IgA- and IgG-antibody titers against enteropathogenic (*Escherichia coli* 125, *Salmonella typhimurium, Shigella sonnei, Klebsiella pneumoniae, Clostridium difficile,* a kind gift of Prof. Rotter, Institute of Hygiene, University of Vienna) and nonpathogenic (*E. coli* 089.H10) bacteria were investigated using indirect immunofluorescence and evaluated with a cytofluorograph.

In brief, 50 µl of a suspension of heat-inactivated bacteria in PBS (approximately 10^7 - 10^8 bacteria/ml) were added to 50 µl of serial two-fold dilutions of the IgA-IgG preparation (one representative lot) and incubated for 60 min at 37°C. The bacteria were then washed twice at 2500 rpm (10 min). Fifty µl of the second step reagent (FITC-conjugated goat anti-human IgA [α chain-specific] or goat anti-human IgG [H+L chain-specific], Jackson Laboratories; final dilution 1:40) were added and the mixture was further incubated for 30 min at 4°C. After washing, the bacteria-associated fluorescence was examined using a cytofluorograph (FACS 440, Becton Dickinson, San Jose, CA). Bacteria and debris were separated by setting a gate on the side scatter histogram (log-amplified with maximal gain). Results are depicted as percent specific fluorescence reactivity, i.e., the percentage of fluorescence-positive bacteria in the experimental sample minus the percentage of background positivity of bacteria stained with the second-step reagent only (mean of quadruplicate samples). For each two-fold dilution of the IgA-IgG preparation the mean of two to three determinations is given.

RESULTS

Prophylaxis of NEC by Oral IgA-IgG

No untoward effects of the immunoglobulin feeding were observed in the treated infants. In contrast, feeding of the oral IgA-IgG preparation significantly reduced the incidence of NEC in our study population (Table 1A). One hundred seventy-nine infants who completed the study were evaluated in great detail. No case of NEC occurred among 88 infants receiving oral IgA-IgG, compared to 6 cases among the 91 control infants (i.e. 6.59%). The clinical diagnosis of NEC was confirmed by abdominal X-ray (N=2), histopathologic examination of specimens obtained during surgery (N=2) or autopsy (N=2). Of the infants withdrawn from the study, two

Table 1. Effect of Oral IgA-IgG Treatment on the Incidence of Necrotizing Enterocolitis (NEC)

A Incidence of NEC during the randomized trial:

	N	Controls	Treatment group
Cases of NEC among the total number of infants enrolled in the study	434	8/223 (3.59%) p=0.0055[a]	0/211
Cases of NEC among the infants who completed the study and had never received any breast milk	179	6/91 (6.59%) p=0.0143[a]	0/88

[a]statistically significant difference between controls and IgA-IgG-treated infants as determined by Chi-square analysis

B Incidence of NEC among low-birth-weight infants (<2500g) - - admitted to the Glanzing Children's Hospital after the randomized trial from March 1989 to August 1990, who all received oral IgA-IgG for the first ten days of life

Total number of infants admitted (N):	606
Cases of NEC:	4 (0.66%)
birth weight (g)	1090,1180,1410,1500
onset of disease	day 2,4,8,10
confirmation of diagnosis at	operation: 2 autopsy: 2

assigned to the control group developed NEC. The different incidence of NEC in the two groups is most likely due to the oral IgA-IgG treatment in group A, since the distribution of several risk factors for NEC (e.g., birth weight, incidence of perinatal complications, choice and date of onset of enteral alimentation) was comparable between IgA-IgG-treated and control infants.

In addition, IgA-IgG feeding seemed to have a beneficial effect on the occurrence of pneumonia in infants who completed the study, since the number of days with clinical symptoms of pneumonia in infants who survived was significantly lower in IgA-IgG-treated group[6]. IgA-IgG feeding also seemed to have a beneficial effect on thriving in low-birth-weight infants. In infants with a birth weight between 1300 and 1700 g, the time required to regain birth weight was significantly lower in IgA-IgG-treated infants (11.3 $\pm$ 0.7 days) compared to the controls (14.6 $\pm$ 1.1 days, mean $\pm$ SEM, $P < 0.02$).

Further support for the beneficial effect of oral IgA-IgG on the incidence of NEC comes from the analysis of the frequency of the disease at our institution after the clinical trial. Since March 1989, all low-birth-weight infants (<2500 g) who are admitted to the Glanzing Children's Hospital neonatal intensive care unit receive 600 mg of oral IgA-IgG per day for the first ten days of life. Until August 1990, 4 cases of NEC were observed among a total of 606 infants admitted (i.e., 0.66%) (Table 1B). The birth weight of the affected infants was between 1090 and 1500 g, the onset of the disease between day 2 and 10 of life. The diagnosis was confirmed in 2 infants by histologic examination of surgical specimens, in two cases at autopsy. We feel that the incidence of NEC observed in infants receiving oral IgA-IgG after the clinical trial is lower than the incidence of the disease in the control group during the study, at it also seems to be lower than the average incidence reported in the literature.

Recovery of Immunoglobulin in the Stool

We found no evidence for resorption of oral immunoglobulin through the gastrointestinal tract of the neonates. As can be seen in Table 2, the number of infants with detectable serum IgA (i.e., IgA above 3 mg/dl) was comparable in IgA-IgG-treated and control infants; serum levels of IgG and IgM tended to be higher in the control group, which might reflect the higher exposure of control infants to environmental antigens through the intestinal tract[6]. However, intact immunoglobulin could be recovered in the stool of IgA-IgG-treated infants. When fecal immunoglobulin was studied in 24 h stool samples obtained from 7 infants treated with oral IgA-IgG and from 6 controls, concentrations of IgA and, to a lesser extent, IgG were significantly higher in stool samples collected from IgA-IgG-treated infants compared to fecal samples from control infants (median [range] mg/g dry feces, first week: IgA-IgG-treated: IgA 7.3 [1.9 - 34.1], IgG 1.3 [0.1 - 12.9]; controls: IgA 0.0 [0.0 - 0.3], IgG 0.4 [0.1 - 3.1]; results for weeks 2-4, see Ref. 6). Fecal IgM levels were comparably low in both groups. In addition, fecal immunoglobulin levels were examined in 3 breast fed infants (Fig. 1). Levels of IgA were comparable in stool samples from IgA-IgG-treated and breast-fed infants. Only small amounts of IgG could be found in the stool of breast-fed infants (fecal immunoglobulin levels in breast-fed infants during the first 2 months of life: IgA 5.8 [3.5 - 24.5], IgG 0.1 [0.02 - 0.6] mg/g dry feces, median and range of 14 determinations).

Table 2. Number of Infants with Detectable Serum IgA Levels During the Study Period

Weeks	IgA-IgG-treatment	Number of infants with detectable serum IgA (>3mg/dl) / total number of infants examined (percent)
1	–	2/82 (2.4%)
	+	3/88 (3.4%)
2	–	7/74 (9.5%)
	+	3/80 (3.8%)
3	–	10/72 (13.9%)
	+	4/72 (5.6%)
4	–	13/65 (20.0%)
	+	14/57 (24.6%)

Serum immunoglobulin levels were determined once a week by single radial immunodiffusion.

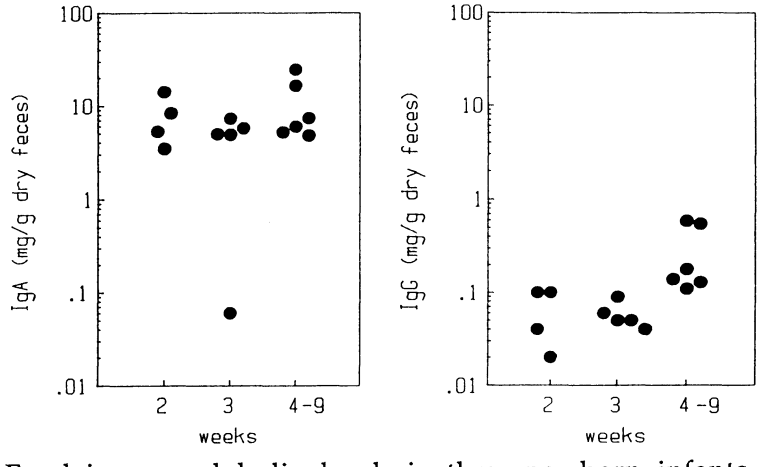

Figure 1. Fecal immunoglobulin levels in three newborn infants receiving breastfeeding.

Antibacterial IgA and IgG Titers of the Immunoglobulin Preparation

Since the oral immunoglobulin preparation contained both IgA and IgG, we were interested in the isotypes of antibodies against enteropathogenic (*E. coli* 125, *Salmonella typhimurium, Shigella sonnei, Klebsiella pneumoniae, Clostridium difficile*) and nonpathogenic (*E. coli* 089.H10) bacteria. Determination of the respective titers of IgA and IgG antibodies of one representative lot of the immunoglobulin preparation was performed using isotype-specific anti-human-immunoglobulin in indirect immunofluorescence, evaluated with a cytofluorograph (Fig. 2). Whereas antibodies against *Salmonella, Shigella, Klebsiella* and *E. coli* 125 were of both the IgA and IgG isotype, predominantly IgA antibodies bound to *Clostridium difficile.*

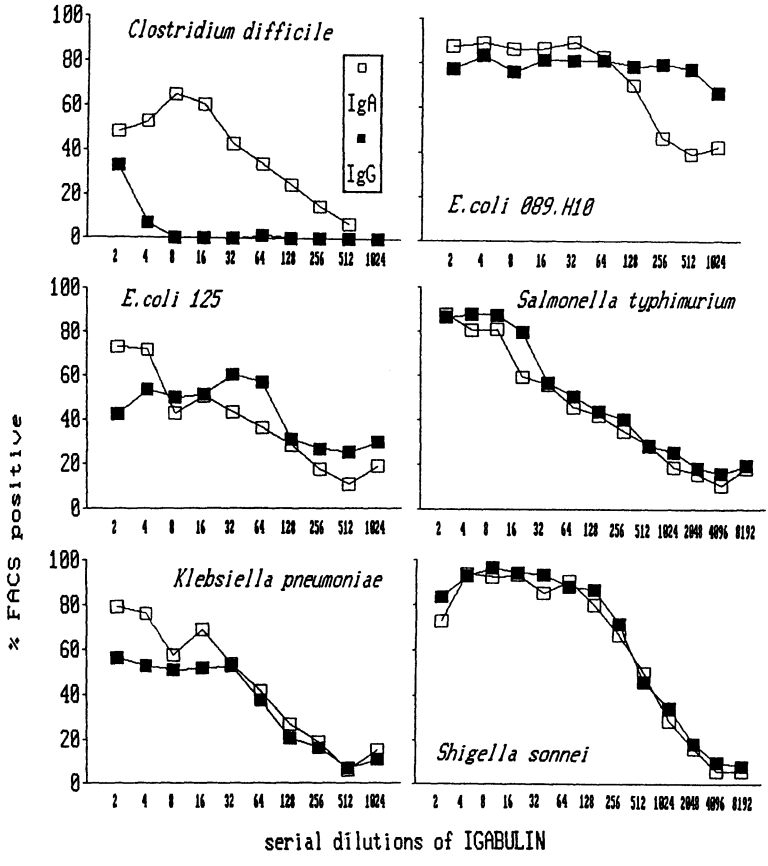

Figure 2. Examination of antibacterial IgA and IgG antibodies of the oral immunoglobulin preparation by indirect immunofluorescence evaluated with a cytofluorograph

In contrast, slightly higher titers of IgG antibodies against *E. coli* 089.H10 were found.

DISCUSSION

Multiple factors play a role in the pathogenesis of NEC. Colonization and invasion of the intestinal mucosa by pathogenic microorganisms as well as the presence of excess protein substrate in the lumen (due to formula feeding) might be essential in the development of NEC. In stressed low-birth-weight infants, clinical conditions associated with perinatal hypoxia might result in ischemia of the gastrointestinal mucosa, which facilitates the invasion of infectious pathogens[7,8]. A variety of infectious organisms by their nature are likely to invade susceptible damaged bowel and/or produce large amounts of toxin. This probably accounts for the general experience that different infectious agents (e.g. *Klebsiella, Salmonella, Clostridia, E. coli,* human enteric coronavirus, rotavirus) have been associated with the disease[9].

Studies performed on the gastrointestinal immunologic defense mechanisms of human neonates generally describe a lack of locally produced antibodies (secretory IgA, i.e. dimeric IgA covalently linked to secretory component) in the gastrointestinal tract of full-term as well as premature neonates[10]. In this condition of inadequate local immunoprotection, alternative mechanisms must function to inhibit overgrowth of potentially pathogenic intestinal flora and prevent invasion. Strong evidence has accumulated for the anti-infectious effect of breastfeeding[11,12]. Due to the immunoprotection provided by breastfeeding, the intestinal flora of breast-fed and formula-fed infants differ with a prevalence of non-pathogenic bacteria in the intestine of breast-fed neonates[13,14]. However, to prevent transmission of infectious agents, pooled human breast milk must be pasteurized, a treatment that significantly reduces not only its content of functional IgA but also its immunoprotective effect[15,16].

The effective prophylaxis of NEC by oral IgA-IgG in our study can best be explained by the well-established immunoprotective effect of orally administered antibodies against infection of the gastrointestinal mucosa in children and adults[17-20]. Results of our study indicate that oral immunoglobulin acts at the level of the intestinal mucosal surfaces. Examination of fecal immunoglobulin in IgA-IgG-treated infants demonstrated that substantial amounts of orally administered IgA and IgG lacking a secretory component can resist proteolytic degradation in the gastrointestinal tract of low-birth-weight infants[6]. Furthermore, the finding of comparable concentrations of fecal IgA in the feces of IgA-IgG-treated and breast-fed infants suggests that we administered physiologic amounts of IgA-IgG as a substitution for the immunoglobulin normally provided by breastfeeding. These data confirm and extend previous reports from others who noted the survival of oral IgG in stool samples of low-birth-weight infants or children with primary immunodeficiency. As assessed by its antigen-binding capacity[21] and the ability to opsonize group B streptococci[22], the fecal immunoglobulin recovered in these studies was functionally intact.

It is interesting to note that different titers of IgA and IgG antibodies against a bacterial strain frequently associated with NEC (*Clostridium difficile*) can be found in our oral immunoglobulin preparation. Whereas the preparation contained comparable amounts of IgA and IgG antibodies against a variety of pathogenic bacterial strains (*Salmonella, Shigella, Klebsiella* and *E. coli* 125), titers of *Clostridium difficile*-specific IgA were significantly higher than IgG titers. Studies performed *in vitro* and in suckling mice prove that secretory IgA from human colostrum can neutralize the cytopathic effect of *Clostridium difficile* toxins A and B[23]. Although both IgA and IgG in our preparation are monomeric, it could be speculated that for at least one bacterial pathogen (*Clostridium difficile*) the IgA antibodies are more effective in protecting from gastrointestinal infection than IgG antibodies.

Analogous to the function of antibodies normally provided by breast-feeding, the immunoprotective effect of oral immunoglobulin (IgA and/or IgG) on the intestinal mucosa can best be explained by the formation of antigen-antibody complexes in the bowel lumen or on the mucosal surface. This hypothesis is supported by the finding of immune complexes formed between orally administered human serum immunoglobulin and endogenous rotavirus in immunodeficient patients with viral gastroenteritis[21]. Binding of functionally intact oral immunoglobulin (IgA and/or IgG) to the antigen (e.g., a bacterial or alimentary constituent) may

cause intraluminal agglutination of potentially pathogenic microorganisms, thereby interfering with the bacterial colonization of the intestinal epithelial surface and by neutralizing bacterial virulence factors or preventing toxic effects of an excess of alimentary protein (i.e. formula feeding) on the intestinal mucosa.

CONCLUSIONS

1. Feeding of human serum IgA and IgG was shown to prevent the development of necrotizing enterocolitis in low-birth-weight infants.

2. The immunoglobulin preparation contained high titers of antibodies against a battery of bacterial and viral pathogens, and comparable amounts of IgA and IgG antibodies against several pathogenic bacteria (e.g., *Klebsiella, Salmonella, Shigella, E. coli*).

3. The preparation's IgA titer against *Clostridium difficile*, a bacterium frequently associated with NEC, was much higher than the IgG titer.

4. Both the inhibition of bacterial colonization and the neutralization of bacterial toxins and/or viral pathogens by IgA and/or IgG antibodies might be responsible for the protective effect of the oral immunoglobulin preparation.

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STRATEGIES FOR THE PREVENTION OF FOOD ALLERGIC ASSOCIATED ATOPIC DISEASE

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INTRODUCTION

Prevention is the most important element that threads the discipline of Pediatrics. Immunization is a case in point where pediatricians have taken the lead in eradication of the common communicable scourges of mankind. In industrialized countries today, allergic disease has become one of the main causes of childhood morbidity. In early infancy, this may be linked causally to sensitization to food proteins or food allergy. The topic remains poorly understood. Food allergy is both underdiagnosed and overdiagnosed. It is important to emphasize that the term "Food Allergy" refers to an immunologically mediated clinical syndrome that develops after the ingestion of a dietary product[1]. This is to distinguish it from the all-inclusive term "Food Intolerance" that also refers to non-immunologically mediated reactions such as symptoms due to enzyme defects, food toxins and microbial contamination, and idiosyncratic reactions. The prevalence of food allergy in the pediatric population is variously estimated to be in the range of 0.5 - 6.0 percent. In selected patients groups, such as children with atopic dermatitis, the prevalence may be as high as 25 percent[3,4].

Once diagnosed, management is straight forward. The food incriminated should be eliminated totally from the diet. In the very young infant whose sole source of nutrient and energy intake is milk, allergy to this food is a serious matter. Substitutes based on hydrolysates of milk, or proteins from either vegetable or meat sources have been employed. Elimination is simpler with foods that are only occasionally eaten. It is prudent to consult a dietitian and devise a nutritionally adequate, palatable and acceptable menu. In patients who fail to respond to food elimination, one should consider non-compliance, other causes of symptoms, and hypersensitivity to other foods.

PREVENTION

Rationale

There are three main reason for thinking of prevention of food allergy. The first is the increasing prevalence of atopic disease, some of which may be

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due to food allergy. Secondly, in spite of effective medicines and inhalation techniques, mortality from asthma has increased in the last 10 years. Thirdly, the management of allergic diseases involves considerable financial and other costs.

In industrialized countries, atopic disease is the most common cause of morbidity and a significant factor in mortality. In our studies, the prevalence of positive skin tests to a panel of common allergens is currently above 10 percent among children 12-16 years of age. Furthermore, there has been a significant increase in atopy in the last 12 years. In Newfoundland, the cumulative incidence of atopic eczema was 2.8 percent in 1976. This has now increased by one-half, to reach a figure of about 4.2 percent. The prevalence of bronchial hyperresponsiveness has increased almost two-fold in the last 12 years. The increase in asthma is even more steep and is currently about 6.4 percent. The reasons for these rapid increases are not clear; increased pollution in the big cities, consumption of processed foods, and stress may be some of the factors. The second reason for thinking of prevention is the cost of treating those with atopic disease. Some of these costs are measurable in dollars, pounds and liras, such as physician fees, hospital expenses, laboratory tests, etc., whereas others cannot be measured in monetary terms, such as school days lost, and physical and emotional isolation.

Identification of Infants at Risk

In order to attempt prevention, the first step is the identification of the infant at high risk of developing atopic disease. If one parent has atopic disease, the risk of developing allergies in the offspring is about 37%. If both parents are affected, this increases to about 62%. Besides family history, there are two additional tests that have been looked at: Cord blood IgE and suppressor T cells. If cord blood IgE is elevated, there is a higher risk, irrespective of positive or negative family history[2,3]. Conversely, with low cord blood IgE the risk is relatively less. Similarly, those infants who develop atopic disease in childhood show a reduction in the number of CD8+ suppressor cells in the first few days of life, again irrespective of family history[4]. This may be because of the important immunoregulatory role of these cells in IgE production. If one combines these tests, there is improved prediction, both positive and negative. In the presence of family history, elevated cord blood IgE and reduced CD8+ T cells, the positive predictive value is almost 95 percent.

Breastfeeding

Having identified the infant who is at high risk of atopic disease, there are several methods that can be considered for prevention. One obvious one is breastfeeding[5-7]. Some of the reasons why breastfeeding reduces occurrence of atopic disease are: reduced exposure to food proteins that would be present in formulas, improved maturation of the intestinal barrier thereby reducing the absorption of macromolecules, reduced frequency of infection which can act as an adjuvant, and finally, the presence of anti-inflammatory factors in human milk.

One cannot overestimate the importance of appropriate study design and analysis[8-10]. The study population must be stratified according to high or low risk based on family history and other factors. Obviously prospective studies are much better than retrospective one, specially when the period of recall is long. In the case of formula-fed infants, randomization is essential.

Sample size should be adequate and should be calculated before the trial begins. There should be a low drop-out rate, certainly less than 20 percent. Criteria for diagnosis and evaluation of severity should be defined. Assessment should be done by observers who are blind to the type of feeding being given. In addition, several confounding factors should be noted and taken into consideration when comparing various groups. For example, when examining respiratory symptoms, it is crucial to note parental cigarette smoking, day care, and presence of animals.

Our cumulative data shown in Table 1 confirm the partial protective effect of breastfeeding. Three points need emphasis. Firstly, the benefits are obvious in those infants who were at high risk because of family history or elevated IgE or both. Secondly, the protection is greater with more prolonged breastfeeding, i.e. more than four months. Thirdly, even among those who are exclusively breast-fed for that length of time, there is still a significant incidence of atopic disease. This led us to consider the role of maternal diet during pregnancy and during lactation.

Table 1. Incidence of Atopic Eczema

Group	Low risk	High risk
Breast-fed		
<4 months	2.8%	32.6%
<4 months	1.6%	22.1%
Cow milk formula-fed	3.8%	48.6%

Role of Maternal Diet

Foods eaten by the pregnant mother do appear in the amniotic fluid which is being swallowed by the fetus. As many as 30 percent of amniotic fluid samples contain small but detectable, and perhaps immunogenic, quantities of dietary proteins. Since the fetus is capable of mounting IgE and other immune responses as early as the 10th week of gestation, it is possible for fetal sensitization to maternal dietary proteins to begin during the first trimester of pregnancy.

