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Immune Activation During Infancy in Healthy Humans

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Immune activity during infancy was investigated using blood samples from 30 neonates and 52 healthy infants between 2 and 15 months of age attending for immunization. The purpose of this study was to assess the total immune activity of T cells using soluble interleukin-2 receptor (IL-2R) and interferon-γ concentrations. These were compared with the proportion of CD4 CD45RO-, IL-2R (CD25)-, and transferrin receptor (CD71)-positive peripheral blood lymphocytes. The median duration of breast-feeding and of introduction of solid feeds was 4.2 and 4.0 months, respectively. Compared to neonates, the mean ± SE soluble IL-2R concentration peaked at 4 months of age (1670 ± 94 vs 3060 ± 252 U/ml; $P < 0.0001$), as did pooled interferon-γ levels. The percentage of CD4 CD45RO T cells remained low and the proportion of activated peripheral blood lymphocytes decreased during infancy. We conclude that noncirculatory immune activity is increased during infancy and this is associated with weaning.

KEY WORDS: Immune activity; infancy; soluble interleukin-2 receptor; T cell; weaning.

INTRODUCTION

After birth, the human infant is exposed to a variety of food, bacterial, and environmental antigens and to cells and other components of milk in those who are breast-fed. This antigenic exposure, particularly during weaning, would be expected to led to activation of the immune system. Indeed, we have found that there is a midweaning peak of activation of both T cells and mucosal mast cells in the infant rat that is localized to the gastrointestinal tract (1–3). Studies of the immune system in humans have indicated that it is immature and has a low activation status (4–6). B-lymphocyte function is immature particularly in neonates but also is impaired in infants until 2 years of age, which is caused in part by an immature T-cell helper function that is defective until at least 6 months of age (5). Infants have naive CD45RA CD4 T cells in blood and have a lower percentage of activated blood lymphocytes expressing interleukin-2 receptors (IL-2R; CD25) than adults (4, 6), while the spleen has poorly developed marginal B-cell zones and immaturity of T cells until after 5 months and extending to 2 years of age (7).

These human studies have not considered the total immune system and, particularly, the large component contributed by the gut-associated lymphoid (GALT) and mucosal tissue that might be expected to be principally affected by weaning. It is obviously difficult to assess the GALT in healthy infants but an indirect method would be to measure total T-cell activity and compare this with the activity of peripheral blood lymphocytes. Any large discrepancy would infer a noncirculatory source, which would implicate the GALT, because it is isolated from the systemic immune system by the liver and by the selective homing properties of mucosal lymphocytes (8).

CD4 T cells express either the CD45RA (naive) or the CD45RO (antigen-primed/memory) phenotype that can be used to assess immune activity (8). Activation of T cells is associated with expression of membrane-bound IL-2R and subsequent release of soluble IL-2R (sIL-2R) systemically (9). Thus, sIL-2R concentration is a measure of total T-cell activity and, importantly, largely survives passage through the portal circulation of the liver (10, 11). Activated T cells also produce interferon-γ (IFN-γ), of which the largest population of IFN-γ-producing cells resides in the upper small intestine (12). A late

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marker of activated T cells is expression of transferrin receptors (CD71, 13). Mast-cell tryptase concentration is a measure of mast-cell activation (14).

Our hypothesis was that healthy human infants have a peak of immunologic activity during weaning. sIL-2R and IFN-γ levels were used to measure T-cell activity and mast-cell tryptase concentration was used to assess mast-cell activation. Flow cytometry was performed on blood lymphocytes to determine their immunologic activity by measuring the proportion of peripheral blood lymphocytes expressing IL-2R or CD71. It is obviously important in such a study to exclude extraneous immune stimuli such as childhood infections and systemic diseases. We have therefore undertaken a study which recruited healthy normal infants from the community attending an immunization clinic.