To examine this issue, we recruited before 10 weeks of pregnancy, mothers with positive history of atopy in themselves or their husbands. They were randomly divided into either the 'Dietary Precautions' group, with avoidance of milk, egg, fish and peanut during the rest of pregnancy, or assigned to the 'Control' group with no dietary precautions. Infants not breast-fed were given a cow milk formula. The infants were followed for 18 months. Among 35 breast-fed infants whose mothers took precautions during pregnancy, 5 developed eczema. Of the 36 breast-fed infants whose mothers did not take these dietary precautions, 11 developed eczema[11]. These differences are significant at 5 percent level. The severity of eczema was assessed by a scoring system that takes into account the extent of skin involvement, its type and severity. The eczema score was lower in the affected infants whose mothers took dietary precautions during pregnancy. Interestingly, this was seen both for breast-fed and formula-fed infants. If the

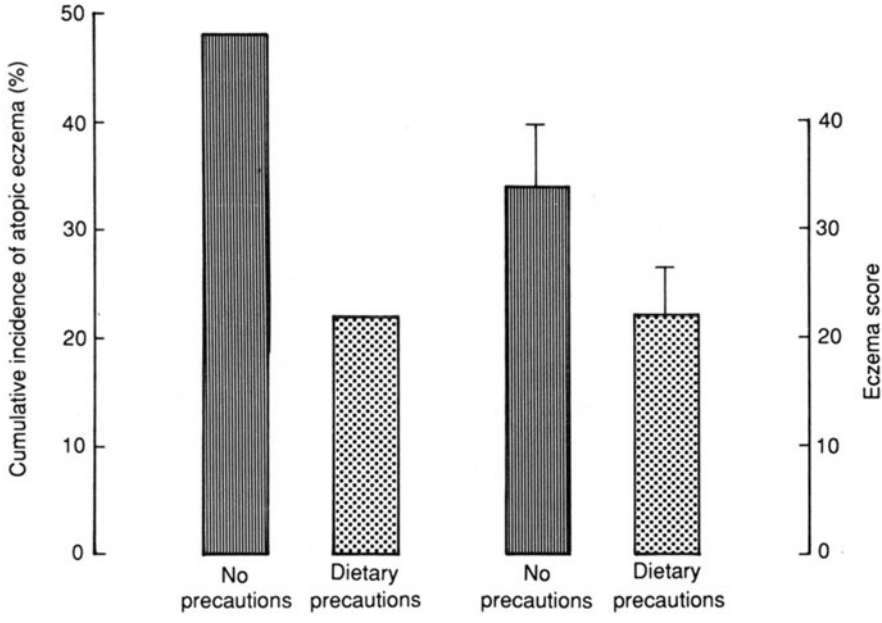

Figure 1. Incidence of atopic eczema in breast-fed infants; in one group, mothers avoided milk, egg, fish and peanuts during lactation.

foods avoided are limited in range and the avoidance is done only during the last trimester of pregnancy, long after the fetus could have been sensitized, the beneficial effects are unlikely to be observed.

Antigens derived from maternal dietary foods do come through into breast milk[12]. If mothers avoid common allergens from their diet, the frequency of breast milk and egg proteins is decreased; only 3 out of 20 samples were positive compared with 15 out of 16 in controls.

This led to our next study, in which breast-fed infants of mothers who has a history of atopic disease in themselves or in their husbands or in a previous child, were randomly divided into two groups - dietary precautions by the mothers who avoided milk, egg, fish and peanut, and others as controls with no precautions[13]. Breast-fed infants whose mothers took dietary precautions during lactation had markedly reduced occurrence of atopic eczema, 22%, compared with the controls in whom the incidence was double, 44% (Fig. 1). The severity of eczema judged by a scoring system was also less in the experimental group. Similar results were obtained in a study conducted in Linkoping, Sweden[14].

Hydrolysate and Soy Formulas

Infants not breast-fed were randomized to three different formula groups: cow milk, soy, and casein hydrolysate "Nutramigen". The incidence of eczema was least in the hydrolysate group[13]. Soy formula did not afford any protection.

In a recent study using a whey hydrolysate formula, "Good Start", which is less expensive and better tasting, we found a reduction in the incidence of eczema at the end of 6 months of follow up (Fig. 2)[15]. Furthermore, the incidence of sensitization as indicated by radioallergossorbent tests was much less in the whey hydrolysate group

compared with the soy and cow milk groups (Fig. 3). Long-term follow up is, of course, important. Preliminary data suggest that the benefits of whey hydrolysate feeding are evident at the age of 18 months and soy formula is still not protective[16].

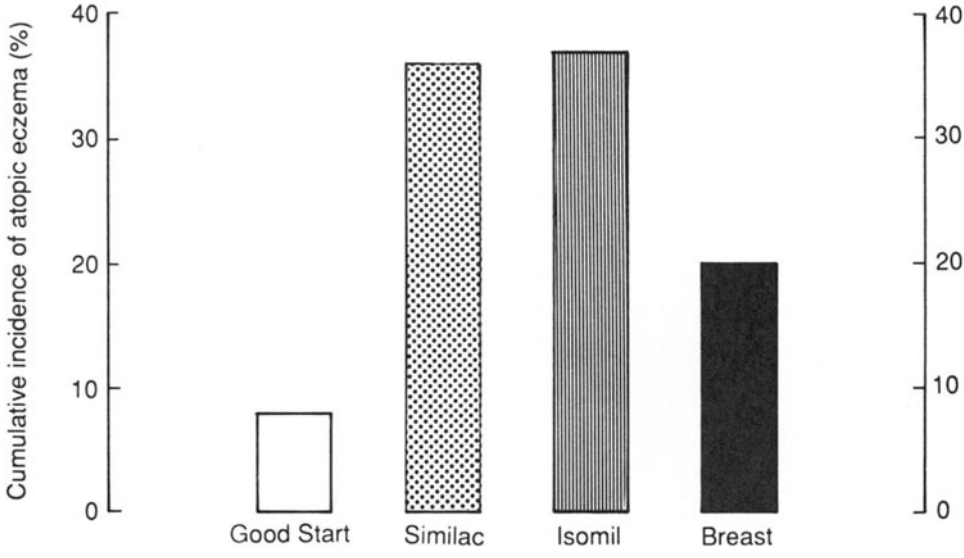

Figure 2. Incidence of atopic disease including eczema, gastrointestinal manifestations and respiratory problems in three groups of high risk formula-fed infants . One group received cow milk (Similac), the second soy (Isomil), and the third a whey-hydrolysate (Good Start). Data are shown until six months of age.

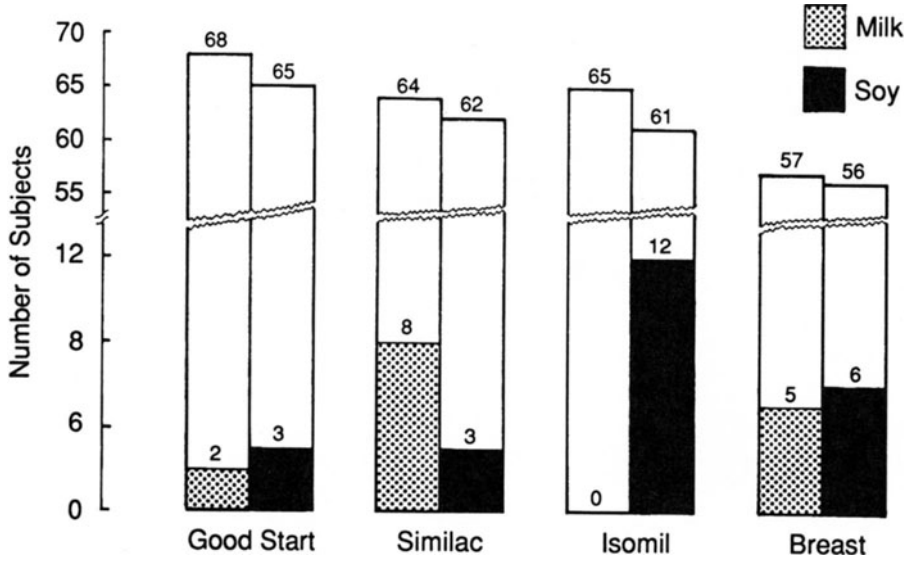

Figure 3. Frequency of positive radioallergosorbent tests for milk and soy proteins in various feeding groups. Other details as in Fig. 2.

CONCLUSIONS

The high and progressively increasing morbidity and mortality due to atopic disease has prompted vigorous attempts at prevention. In high risk infants identified by positive family history and/or elevated cord blood IgE, several preventive strategies may be adopted. These are likely to have synergistic effects, and include: dietary precautions by the mother during pregnancy and lactation; prolonged breastfeeding; in those not breast-fed the use of a hydrolysate formula, both casein and whey hydrolysate being equally effective; and reducing the exposure to inhalants such as cigarette smoke, dust mites and animal dander. These measures are practical and cost-effective.

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SECRETORY ANTIBODIES TO COW MILK PROTEINS AND TO RESPIRATORY SYNCYTIAL VIRUS

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INTRODUCTION

The failure of early studies to detect IgA on mucosal membranes and the lack of a reason to assume the presence of secretory antibody for many years preserved the dogma that mucosal membranes are without specific protection at the time of birth. In recent years secretory IgA (S-IgA) and IgM antibodies to *Escherichia coli*, to poliovirus type I and to milk proteins have been identified in the saliva of newborn infants[1,2]. S-IgA anti-β-lactoglobulin (BLG) and S-IgA anti-casein (Cas) were found independently of the mode of feeding, could be specifically blocked but were not blocked by preincubation with unrelated protein. In a subsequent study in which 158 infants were investigated, such antibodies were shown to be consistently present[3]; IgA accounted for the majority of specific binding. Comparison of infants with and without risk of allergy showed a significantly higher salivary S-IgA anti-Cas in infants at risk for allergy. In a follow-up of these 158 infants over 3 and 6 months of age, breast-fed infants had significantly lower antibody titers than formula-fed infants. This was unrelated to the amount of cow protein antigen received by the infants; breastfeeding of even 1 - 3 weeks duration resulted in suppression of S-IgA antibody formation[4]. The following paper will describe the relationship between elevated concentrations of anti-Cas antibodies at birth and the appearance of atopic disease during the first year of life. Data will also be shown from a second group of 108 infants whose secretory antibody response to respiratory syncytial virus (RSV) was determined at birth, 3 months, 6 months and 1 year of age.

MATERIALS AND METHODS

Study Design

One hundred and fifty-eight healthy newborns were divided into 3 groups according to their risk of allergy. Sixty-two infants (group A) had a

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negative family history of allergy and a cord blood IgE level <0.9 IU/ml. Thirty infants had parents without atopic disease but with elevated serum IgE, positive skin prick-tests or positive family history for atopic disease in first degree relatives (group B). Group C comprised 66 infants from parents with clinical manifestation of atopy or infants having a total cord blood IgE ≥0.9 IU/ml (group C, allergy risk). The groups were matched for sex, social background, smoking parents, and milk consumption of mothers.

In the second study, infants were excluded who fit the characteristics of group B infants of the first study as the results of this group had not differed from group A. In this study, 108 infants were included. Group A (28 infants) was defined in the same way as the no risk group of the first study, group B (n = 43) had one parent with a manifest atopy, group C (n = 34) had either an elevated cord blood IgE levels (20 infants) or two parents with manifest allergies (14 infants).

Collection of Saliva

Unstimulated saliva was obtained over 20 - 30 min. using a saliva absorbent cellulose swab. Collection was carried out during the first 36 h after birth and at least 2 h after the last meal in order to avoid contamination of saliva with milk. Samples were heated for 30 min. at 56°C, centrifuged at 5000 rpm for 15 min. and stored at -20°C until tested.

Collection of Milk Specimens

During the first week after delivery breast milk was collected in sterile covered glass jars. Immediately after collection samples were centrifuged at 9000 rpm for 15 min. at 4°C to remove both fat and cellular elements. The milk samples were then divided into aliquots and stored at -20°C until tested.

Determination of Secretory Antibodies Against Cow's Milk Protein in Saliva and in Breast-Milk

S-IgA anti-Cas and anti-BLG were determined by direct ELISA technique as described[3]. ELISA-units were expressed as a reciprocal value of the dilution which yielded an optical density of 0.5.

Determination of S-IgA Anti-RSV

Preparation of RSV was performed according to Sarkkinen *et al*[5]. Nunc plates were coated with antigen and with control antigen consisting of non-infected cell lysates. A direkt ELISA was used for S-IgA determination[3].

Determination of Antibody Affinity

Quadruplicate assays of antibody determinations were performed as described above. Following incubation in the antigen-containing microtiter plate, one duplicate was washed with a buffer containing 8M urea. The assay was finished in the usual way and ELISA-units were calculated for both assays. The ratio of the assay washed with 8M urea divided by the "normal" assay

gave a reflection of antibody affinity. The closer the value of the end point ratio (EPR) was to 1.0, the higher the affinity of the antibody.

RESULTS

Infants in the first study were seen at 3 months and at 6 months of age by one of the investigators who had no knowledge of the infants' immunological parameters at birth. At one year of age, a questionnaire was sent to the parents which contained questions concerning the course of previously diagnosed allergic disease and the development of new signs or symptoms. In addition, parents were called by phone to discuss the infant's history and details of the questionnaire. Atopic dermatitis of at least 6 weeks duration, wheezing and cough for at least 6 weeks, vomiting, colics, and diarrhea in connection with feeding and clearing after elimination of the incriminated food, urticarial reactions in connection with such episodes or a positive skin test were recorded as manifestations of atopic disease. By these criteria, 81 of 158 children developed allergic manifestations (59 had atopic dermatitis, 11 wheezing with cough, 11 had allergic symptoms related to the gastrointestinal tract).

When the frequency of atopic disease during the first year of life was correlated to the concentrations of salivary S-IgA anti-Cas at birth, a positive relationship was found. Infants of the first two groups (no or low risk) who had had anti-Cas concentrations of <20 EU/ml had a 20% incidence of atopic manifestations, infants with concentrations of 20 - 249 EU/ml a 44% incidence, and infants with S-IgA values >250 EU/ml a 66% incidence of atopic manifestations during the first year. When all 3 groups were combined, the respective figures were also significantly different. In this case, S-IgA anti-Cas >250 EU indicated a 54% incidence of atopic disease during the first year (Fig. 1a and 1b).

Secretory antibodies to RSV in breast-milk, maternal serum and infants' saliva were determined in a second study of 108 infants at birth. IgG anti-RSV could be readily detected in 90% of maternal sera and in 72 of 84 breast milk samples. However, measurable amounts of S-IgA anti-RSV were present in only 12 infants. Nineteen samples of neonatal saliva contained antibody in concentrations that were too low for quantitative determination (Fig 2). Determination of salivary anti-RSV was repeated at 3, 6, and 12 months of age. Towards the end of the first year, the number of samples with measurable amounts of antibody increased slightly but the median of the groups did not change. At the age of 1 year, 7 infants of the high risk group (both parents with manifest allergic disease or elevated cord blood IgE) had developed manifest symptoms of allergy. None of these infants had measurable amounts of S-IgA anti-RSV in their saliva. In comparison, 10 of the remaining 27 high risk infants did have such antibody.

The affinity of S-IgA anti-RSV in those neonatal salivary samples available for study was significantly higher than affinity determined in breast milk S-IgA but comparable to the affinity of serum IgG (Fig. 3). Follow-up at 3 months, 6 months, and 1 year showed a continuing decrease of affinity towards the end of the study.

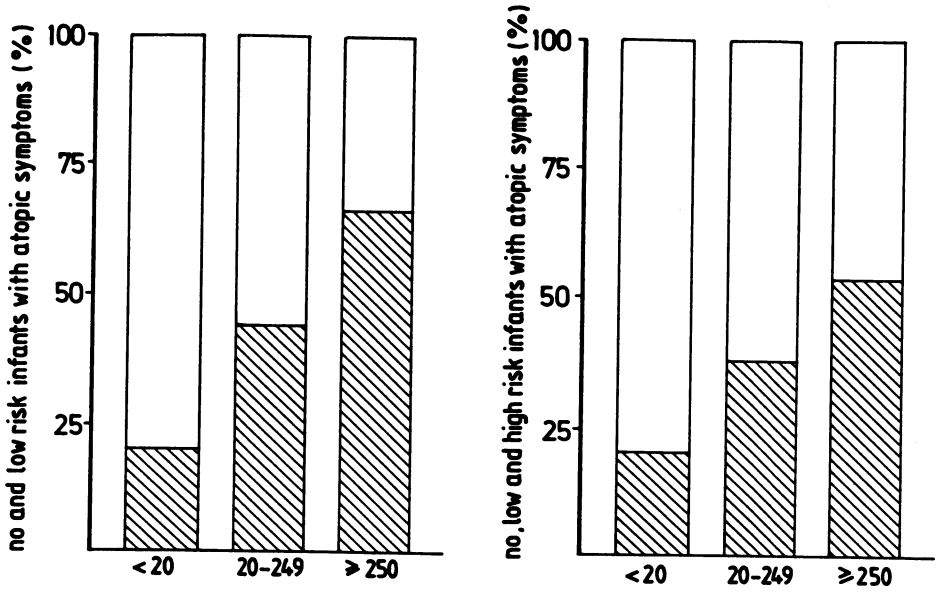

Figure 1a and 1b. Salivary S-IgA anti-casein (ELISA units) at birth in relation to atopic disease in infants with no/low risk and in infants with high risk.

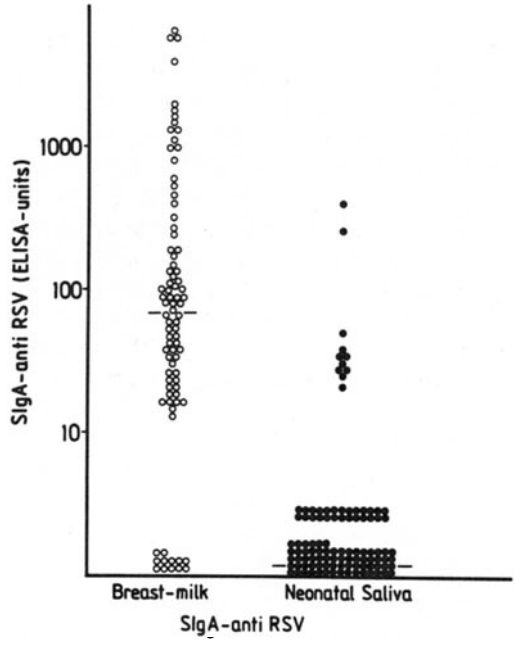

Figure 2. S-IgA anti-RSV in breast-milk and neonatal saliva

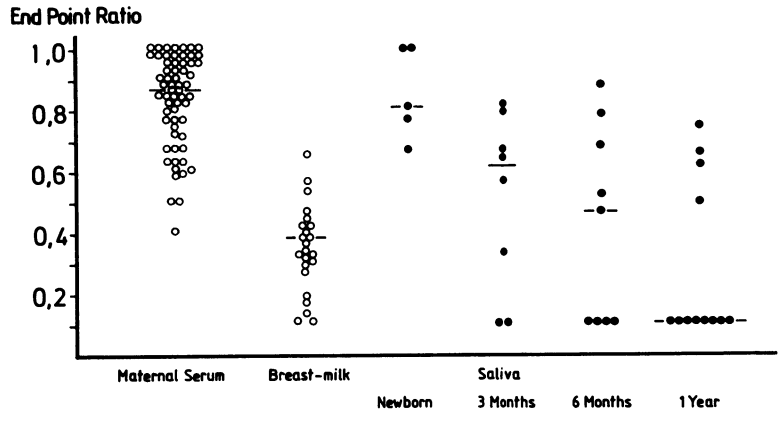

Figure 3. Affinity of anti-RSV IgG antibodies in maternal serum and of S-IgA in breast milk and infantile saliva.

DISCUSSION

S-IgA and S-IgM antibodies against viral, bacterial, and protein antigens are present in the secretions of neonates. These antibodies are not passively acquired but actively secreted by the infant. They appear to be characteristic of the neonatal period and wane during the first year of life. Anti-BLG and anti-Cas concentrations are highest at birth and decrease rapidly during the subsequent months; they also loose their association with IgA in favor of IgM[2]. In addition, it was found in this study that the initial high affinity of anti-RSV in neonates, which exceeds the affinity of breast milk S-IgA anti-RSV, decreases constantly towards the end of the first year. The origin of neonatal secretory antibody is still speculative. Exposure of the fetal immune system to antigen *in utero* would be one possible explanation. The demonstration of cow proteins in the circulation of healthy adults would support this concept[6] although antigen has not yet been directly demonstrated in amniotic fluid. Other antigens, such as polio- or RS-viruses, are unlikely to pass into amniotic fluid. Therefore, antigenic exposure *in utero* is unlikely to account for all S-IgA present at birth. Anti-idiotypic antibody transferred from maternal serum and acting as a template for active S-IgA synthesis has been suggested as an alternative explanation[1,7]. The high affinity of anti-RSV seen in this study and its similarity to the affinity of maternal serum IgG anti-RSV support the concept that at least some neonatal S-IgA may be formed in response to anti-idiotypic antibodies rather than to antigen present in amniotic fluid.

The development of antibodies to cow protein and of S-IgA antibodies to RSV over the first year are different in that the former show a rapid decrease during the first months of life whereas little change was seen with S-IgA anti-RSV. Either finding is surprising since an increase towards the end of the first year would be expected in view of the exposure to cow protein in all infants in the study, an infection with RSV in presumably 60% of infants,

and the known increase of total IgA during the first year. S-IgA anti-cow protein, however, is significantly suppressed by breastfeeding[4] which was practiced by over 90% of study mothers. Thus, this antibody may contribute a comparatively small amount of total IgA in breast-fed infants. The secretory response to a single RSV-infection is vigorous but of short duration[8]; therefore, the titers seen by the end of the first year are unlikely to reflect much natural exposure. The low affinity seen in this group also argues against a significant boost during the first year.

Two types of abnormal antibody responses were seen in the two studies reported here: elevated S-IgA anti-Cas in infants who later developed atopic disease and a deficient S-IgA anti-RSV response in symptomatic high-risk infants at 1 year of age. The former is reminiscent of the elevation of cord blood IgE in infants at risk of atopy. It was, however, also present in infants without a family history of allergy and with normal cord blood IgE. Since it correlated closely with the development of actual atopy, it may represent a means of identification of atopic infants. Further follow-up of the study infants will be necessary to determine the usefulness of this parameter. The deficient S-IgA anti-RSV in symptomatic high risk infants fits reports of an increased risk of bronchiolitis in atopic infants and earlier findings of a deficient total S-IgA in this group[9].

CONCLUSIONS

Secretory antibodies of the S-IgM- and the S-IgA-isotype are actively secreted into the saliva of neonates. Although their antigenic stimulus is unclear, atopic disposition appears to upregulate their production. The decrease of antibody concentrations to milk proteins and the loss of avidity, as seen with RSV antibodies, suggest that this phenomenon is closely linked to the newborn period and may represent a transient mechanism of surface protection during this time.

ACKNOWLEDGEMENT

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CHARACTERIZATION OF COW MILK PROTEINS IN HUMAN MILK: KINETICS, SIZE DISTRIBUTION, AND POSSIBLE RELATION TO ATOPY

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INTRODUCTION

Human milk contains a number of substances immunologically important to the infant[1], such as leukocytes, antibodies, and complement factors. In addition, it has been known for many years that dietary antigens may pass from the mother into the breast milk and cause allergic reactions in the infant[2]. Dietary antigens have been directly demonstrated in breast milk by sensitive immunoassays[3,4]. The level of β-lactoglobulin (BLG) in breast milk after maternal intake of cow milk was related to the presence of cow milk allergy (CMA) as colic in the infant[5]. The milk level of ovalbumin after the intake of hen eggs, however, did not correlate with the worsening of atopic eczema in the infants studied[6].

We have conducted an investigation of the prevalence of CMA in a cohort of infants[7] and observed a frequency of approximately 2% of babies fed cow milk products and of approximately 0.5% (9 of 1,749) of breast-fed infants. BLG was assessed in the mother's milk given to the latter 9 infants. As BLG was not measurable in the milk samples from 6 of 9 women, further studies of BLG in consecutive milk samples from 10 atopic and 10 healthy lactating women were initiated[8]. The present report summarizes our results.

MATERIALS AND METHODS

Subjects

Study 1. Nine lactating women with infants with CMA, originating from the cohort study of CMA[7], drank 0.5 l of cow milk and 4 h later gave a 10 ml sample of breast milk. Similar samples from 10 healthy women with healthy infants were obtained as controls.

Study 2 (pilot study). Three healthy women drank more than 0.5 l of cow milk per day for a week. After the intake of 500 ml of cow milk samples of breast milk were taken at 0, 8, 12, 24, and 72 h, during which the women did not take milk or milk products. After 72 h they consumed another 500 ml of cow milk and a milk sample was obtained after a further 4 and 12 h.

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Study 3. Based on the results of Study 1 and Study 2, ten lactating women with atopy (asthma, rhinitis, or atopic eczema) and 10 healthy mothers consumed more than 1 l of cow milk daily for six days. After the intake of 0.5 l cow milk, samples of breast milk were taken at 0, 4, 8, 12, and 24 h. This schedule was repeated for 3 consecutive weeks with either homogenized (HOM) or unhomogenized (UNH) cow milk for one week at a time, so that 5 women of each group had HOM, UNH, and HOM, and 5 women from each group had UNH, HOM, and UNH for 1 week at a time.

Sampling of Human Milk

All breast milk samples (about 10 ml) were taken after 2-4 min of sucking, expressed by hand into sterile test tubes and frozen at -20° C. When all samples from 24 h had been collected they were transferred to -60° C. Before analysis, the samples were centrifuged at 2200*g* to discard fat and debris.

Analysis of BLG

The ELISA method for BLG determination in serum has been described in detail previously[9] and was followed with minor modifications[7]. All standards and controls were prepared in human milk without BLG, due to matrix effects using serum. The coefficient of variation (CV%) were estimated from control samples at 10, 3 and 1 μg/l of BLG. The between-days CV% were 14, 22, and 29%, respectively, and the within-assay CV% were 6, 7, and 7%, respectively.

High-performance liquid gel-permeation chromatography was combined with ELISA analysis for BLG (HPLC-ELISA) as described[9]. A Toyo Soda TSK G 3000 SW size separation column was used. To determine the Mr, the following purified protein markers were used; thyroglobulin (Tg, Mr 660 kDa) that appear in the void volume, IgG (Mr 150 kDa) and myoglobin (Mg, Mr 17 kDa).

RESULTS

Study 1. BLG was detected in 3/9 milk samples from mothers with breast-fed infants with CMA, and in 1/10 samples from mothers of healthy infants. The maximal concentration was 45 μg/l. All samples were further analyzed by HPLC-ELISA in order to estimate the size distribution of BLG and to reveal possibly hidden BLG-determinants in immune aggregates. The size of the BLG in the 4 milk samples with detectable BLG by ELISA corresponded to monomeric BLG at 18 kDa (Fig. 1). No additional BLG was revealed in the other samples.

Study 2. In breast milk from 2/3 women, BLG was detectable even 72 h after the intake of cow milk, indicating a slow turnover of BLG (data not shown).

Study 3. The breast milk from 19/20 women contained BLG at least at one time point. The maximal values (Table 1) ranged from 0.9 - 150 μg/l and

were reached 4 - 24 h after the intake of cow's milk. No differences were observed between the BLG levels from the atopic and the non-atopic women. Furthermore, no difference was seen between the values after consumption of HOM or UNH cow milk. The BLG pattern during the three occasions was fairly constant for each woman, and a group with slow (Figs. 2a and 2c) and a group with fast (Figs. 2b and 2d) elimination of BLG could be separated within both the atopic and the healthy women.

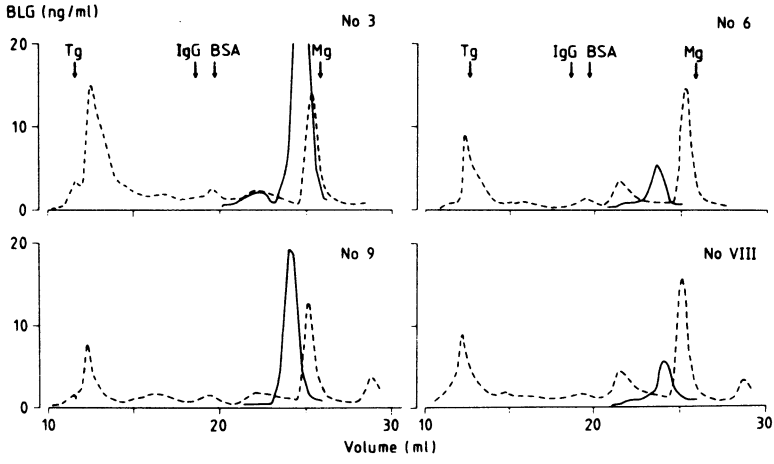

Figure 1. Estimation of the size distribution of β-lactoglobulin (BLG) in human milk by HPLC-ELISA. Samples obtained from three women with infants allergic to cow milk (nos. 3, 6, 9) and one woman (no. VII) with a healthy infant. The elution volumes of thyroglobulin (Tg), IgG and myoglobin (Mg).

DISCUSSION

Our studies show that the appearance of intact BLG in human breast milk is a common finding. The BLG may be present for several days after the intake of cow's milk and the peak levels varied widely. In study 1 of milk given to breast fed babies with CMA, the dietary protein was only measurable in 3/9 samples. Size separation did not reveal additional BLG in the other samples, nor were any antigen fragments or BLG containing immune aggregates observed. This observation is remarkable, as antibodies to cow's milk may be found in breast milk[10,11]. Lower antibody levels were reported in milk given to infants with CMA[11], but were not evaluated in the present studies.

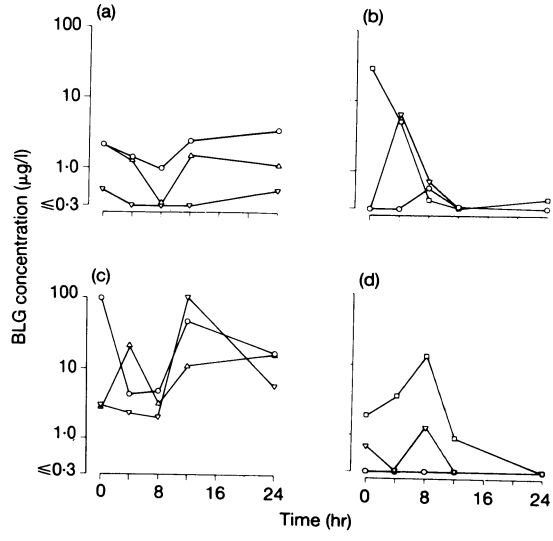

Figure 2. Examples of the time course for the appearance of β-lactoglobulin (BLG) up to 24 h after the intake of 500 ml of cow milk (homogenized milk ○ and □ and unhomogenized milk ▽ and △). Samples from two atopic women (a and b) and two non-atopic women (c and d).

In order to optimally secure the detection of BLG, the subjects of Study 2 and Study 3 had a daily intake of at least 0.5 l of cow milk for one week before the breast milk samples were taken for analysis. Thus, a clear-cut kinetic profile was not obtained. However, the data do show (Fig. 2) a fairly rapid disappearance of BLG from the milk of 9 women and a slower disappearance from another 9 women. A large inter-individual variation was apparent (Table 1).

Increased gut permeability to macromolecules has been suggested as a pathogenic factor in atopy[12,13]. Also, a positive correlation between the levels of BLG in breast milk and CMA with infant colic was reported[5]. In our study, the levels of BLG in the breast milk did not differ between atopic and healthy women, thus not pointing to an increased antigenic load in the babies of atopic mothers. In high-risk infants, a potential beneficial effect of breast-feeding has been studied for many years with conflicting results[14]. Recent investigations of a maternal hypo-allergenic diet during lactation have not settled the controversy[15,16,17]. Possibly, the development of clinical allergy depends more on the genetic predisposition of the high-risk infant than on the amount of dietary antigen to which the infant is exposed in the breast milk. However, the amounts of dietary antigen in cow milk products is is evidently very large as compared to the amount in breast milk. In our Study 1, based on 1,749 babies, all 9 breast-fed babies with CMA had been given cow milk formula in the nursery during the first days of life, suggesting a deletterious effect of early exposure to large amounts of cow milk protein.

Table 1. Maximal Concentration (μg/l) of BLG in Human Milk from Atopic Mothers (Group A) and Non-Atopic Mothers (Group B) and the Interval (h.) Between Ingestion of 500 ml Homogenized Milk (HOM) and 500 ml Unhomogenized Milk (UNH) and Maximal Concentration of BLG.

	Group A ($n = 10$)				Group B ($n = 10$)			
	HOM		UNH		HOM		UNH	
No.	Maximum concentration	Interval	Maximum concentration	Interval	Maximum concentration	Interval	Maximum concentration	Interval
1	<0·3	—	<0·3	—	4·8	4	40·0	4
2	9·0	12	5·0	12	135·0	8	150·0	8
3	2·9	24	1·9	8	5·0	4	7·8	4
4	100·0	4	50·0	12	36·0	4	24·0	8
5	29·0	4	6·5	4	13·0	8	1·3	8
6	4·8	8	5·5	12	<0·3	—	1·4	12
7	3·6	24	1·5	12	1·2	12	0·9	24
8	3·4	12	3·0	8	1·1	8	1·2	8
9	<0·3	—	19·0	8	46·0	12	100·0	12
10	<0·3	—	1·0	8	<0·3	—	1·4	8

CONCLUSIONS

1. Intact, monomeric BLG occurs at low levels as a common finding in human breast milk. The protein may be detected up to at least 3 days after the mother's intake of cow milk.

2. No differences were observed in BLG levels after the mother consumed HOM or UNH cow milk.

3. There was no indication of higher BLG levels in the breast milk given to infants with CMA. No significant difference was observed between BLG levels in the breast milk from atopic and non-atopic women.

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TRANSFER OF ENTERALLY ADMINISTERED PROTEINS FROM LACTATING MOUSE TO NEONATE: THE POTENTIAL ROLE OF ENVIRONMENTAL CONTAMINATION

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INTRODUCTION

Although maternal dietary proteins were first demonstrated in breast milk in 1930, interest in the presence of dietary protein antigens in milk has revived in recent years. Human studies have clearly demonstrated that various dietary antigens are present in milk in quantities likely to be of immunologic significance[1,2]. Human clinical studies[3-8] and animal experiments have been carried out to explore the influence on the infant of exposure to antigen via the milk[9,10].

In previous studies, we investigated the uptake of immunologically intact ovalbumin (iOVA) from the intestine of the lactating mouse into her systemic circulation[11] and the transfer of intravenously administered OVA, bovine serum albumin (BSA), bovine γ-globulin (BGG), and bovine β-lactoglobulin (BLG) from the maternal circulation into the milk[12]. In the studies described herein, we examined the combined processes of intestinal uptake of OVA and BSA administered by gavage and then transferred into milk of lactating BDF_1 mice. Lactating mice were also given OVA or BSA in drinking water for several days; in these experiments, the concentrations of protein in maternal serum and milk and in neonatal serum were determined. Because of the finding of high concentrations of iOVA and iBSA in the serum of the neonates, we tested the possibility that these proteins might have reached the neonate by routes other than breast milk.

MATERIALS AND METHODS

Dietary Protein Uptake From the Intestine and Transfer Into Milk and Neonatal Serum

Administration of protein by gavage. Lactating BDF_1 mice were separated from their litters at various times between 4 and 10 days postpartum, and were given a single gavage of 75 mg of BSA (n = 6) or 100 mg

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of OVA (n = 4). Four or 24 hr later a blood sample was obtained from the retro-orbital venous plexus. Thereafter, the mice were injected with phenobarbitone and oxytocin and milk was obtained by manual expression.

Administration of protein in drinking water. Lactating BDF_1 mice received equimolar concentrations of BSA (23 mg/ml; n = 10) or OVA (15 mg/ml; n = 10) in their drinking water starting in the first week postpartum and continuing for a total of 5 or 6 days. They were then separated from their litters, and 4 hr later milk and blood samples were obtained as above. Blood samples were obtained from the neonates by cardiac puncture immediately after their separation from the mother.

Examination of the environment for possible sources of contamination with OVA or BSA. Lactating BDF_1 mice were given a single dose of 400 mg of BSA by gavage; non-lactating mice received two 300 mg doses of OVA by gavage. These doses of BSA and OVA were approximately equal to each animal's daily intake during the experiments in which the proteins were added to the drinking water. Twenty-four hr later, the surface (skin and fur) of the adult mice and neonates was thoroughly swabbed with a moistened cotton swab. Samples of cage bedding were also obtained. Both the swabs and the bedding were eluted in phosphate-buffered saline containing 10% normal mouse serum. iBSA or iOVA concentrations in the milk and serum and in the swab and bedding eluates were then measured. iOVA and iBSA were estimated in all samples by an enzyme-linked immunosorbent assay (ELISA). This assay had a lower limit of sensitivity of 10 to 20 ng/ml.

RESULTS

Antigen Uptake and Transfer Studies

Following a single gavage of BSA or OVA, iBSA or iOVA was detected in the serum of lactating mice at 4 hr (Table 1). By 24 hr, the serum iBSA concentration had increased, but iOVA was markedly reduced. Both iBSA and iOVA were present in milk at 4 hr; at 24 hr the concentration of iBSA was enhanced while that of iOVA had diminished.

Following 5 or 6 days of administration of BSA or OVA in the drinking water, these proteins were detected in milk and in both maternal and neonatal serum. The levels detected in maternal serum following BSA administration were much higher than those detected following administration of OVA. The maternal serum concentration of iOVA was lower than the level measured in milk. The concentrations of iBSA and iOVA measured in the neonatal serum samples were higher than those of either the maternal serum or milk. Because of these findings, we tested the possibility that non-milk sources might contribute to the high serum levels of iOVA and iBSA.

Environmental Contamination Studies

Twenty-four hr after administration of BSA or OVA by gavage, neither of these proteins could be detected on the surface of the recipient adult mice. In contrast, high levels of iBSA were detected on the skin of neonatal mice whose mothers had received BSA by gavage, and high levels of iOVA were detected in the bedding of adult mice who received OVA by gavage.

Table 1. Concentration of iOVA and iBSA in Serum and Milk of Lactating BDF_1 Mice and in the Serum of the Suckling Neonate

a) After single gavage

	Maternal			
	Serum		Milk	
	4 hr	24 hr	4 hr	24 hr
iBSA (ng/ml)	39 ± 6	53 ± 11	13 ± 6	58 ± 18
iOVA (ng/ml)	26 ± 6	ND	19 ± 2	ND

b) After chronic administration

	Maternal		Neonatal
	Serum	Milk	Serum
iBSA (ng/ml)	928 ± 153	271 ± 64	1088 ± 227
iOVA (ng/ml)	75 ± 12	154 ± 35	516 ± 157

Results expressed as mean (± SEM).
ND – Not detected

DISCUSSION

In a previous study, we demonstrated increased intestinal uptake of OVA during lactation[11]. We also showed that the clearance of intravenously administered OVA from the serum of lactating mice was more rapid than that of BSA[12]. Moreover, 4 hr after the intravenous administration of these proteins, the concentration in the milk of iBSA was 10-fold higher than that of iOVA, suggesting that transfer may be dependent on maternal serum concentration. The results of the studies of enterally administered proteins described here appear to be consistent with the previous observations. Following the administration of a single dose of BSA by gavage, serum levels of iBSA rose between 4 and 24 hr, presumably reflecting continued absorption of the protein. During this same time period, levels of BSA in milk also rose markedly. Although roughly comparable concentrations of iOVA were detected in the maternal serum at 4 hr, these levels were markedly reduced by 24 hr. The concentration of iOVA present in the milk at 4 hr was similar to that of iBSA, but by 24 hr the concentration of iOVA was also markedly reduced. The results of the chronic feeding experiments are also consistent with the hypothesis that milk levels of dietary proteins are influenced by the persistence of the dietary protein in the maternal serum. Higher levels of both iBSA and iOVA were detected in milk from mothers subjected to prolonged protein feeding compared to those of mothers given a single feeding. The maternal serum concentration of iBSA was high, while that of iOVA was disproportionately lower, presumably reflecting its faster clearance from the circulation. Neonatal serum concentrations of both iBSA and iOVA were both very high; the concentration of iBSA was close to that of maternal serum, and that of iOVA was seven-fold greater than the maternal serum level.

The high concentration of iBSA and iOVA in the neonatal circulation might reflect enhanced uptake by the immature intestine or alternatively, slow clearance of these dietary antigens from the circulation[13]. We also considered the possibility that the high level of these proteins in the serum of the neonate might have resulted from contamination of the environment by BSA and OVA. Contamination might arise from the feeding bottle dripping onto the animals and the bedding, from the maternal urine which may contain the fed proteins, or from fecal excretion of the intact proteins. Any and all of these processes might provide BSA and OVA that enters the neonatal circulation because the animals lick their fur or bedding or because the needle used to obtain blood is contaminated as it penetrates the fur and skin. Adult mice appear to minimize the contamination of their fur (Fig. 1) probably because of grooming; the neonates do not appear to do so. The acquisition of BSA or OVA from the environment may lead to overestimation of the amount of these proteins transferred via the milk. This possibility may make it necessary to re-examine the work of other investigators who failed to take potential contamination into account in their experiments[14,15]. Meticulous cleaning of the surface of the neonate before introducing the needle into the heart and repeated changes of bedding may minimize, but not entirely avoid the transfer of dietary protein to the neonate by routes other than the milk.

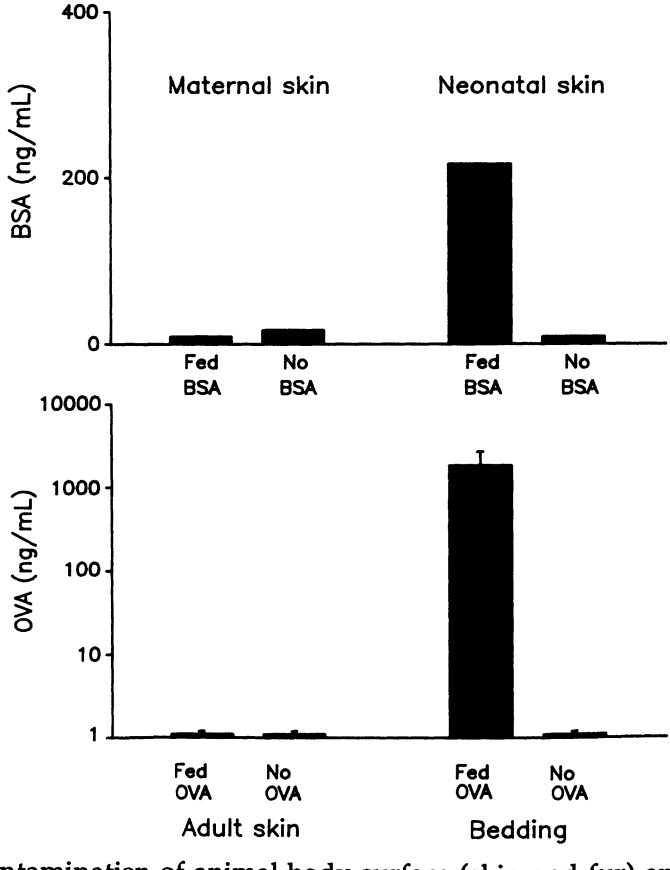

Figure 1. Contamination of animal body surface (skin and fur) and of bedding following administration of BSA or OVA by gavage.

CONCLUSIONS

Following a single gavage feeding of BSA or OVA to lactating BDFI mice, immunoreactive BSA and OVA were detected in the blood and milk. After chronic administration of BSA and OVA, higher levels of these proteins were detected in the serum and milk of the lactating mice and unexpectedly high levels of iBSA and iOVA were detected in the serum of the neonates. The cage bedding and fur of the neonates were found to be contaminated by food proteins suggesting that contact with these sources might account for elevated levels of iBSA and iOVA detected in the blood of the neonates leading to overestimation of protein transfer via the milk.

ACKNOWLEDGEMENTS

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LEVELS OF IgA AND COW MILK ANTIBODIES IN BREAST MILK VS. THE DEVELOPMENT OF ATOPY IN CHILDREN. LOW COLOSTRAL IgA ASSOCIATED WITH COW MILK ALLERGY

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INTRODUCTION

Little attention has been paid to the possibility that the immunoprotection conferred by breast milk may vary from mother to mother and that in some cases the milk may even be deficient in protective factors, which might result, for instance, in the development of food allergy. An earlier study showed that milk samples from mothers whose infants showed symptoms suggestive of cow milk (CM) allergy had low total IgA contents and low levels of IgA antibodies to cow milk[1].

We have followed 198 healthy full-term infants from birth to the age of 5 years: 7 of them developed CM allergy and the colostral samples from the mothers of these infants had lower total level of IgA than the samples from non-atopic mothers of non-atopic infants. No association appeared between the development of later allergy and the levels of IgA or antibodies to CM.

PATIENTS

Mothers who delivered healthy babies at the Department of Obstetrics, Helsinki University Hospital, were enrolled in a study of infant feeding and its effects on the baby's health[2]. The goal was to obtain 200 newborns for the study. Two families dropped out during the first year, leaving 198 infants.