SUBJECTS AND METHODS

Subjects

Normal healthy infants who attended the Immunization Clinic of the Women's and Children's Hospital (Adelaide Children's Hospital, Adelaide, South Australia) were recruited for the study. These subjects were aged 2, 4, 6, and 15 months. Oral polio and diphtheria/tetanus/pertussis vaccines were given at 2, 4, and 6 months of age, and measles/mumps/rubella vaccines administered at 15 months. Each age interval had 11–14 infants in a cross-sectional design. Two sets of twins were present in the 2-month group. After parental consent was obtained, a medical and feeding history was taken and capillary blood was collected from the heel or finger. Infants were excluded if there was a history of vaccination or infection within the preceding 6 weeks or systemic illness (for example, wheezing or congenital heart disease). Neonatal blood from 30 further infants was obtained from the residuum after determination of physiologic hyperbilirubinemia from samples at the Department of Clinical Chemistry of the Queen Elizabeth Hospital, Woodville South, South Australia. Only samples from otherwise healthy neonates were used. Blood samples were also collected from 40 healthy adult control subjects. These subjects had no history of infection in the preceding 4 weeks and had no systemic disease. The exact number of samples from these neonates and adults varied in some assays, as these measures were performed at different times during the study. This study was approved by the relevant Human Ethics' Committees of the Women's and Children's Hospital and of The Queen Elizabeth Hospital.

Medical and Feeding History

Accompanying parents of infants were asked about breast- or bottle-feeding and the time of commencement of supplementary and solid feeds. These data were used to calculate the duration of breast-feeding, the age at which an infant first received a supplementary milk feed, and the age at which an infant was first given solid food. As these data were skewed, the medians were used to summarize the central tendency. Feeding data from infants aged 2 and 4 months were not used in these calculations if they still had not received either a first supplementary feed or a solid food. A general health history was taken of any maternal and perinatal problems, and the current health of the infant was assessed. Specific questioning was directed to any cause (recent infections, dermatitis, vaccination) that might activate immune responses.

sIL-2R and IFN-γ Assays

sIL-2R concentration was measured in plasma using an enzyme-linked immunosorbent assay (ELISA; CellFree IL-2R, T Cell Sciences, MA). The assay sensitivity is 50 U/ml. Plasma aliquots of 25 μl were diluted 1:2 before being measured. Plasma IFN-γ was measured using ELISA (Intertest-γ, Genzyme, MA). The sensitivity of IFN-γ assay was 100 pg/ml. As there was inadequate plasma available for measuring individual samples, 10-μl plasma aliquots from each sample were taken and pooled for each age group in infants. However, plasma IFN-γ was measured in 28 adults.

Plasma Mast-Cell Tryptase Assay

Plasma mast-cell tryptase was measured using an immunoradiometric assay (Pharmacia Diagnostics AB, Pharmacia Australia, Sydney, Australia). Plasma from each subject in each age group was pooled for this tryptase assay using 10-μl aliquots as well as from 12 healthy adult subjects. The assay was repeated after 10 days and individual samples of adult plasma were measured. Levels of plasma tryptase were expressed as counts per minute (cpm) (14). Each sample had the background reading subtracted.

Flow Cytometry of Blood

Peripheral blood cells were stained with mouse IgG monoclonal antibodies against CD3, CD4, CD8, CD45RA, CD45RO, IL-2R, and CD71 (Becton and Dickinson, Sydney, Australia) and human mucosal lymphocyte (HML-1; Immunotech, Marseilles, France). Antibody labeling was detected either using direct fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugates or using a secondary goat FITC $F(ab')_2$ anti-mouse antibody (Cappel, Organon Teknika, Veedijk, Belgium). A direct lysis technique for removing red blood cells was employed. Flow cytometry was performed using the FACScan (Becton-Dickinson). Cells were selected on forward- and side-scatter characteristics for lymphocytes. Dual-color analyses were performed for CD4 versus CD45RA and CD45RO and IL-2R versus CD3, CD4, CD8, CD45RA, CD45RO, CD14, and CD19. A negative control used a mouse isotypic IgG monoclonal antibody. For dual-color analyses, cursors were set at the same value as those using the relevant single label. All results had negative values subtracted as obtained from single histogram analysis or from the relevant quadrants in the case of dual-color analyses.

Statistics

Peritz' multiple *F* test was used to compare means of sIL-2R and to compare percentage readings from flow cytometry of blood cells (15). To test the hypothesis of immune activation in infancy, the principal comparison was made between neonates and infants 2, 4, and 6 months of age. However, the infant groups were also compared to healthy adult subjects.