From each mother, a sample of colostrum was obtained when she agreed to participate in the study. That occurred within 5 days of delivery, with median age of 3 days. Infants visited our clinic at ages 2, 4, 6, 9, and 12 months. We provided all the medical care needed during the first year[2]. A venous blood sample was taken from the infant at each visit; most mothers gave a sample of mixed breast milk collected during the preceding 24 h that consisted of equal amounts of fore- and hind-milk. The diet of the mothers was not restricted in any way. All samples were stored in small aliquots at -20°C until used.

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Infants with Cow Milk Allergy

Of the 198 infants, seven showed symptoms and signs suggestive of CM allergy during the first year of life (Table 1). The elimination of CM-containing foods, and beef in one case, from the infants' diet or from the diet of the mothers of two infants resulted in disappearance of the symptoms. An open challenge test was later performed on every infant at the hospital. First a drop of CM formula was placed on the lips. If this caused no reaction, 5 ml of the formula was given orally. If no symptoms developed within 4 h, the amount of formula was doubled at each feed during the daytime, reaching the full amount at the end of second day of the test. Signs compatible with CM allergy were observed in the seven infants after variable amounts of formula (Table 1).

Atopy During Infancy and Early Childhood

Altogether 13 infants showed symptoms and signs of atopy during the first year of life. Atopic symptoms between years 1-2 were surveyed; 187 returned the questionnaire. During the first 2 years, 44 had symptoms or signs of atopy[3].

At the age of 5 years, 168 children visited the outpatient clinic for a careful history and a physical examination. A standard series of skin prick tests with 9 allergens was done; a test was interpreted as positive if mean diameter of flare was equal or greater than 1/2 of that caused by histamine.

By history or signs, a total of 60 children had atopy. Of the 168 children tested, 29 had a positive skin prick test to one or more allergens. These groups were compared with 102 children who showed no clinical evidence for atopy and had negative skin tests (comparison group I).

Non-Atopic Mothers with Children without Atopy During Infancy

Fifty-one mothers had a history of atopic symptoms. The findings in the breast milk of mothers of CM-allergic infants were compared to those in the milk of non-atopic mothers of infants who to the age of 2 years showed no symptoms of atopy (comparison group II). From 102 such mothers a colostral sample was available, from 100 a milk sample at 2 months, from 65 at 6 months and from 39 at 9 months postpartum.

Measurement of IgG, IgA, IgM, and IgE Concentrations

The plasma IgG, IgA, and IgM levels in the infants were measured by a nephelometric method. IgM and IgG in the breast milk samples were measured by the single radial immunodiffusion method. For measurement of IgA in breast milk, samples were allowed to diffuse for 72 hours: a breast milk sample with a known content of secretory IgA was used as a reference (a gift from Dr. B. Carlsson, Gothenburg, Sweden).

Plasma IgE levels were measured by the paper radioimmunoabsorbent test (Prist^{R}, Pharmacia, Uppsala, Sweden).

Measurement of Levels of Cow Milk- and β-Lactoglobulin-Specific Antibodies

CM specific IgE in the plasma was measured by the radioimmunoabsorbent test (RAST^{R}, Pharmacia, Uppsala, Sweden). Levels of IgA, IgM, and IgG antibodies specific to CM and bovine β-lactoglobulin (BLG) were measured by the enzyme-linked immunosorbent assay (ELISA) as described earlier[4]. Breast milk samples were diluted 1:2.

Measurement of β-Lactoglobulin Concentrations in Breast Milk

A solid-phase fluoro-immunoassay was used to measure concentration of BLG in breast milk samples[5]. Polystyrene microtitre strips were coated with rabbit gammaglobulin to BLG; bound BLG from the samples was detected with Europium-labelled rabbit BLG antibodies. Fluorescence was measured with a Arcus Fluorometer (LKB, Wallac, Turku, Finland). The measuring range was from 0.1 to 50 μg/1. The dose-response curve was nearly linear.

RESULTS

Infants with Cow Milk Allergy

Characterization of infants with CM allergy. The seven infants with symptoms suggestive of CM allergy had positive results in the provocation test (Table 1). All seven developed skin manifestations and one had rhinitis within 1 h after the last challenge dose. Two of the infants developed a skin eruption during exclusive breast-feeding; at the time of appearance of the allergy both had increased plasma levels of IgE CM antibodies. Altogether 6 had elevated levels of IgE CM antibodies at the time of appearance of symptoms. Total IgE levels were increased in four of the infants (Table 1).

Table 1. Symptoms, Signs, and Levels of IgE and IgE CM Antibodies in Infants with Cow Milk Allergy

Age at appearance of symptoms (mo) /challenge	Symptoms at challenge	IgE CM antibodies (RAST score)	Level of total IgE IU/ml	Amount of CM causing symptoms
7/8	urticaria	–	7	touch
10/11	erythema	+ (4)	180 ↑	150 ml
3/11	perioral erythema	+ (2)	250 ↑	5 ml
7/14	eczema rhinitis	+ (3)	10	150 ml
4/5	perioral erythema	+ (3)	17 ↑	15 ml
3/4	urticaria	+ (3)	10	10 ml
5/12	eczema urticaria	+ (2)	23 ↑	160 ml

β-Lactoglobulin concentration in breast milk. The concentration of BLG in the breast milk samples varied widely (Fig. 1). There was no difference between the samples from the mothers of the infants with CM allergy and from the comparison group II: The geometric mean was 1.7 μg/1 for 10 samples taken during the first 3 months of lactation from the former group and 2.7 μg/1 for 20 samples from the comparison group II. The latter value is one millionth of the concentration in the cow milk.

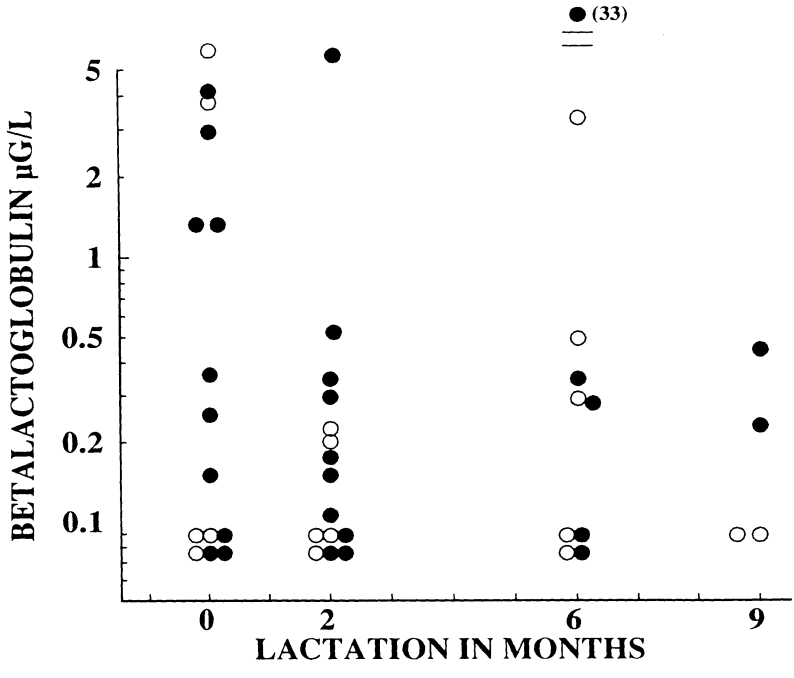

Figure 1. BLG concentrations in colostrum and milk from mothers of infants with CM allergy (o) and of mothers in comparison group II (●).

Total immunoglobulin levels in breast milk. The mean level of IgA in colostrum from the mothers whose infants later became allergic to CM was markedly lower than that from comparison group II (Fig. 2). There was a decrease in the IgA concentration in comparison group II at 2 months compared with levels in colostrum ($p<0.001$). Breast milk from the mothers of infants with CM allergy contained less IgA than milk from the mothers in comparison group II through the lactation, and the 95% intervals of the groups did not overlap at any observation point (Fig. 2).

IgM levels were similar in colostrum from the mothers of the infants with CM allergy and from comparison group II (geometric mean in both groups 0.07 g/l). In samples taken at 2, 6, and 9 months of lactation the amounts were below the level of measurement in most samples (<0.03 g/l) and for IgG even in the colostral samples (<0.08 g/l).

CM- and BLG-specific antibodies in breast milk. The levels of IgA CM- and BLG-specific antibodies did not differ significantly in the breast milk from mothers of the infants with CM allergy and from comparison group II (Fig. 3). In comparisons between colostrum and breast milk sampled at the age of 2 months, there was a significant decrease in titre of both CM-, and BLG- IgA-antibodies in comparison group II ($p<0.01$ in both comparisons). The levels remained then steady at 6 and 9 months of lactation. The geometric mean level of IgA BLG antibodies in colostrum of mothers whose infants later became allergic to CM was 0.23% (95% confidence limits 0.16-0.32%); 0.22%

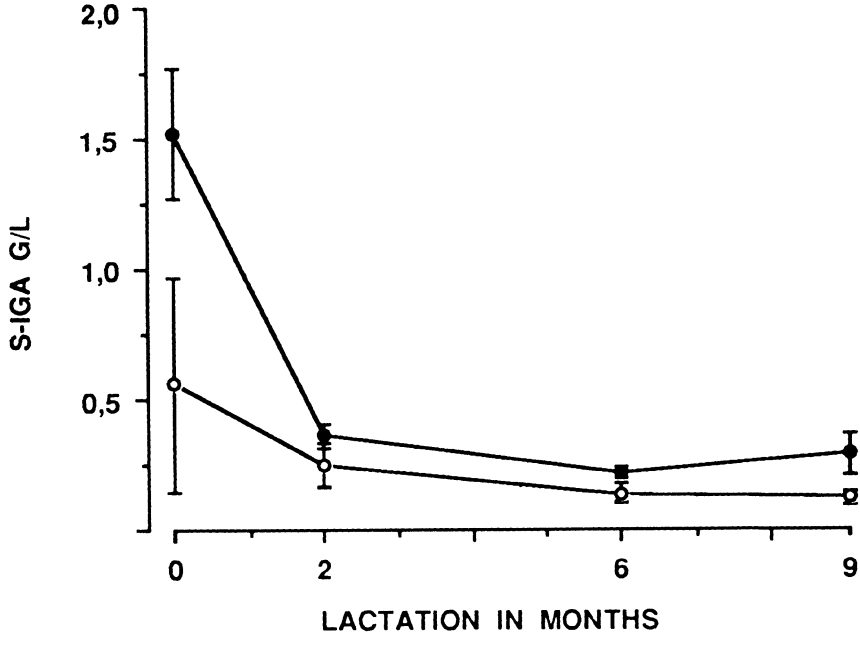

Figure 2. Concentration of IgA in the colostrum and milk of mothers of infants with CM allergy (o) and of mothers in comparison group II (●). The mean and 95% confidence limits are given.

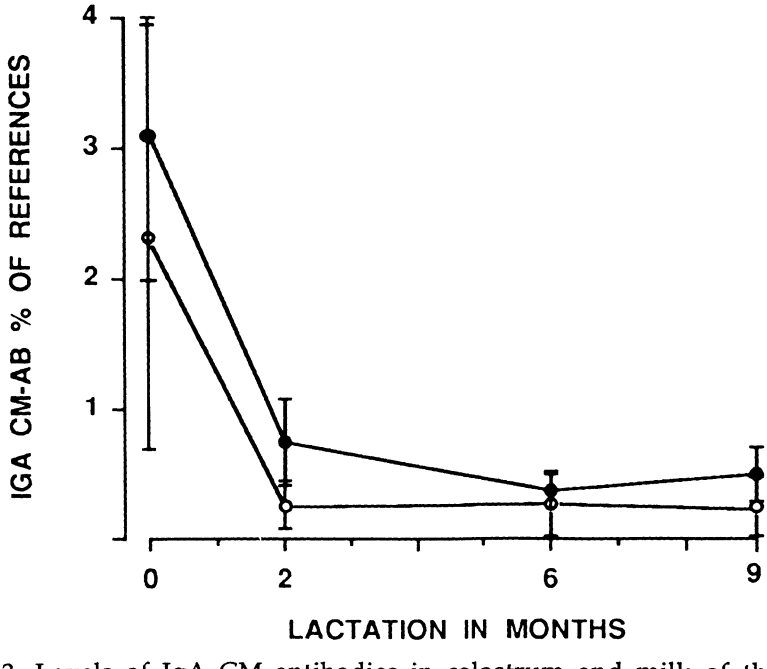

Figure 3. Levels of IgA CM antibodies in colostrum and milk of the same groups as above, symbols as in Fig. 1.

(0.21-0.23%) in comparison group II. In the samples taken later during lactation, the mean levels were low and similar in both groups.

Amounts of IgM and IgG CM antibodies were below the sensitivity of the measurement even in colostral samples.

Comparison Between Groups Developing Atopy Later and Non-Atopic Children

Total IgA in colostrum and breast milk. The mean level of IgA in the colostral and breast milk samples did not differ between mothers of children who later developed atopic symptoms and mothers of 102 non-atopic children (Fig. 4).

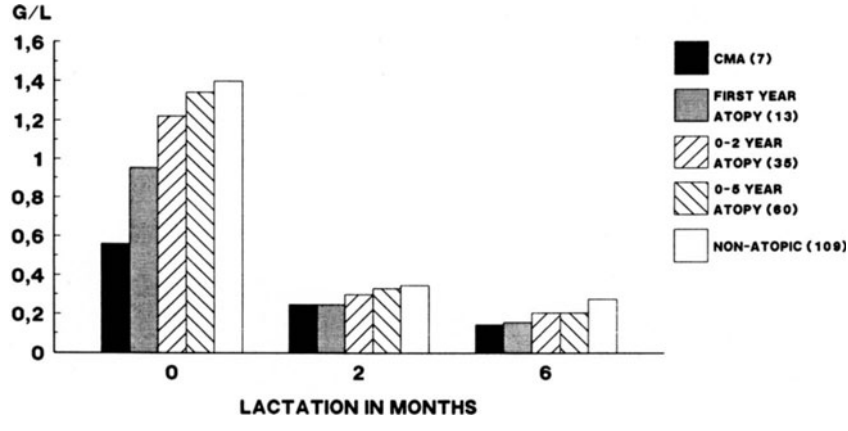

Figure 4. The mean levels of IgA in colostrum and milk from mother of infants developing allergy at various ages and from mothers of non-atopic children.

IgA CM antibodies in colostrum and breast milk. The mean colostral levels were lowest in the mothers of infants developing CM allergy (Fig. 5), but in their 95% confidence interval they overlapped with that of non-atopic infants (Fig. 3). In their colostral or breast milk levels, mothers of children developing allergy by the age of 1, 2 or 5 years did not differ from mothers of non-atopic children (Fig. 5).

IgA in the colostrum and breast milk of atopic and non-atopic mother. Total concentration of IgA did not differ between colostrum and breast milk of mothers with history of atopic symptoms (51 mothers) and mothers without such history (118) (Fig. 6). Neither was there any difference between these groups in the levels of IgA CM antibodies (data not shown).

DISCUSSION

CM allergy may develop through many mechanisms[6]. The clinical picture of CM allergy was homogenous in this study: in all 7 infants the

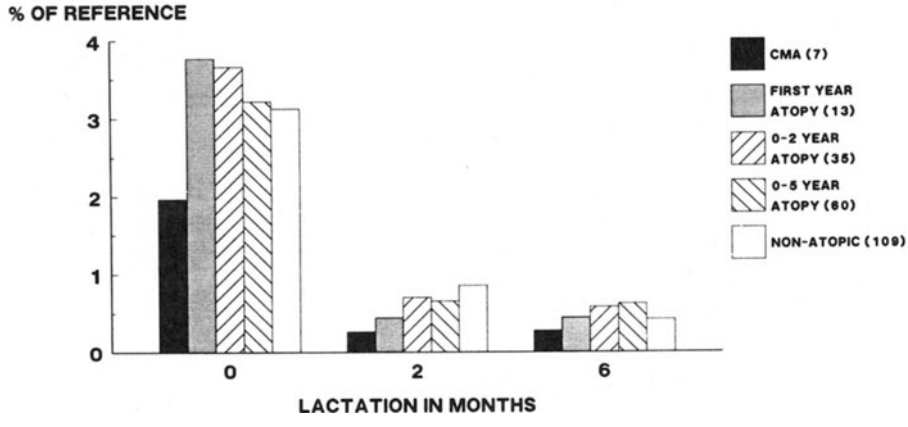

Figure 5. The mean level of IgA CM antibodies in colostrum and milk from mothers of the same groups as in Fig. 4.

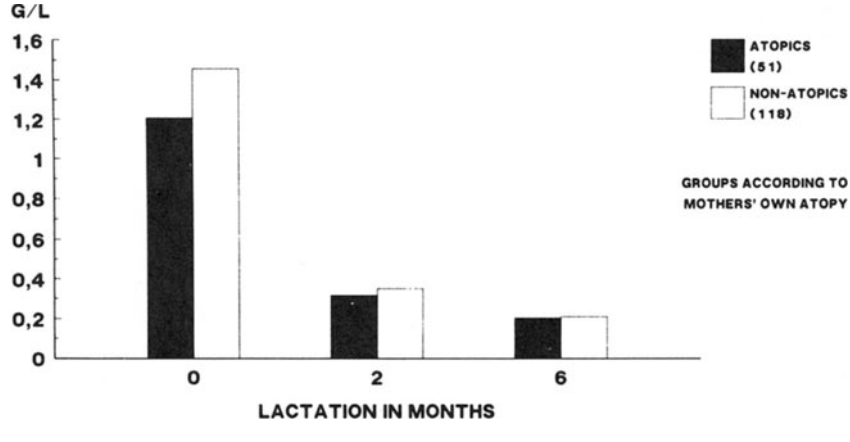

Figure 6. Mean levels of IgA in the colostrum and milk from mothers with own atopic symptoms (by history).

illness began with acute cutaneous symptoms and none had any gastrointestinal trouble. This may be due to long exclusive breastfeeding in every case: it ranged from 6 weeks to 6 months. Six infants had elevated levels of CM-specific IgE antibodies. As described earlier[7], this type of CM allergy may be seen even during exclusive breastfeeding, as in two cases of this study.

Small quantities of food proteins or their breakdown products appear in breast milk and are detectable by immunological techniques[1,8]. In our series, the quantity of BLG or its split products secreted into the milk was not related to the development of CM allergy.

IgA antibodies specific for a food antigen bind to it in the gut lumen. These complexes are digested by proteolytic enzymes and the absorption of the antigen prevented[9]. A deficiency of specific IgA antibodies in breast milk might predispose an infant to sensitization during both breastfeeding and mixed feeding; evidence for this has in fact been reported[1]. The mean levels of CM and BLG antibodies were lower in the colostral samples of mothers whose infants became later allergic to CM; the difference to the comparison group was not significant, however.

The total IgA concentration in the colostrum of mothers whose infants later became allergic to CM was strikingly low; a smaller difference persisted through the lactation. The proportion of CM-specific antibodies may contribute only to a small percentage of this difference. IgA antibodies in colostrum and breast milk act in concert with several types of non-specific defense factors[8], such as lysozyme, lactoferrin, lipids, and oligosaccharides. Specific antimicrobial IgA antibodies, together with oligosaccharides, prevent adherence of infective bacteria and viruses to the intestinal epithelium and so act as a barrier to infection. Further, secretory IgA neutralizes toxins and virulence factors of microbial pathogens[9]. The major factor of secretory IgA in breast milk may be the ability to reduce inflammation in the intestine of the infant[9]. When inflammation occurs, it may increase the permeability of the intestine and predispose to IgE responses and atopy. Even though we did not see any gastrointestinal infections in these infants with CM allergy, the lower total IgA concentration in the colostrum and mature milk of their mothers may be associated with more frequent and severe intestinal inflammation in these babies, predisposing to sensitization to antigens with which they are in contact.

We could not demonstrate any association of the appearance of atopy at later age with the levels of IgA or CM antibodies in the colostrum or breast milk. This may suggest that early sensitization through the intestine is not important for later development of atopy. The antibodies in breast milk might protect against sensitization by an antigen to which the infant is exposed during breastfeeding; quantitatively, the most important antigens are proteins in cow milk and cow milk formulas.

CONCLUSIONS

An infant was more likely to develop CM allergy if the mother's colostrum had a low total IgA content. Levels of colostral or milk antibodies did not associate with the manifestaiton of other types of allergies during the first 5 years of age.

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SENSITIZATION VIA THE BREAST MILK

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INTRODUCTION

The relationship between appearance of allergy during infancy and breastfeeding is not a simple one. Several studies indicate that breastfeeding prevents, or at least delays, the onset of allergic disease[1-6]. However, in other studies, the development of food allergy was not influenced by type of feeding or was even more common in breast-fed babies[7-10]. The divergent findings may in part be explained by choice of study groups because any protective effect seems to be limited to infants with a genetic propensity to allergy (reviewed in [12]).

As pointed out in several reviews of the most recent prospective studies, breastfeeding, compared to early administration of infant formula, is associated with a moderately lower risk for allergy[11-12], particularly if breast-feeding is combined with avoidance of solid foods for the first 4 months[3]. Several mechanisms could possibly explain the protective effect. It is widely thought that the fact that human milk is low in foreign proteins reduces exposure and the risk of sensitization to ingested allergens. But on the other hand, low doses of antigen administered to experimental animals are particularly effective in triggering the production of IgE-antibodies[13]. Thus, the relation between infant allergy and the presence of foreign antigens in human milk is not fully understood.

In this review the presence of food allergens in human milk and sensitization in exclusively breast-fed infants will be discussed.

PRENATAL SENSITIZATION

It has been demonstrated that IgE is produced by the 11th week of gestation[14]. As the placental barrier does not permit IgE antibodies to cross over to the fetus, any IgE present in cord blood would be of fetal origin. Prenatal sensitization is, however, not common and many studies have confirmed that presence of specific IgE antibodies to cow's milk or egg proteins are rarely encountered in newborn infants, even in babies with elevated IgE

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levels in the serum (Table 1). The lack of intrauterine sensitization despite the fact that the fetus has the capability to IgE-production is not fully understood. Possible explanations include, poor transfer of, for example, immunologically intact allergens over the placenta, intrauterine immune suppression, and presence of maternal blocking antibodies. The absence of IgE antibodies with known specificity at birth suggests that sensitization *in utero* occurs only rarely and that the IgE present in cord blood is polyclonal.

Table 1. Specific IgE in Cord Blood as a Marker of Prenatal Sensitization

Author	Year	Incidence	Specificities tested
Kjellman & Johansson[15]	1976	0/68	Pollens, dust
Michel *et al.*[16]	1980	3/136	Milk
Croner *et al.*[17]	1982	0/120	Milk, egg, fish
Businco *et al.*[18]	1983	1/101	Milk
Delespesse *et al.*[19]	1983	2/96	Milk, grass
Hamburger *et al.*[20]	1983	0/55	Egg, milk
Hattevig *et al.*[21]	1984	0/86	Milk, egg
Fälth-Magnusson *et al.*[22]	1987	0/195	Milk, egg
Lilja *et al.*[23]	1988	0/170	Milk, egg
Hattevig *et al.*[24]	1990	0/115	Milk, egg
Total for "allergens"		6/1141	
Weil *et al.*[25]	1983	14/57	Microfilaria

In two recent Swedish studies the possible importance of maternal diet during pregnancy for the prevention of allergy in infants was analyzed. The studies clearly showed that even large variations in maternal intake of egg and cow's milk during the last trimester of pregnancy did not influence cord blood IgE levels, the presence of IgE antibodies directed against foods, nor the incidence of allergic disease during the first 18 months of life[26,27]. A follow-up performed in one of the studies when the children were 5 years old confirmed the lack of preventive effect (Fälth-Magnusson & Kjellman, unpublished).

Although intrauterine sensitization to foods giving rise to IgE antibody production in the fetus is rare and fetal IgE antibody production to inhaled allergens has not been proven, intrauterine production of specific IgE antibodies against parasites has been reported to be common. Thus, in infants born to helminth infested mothers, IgE antibodies to *Filaria* antigens were demonstrated in 57% of the cord sera[25], strongly suggesting prenatal sensitization. Cord blood white cells have also been shown to give a strong proliferative response against idiotypes expressed on antibodies with

specificity for *Trypanosoma* and *Schistozoma* antigens, suggesting transfer of cellular immunity *in utero*[28].

ALLERGY IN EXCLUSIVELY BREAST-FED INFANTS

Food proteins dominate as allergens in infants. Clinically important antigens include cow milk, egg, soy and peanut and, to a lesser extent, fish and wheat flour[29]. Sensitization to food and clinical food allergy have been repeatedly reported in exclusively breast-fed babies. Symptoms include eczema, diarrhoea, vomiting, allergic colitis, and even anaphylactic shock. Infantile colic has also been associated with food sensitivity, *e. g.*, to cow milk[30]. The symptoms appear within a few hours after breastfeeding.

Furthermore, several studies have revealed that exclusively breast-fed infants may have positive skin prick tests and positive RAST against cow milk and ovalbumin[31-33] or react on challenge tests[32-35]. These findings do not, however, prove that sensitization has occurred via the breast milk. It is a world wide practice to administer formulas containing cow milk as supplementary feeding in maternity wards and often the mothers are not even aware that their babies have been exposed to infant formula during the first days of life. The "hidden bottle" is therefore an important confounding factor. To clarify the possible sensitizing role of breast milk, prospective studies are required.

As an example of the difficulty in proving sensitization via human milk, Høst *et al.*[36] found, in a prospective study, nine exclusively breast-fed infants who developed hypersensitivity symptoms to cow milk. But, by examining the maternity records, they could confirm that all of them had been exposed to cow milk during their first days of life. Thus, these infants may not have been sensitized via the breast milk.

Jacobsson *et al.*[37] reported four exclusively breast-fed infants with adverse symptoms to cow milk proteins in a prospective study of cow milk intolerance. They also found another three with symptoms on their first known cow milk-containing meal. The symptoms in the breast fed babies disappeared with the mothers on a cow milk-free diet and returned with the mother on an unrestricted diet. Similarly van Asperen *et al.*[38] have reported 8 children with allergic symptoms on their first known exposure to the food.

The conclusion from all these studies is that many infants with allergic symptoms while still breastfeeding probably have been sensitized by exposure to small amounts of cow milk-containing formula in early infancy in the maternity ward and then develop clinical symptoms when exposed to small amounts of antigen present in the breast milk.

There are, however, reports clearly indicating sensitization via breast milk (Table 2). In these prospective studies performed by our group, no cow milk-containing formulas were given at home for the first 3-6 months of life and no cow milk-containing formulas were given at the maternity wards. Thus, the only fluid other than breast milk given to babies in the maternity ward was water or a casein hydrolysate (Nutramigen). Despite these precautions, IgE antibodies were detected in 27 of 204 babies against egg and in 7 of 204 against cow milk proteins at six months of age in one of the other studies[26] and in 8 of 107 to egg and 4 of 104 to milk in the other study[24]. In both studies each family was advised individually and in group sessions, by an experienced dietician, how to avoid even trace amounts of egg and cow milk in the food.

Table 2. Presence of IgE-Antibodies Against Food Antigens in Sera of Exclusively Breast-fed Infants. In two of the studies the mothers either avoided all cow milk- and egg-containing foods, or they were on a regular diet. None of the babies had detectable IgE antibodies in the cord blood.

Author	Year	Method	Incidence diet	Incidence no diet	Antigen
Hattevig *et al.*[21]	1984	RAST		2/86	Milk
				7/86	Egg
Fälth-Magnusson *et al.*[26]	1987	SPT	2/79	2/99	Milk
			6/79	17/99	Egg
Hattevig *et al.*[24]	1990	RAST	0/59	4/45	Milk
			2/61	6/46	Egg
Total			2/138	8/230	Milk
			8/140	30/231	Egg

In a third study from our group, 9 of 86 children developed IgE antibodies to food antigens before eating the food in question[21]. In all the three prospective studies the families were well informed about the purpose of the study and they cooperated fully. It is therefore very unlikely that all infants with demonstrable IgE antibodies against egg or cow milk had inadvertently been given foreign proteins.

FOOD ANTIGENS IN BREAST MILK

Many studies have demonstrated that human breast milk may contain food antigens, including the cow milk proteins Beta-lactoglobulin (BLG) and casein[36,39-44], and the egg proteins ovalbumin and ovomucoid[31,40] (Fig. 1, Table 3). Kilshaw & Cant[40] demonstrated the presence of BLG in 53% of lactating women drinking cow milk. The concentrations ranged from 110 ng - 6.4 µg/l. Maximum concentration in the maternal sera appeared 2-3 hr after ingestion and in the breast milk 4-6 hr after ingestion.

Stuart *et al.*[39] reported even higher concentrations, up to 16 µg/l and showed that BLG is usually still detectable in breast milk 12 hours after ingestion and in a few mothers for even longer (up to 3 days). Similar findings have been reported for ovalbumin and ovomucoid[42].

The immunological studies do not determine whether the intact protein or immunologically active peptides are absorbed in the gut and excreted into the breast milk. Using High Pressure Liquid Gel Permeation Chromatography (HPLC) and gel filtration, however, it has been demonstrated for BLG and ovalbumin that there may be intact protein in breast milk[36,40].

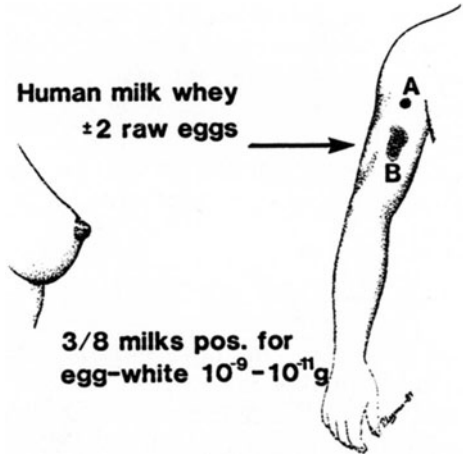

Figure 1. Demonstration of egg proteins in human milk in 1930[44]. The milk donor had eaten two raw eggs and the milk whey was then used as antigen in a Prausnitz-Küstner test.

Table 3. Concentration of the Food Antigens Beta-Lactoglobulin (BLG), Casein, Ovalbumin (OA) and Ovomucoid (OM) in Human Breast Milk

Author	Year	Antigen	n	Concentration µg/l	Method
Stuart *et al.*[39]	1984	BLG	3/28	1-16	ELISA
		Casein	13/28	1-19.5	ELISA
Kilshaw & Cant[40]	1984	BLG	10/19	0.11-6.4	RIA
		OA	13/22	0.26-6.17	RIA
		OM	7/9	0.44-2.88	RIA
Cant *et al.*[31]	1985	OA	14/19	0.2-4.0	
Axelson *et al.*[41]	1985	BLG	93/232	5-800	RIA
Machtinger & Moss[42]	1986	BLG	26/57	0.2-6.4	ELISA
Høst *et al.*[36]	1988	BLG	4/19	0.5-45	ELISA
Cavagni *et al.*[43]	1988	BLG	8/13	"positive"[a]	RIA

[a]Lower detection limit 0.01 µg/l

Of obvious interest, from a clinical point of view, are the reports that allergic infants improve clinically and show fewer symptoms when the mother avoids cow milk or egg in the food[36-38,43]. In a recent study, Cavagni *et al.*[43] showed that detectable amounts of foreign protein persisted in breast milk when allergic symptoms in breast-fed children did not improve and that their mothers continued to ingest small amounts of cow milk-containing

foods like cookies, without being aware of it. A more effective elimination of cow milk and egg antigens gave an improvement even in those cases. The authors also showed that immunologically intact food antigens may pass into the circulation of the breast-fed baby as proven by the presence of up to 400 μg/l circulating BLG in the infant.

In conclusion, there is ample evidence that foreign proteins are present in the human milk in mothers eating the specific food in question.

ALLERGY PREVENTION BY MATERNAL DIET

The demonstration of foreign antigens has prompted clinical studies in which the diet of lactating mothers was modified. The study populations in these prospective studies included babies with a family history of allergic disease. The mothers in all the studies carefully avoided egg and cow milk. In addition, local potent food allergens like peanut and soy were avoided in one study in the USA[45], peanuts, soy beans and fish in a Canadian study[46], and fish in a Swedish study[24,47].

Zeigler *et al.*[45] studied two groups of mothers with similar smoking habits and heredity for atopic disease in their babies. The mothers of the study group had a diet free of egg, milk, and peanuts during the last 3 months of pregnancy and their babies (n=103) were either exclusively breast-fed or were given a casein hydrolysate (Nutramigen) for the first 6 months. No solid foods were given until 6 months of age. Cow milk, soy, corn, wheat, and citrus were avoided for the first 12 months and fish, peanut, and egg for 24 months. The mothers and their infants in the control group (n=185) had no dietary restrictions. The cumulative rate of dermatitis, urticaria, and gastrointestinal symptoms was significantly lower in the diet group at 12 months (5.1% vs 16.4%). However, the prevalence of rhino-conjunctivitis and asthma were similar.

A Canadian study compared 97 infants with a family history of allergy who were exclusively breast-fed for more than 5 months (mean time)[46]. At 18 months of age, eczema was seen less often and was milder in babies whose mothers were on restricted diets.

In three Swedish studies, maternal diet during pregnancy and lactation was studied. Fälth-Magnusson *et al.*[26] and Lilja *et al.*[27] found no effect of maternal diet during the last three months of pregnancy. Hattevig *et al.*[47] compared two groups of infants with a heredity for atopy. In one group (n=65) the mothers carefully avoided even minute amounts of cow milk and egg, while the mothers in the control group (n=50) had an unrestricted diet. All the babies were either fully breast-fed or weaned with casein hydrolysate (Nutramigen), and solid foods were avoided for up to 6 months of age, cow milk-containing products for up to 9 months, and fish and egg for up to 12 months. The cumulative prevalence of atopic dermatitis up to 6 months of age was significantly lower in the diet group than in the non-diet group (10.8% vs 28%) and there was a tendency, although not statistically significant, towards lower prevalence up to 18 months. Also, the severity of disease at that age was significantly milder in the diet group. The prevalence of respiratory symptoms was similar in the two groups, supporting the findings by Zeigler *et al.*[45].

The clinical results were supported by the finding that less babies of mothers adhering to the diet had IgE antibodies against cow milk and egg at 3

months of age than breast-fed babies whose mothers did not avoid cow milk and egg in the food.

Thus, in conclusion, studies showed that avoidance of food allergens by mothers during lactation, rather than during the last trimester of pregnancy, does not prevent but delay the onset of allergic symptoms during the first 6-12 months of life.

MECHANISMS FOR ALLERGY PREVENTION BY BREASTFEEDING

There are several possible mechanisms by which breastfeeding could influence the likelihood of allergic disease (Table 4). As previously mentioned the lower content of foreign proteins compared to infant formula does not automatically lead to less allergy, as low antigen doses may be more sensitizing than massive exposure. Thus, Jarrett *et al.*[13] showed in experimental animals that low amounts of antigen stimulate the IgE response, while high amounts preferentially stimulate the IgG antibody production. The same is probably true also for man.

Breast milk contains may specific and non-specific components (reviewed in [48]), that protect the baby against gastrointestinal and respiratory infections. As infections have been suggested to play a role for the development of allergy by enhancing sensitization[49], breastfeeding may indirectly protect against allergy.

The dominating immunoglobulin in the milk is secretory IgA, which is transferred in large quantities to the baby. There is also evidence of production or active transport to the infant of IgG4 via the milk[50]. Both these immunoglobulins bind to microbial antigens, thereby preventing bacteria and viruses from binding to the mucosal wall. Similar mechanisms have been proposed for the protection from allergy to food proteins[48]. In fact, a recent study showed a lower content of IgA antibodies to cow milk proteins in the breast milk of mothers of infants with high scores of allergic disease[42], although this could not be confirmed by our group[51].

Human milk contains factors that enhance the maturation of the gastrointestinal mucosa[52], which in turn would reduce the uptake of intact antigens. There is also evidence that breast milk may enhance the maturation

Table 4. Possible Mechanisms for Protection by Exclusive Breastfeeding Against Allergic Disease in Genetically Predisposed Babies

Low content of foreign proteins
Reduces the number of infections
Antibodies block absorption of antigens
Enhances maturation of intestinal mucosa, thus reducing absorption of intact antigens
Enhances maturation of infant immune functions
Effect on intestinal flora

of the immune system in the infant, *e.g.*, synthesis of secretory IgA and cell-mediated immunity[53,54]. Breast milk may also have a regulatory effect on the IgE response, as indicated by studies in newborn rodents[55,56]. There is some evidence for a similar effect in humans[57,58]. As atopic disease appears to be related to defective immunoregulation of IgE) and antibody formation (reviewed in [59]), all these factors may be particularly important only for the infant with a genetically determined propensity for allergic disease. This would explain why the clinical studies indicate that exclusive breastfeeding is associated with less allergy only in risk babies.

CONCLUSIONS

Intrauterine sensitization is rare. Sensitization occurs readily through postnatal administration of small amounts of infant formula in maternity wards. This may partly explain the appearance of food allergy in exclusively breast-fed babies when they react to the small amounts of food antigens that are present in the human milk. Babies with adverse symptoms often improve when their mothers avoid the antigen. It has even been shown that intact cow milk proteins may appear in the circulation of breast-fed babies.

There is, however, also evidence that sensitization may occur in breast-fed babies who have not been otherwise exposed to foreign food proteins. Several studies have shown that specific IgE antibodies are produced by breast-fed babies not previously exposed to cow milk. Thus, sensitization to food antigens via the breast milk is possible in atopic individuals. Even if such sensitization is possible, most controlled prospective studies of allergy prevention that have been performed in infants with a high risk for allergic disease indicate that exclusive breastfeeding is associated with less allergy. Maternal avoidance of particularly allergenic foods like cow milk, fish, and peanuts may be considered in families with a high motivation. Independently of any maternal diet, however, breastfeeding should be recommended for the allergy risk infant, at least for the first four months of life.

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MANAGEMENT OF INFANTS WITH COW MILK ALLERGY

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INTRODUCTION

For centuries, human milk has been the only way of feeding the human newborn, and cow milk allergy (CMA) was virtually unknown in infants. Since the beginning of this century, cow milk (CM) formulas have become increasingly more common as breast milk substitutes when mother's milk is unavailable, and CMA has thus gradually become a more common disorder.

The management of infants with CMA is a challenge to both pediatricians and allergologists. In the first year of life of many children, CM provides almost the entire dietary supply of proteins, carbohydrates, and fat; its high nutritional value and low cost should be noted. For a 2-year-old child, 500 ml of CM provides 100% of the daily requirement of calcium, 50% protein, 100% riboflavin, and 24% energy. However, children with CMA can avoid CM without nutritional loss if nutrients are provided by other food such as meat, fish, vegetables, and fruit. Nevertheless, the choice of an adequate CM substitute is mandatory for infants with CMA. The ideal CM substitute should satisfy the following criteria: it should be hypoallergenic; have an adequate nutritional value according to the infant's age; be easily available and inexpensive; and be palatable in order to obtain a good compliance. The CM substitutes now available are soy protein formulas (SPFs) and hydrolysate formulas (HFs). As a result, pediatricians are now bombarded with a large variety of information on new special formulas named "hypoallergenic" and are confronted with the difficult choice among all these formulas.

SOY PROTEIN FORMULAS

Since 1929, SPFs have been used for feeding infants with CMA. They contain purified soy proteins, the fat is a mixture of vegetable oils, and carbohydrates are represented by maltodextrines, starch, or saccharose. SPFs are well accepted by most infants, and their nutritional adequacy is comparable to that of CM formulae. Studies recently done in infants fed exclusively SPFs failed to confirm immunologic abnormalities, or increases in

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Table 1. Measures for the Prevention of Atopic Disease

Exclusive breastfeeding for the first six months of life; soy-protein formula when breast milk is unavailable.
Selected weaning after the 6th month of life.
Egg and CM free diet for the nursing mothers.
No smoking or pets in the house.
Day-care center attendance only after 3 years.

infection morbidity as previously reported[1]. The use of SPF for the prevention of atopy is rather controversial. Some studies have shown that SPF, or breastfeeding supplemented with SPF for the first six months of life significantly reduced the prevalence of atopic disease (AD)[2,3], but others did not confirm this[4,5]. Over the last decade, we have investigated the possibility of AD prevention in high-risk babies, following the measures reported in Table 1.

The results of our studies show that the onset of AD is significantly reduced with the preventive measures above reported (Fig. 1).

More recently, we have shown in a multicenter study comprising 2,291 babies with the cooperation of many Italian Maternity Hospitals, that babies fed breast milk and/or SPF and whose parents strictly followed the environmental measures had at one year of age a prevalence of atopic

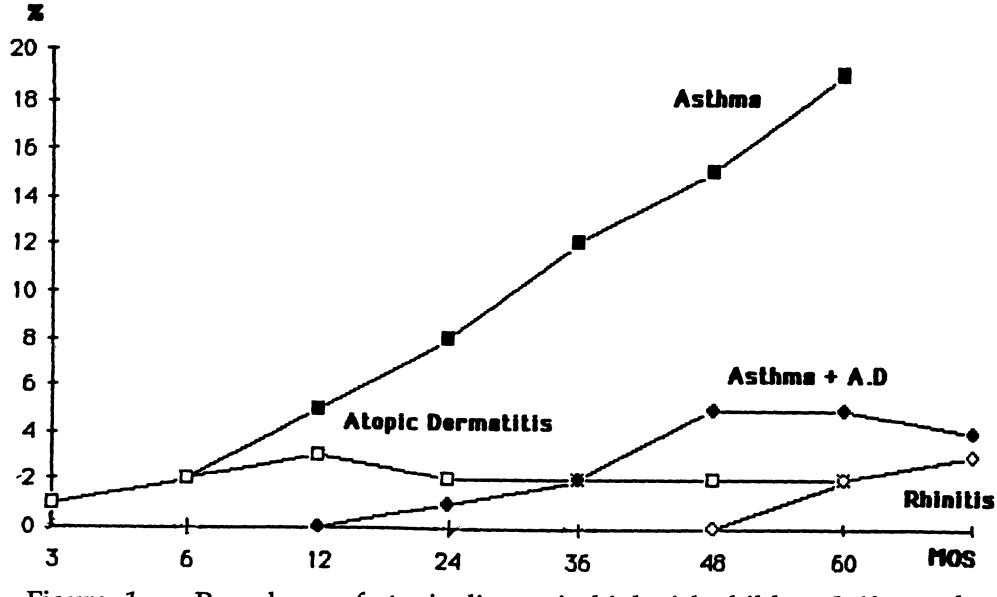

Figure 1. Prevalence of atopic disease in high risk children 3-60 months.

diseases (5%) in comparison with CM-fed babies (13%). Summing up our studies using a SPF when breast milk was not available, we did not see an increased prevalence of soy sensitization[6-9].

However, in the last decade, soy allergenicity has been emphasized in the literature without documenting with double-blind-placebo controlled food challenges (DBPCFC). Only 5% of 204 children with atopic dermatitis showed soy sensitivity, as demonstrated by DBPCFC[10]. We have confirmed that only 4% of 143 children with atopic dermatitis showed positive DBPCFC to soy[11].

In conclusion, SPFs are nutritionally adequate and well accepted by many infants. A variety of foods such as cakes, biscuits, ice cream, desserts, and beverages can be made with SPFs thus offering children with CMA a varied diet. It should be taken into consideration that SPFs are less expensive and have a more pleasant taste than other CM substitutes. Although soy proteins can be sensitizing, they are less allergenic than CM proteins.

HYDROLYSATE FORMULAS

The use of these formulas is based on the premise that predigested protein, when fed as amino acids and peptides, provides nutrients in a non-antigenic form. Thus, protein HFs have been defined as "hypoallergenic". HFs are processed using two main techniques: heat denaturation and enzymatic hydrolysis to reduce the molecular weight (mw) of peptides. Heat-treatment eliminates conformational antigenic determinants, while enzymatic hydrolysis affects sequential determinants. These different technical procedures are necessary to obtain an acceptable palatability. The reduction of the antigenicity (peptides with very low mw) is associated with a reduction of the palatability. The allergenicity of these formulas is dependent on the degree of digestion, post-hydrolysis processing, elimination of the enzymes used for the hydrolysis and protein source. According to the protein source, there are several types of HFs (Table 2).