RESULTS

Medical and Feeding History

The mean ± SE ages of neonates and infants were 0.1 ± 0.02, 2.3 ± 0.08, 4.3 ± 0.1, 6.1 ± 0.2, and 14.5 ± 0.7 months. The infant population in our study was relatively homogeneous and stable over time with respect to the initial rate of breast-feeding, with individual group rates of 90 to 92%, despite the different ages of recruitment. Only four infants were totally bottle-fed from birth, with one infant in each of the 2-, 4-, 6-, and 15-month age groups. Hence, the remaining infant population was pooled to determine the duration of breast-feeding, the initial age at introduction of a liquid supplement, and the age at introduction of solid feeds. The median duration of breast-feeding was 4.2 (range, 0.1–13) months. The median age at which the first supplementary feed was introduced was 1.7 (range, 0–11) months, and the median age at which solid feeds were introduced was 4.0 (range, 0.7–10) months.

sIL-2R and IFN-γ Assays

Total T-cell activity was measured by plasma sIL-2R concentration. sIL-2R levels showed a broad peak from 2 to 6 months of age, with a mean value at 4 months of 3060 U/ml, compared to 1670 U/ml in neonates (Fig. 1). In addition, sIL-2R concentrations at 2, 4, 6, and 15 months of age were significantly higher than those of adults. In contrast, the proportion of IL-2R+ blood lymphocytes declined during infancy (Fig. 1), indicating that the source of the elevated sIL-2R was noncirculatory. Plasma IFN-γ concentrations in pooled samples peaked at 4 months of age (Fig. 2). Thus, changes in pooled IFN-γ concentrations were similar to those of sIL-2R concentrations and supported the notion that total T-cell activity is increased.

Plasma Mast-Cell Tryptase Assay

Plasma mast-cell tryptase was measured in pooled plasma from each group (Fig. 2). Plasma tryptase showed a broad peak from 2 to 6 months of age before declining at 15 months. The peak represented a 13-fold increase over the background level. The assay was repeated using the same neonatal and infant samples but individual adult aliquots. Once again, the infant groups had higher readings than those of either neonates or each individual adult.

Phenotype and Activation Status of Peripheral Blood Cells

The phenotype and activation status of peripheral blood cells is given in Table I. Neonates had a higher proportion of CD3 lymphocytes than the other age groups. Neonates had the highest and adults the lowest values for CD4 cells. Neonates and infants 2, 4, 6, and 15 months of age had persistence of the CD45RA rather than the CD45RO phenotype, which indicates a lack of peripheral antigen activation.