Moreover, a partly hydrolyzed bovine whey HF with lactose has been developed. These formulas are integrated with vegetable lipids, and Alfa-Ré, Alimentum, and Pregestimil contain also medium chain triglycerides. All HFs, except Good Start and similar products, are lactose-free, contain small amounts of carnitine and are rather unpalatable (except for Good Start) and

Table 2. Cow Milk Protein Hydrolysate Formulas

Highly hydrolyzed:	
Casein:	Alimentum, Nutramigen, Pregestimil
Whey:	Alfa Ré, Prophylac
Soy + Beef Collagen:	Pregomin
Partly hydrolyzed:	
Whey:	Beba HA, Good Start, Nidina HA

compliance is therefore poor. Although HFs are considered nutritionally adequate and infants generally gain weight until they refuse the formula because of its bad taste, recently it has been reported that the total essential amino acid (EAA) concentration and the ratio of E to total AA concentration were higher in term infants fed a whey HF (Good Start) in comparison with breast-fed infants; in addition, they showed higher threonine and lower proline and tyrosine values[12]. Caution should be taken when HFs are given for prolonged periods; no data are available on nutritional assessment of infants exclusively fed with HFs for many months.

Extensively hydrolyzed formulas are considered the most hypoallergenic, whereas partly hydrolyzed formulas are considered less hypoallergenic and even dangerous in children with CMA[13,14]. Mw profiles of HFs are an index of the extent of hydrolysis. Fig. 2 summarizes the mw distribution of some HFs.

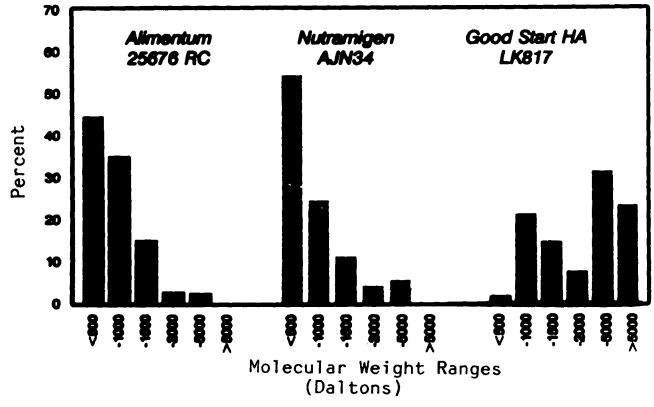

Figure 2. Molecular weight distribution of some protein hydrolysates. Two formulas contain casein, highly hydrolyzed: Alimentum and Nutramigen are very similar. Good Start, a whey-based low hydrolyzed formula contains a considerably greater percentage of peptides greater than 1500 mw.

Good Start contains a greater percentage of peptides, more than 1,500 mw. The analysis of the components of different HFs is very surprising, since casein is present in a larger amount in Good Start HA but it is not present in two casein hydrolysates (Alimentum and Nutramigen) (Fig. 3).

We first reported anaphylactic reactions in 5 infants with IgE-mediated CMA fed a whey HF (Alfa-Ré). All these infants were successfully fed with a SPF (Isomil). All the infants had positive skin tests and RAST to CM proteins and to Alfa-Ré. These data show that whey HFs can trigger severe anaphylactic reactions in children with IgE-mediated CMA[15], as confirmed by a recent study which demonstrated residual casein epitopes in all the HFs tested, such as Alfa-Ré, Pregomin, Beba HA[16]. We have recently shown that IgE to a partially hydrolyzed formula (Good Start) were detectable in 7/20 babies with IgE-mediated CMA and to an extensively hydrolyzed (Prophylac) in 4/20 babies (Table 3).

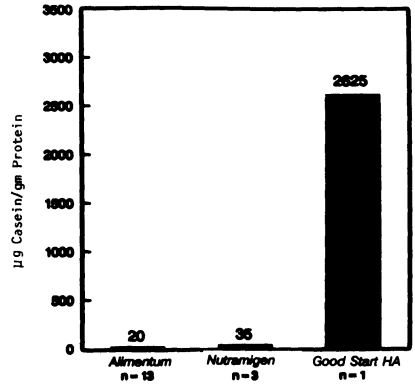

Figure 3. Proportion of casein in a partial whey hydrolysate (Good Start) and in two extensively hydrolyzed casein formulas (Alimentum and Nutramigen).

The above data agree with a study by Strobel *et al.*[13] showing that antibodies raised against a CM formula recognized epitopes displayed by peptides of some HFs. In addition, the same investigators showed that HFs in experimental animals induce cell-mediated immunity, and that cross-reactivity exists also between IgE antibodies to CM and peptides of HF in this limb of the immune response. Bauer confirmed that HFs contain protein fractions which resulted in a specific IgE-binding after incubation with serum samples from patients allergic to CM[14]. In conclusion, although the proteins of HFs have been processed by heat and enzymatic hydrolysis and therefore contain peptides of lower mw than the native protein source, the peptides still have allergenic potency and can be recognized by the cell-bound IgE of a child allergic to CM. As shown by an elegant study, 9/15 children sensitive to CM and with a positive histamine release from mixed leukocytes also had a

Table 3. Number of Children with CMA[20] and IgE to CM and to Whey-Proteins HFs

IgE to	No. of Children
Cow milk	19
Alpha-lactalbumin	20
Beta-lactoglobulin	19
Casein	16
Nidina HA	7
Prophylac	4

positive histamine release to at least one of five tested HFs[17]. It was also shown that basophils of patients with IgE-mediated CMA incubated with HFs release histamine thus strongly suggesting that HFs still have epitopes recognized by IgE bound to basophils. Recently, Ugazio *et al.* have shown that children with CMA have IgE to CM proteins and to numerous HFs[18]. It has been reported by Schwartz[19] that children with IgE-mediated CMA had positive skin test responses to both whey and casein hydrolysates, however, the wheal diameter to the whey hydrolysate was significantly higher. A recent elegant study by Sampson[20] showed that DBPCFC with two casein extensive hydrolysates (Alimentum and Nutramigen) were negative in children with documented IgE-mediated CMA.

In Table 4, the antigenicity, the allergenicity, and the cross-reactivity of SPFs and HFs are reported.

Table 4. Immunogenicity and Cross-Reactivity of HFs

	Hydrolysate			
	Soy	Casein highly	Whey partly	Whey highly
Antigenicity	+	–	+	–
Allergenicity	+	?	?	?
Cross-reactivity with IgE Abs to CM	–	+	+++	+

Soy is antigenic, can be allergenic, but does not cross-react with IgE antibodies to CM. HFs are less antigenic and allergenic, however, they do cross-react with IgE antibodies to CM. Therefore, SPFs should be used in babies with IgE-mediated CMA and whey partly HFs should not be used in infants with IgE-mediated CMA. Further studies are needed to evaluate the nutritional adequacy of HFs in babies exclusively fed such formulas for many months.

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BREAST MILK AND SPECIAL FORMULAS IN PREVENTION OF MILK ALLERGY

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INTRODUCTION

Cow milk allergy (CMA) has been reported to affect 0.3-7.5% of children, with a higher prevalence in infants than in older children[1]. In infants in Western countries, a prevalence for CMA of 3% would be a reasonable estimate. Accordingly, in the United States with over three million live births per year, about 90 thousand new infants each year would suffer from CMA. The clinical manifestations might affect one or more of several body systems, particularly the gastrointestinal tract, skin and respiratory tract, and often persist for several months or years[2]. Prevention or reducing the prevalence of CMA would, therefore, have medical, social, and economic advantages. In one study[3], the percentage of infants with CMA who required three or more visits to physicians for illness during the first year of life was three times more than that for nonallergic infants.

In infants who are genetically at high risk of atopy, the method of feeding is one of the most important factors in determining the development of allergy, at least during that age[4]. Breastfeeding and bottle-feeding are the principal methods. Infant formulas derived from cow milk are much more commonly used than those derived from soybean.

Breast Milk Versus Cow Milk Formula

Several studies have shown a lower incidence of allergy, particularly to foods, in breastfed infants than those fed cow milk formula. The difference was most prominent when infants of atopic families were fed exclusively breast milk for six months or more[5].

Table 1 presents a summary of the findings of 14 studies on allergy incidence in breastfed versus milk-formula-fed infants. Twelve of these studies showed that the incidence of allergy in the breastfed was lower than in the formula-fed. In only two studies, the difference was not statistically significant. Differences in study design, duration of breastfeeding, duration of observation, and data analysis might account for such contradictory observations[6]. It is also difficult to control for numerous factors, other than

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feeding, that might be involved. Nevertheless, the available evidence is overwhelming in favor of breastfeeding for allergy prevention.

Soybean Formula Versus Cow Milk Formula

Soy-based formula was developed much later than cow milk formula and because it was well tolerated by the majority of milk-sensitive infants[3], it was perceived as less allergenic. However, a formula that may be generally useful in therapy may not be so useful in prevention.

Table 1. Allergy Incidence in Breast-Fed vs. Milk-Formula-Fed Infants

Study	Duration of breastfeeding	Duration of study	Allergy in breast-fed
1. Matthew *et al.*, 1977[7]	3 mo	6 mo	less
2. Chandra, 1979[8]	6 wk	2 yr	"
3. Saarinen *et al.*, 1979[5]	2 mo	3 yr	"
4. Fergusson *et al.*, 1981[9]	4 mo	2 yr	"
5. Hide & Guyer, 1981[10]	6 mo	1 yr	"
6 Kaufman & Frick, 1981[11]	6 wk	2yr	"
7. Juto *et al.*, 1982[12]	1 mo	1 yr	"
8. Businco *et al.*, 1983[13]	6 mo	2 yr	"
9. Duchateau & Casimir, 1983[14]	1 mo	1 mo	"
10. Miskelly *et al.*, 1988[15]	4 mo	1 yr	"
11. Vandenplas *et al.*, 1988[16]	4 mo	4 mo	"
12. Chandra *et al.*, 1989[17]	6 mo	6 mo	"
13. Halpern *et al.*, 1973[18]	6 mo	0.5-7 yr	same
14. Gordon *et al.*, 1982[19]	1 mo	6 mo	same

Table 2 presents a summary of the findings of seven studies on allergy incidence in soy-formula-fed versus cow-milk-formula-fed infants. In only two of these studies, soy formula was associated with a lower incidence of allergy. On the other hand, the majority showed no significant difference between these two methods of feeding. Soybean protein has been shown in human to be as antigenic as bovine milk protein[20]. It seems, therefore, that soy formula, though might be tolerated by milk-sensitive infants, would not be an appropriate choice for prevention of allergy in high-risk infants.

Allergy in Breastfed Infants

Breastfed infants are not exempt from developing food allergy even while their sole diet is breast milk[24]. Infants at high risk of atopy can be sensitized to bovine milk or other foods ingested by the mother and reach the infant, though in minute quantities, across the placenta[25-27] or the mammary gland[28-30]. Details in this subject are mentioned in two relevant articles in this volume; one by Harmatz on dietary protein antigen transfer in breast milk and the other day Björksten on sensitization of babies via the breast milk.

Table 2. Allergy Incidence in Soy-Formula-Fed vs. Milk-Formula-Fed Infants

Study	Duration of formula feeding	Duration of study	Allergy in soy-fed
1. Johnstone & Dutton, 1966[21]	9 mo	10 yr	less
2. Businco *et al.*, 1983[13]	6 mo	2 yr	less
3. Brown *et al.* , 1969[22]	3-4 mo	1-2 yr	same
4. Halpern *et al.*, 1973[18]	6 mo	0.5-7 yr	same
5. Kjellman & Johansson, 1979[23]	9 mo	4 yr	same
6. Miskelly *et al.*, 1988[15]	4 mo	1 yr	same
7. Chandra *et al.*, 1989[17]	6 mo	6 mo	same

Pediatricians are only partially aware of the frequency of giving bottle feeding by error to the breast-fed infants while they are still in the newborn nursery. The parents are vastly unaware of such incidents and are often not aware either of the frequent introduction of various foods in the baby's mouth by other family members or baby sitters. The magnitude of this problem is difficult to estimate but is certainly much more than is realized. Such unrecognized exposure undoubtedly affect the results of studies in this area.

Maternal Diet During Pregnancy and Lactation

Only a few studies explored the effect of dietary elimination on the incidence of allergy in infants of atopic families. Fälth-Magnusson *et al.*[31] reported that avoidance of bovine milk and egg from the 28th week of gestation until delivery did not reduce the incidence of allergy at 6-18 months in the infants of those mothers. Chandra *et al.*[3] noted that avoidance of milk, egg, fish, and peanuts during pregnancy and lactation (for about 6 months) resulted in reduction in the incidence as well as the severity of infantile atopic dermatitis. In that study, the combination of breastfeeding and maternal dietary elimination was about twice as effective as breastfeeding while the mother was on a normal diet. In the study by Hattevig *et al.*[32], breast-fed infants whose mother avoided milk, egg, and fish during the first three months of lactation developed less atopic dermatitis at three and six months of age than those whose mothers were on normal diet. Zeiger *et al.*[33] noted that the combined maternal and infant avoidance of food allergens resulted in a lower incidence of gastrointestinal and skin allergic disorders during the first year of life then the difference became less marked by two years of age. The mothers avoided milk, egg, and peanuts during the third trimester and lactation. Infants were fed breast milk and/or casein hydrolysate formula until six months of age; avoided cow milk, corn, soy, citrus and wheat until one year of age, and egg, peanuts and fish until two years of age.

Three conclusions may be drawn from the above studies. First, the effect of dietary elimination during pregnancy is not clear yet. Second, the beneficial effect of breastfeeding would be most prominent when it is combined with dietary elimination by the lactating mother. Third, the benefit

of prophylaxis noted during the first 6 to 12 months will probably be gradually lost after the prophylactic measures are discontinued and the child becomes exposed to normal diet and environment. Allergy at that later age, however, usually causes less morbidity than during infancy.

Special Infant Formulas

The increasing recent interest in allergy prevention in high-risk infants led to exploring the potential benefit of prophylactic feeding of special formulas. Initially, this class of formulas was introduced mostly for use in milk-sensitive infants, hence they were coined the term "hypoallergenic."

To reduce the antigenicity/allergenicity of native protein molecules, formula manufacturers have utilized a variety of methods. Heat treatment was found to have a limited effect[34]. By changing the molecular configuration, heat affects the conformotional epitopes which might render the protein molecule less antigenic. Occasionally however, heat increases the molecule's antigenicity by exposing previously hidden antigenic determinants. On the other hand, enzymatic hydrolysis by endopeptidases (such as trypsin and chymotrypsin) splits the molecule into small peptides; this changes the sequential epitopes. Compared to heat treatment, this method is more effective in reducing the antigenicity and in preserving the nutritional quality[35-36]. A combination of the two methods or the additional use of ultrafiltration to remove large peptides might provide optimal preparations. On using extensive processing, however, the manufacturers confront some major problems, such as cost, physico-chemical properties, hyperosmolarity, imbalance of nutrients, and poor taste or smell.

The currently available special formulas are derived from bovine casein, bovine whey, soybean, or elemental diet (Table 3). Casein hydrolysates were developed first, with *Nutramigen* (Mead Johnson) being available since 1942. In 1971, the same manufacturer introduced a similar preparation, *Pregestemil*, that contains medium-chain triglyceride oil (60% of fat) to be used in malabsorption states. *Alimentum* (Ross) was introduced in 1988 and also contains medium-chain triglyceride oil (60% of fat); it is basically similar to *Pregestemil* except for the sources of carbohydrate and fat.

The whey hydrolysate preparations were introduced in the mid 1980's, with *Alfaré* (Nestlé) being the first and more hydrolyzed than its counterpart by the same manufacturer (*Nan HA , Nidina HA, Beba HA, Good Start*). Other recent whey hydrolysate formulas are *Prophylac* (ALK) and *Almiron Pepti* (Loma Linda/Nutricia).

Aptamil (Milupa) is composed of hydrolysates of both casein and whey serum proteins in equal parts. The same manufacturer produced a preparation (*Pregomin* or *Hydrolac*) of soybean hydrolysate plus bovine collagen.

An elemental diet whose protein consists of synthesized amino acids is exemplified by *Vivonex* or *Tolerex* (Norwich Eaton) and *Vivasorb* (Phrimer A/S. This formula was originally devised for postoperative nutrition of patients with major intestinal surgery and became occasionally used as a temporary elimination diet in patients with multiple food sensitivity[37,38].

Trials of these special formulas in prevention of allergy, particularly of CMA, in high-risk infants have been few so far and mostly limited to the casein hydrolysate formula by Mead Johnson and the partially hydrolyzed whey formula by Nestlé. Studies of this type are not easy to conduct, not only because of the high cost, but more importantly because of the difficulty in

Table 3. Special "Hypoallergenic" Formulas

A. Bovine casein hydrolysate
   1. Nutramigen (Mead Johnson)[a]
   2. Pregestemil (Mead Johnson)[a]
   3. Alimentum (Ross)[a]

B. Bovine whey hydrolysate
   1. Nan HA, Nidina HA or Beba HA (Nestlé)
      Good Start (Carnation/Nestlé)[a]
   2. Alfaré (Nestlé)
   3. Prophylac (ALK)
   4. Almiron Pepti (Loma Linda/Nutricia)

C. Combined casein & whey hydrolysates
   1. Aptamil (Milupa)

D. Soy hydrolysate + bovine collagen
   1. Pregomin or Hydrolac (Milupa)

E. Elemental Diet
   1. Vivonex or Tolerex (Norwich Eaton)[a]
   2. Vivasorb (Phrimer A/S)

[a] Currently available in the United States

controlling and monitoring the numerous factors that determine the development of allergy[4].

High-risk infants who were fed *Nutramigen* for the first six months of life and followed until 18 months of age showed lower incidence and severity of atopic dermatitis than those fed conventional milk-based or soy-based formula[39]. Similarly, in the study by Zeiger *et al.*[33], the incidence of both gastrointestinal and skin allergies was reduced during the first year of life.

When 15 infants of atopic families were exclusively fed the partially hydrolyzed whey formula (Nestlé) for four months, allergy developed during that time in none of that group, compared to one in the exclusively breast-fed group and eight in the group fed cow milk formula[16]. In a larger study[17], exclusive whey hydrolysate feeding for six months resulted in a much lower incidence (7%) of probable allergy than in the group fed breast milk (20%), soybean formula (37%), or conventional cow milk formula (36%). In both of these studies, it seems that breastfeeding without maternal dietary elimination is more likely to be associated with allergy than exclusive feeding of a hydrolysate formula.

More studies are needed to demonstrate the degree of clinical hypoallergenicity of each of these various special formulas and their long-term prophylactic and nutritional adequacies.

CONCLUSIONS

Cow milk allergy affects an estimated 3% of infants and can present with a wide variety of manifestations. Its prevention should be worth considering, particularly in infants of atopic families. Exclusive breastfeeding, whenever possible, for six months or more is the most natural and economic method. Its effect can be more marked when the lactating mother avoids the most common allergenic foods. Soy-based formula can be as allergenic as cow milk formula. If breastfeeding cannot be provided, special "hypoallergenic" formulas seem to be the next best choice. The limited trials of casein or whey hydrolysate formulas show promising results. The long-term benefit from their use during the first months of life remains to be determined.

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ATOPY PROPHYLAXIS IN HIGH-RISK INFANTS

Prospective 5-Year Follow-Up Study of Children with Six Months Exclusive Breastfeeding and Solid Food Elimination

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INTRODUCTION

Prophylaxis of atopic diseases has been a topic of interest for several years. The expression of allergic illness results from an interrelationship between the atopic genetic constitution and the encountered environment. In early life, infants and young children are exposed to a range of environmental allergens that may stimulate a local or systemic immune response[1]. Children with positive family history for atopy may develop allergic symptoms[2,3].

The mode of feeding during the first few months of life has been shown to affect the subsequent development of atopic disease. Prolonged breastfeeding without any cow milk reduced eczema during the first three years[4] and late introduction of solid foods has shown a prophylactic effect on food allergy and atopic eczema for the first year of life[5].

The purpose of this five-year prospective follow-up study of allergy prophylaxis was to determine the benefits of exclusive six months breast-feeding combined with delayed introduction of solid foods in children at high risk for atopic disease.

SUBJECTS AND METHODS

The original study group consisted of 135 infants of atopic parents. All children had positive atopic heredity (Table 1). All children received breast milk without any cow milk supplements for the first six months. Half of the children, 65 infants, were exclusively breast-fed without any solid food up to 6 months of age, and 70 infants started with solid feeding at three months of age[5].

Fifty-one children of the exclusive breast milk group and 62 of the solid food group could be reached for re-examination at the age of five years.

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Examination at the Age of 5 Years

- history of food allergy
  (repeated skin rash, urticaria or heavy vomiting after ingestion)
- history of seasonal symptoms or symptoms associated with animal contacts
  (nasal discharge, itching conjunctivitis, wheezing during pollen season or at animal contacts)
- skin prick tests
  (birch, alm, timothy, ragweed, dog & cat epithelium, fish, milk & wheat)

Diagnosis of Atopic Disease

1. Atopic eczema at the time of examination (6)
   or
2. Diagnosis of asthma (7)
   or
3. Prick-test positive corresponding with the history of food, pollen or animal dander allergy, respectively

Suspicion of Atopic Disease

1. Positive history but negative prick tests
   or
2. Positive prick test but negative history

Table 1. The Similarity of Atopic Heredity in the Study Groups

	GROUP I exclusive breast feeding	GROUP II solid food
biparental atopic heredity	18%	8%
atopic mother	82%	61%
atopic father	36%	47%
atopic eczema	51%	37%
seasonal rhinitis	57%	56%
asthma	24%	18%

RESULTS

Atopy was common in these 5-year-old children, 39 (33%) children out of the total 113 were regarded atopic. Atopic disease was more common in the solid food group, occurring in 40% compared to 27% in the exclusive

breast milk group (p=0.15) (Fig. 1). If suspicion of atopy also was considered, the portions were 50% and 35% (Fig. 1). Atopic eczema was discovered equally in both groups, 24%, at the time of examination.

The first examination at the age of one year showed a significant difference between the child groups, atopic eczema and history of food allergy occurring more frequently in the solid food group (Fig. 2)[5].

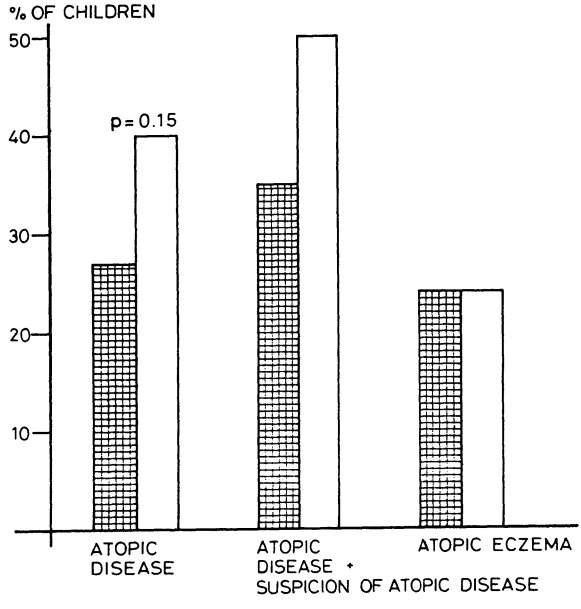

Figure 1. Atopic disease in the 5-year-old children. Shadowed area indicates children of the exclusive breastfeeding group.