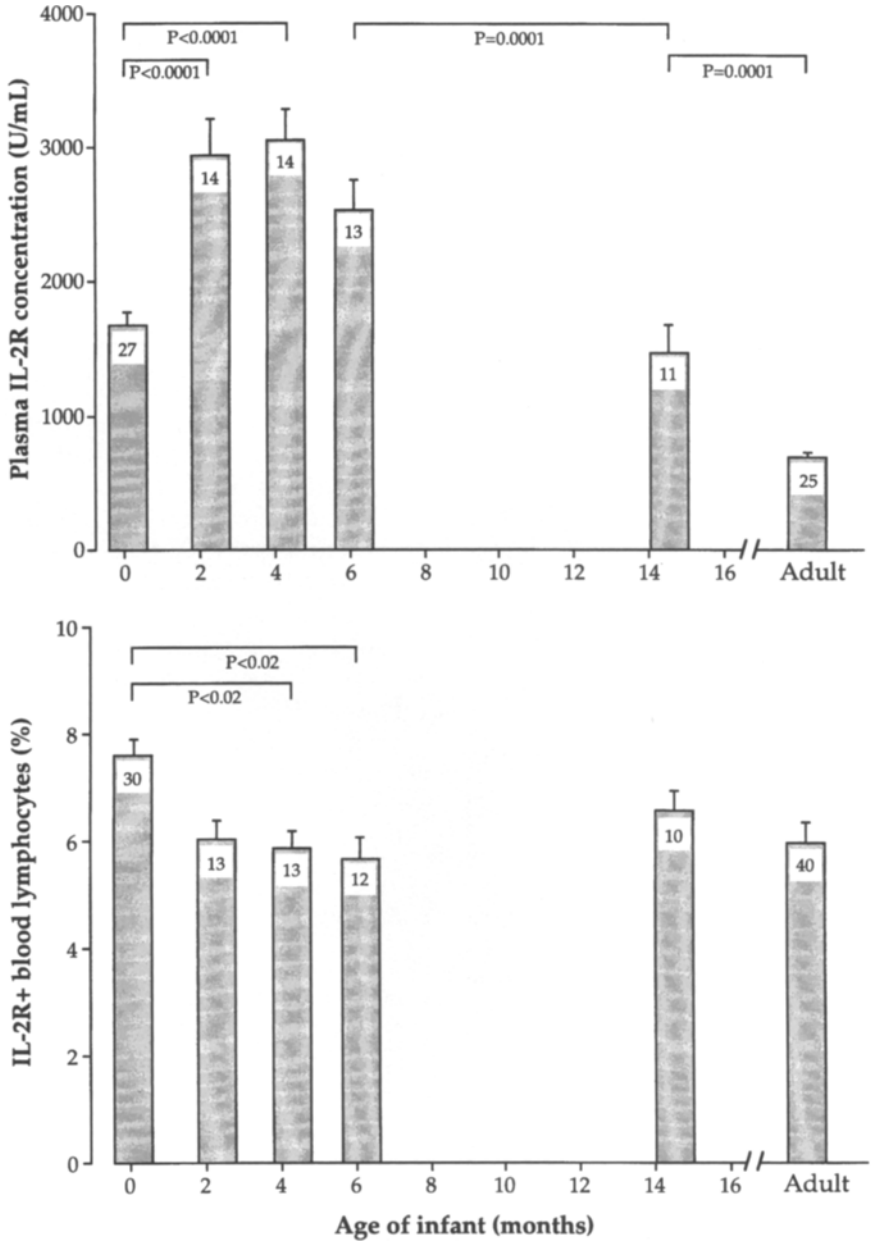

Fig. 1. Total T-cell activity was measured by plasma sIL-2R concentration (top) and is contrasted with the proportion of IL-2R+ blood lymphocytes (bottom). Data are given as mean + SE, with the number in a group indicated within the bar. sIL-2R concentration increased in infants compared to neonates and reached a peak at 4 months of age.

Neonates and infants at all ages had significantly lower percentage values of CD8 cells than adult subjects. The proportion of CD4 CD45RO cells remained low in neonates and infants, presumably indicating a lack of antigenic stimulation. Some coexpression (10–30%) of CD45RA and CD45RO was present on CD4 T cells, especially in adults. Neonates had the highest proportion of activated IL-2R+ or CD71+ blood lymphocytes and this decreased in infancy (Fig. 1, Table I). Certainly, there was no increase in activated blood lymphocytes in any of the infant groups that would be concordant with the elevated sIL-2R levels. CD3, CD4, and CD8 lymphocytes comprised 80–90, 65–76, and 14–27% of IL-2R+ cells, respectively. There was essentially no contribution of IL-2R+ cells from either macro-

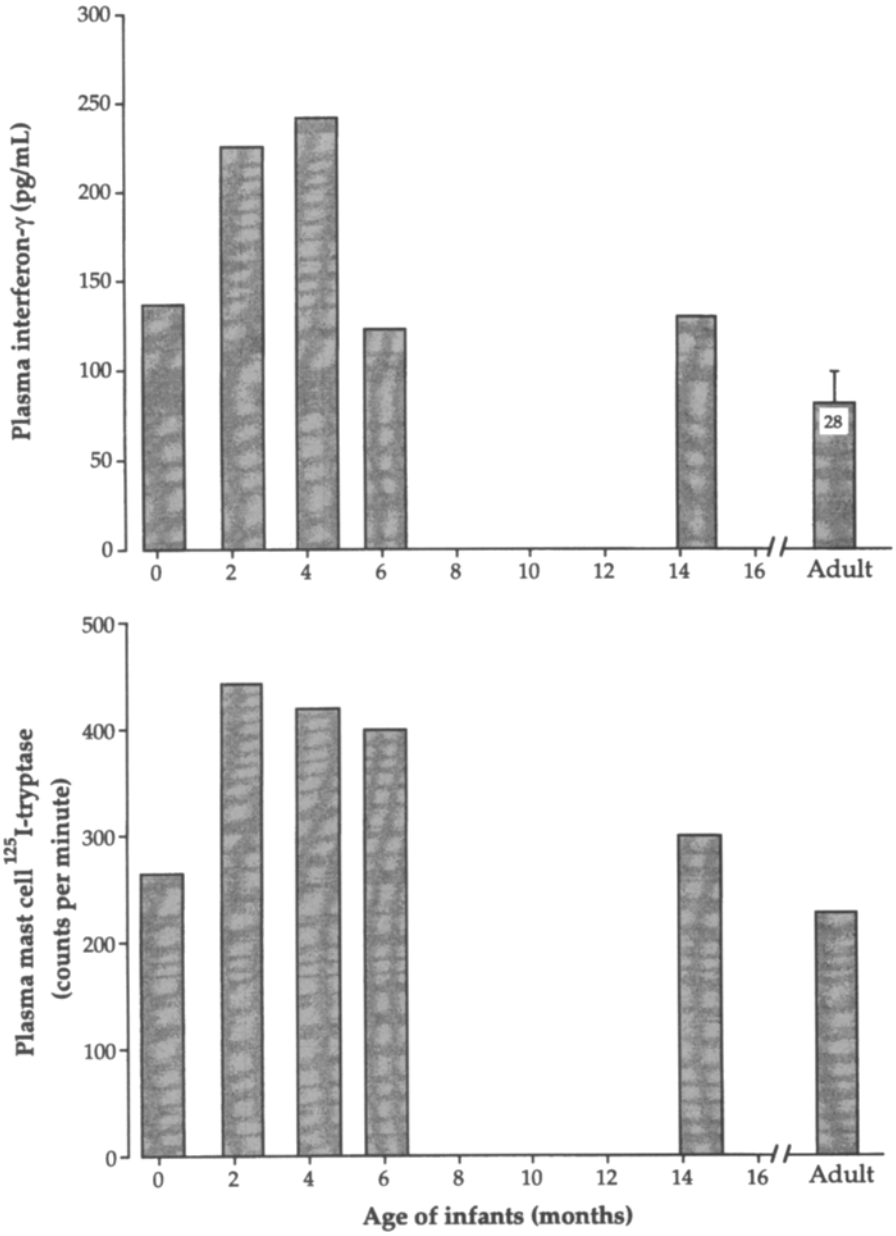

Fig. 2. Plasma IFN-γ concentration was used as a supporting measure of total T-cell activity (top). Neonatal and infant samples were pooled but individual samples were from 28 adults. Activation of mast cells was measured by plasma mast-cell tryptase concentration in these pooled samples (bottom).

phages (CD14) or B lymphocytes (CD19). Neonates also had a higher percentage of lymphocytes expressing the HML-1 antigen.

DISCUSSION

Previous studies of the immune system in human infants have shown that it is immature and has low activity (4–7). As we have observed a physiologic midweaning peak of T-cell and mast-cell activity in infant rats (1–3), we hypothesized that the human infant also has a similar midweaning peak that would be evident if measures of total immunologic activity were used instead of those relating only to the circulating peripheral immune system. This comparison relied on the principle that elevated

Table I. Phenotype and Activation Status of Peripheral Blood Lymphocytes from Neonates, Infants, and Adults

	Mean percentage of positive cells ± SE					
		Infants				
Determinant	Neonates (n = 14–28)	2 mo (n = 13)	4 mo (n = 13)	6 mo (n = 12)	15 mo (n = 10)	Adults (n = 8–38)
CD3[a]	82.3 ± 1.3	69.1 ± 1.5	66.6 ± 2.1	73.8 ± 1.0	71.2 ± 1.5	68.6 ± 1.2
αβ-TCR[b]	81.0 ± 1.5	66.1 ± 1.0	65.6 ± 2.7	71.8 ± 0.8	66.9 ± 2.1	67.4 ± 1.3
γδ-TCR	2.4 ± 0.3	3.5 ± 0.3	3.1 ± 0.3	3.3 ± 0.5	4.3 ± 0.9	2.6 ± 0.3
CD4[c]	59.6 ± 1.2	50.3 ± 1.9	48.0 ± 2.3	53.9 ± 1.0	48.8 ± 2.5	41.9 ± 1.2
CD45RA[d]	57.0 ± 1.6	43.5 ± 2.4	42.3 ± 1.0	52.2 ± 2.5	39.1 ± 2.2	16.8 ± 1.8
CD45RO[d]	7.6 ± 0.9	5.0 ± 0.5	6.0 ± 0.6	5.5 ± 0.6	8.0 ± 2.0	27.5 ± 1.1
CD8[e]	21.4 ± 1.1	19.3 ± 1.7	18.4 ± 1.1	19.4 ± 1.5	21.1 ± 1.5	25.9 ± 1.1
CD45RA (Leu18)[d]	88.7 ± 1.2	90.7 ± 0.7	89.2 ± 1.0	91.3 ± 0.5	86.3 ± 1.1	58.7 ± 1.8
CD45RO (UCHL1)[d,f]	19.5 ± 1.9	16.3 ± 2.6	23.5 ± 3.3	19.8 ± 2.7	32.6 ± 2.0	51.1 ± 2.2
IL-2R (CD25)[g]						
CD3	6.5 ± 1.7	5.1 ± 0.3	5.0 ± 0.3	5.2 ± 0.3	5.3 ± 0.3	5.3 ± 0.4
CD4[b]	5.8 ± 1.5	4.3 ± 0.3	4.2 ± 0.2	4.0 ± 0.3	4.4 ± 0.3	4.2 ± 0.4
CD8	1.2 ± 0.2	1.0 ± 0.2	1.6 ± 0.3	1.4 ± 0.2	0.9 ± 0.2	1.3 ± 0.1
CD45RA	4.4 ± 0.2	ND[h]	ND	ND	ND	1.4 ± 0.4
CD45RO	2.7 ± 0.3	ND	ND	ND	ND	5.8 ± 1.0
CD14	0.3 ± 0.04	0.2 ± 0.03	0.3 ± 0.6	0.3 ± 0.03	0.3 ± 0.06	0.1 ± 0.02
CD19	0.5 ± 0.08	0.8 ± 0.06	0.8 ± 0.1	0.7 ± 0.06	1.1 ± 0.1	1.7 ± 0.2
CD71[i]	9.1 ± 1.3	1.1 ± 0.2	1.2 ± 0.3	0.9 ± 0.2	1.4 ± 0.2	1.5 ± 0.3
HML-1[j]	3.1 ± 0.4	2.7 ± 0.3	2.5 ± 0.4	2.0 ± 0.3	1.5 ± 0.2	1.1 ± 0.1
CD19[k]	13.1 ± 1.1	26.1 ± 2.0	28.1 ± 2.2	27.1 ± 3.1	24.5 ± 1.1	12.7 ± 0.7