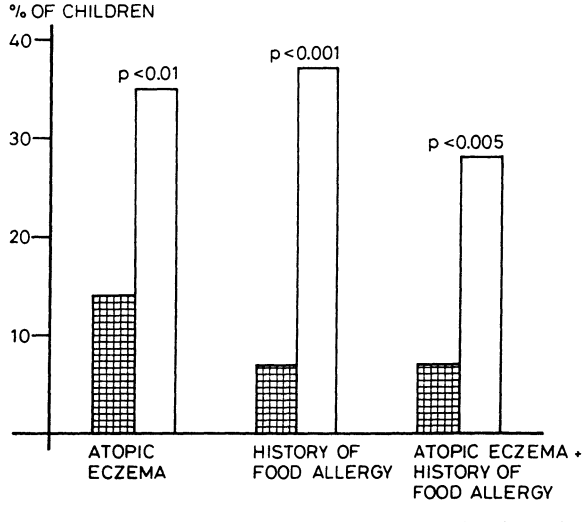

Figure 2. Atopic eczema and food allergy in the studied children at one year of age. Shadowed area indicates children of the exclusive breastfeeding group.

The growing importance of pollen allergy and atopic airway disease was observed at the age of five years. Pollen allergy was diagnosed in a total of 28% of the children, 37% of the solid food group, and 20% of the exclusive breast milk group (p=0.04) (Fig. 3). Asthma was verified in a total of 13 children, 8% versus 15%. The exclusively breastfed group showed the benefit but the difference did not reach statistical significance (Fig. 3). All children with asthma in both study groups also had pollen allergy.

In this age range, food allergy was of little importance and no differences were seen between the child groups. Four of the exclusively breast-fed group and 6% in the solid food group also suffered from fresh fruit allergy associated with birch allergy. Allergy to animal dander also occurred in similar proportions in both groups, 8% and 10%, respectively (Fig. 4).

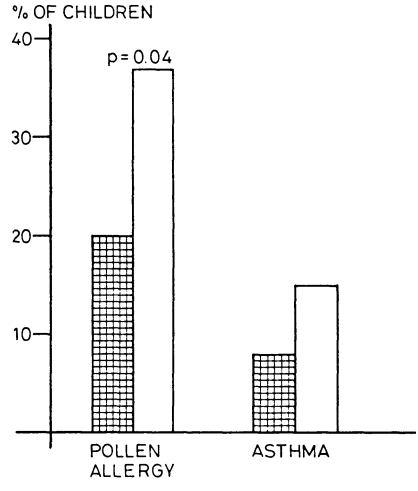

Figure 3. Asthma and pollen allergy at the age of 5 years. Shadowed area indicates children of the exclusive breastfeeding group.

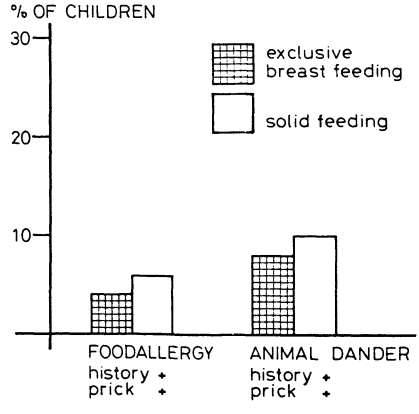

Figure 4. Food allergy and allergy to animal dander at the age of 5 years. Shadowed area indicates children of the exclusive breastfeeding group.

DISCUSSION

The late introduction of solid feeding besides breastfeeding seems to have long-lasting benefits on the development of atopic disease, and especially on pollen allergy in high-risk children (Fig. 3). Atopic disease was common in both child groups indicating a strong hereditary influence. The study groups differed only in respect to solid feeding during 3 to 6 months of age.

The mode of feeding during the first few months of life is shown to influence the atopic presentation[4,8] but a distinction must be made between infants with or without atopic heredity and the emphasis directed at children of atopic families[4,10,12]. The burden of genetic forces is significant[9,11]. The risk of allergy is estimated to be 58% in unilateral heredity and up to 67-100% in bilateral atopic heredity[2].

No previous work has evaluated a prospective study with a 5-year follow-up, a strict dietary design, and hereditary basis.

Food allergy and atopic eczema were common in the solid feeding group at the age of one year (Fig. 2), probably serving as a preceding symptom for further atopic manifestation. At the age of five years, food allergy was only a minor complaint in both groups. Atopic eczema was equal in both groups, 24%. The significant difference regarding pollen allergy, 37% vs 20%, and the distinction between the occurrence of asthma, 15% vs 8%, showed the long-lasting benefit of exclusive breastfeeding combined with the postponement of solid feeding (Fig. 1). According to another recent study, a high risk of allergic airways disease was found after early eczema in infancy[3].

CONCLUSIONS

The development of atopic disease is strongly predisposed by genetic determination. However, according to the present study, promising prolonged protection can be achieved by exclusive 6 months breastfeeding without solid foods. In atopic families, mothers with abundant breast milk supply should be encouraged to continue exclusive breastfeeding without solid foods up to 6 months.

ACKNOWLEDGEMENTS

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NATURAL HISTORY AND IMMUNOLOGICAL MARKERS IN CHILDREN WITH COW MILK ALLERGY

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INTRODUCTION

Cow milk allergy (CMA) affects between 2 and 7.5% of infants. Our previous studies have described the clinical and immunologic features of CMA in infants and young children[1]. From the first 100 patients with challenge proven CMA, three clusters of patients were identified. Group 1, or immediate reactors, developed anaphylaxis and/or exacerbations of eczema and/or urticaria within 45 min. of ingestion of small volumes of cow milk. Group 2, or intermediate reactors, developed symptoms of vomiting and/or diarrhoea within several hr of ingestion of larger volumes of cow milk. Group 3, or late reactors, developed eczema and/or bronchitis and/or diarrhoea following the ingestion of normal volumes of cow milk over 24 to 72 hours.

Compared with a control population, all patient groups had low total serum concentrations of IgG and IgA, but only the Group 1 patients had elevated IgE[2]. IgA and IgM anti-cow milk specific antibody levels were similar to those in a control population, but all patient groups had low IgG anti-cow milk antibodies, a phenomenon we attributed to exclusion of cow milk from their diet. IgE anti-cow milk antibody levels were increased in the immediate reacting Group 1 patients only[2]. Leukocyte migration-inhibition factor (LIF) production was measured as a marker of *in vitro* delayed-type hypersensitivity. The lymphocytes of the Group 2 and Group 3 patients produced more LIF in response to α-lactalbumin, β-lactoglobulin, and α-casein than the lymphocytes from the Group 1 patients[3].

In this longitudinal study of our patients with challenge proven CMA, there were two broad aims. First, we wished to study the clinical outcome of children with CMA with regard to, the effect of prolonged cow milk avoidance on clinical features, the reported frequency of adverse reactions to other foods, and the development of other atopic disorders. Secondly, the immunological outcome of children with CMA was examined.

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METHODS

Patients

Clinical studies. Of the initial cohort of 100 CMA patients, 34 girls and 63 boys were reviewed after an average period of 5 years; three children could not be located. From the time of diagnosis, patients had been advised to avoid all milk-containing foods until clinical tolerance had been demonstrated by formal challenge. The mean age of children at initial challenge was 16 months (1-98 months) and at the final challenge was 60 months (11-135 months). The mean age of all the patients at the final review was 99 months[4].

Immunological studies. There were 47 patients (30 boys) in this study. They were part of the 100 patients with CMA whose clinical response to formal challenge and immunological features have been described as elsewhere[1,5]. Patients had serum samples taken at the time of initial and final challenges. The mean interval between samples was 16 months.

Milk Challenge Procedure

The diagnosis of CMA was determined by cow milk challenge according to a previously published protocol[1]. This required patients to be free of milk-allergy-related-symptoms when on a milk-free diet, exclusion of disaccharidase deficiency as a cause of milk intolerance, and the demonstration of an unequivocal relapse of symptoms following milk challenge. The milk challenge was not placebo controlled. It was conducted in a hospital setting on days 1, 2, and 3. Subsequent milk ingestion was continued at home. Patients were reviewed at the time of any adverse reaction, and one week and one month after commencing cow milk.

Conduct of Study

Clinical studies[4]. After the diagnosis of CMA by challenge, all patients remained under the care of the chief investigator who reviewed them 4 to 6 weekly until a stable diet was achieved, and then 3 to 6 monthly. Formal milk challenges were conducted in the Allergy Unit at yearly intervals until tolerance was demonstrated. If milk tolerance developed, patients continued to be assessed by the chief investigator at 3 to 6 month intervals if they had evidence of adverse reactions to other foods, or other atopic diseases.

Five years after the commencement of the investigation, 30 of the original cohort of 100 CMA patients remained under the care of the chief investigator. At the 5-year follow-up, the parents of 97 of the initial cohort of 100 patients were interviewed. This interview documented: 1) the volume of dairy products ingested daily; 2) the incidence and nature of adverse reactions to other foods; 3) the presence or absence of atopic diseases like eczema, asthma, rhinitis, and uriticaria; and 4) the results of the most recent formal cow milk challenge conducted in the Unit. This, and other information, was recorded on a standardized questionnaire and entered into a computer data base. Patients who were CMA-positive at challenge within 12 months of the current study, were regarded as still suffering from the disease at the time this data was collected.

Immunological studies[5]. Serum samples were collected immediately before each milk challenge. Total serum immunoglobulin, and cow milk-specific antibody values were those measured in samples collected at the time of the initial and final follow-up milk challenges. These serum samples were from the same 47 patients who were the subjects of an earlier report that

examined the relationship between the time of clinical response to milk challenge and the humoral immune response to cow milk.

Statistical Analysis

The three clusters of patients with CMA previously identified[4] were, for the purposes of analysis, retained in their original groupings. These three groups were defined on clinical grounds. In summary, certain historical information and the effect of formal cow milk challenge conducted in the Unit was noted, entered into a computer data base, and the 3 groups of patients with common features were identified using a K-means algorithm[4]. Once patients were classified into Group 1, 2, or 3 at the outset, they remained in those groups throughout the longitudinal studies of both the clinical and immunological outcome.

Comparison between groups of patients was made using appropriate non-parametric statistical methods.

RESULTS

Clinical Outcome

Remission of CMA. Nineteen (6F,13M) of the 97 children had persistent CMA at the 5-year follow-up; eleven of these patients were less than 6 years of age, six were aged from 6 to 8 years, and two were older than 8 years. Of the 27 patients in Group 1, 9 had persistent CMA (33%), of 52 in Group 2, 7 had persistent CMA (13%), and of 18 patients in Group 3, three had persistent CMA (17%). The incidence of persistent CMA was significantly greater in Group 1 compared to Group 2 ($P = 0.04$, Chi-squared test). There was no difference in the incidence of remission between the other group comparisons. The mean age of the 19 patients with persistent CMA at follow-up was 91 months (66-129 months) whereas the 78 children who became milk tolerant became so at a mean age of 46 months (11-144 months).

Adverse reactions to non-cow milk foods in children with CMA. Only 25 of the 97 children were reported to be allergic to cow milk alone. The frequency of adverse reactions to a range of foods, as reported by parents, is shown in Table 1. A high incidence of soy milk intolerance and peanut allergy was noted. However, of the 34 children said to have peanut allergy, 19 were clinically tolerant to soy milk (Table 1).

There were more patients in Group 1 reported to have adverse reactions to egg, orange, and peanut compared to Group 2 (Chi. squared $P<0.05$). More children in Group 3 had egg allergy than in Group 2 (Chi. squared $P<0.05$) but no other difference in the incidence of other food allergies was reported.

There were 56 patients who reported symptoms of egg allergy. Symptoms developed within 1 hr in 45 patients including urticaria/ angioedema (45 cases), vomiting and diarrhoea (7 cases), and stridor (2 cases); 11 reported symptoms including diarrhoea (6 cases), vomiting (3 cases) and eczema (4 cases) more than 1 hr but within 24 hr after egg ingestion.

A total of 37 children were reported to have adverse reactions to soy milk. Of thirteen who reacted within 1 hr of soy milk ingestion, symptoms of urticaria (3 cases), vomiting (8 cases), diarrhoea (4 cases), eczema and morbilliform rash (1 case), wheeze and collapse (1 case) were observed; 24 parents reported symptoms which developed over several hours including

Table 1. The Percentage of CMA Children with an Adverse Reaction to Individual Foods is Shown. The Total Number of Children Reported to React to each Food Compared to the Total Number Exposed is Shown.

Egg	56/97	(58%)
Wheat	16/97	(16%)
Soy milk	37/78	(47%)
Casein hydrolysate	13/58	(22%)
Banana	17/93	(18%)
Apple	5/97	(5%)
Pear	8/96	(8%)
Orange	33/93	(35%)
Strawberry	10/90	(11%)
Tomato	11/90	(12%)
Fish	12/95	(13%)
Peanut	34/97	(34%)
Lamb	7/97	(7%)
Beef	14/96	(14.5%)
Chicken	9/96	(9%)

diarrhoea (18 cases), vomiting (5 cases), eczema (5 cases), irritability, cough, and wheeze (1 case each).

Sixteen patients were reported to develop adverse reactions to wheat. In five children, symptoms of urticaria (2 cases), diarrhoea (3 cases), vomiting (2 cases), and rhinitis (1 case), developed within 1 hr of wheat ingestion; eleven children were reported to develop symptoms, including diarrhoea (8 cases), eczema (2 cases), vomiting (2 cases), rhinorrhoea, cough and wheeze (1 case each), several hours after ingestion of wheat products. Four children with wheat-induced diarrhoea had normal duodenal biopsies while suffering from diarrhoea induced by wheat ingestion.

In thirteen children, casein hydrolysate was excluded from their diet because of reported adverse reactions. In 4 cases, reactions were reported within 1 hr of ingestion; symptoms of urticaria (2 cases), colic, distress and diarrhoea (1 case), and vomiting (1 case), were noted. Nine children were reported to develop symptoms, of diarrhoea (8 cases), vomiting (3 cases), face erythema and eczema (1 case), and irritability (2 cases), within hours of ingestion. One of the patients with adverse reactions to casein hydrolysate tolerated soy milk satisfactorily.

Atopic diseases associated with cow milk allergy. When the results were analyzed according to whether patients were milk tolerant or not at final follow-up, higher incidences (Chi squared or Fishers Exact Test as appropriate) or asthma ($P<0.001$), eczema ($P2=0.023$), urticaria ($P=0.045$) was seen in patients with persisting CMA.

Milk intake of cow milk tolerant patients. Nearly 20% of the patients who achieved tolerance to cow milk on challenge were ingesting less than 100 ml. of cow milk per day, and only a third of the tolerant patients were ingesting more than 300 ml. of cow milk per day.

Immunological Outcome

Samples from 47 patients taken over a mean period of 16 months were analyzed. Because small volumes of serum were obtained and some tests were repeated, complete sets of results for the eight immunologic measurements were not available at each assessment of some patients. Of the possible 752 assays (16 individual assays of 47 patients), the results of 727 assays were available to be analyzed. Four of the missing assays were of anti-cow milk antibody levels, and 21 were of serum immunoglobulin levels.

Group 1: immediate reactors. Tolerance to cow milk developed in six patients (median age 24 months), and nine remained milk intolerant (median age 24 months). There was no significant difference in the ages of these two patient subgroups at the final assessment. Serum IgM percentile values were significantly higher in the patients with persistent CMA in comparison with the initial sampling ($p<0.05$), but there were no changes in the IgG, IgA, or IgE levels between these two sampling times, irrespective of whether or not milk tolerance was achieved (Table 2). Anti-cow milk antibody levels of the IgG, IgA, IgM, or IgE isotypes did not change between the initial and final sampling regardless of whether CMA persisted. There was no difference in levels of immunoglobulins or anti-cow milk antibodies, at either the initial or the final assessments, between patients who acquired milk tolerance and those who had persisting disease (Tables 2 and 3).

Group 2: intermediate reactors. At the final assessment, 10 patients were milk tolerant (median age 27.5 months) and 14 had persistent CMA (median age 35 months). There was no significant difference in the ages of these two patient groups (Table 3). No difference was observed in the serum IgG, IgA, and IgM percentiles, IgE levels, or anti-cow milk antibody levels of the IgG, IgA, IgM, or IgE isotypes between the initial and final assessments irrespective of whether or not milk tolerance was achieved (Tables 2 and 3). However, at the final assessment alone, the serum IgA percentiles were higher and the IgG anti-cow milk antibody levels were lower in patients with persistent CMA in comparison with those with tolerance to cow milk.

Although for the group there was no significant difference in the levels of IgE anti-cow milk antibodies over the study period, six patients had a greater than fourfold increase in serum IgE anti-cow milk antibodies during this time; two of these patients developed clinical tolerance.

Group 3: late reactors. Only two of eight patients in this group acquired tolerance to cow milk. Analysis was confined to the six patients (median age 36-1/2 months) with persistent CMA, but there was no significant difference between the initial and final values for any of the immune system measurements.

Comparison of immune system measurements between the different patient groups demonstrated higher serum total IgE and IgE anti-cow milk antibody levels for Group 1 over Group 2 at the initial and final assessments.

Table 2. Median Age in Months, Serum IgG, IgA, IgM Percentiles and Total IgE Levels in IU/ml for Immediate or Late Reacting CMA Groups at Initial (I) and Final (F) Times

		Age		IgG		IgM		IgA		IgE	
	Pt.	I	F	I	F	I	F	I	F	I	F
Immediate											
Intolerant	9	7	33	17	14	31[a]	44[a]	17	26	105[c]	124[d]
Tolerant	6	11.5	24	21	24	24	25	19	21	24	35
Intermediate											
Intolerant	14	16	35	15	26	21	40	10	30[b]	12[c]	13[d]
Tolerant	10	6	27.5	13	17	44	50	5	18[b]	7	25
Late											
Intolerant	6	17.5	36.5	28	19	62	46	40	25	223	525
Tolerant	2	Insufficient data				-		-		-	

Data recorded according to clinical features at initial assessment, and whether or not clinical tolerance to cow milk was achieved at final follow-up.
a,b,c,d - indicate $P<0.05$ between each of the values indicated.

Table 3. Median Anti-Cow Milk IgG, IgM, and IgA Levels in Arbitrary Milk Antibody Units, and Anti-Cow Milk IgE Antibody Levels in Absolute Counts Per Minute of Isotope Bound, for Immediate, Intermediate or Late Reacting CMA Groups.

		IgG		IgM		IgA		IgE	
	Pt.	I	F	I	F	I	F	I	F
Immediate									
Intolerant	9	27	17	200	925	32	90	1954[b]	4345
Tolerant	6	21.5	18	925	550	55	42	2718	791[c]
Intermediate									
Intolerant	14	16.5	16.5[a]	325	725	38	71	309[b]	283
Tolerant	10	14	27.5[a]	438	538	57.5	140	382	375[c]
Late									
Intolerant	6	31	21	196	462	78	78	623	246
Tolerant	2	Insufficient data				-		-	

Data recorded according to clinical features at initial assessment (I), and whether or not clinical tolerance to cow milk was achieved at final (F) challenge.
a,b,c - indicates $P<0.05$ between each of the values indicated.

CONCLUSIONS

1. CMA is not a transient disease; in our study nearly a third of patients are milk tolerant by the age of 2, two thirds by the age of 4, and nearly 80% by the age of 6 years[4]. However, because this is a referred population, it is likely these children represent the more severe end of the spectrum of this disorder.

2. Only 25% of the children were allergic to cow milk alone with adverse reactions to - egg, peanut, soy milk, casein hydrolysate, and wheat are common in CMA patients[4].

3. Children with CMA frequently develop disorders like eczema, rhinitis, asthma, and urticaria. The incidence is greatest in children with persisting CMA at the final follow-up. Some patients with non-IgE associated CMA subsequently develop atopic diseases[4].

4. As a group, patients with IgE-type hypersensitivity to cow milk maintained this response despite the development of clinical tolerance. As a group, patients with non-IgE cow milk enteropathy do not generate IgE responses to cow milk.

5. The small number of patients may have obscured a real difference in the immune parameters, through a Type-2 error, but the possibility that development of clinical tolerance in CMA may not be associated with primary immunological events warrants further investigation[5].

ACKNOWLEDGEMENTS

This report is based on a series of studies conducted in this Department over the last 15 years. We wish to thank the co-authors of those studies and the staff of the Department of Allergy, Royal Children's Hospital, Melbourne, for their assistance.

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IgG, IgA, AND IgE ANTIBODIES TO COW MILK PROTEINS IN AN ALLERGY PREVENTION STUDY

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INTRODUCTION

In the last few years, several trials have been carried out to determine if certain feeding regimens in neonates prevent atopic diseases[1-13]. In our country, there is a continuing debate about nutritional supplementation of breast milk with hydrolysate formulas: in the first days of life should only newborns at allergy risk[8,9,11,14], or should all newborns be fed only breast milk? This age is claimed to be an especially vulnerable period for a sensitization against foreign proteins because of intestinal immaturity and inexperienced gut-associated lymphoid tissue[15]. A prospective Danish study revealed cases of cow's milk allergy only among those breastfed infants who had received cow's milk formula as a "night-bottle"[16]. Others reported allergic manifestations in infants after a sensitization by small quantities of antigens via human milk[17,18]. The milk industries have provided pediatricians with several new "hypoallergenic" (HA) hydrolysate formulas of different origin and of different molecular size promising allergy prevention not only in newborns at risk.

In the following, we present the initial results of our prospective study comparing four feeding regimens in newborns with and without allergy risk. The inclusion of the latter is based on the findings of Croner[19] that 35% of children with obvious allergic diseases passed a neonatal screening, i.e., had a negative family history and a normal cord blood IgE[1,20]. The aim of our study was first to determine the frequency of allergic manifestations of the skin, the gastrointestinal tract and the respiratory tract in children with different feedings during their neonatal age. Because cow milk is usually the first foreign protein introduced in infancy, this study was used secondly to determine the degree of sensitization against various cow milk proteins. Furthermore, it was possible for the first time to compare the levels of these antibodies in infants fed different formulas, including cow milk derived HA formulas, and in infants with different allergy risks.

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PATIENTS AND METHODS

Study Design

Allergy risk was defined as high if there was a positive family history in at least two first-degree relatives, or in one first-degree relative and elevated cord blood IgE; as moderate if one first-degree relative was allergic or cord blood IgE was elevated. A newborn without allergy risk was defined by a negative family history (first-degree relatives) and normal cord blood IgE.

Mature newborns with and without allergy risk were recruited from two obstetric departments and assigned to four different groups:

A. Exclusively breastfed (for at least 4 weeks, without maternal food allergen avoidance).

B. Breastfed (for at least 4 weeks) with supplementation with a hydrolyzed formula in the first few days (casein hydrolysate [60%] and hydrolyzed whey proteins [40%]: Hydrolac; Milupa, Friedrichsdorf, Germany).

C. Breastfed (for at least 4 weeks) with initial supplementation with conventional cow milk formulas.

D. Formula fed or breastfed with continuous supplementation with a cow milk formula (mixed feeding).

Times of examination. Birth (groups A-B), 4 weeks after additional feeding of cow milk during weaning (groups A-C) or after 3-4 months (group D), after one year (groups A-D) and after two years (groups A-D).

Serological investigations included whole serum IgE (EIA, Pharmacia) and IgG, IgA, and IgE antibodies against casein, α-lactalbumin, β-lactoglobulin, and bovine serum albumin (fluorescent immunosorbent test). Because of wide individual variations in antibody production against these four proteins and the large number of possible combinations, a geometrical mean titer (gmt) was used in order to obtain a single figure characterizing the quantity of antibodies[21]. Maternal contamination of cord blood was estimated by determination of serum IgA levels by radial immunodiffusion (Behring, Marburg, Germany). Cord blood samples with demonstrable IgA (IgA > 3,9 mg/dl) were excluded.

CLINICAL RESULTS

The population now consists of 471 children followed for two years: 22% with high, 43% with moderate and 35% without allergy risk (Table 1).

Twenty-five percent of the infants in each risk group of group A presented allergic manifestations, mainly cutaneous signs. This percentage was lower in the HA supplemented group B, whereas especially high risk infants with initial formula supplementation and nearly half of the high risk infants with continuous formula feeding developed allergic signs (groups C and D) (Table 2). This distribution persisted into the second year of life. Only no-risk children of group D showed less allergic symptoms in the second year of life (14%). Predominant allergy problems in all groups were cutaneous manifestations (Group A: 21%, B: 8%, C: 17%, D: 15%), followed by gastrointestinal disorders (Group A: 5%, B: 6,6%, C: 9%, D: 9%) and some respiratory signs (wheezy bronchitis more than 3 times) (A: 2,5%, B: 0, C: 0, D: 3%).

Table 1. Distribution of Allergy Risk (n/%) (++ = high, + = moderate, - = no risk)

Groups	N	++	+	−
A	181	43/(24%)	81/(45%)	57/(31%)
B	79	25/(32%)	34/(43%)	20/(25%)
C	60	11/(18%)	29/(48%)	20/(33%)
D	151	23/(15%)	59/(39%)	69/(46%)
Total	471	102/(22%)	203/(43%)	166/(35%)

Table 2. Allergy Manifestations in the First Two Years of Life

Groups	N	Allergy risk (N/%) 1st year			Allergy risk (N/%) 2nd year		
		++	+	-	++	+	-
A	177	11/27%	19/25%	13/25%	12/29%	20/25%	13/25%
B	71	2/10%	5/17%	3/16%	2/10%	5/15%	4/24%
C	58	3/30%	3/13%	6/33%	5/45%	5/18%	5/26%
D	146	10/45%	12/21%	14/21%	8/38%	13/23%	9/14%

LABORATORY RESULTS

The median values of the gmt IgG were lowest in the exclusively breastfed infants and highest in those with continuous formula feeding. Initial supplementation with HA formula resulted in low gmt values similar to breast feeding. Only in group C (initial supplementation with cow's milk formula) can a difference between infants with and without allergic symptoms be seen (Fig. 1). The investigations in the second year of life revealed an increase of gmt levels in groups A and B, but a decrease in groups C and D. The highest values were found in symptomatic children of group B (Fig. 2). Comparable results were obtained concerning gmt IgE values (not shown), whereas IgA levels did not change at all (omitted). The gmt IgE levels assigned to the three risk groups revealed the lowest results in the breastfed children, followed by groups B and C, and the highest in Group D. The evaluation of the gmt IgG levels gave similar results without differences between the three risk groups.

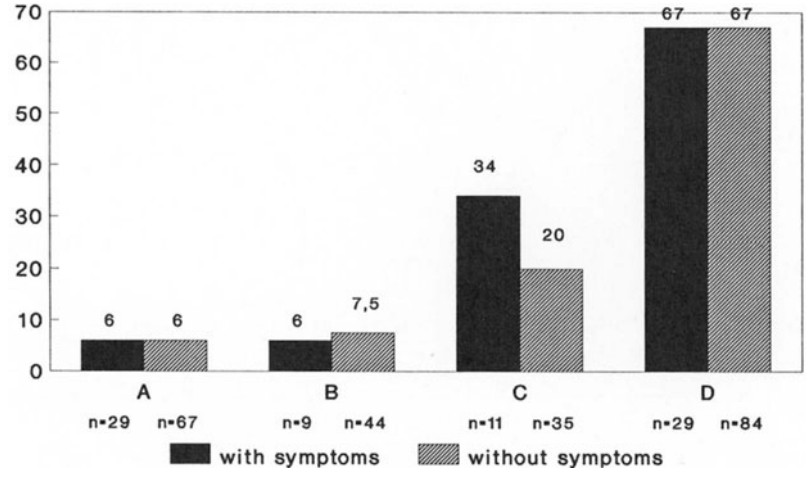


Figure 1. Median values of the gmt IgG in the 1st year of life (E3).

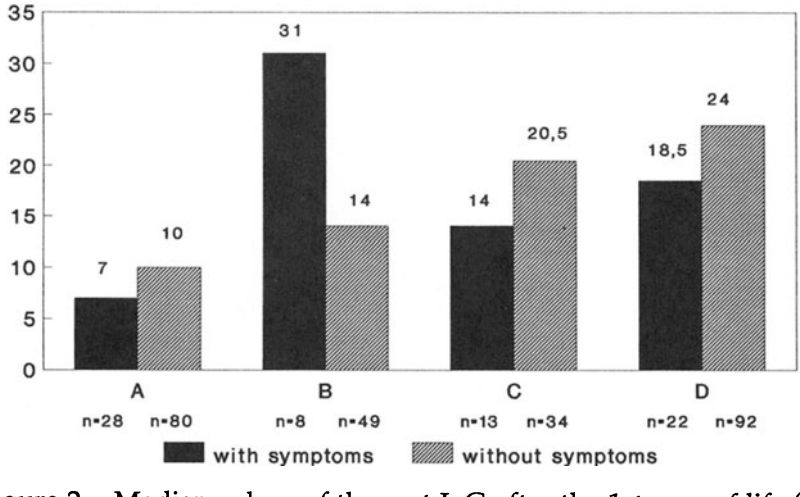
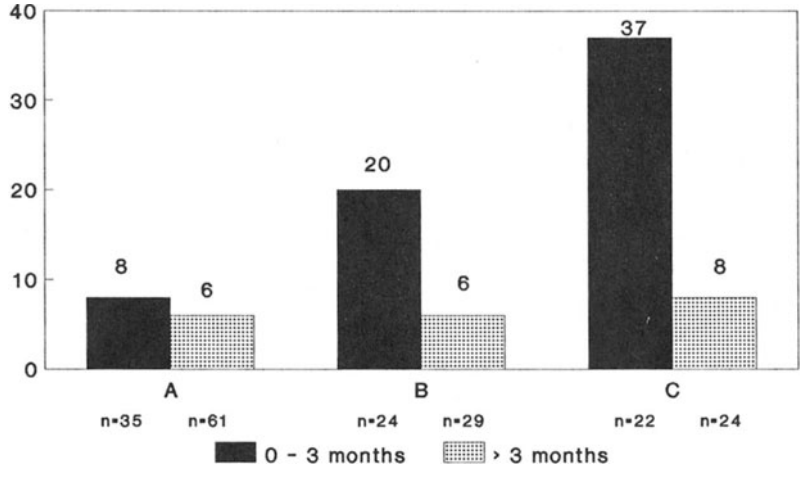


Figure 2. Median values of the gmt IgG after the 1st year of life (E4).

The beginning of formula feeding within the first three months effected higher gmt IgG levels than later introduction of cow's milk formulas. Again, the lowest values were detected in breastfed, the highest in initially formula supplemented and thereafter fully breastfed infants. However, a difference between the three groups was not demonstrated, when cow milk was introduced in the second trimenon (Fig. 3).



FF = Formula Feeding

Figure 3. Median values: gmt IgG (E3) 1st year of life - start with FF (mo).

DISCUSSION

The clinical results of our study may be cautiously interrupted to confirm with cautious interpretation the results of Chandra[8,9] and Zeiger[11], that high risk infants may benefit from HA supplementation in infancy. This advantage may lessen after the first year of life[11]. Several newborns of our study needed nutritional supplementation. High risk breastfed infants revealed less allergic symptoms during the first two years if supplemented with HA initially than with cow milk formulas (Table 2). This contrasts with the findings of Lindfors[4]. Artificially fed high risk infants developed allergies in a high percentage of cases (Table 2). The primary allergy manifestations were atopic dermatitis and urticaria. Only a few cases were correlated to cow milk formula. Fruit juices and the introduction of beikost played a more important role, as pointed out previously[2]. Gastrointestinal cow's milk protein intolerance occurred in four formula fed and in two fully breastfed infants. This is in contrast to the findings of Host[16], who found this disease only in breast-fed, but initially supplemented ("night bottle"), children.

Antibodies against cow milk proteins can be shown very frequently even in healthy children[21-27]. The present investigation with all breastfed infants showing lower IgG antibody titers compared to fully formula fed infants (Fig. 1) has confirmed previous studies[22-25]. The influence of mixed feeding of human milk and cow milk was not investigated[24]. In contrast to

that study, our results suggest an influence of age at the introduction of formula feeding on the antibody levels (Fig. 4): late introduction of cow's milk resulted in low gmt IgG and IgE values disregarding the initial feeding, but early introduction of formula was followed by higher gmt levels, relatively moderate in the fully breastfed group and more pronounced in the formula supplemented breastfed children. Interestingly, addition of HA formula (whey/casein product) resulted in elevated gmt values. We think, this kind of formula may still contain sufficiently large proteins with enough allergenic epitopes to sensitize infants against cow's milk proteins[28,29].

Normally, antibodies against cow milk proteins have peak levels during the first 3-6 months of life[21-23,27], that lower during the next years of life. This can be seen in our formula fed children (Fig. 2), but not in the three breastfed groups. Surprisingly, infants supplemented initially with HA formula presented the highest gmt levels after the first year of life, with allergic symptoms even more pronounced. The diagnostic significance of these results is still unclear, but it seems possible that small quantities of hydrolyzed whey/casein proteins fed neonatally may sensitize an infant at risk to cow milk proteins with a boostering effect of antibody production[29]. However, differences in the gmt IgG and IgE levels of cow milk antibodies among the three allergy risk groups could not be substantiated.

Low level antibodies against cow milk proteins can be attributed to different factors: 1) degree of gut permeability (maturation with age) (Fig. 3), 2) intestinal protection by maternal milk sIgA (prolonged breastfeeding, even mixed feeding[24] (Fig. 1), 3) lack of initial sensitization during neonatal age (possibly maternal diet, supplementation with conventional or HA formulas) (Fig. 2).

CONCLUSIONS

From a clinical point of view, high risk infants may benefit from supplementation with HA formula if human milk is lacking. It is unclear how long this possible advantage persists. The earlier the introduction of cow milk and the shorter the breastfeeding period, the higher the antibody levels against cow milk proteins. It is obvious that HA formulas also may lead to a sensitization against cow's milk, possibly at a later age. The diagnostic significance of these antibodies may be limited to excessively elevated titers in young infants with gastrointestinal cow milk protein intolerance[21] but we have to observe the elevated antibody titers in HA fed children to determine, if they are indicative of a pathologic condition.

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THE CLINICAL EXPRESSION OF ALLERGY IN BREAST-FED INFANTS

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INTRODUCTION

It was long held that some protection against atopic disease could be included among the many benefits of breastfeeding. Reports from many parts of the world seemed to confirm this although few whole population studies were made. There are major problems inherent in such research[1]. Ascertainment of family history, or accurate recall of changes in infant feeding practice, may be imprecise. The greatest source of error is to believe that comparing a population of infants fed at the breast and a population formula fed is a comparison of the two methods of nutrition. In fact, as Sauls[2] pointed out, in the Western world at least, there tend to be many socioeconomic differences between breast- and formula-feeding mothers. A recent study by Howie *et al.*[3] has confirmed that such differences persist in Scottish mothers. Unless these multiple confounding factors are heeded, any direct conclusions are of doubtful validity. Furthermore, the researcher in this field has no option to allocate randomly those being studied into breast/formula-feeding groups. In spite of these limitations, an attempt has been made on the Isle of Wight to study the manifestations of allergy in cohorts of the whole child population.

The Isle of Wight is situated some five miles off the mainland of Southern England. It has a population of 130,000 and is mainly a farming and holiday area. The population reflects the general social class scatter of the United Kingdom but probably there is less migration of families or individuals compared with many mainland areas. Thus, it is an appropriate area for epidemiological study. About 98% of the 1500 births per year take place in one Maternity Unit at St. Mary's Hospital, Newport, and in a small community good communication exists between family and hospital doctors, midwives, and health visitors. This paper summarizes the studies into the epidemiology of allergic disease carried out on the Isle of Wight over the last twelve years.

Immunology of Milk and the Neonate
Edited by J. Mestecky *et al.*, Plenum Press, New York, 1991

RESULTS

In 1977, Hide and Guyer attempted to determine the manifestations of allergy in the whole population born during a twelve month period. As expected, the study confirmed the predisposition of children of allergic parents to themselves develop allergic disease[4]. The study was unable to demonstrate that exclusive breastfeeding reduced the prevalence of atopic eczema. Indeed, after four years more, eczema was recorded among the exclusively breast-fed but this may reflect the preference of mothers with a family background of allergy to at least initiate breast feeding (76 compared with 67%, $p<0.01$)[7] (Table 1).

Table 1. Allergy Related to Feeding Method - 1977 Study

	1 Year		2 Years		4 Years	
	breast	Other	breast	Other	breast	Other
Number	155	688	138	471	115	371
Eczema	8.4%	8.1%			16.5%	12.4%
Asthma	3.9%[a]	10.3%[a]			7.0%	7.3%
Rec. croup					8.7%	5.7%
Cow's milk intol.			2.9%	4.0%		

[a] $p<0.02$

Initially, it was concluded that exclusive breastfeeding protected against the development of asthma. The diagnosis of asthma in early childhood is generally a clinical one and any single episode of lower respiratory tract obstructive disease was recorded as asthma. After the first year, there appeared a difference between the exclusively breast-fed (3.9%) and all other infants (10.3%) and this was significant at the $p<0.02$ level. However, the criteria were revised to 'recurring wheezing episodes' at the 4 year followup and the prevalence at that time was almost identical in the breast- and formula-fed populations[5] (Table 2). It was considered that in the first year, breastfeeding had protected against infections such as bronchiolitis.

A surprising consequence of the 1977 study was the identification of recurring croup as a manifestation of allergy[6]. This had a prevalence of 6.4%, similar to that of asthma. It, too, has a male predominance and is related to a family history of atopic disease. The mode of infant feeding did not appear to be a significant factor.

Precise identification of allergic disease as the cause of gastrointestinal symptoms requires double-blind placebo controlled food challenge and this is not often feasible in clinical practice. The Isle of Wight study recorded signs attributed to food and reproduced on open challenge. Intolerance to cow's milk was identified in 3.8% of children by the age of two years[7]. It was related significantly to family history of atopy but not to mode of feeding. Colic has been reported as a manifestation of cow's milk allergy although others have

suggested colic occurs more often in the breast-fed. The inter-relationships between parental allergy, social class, feeding methods and colic were studied by log linear modelling. In our population colic was related to social class, occurring in the group predisposed to breastfeed[8]. The prevalence of colic in initially breast-fed infants was 15.4% and in those initially formula-fed was 13.8% so this was considered an insignificant difference. That is not to deny that colic may be an important symptom of food allergy or intolerance for we and others have been impressed that some infants are dramatically relieved of the disturbing problem by change in their, or their mother's, diet[9].

Table 2. Allergy Related to Parental History - 1977 Study

	1 Year		2 Years		4 Years	
Atopic family history	+ve	-ve	+ve	-ve	+ve	-ve
	266	577	202	407	167	319
Eczema	10.5%	7.1%			15.6%	12.2%
Asthma	11.7%	7.3%			10.8%[a]	5.3%[a]
Rec. croup					10.8%[b]	4.1%[b]
Cow's milk intolerance			6.4%[c]	2.5%[c]		

[a] $p < 0.05$
[b] –0.01
[c] $p < 0.02$

When this data was first presented it opposed the then concensus that breastfeeding protected against the development of allergic disease. However, a number of studies in the last ten years have come to a similar conclusion. For more than fifty years suggestions have been made that infants might react to items from mother's diet transmitted in her milk[10]. In 1983, Dr. Peter Kilshaw of the National Institute for Research in Dairying provided us with an ELISA method for detecting cow's milk protein in human milk[11]. In 13 of the first 28 samples of milk examined either bovine β–lactoglobulin or casein or both were found[12]. Others have since been able to detect such protein in close to 100% of samples[13]. The quantities vary but it seems at least feasible that the newborn could be sensitized from this source via the breast milk.

In the decade since we embarked on this work, emphasis has been placed upon other triggering factors such as aero-allergens, the house dust mite[14], tree and grass pollens, animal danders and the effect of atmospheric pollutants - particularly tobacco smoke, in the genesis of clinical allergy. Many but not all, consider that the prevalence of asthma, eczema, and allergic rhinitis is rising. In the United Kingdom this has been linked to our desire to make the internal climate of our homes more comfortable for the house dust mite *Dermatophagoides pteronyssinus* by providing central heating, wall to wall carpeting, double glazing, etc. Additionally, we have maintained the

British trait of sharing our homes with cats, dogs, and a range of potentially highly allergenic birds and mammals. Sixty-two percent of Isle of Wight homes with a new infant contain one or more pets. Fifty-six percent of these are cats and dogs with virtually the same number of pets in homes of mothers who sustain breastfeeding as in the formula-feeding group[15]. We have lagged behind the United States of America in discarding the addiction of cigarette smoking and still expose 45% of new born to passive smoking in the home[15].

In the past two years, a whole population study has been repeated to try and discern any changing trend of expression of allergic disease in childhood. One thousand five hundred thirty-four infants were born on the Isle of Wight in 1989 and so far 910 have been followed for between nine and twelve months. Preliminary analysis of the data shows no difference in the prevalence of atopic eczema between those exclusively breast-fed for three months and those breast-fed for a shorter time or formula-fed (Table 3). The 1989 study uses rather stricter criteria for the diagnosis of asthma and eczema than in 1977. In spite of this, there is a trend for an increasing prevalence of asthma, eczema, and food allergy in 1989. Exclusive breastfeeding for three months is again associated with less 'asthma' as was the case in the earlier study. The criterion for inclusion in this later study is three or more episodes of wheeze. Either breastfeeding is protecting against true asthma or perhaps the sustained bronchial hyperreactivity that is a sequelae of viral bronchiolitis in some infants occurs less often in the breast-fed. A recent report from Wright *et al.* in Tucson[16] concludes that breastfeeding seems to protect against wheezing in the early months of life. This study found other risk factors for the infant to be: sharing a bedroom, male sex, belonging to an ethnic minority, and lower maternal educational attainment. The protective effect of breastfeeding was only found in the first four months of life suggesting that the benefits might end with breastfeeding. The Arizona workers suggested that the increased illness and beneficial effect of breastfeeding they found in their non-white, particularly Hispanic, population might not be found in studies among white subjects. Our population, which is 99% caucasian, does show the same effect although it is too early to say how long it is sustained. Smoking is a confounding factor. Our data suggests that both sustained breastfeeding and a non-smoking mother are associated with less wheezing in infancy (Table 4). A smoking father is not revealed by these figures as an adverse factor. There may be a true protection against atopic asthma as positive skin prick tests were almost twice as common in formula-fed infants with recurring wheeze.

This infant population will be followed for at least the next four years. The appearance of manifestations of atopy is being related to family history and to the level of IgE in the cord blood. This year, we have embarked on a study in which a group of infants considered by their family history or cord IgE level to be at high risk of developing atopic disease is being protected as much as possible from dietary and aero-allergens in the early months of life. Infants are either being breast-fed with strict control of mother's diet or given a hypoallergenic soy hydrolysate. Particular attention is being paid to reducing the exposure to the house dust mite and its faecal material. A control group of high-risk infants is being fed and managed conventionally. The hope of these studies is that the vulnerable infant may obtain the undoubted benefits of human milk without running the risk of sensitization by the same medium.

Table 3. Atopy and Feeding Method - 1989 Cohort

	Breast	Formula	Significance
	N = 380	n = 530	
Asthma	7%	13%	p = 0.0065
Eczema	11%	8%	n.s.
Rhinitis	3%	3.5%	n.s.
Food all./intolerance	8%	6%	n.s.
Cow's milk intolerance	5.4%	5.3%	n.s.
Skin prick +ve	6.5%	7.2%	n.s.

Table 4. Feeding Method, Smoking, and Asthma - 1989 Cohort

Smoking	No. parent	Father only	Mother only	Both
Number	498	191	59	153
Breast	254	71	14	33
Asthma	15 (5.9%)[a]	6 (8.4%)	2 (14%)	4 (12%)
Formula	244	120	45	120
Asthma	27 (11%)[c]	10 (9%)[c]	8 (18%)	24 (19%)[b,c]
Total	8.4%[d]	8.3%[e]	17%	17%[d,e]

[a] $p < 0.04$
[b] $p < 0.03$
[c] $p < 0.01$
[d] $p < 0.01$
[e] $p < 0.01$

CONCLUSIONS

The pattern of allergic disease affecting infant populations in one area of the United Kingdom has been studied during the past twelve years. On a population basis, breastfeeding does not appear to offer any protection against atopic eczema. It does reduce the frequency of wheezing in infancy but whether a sustained lessening of asthma in older children occurs remains uncertain. Atopy appears to be triggered by dietary and environmental antigenic stimulation and efforts to restrict exposure of the new born to such challenge offers the prospect of a reduction in the morbidity caused by these ubiquitous and tedious disorders.

ACKNOWLEDGEMENTS

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