[a]Neonates versus each infant group and adults, all contrasts: $P < 0.0002$.
[b]Neonates versus each infant group and adults, all contrasts: $P < 0.01$.
[c]Neonates versus each infant group and adults, all contrasts: $P < 0.005$.
[d]Adults versus neonates and each infant group, all contrasts: $P < 0.0001$.
[e]Adults versus neonates and each infant group, all contrasts: $P < 0.02$.
[f]Fifteen-month-old infants versus neonates and 2-, 4-, and 6-month infants, all contrasts: $P < 0.05$.
[g]Total IL-2R reading is given in Fig. 1.
[h]Not done.
[i]Neonates versus each infant group and adults, all contrasts: $P < 0.0001$.
[j]Adults versus neonates and 2-, 4-, and 6-month (not 15-month)-old infants, these contrasts: $P < 0.006$.
[k]Neonates versus 2-, 4-, and 6-month-old infants, all contrasts: $P < 0.0001$. Adults versus 2-, 4-, 6-, and 15-month-old infants, all contrasts: $P < 0.0001$.

sIL-2R levels must originate from an elevated number of IL-2R+ lymphocytes (or possibly other immune cells) which are either circulating or noncirculating. sIL-2R and pooled IFN-γ levels were elevated, which indicated that total T-cell activity was increased. We confirmed the previous studies that peripheral blood lymphocytes from infants had a lower activation status and that CD4 cells had the CD45RA naive phenotype and low expression of the CD45RO antigen-primed/memory phenotype. Mast-cell tryptase pooled levels increased during infancy, which suggests that mast cells were activated (1, 14).

We believe that it is futile to attempt to dissociate the nexus of infancy and weaning and their relative effects on immunologic activity, as the two are inextricably linked. Weaning is a complex process that involves a slow exponential decline in milk-feeding and reciprocal replacement with liquid feeds and, equally, a prolonged period of exposure to a variety of solid feeds with increasing antigenic diversity. This process is likely to extend over many months and even into the first 1 or 2 years of life. In the present study, sIL-2R levels were still significantly elevated in the 15-month-old group over those of adult subjects, implying continuing antigenic stimulation even at this age. Thus, bottle-fed infants cannot be considered to be divorced from the weaning process, and we did not attempt to compare our infants with such a group, as there is likely to be a considerable overlap. The only comparison would be with the highly contrived situation of patients with severe short bowel syndrome on total parenteral nutrition in a stable

nutritionally adequate nonseptic state. Equally, we did not undertake a longitudinal study, mainly because of the reluctance of parents, but also because this does not control feeding variables any better than a careful cross-sectional study. We see particular advantages of our study in using a community population which had a high breast-feeding rate, which was naturally selected to present in a healthy state, and which was further screened by interview for good health. This study design ensured that extraneous causes of immune stimulation (allergies, infections, other childhood diseases) were unlikely, and this was confirmed by the persistence of CD4 CD45RA cells and by the low activation status (IL-2R and CD71) of peripheral blood lymphocytes.

The finding of elevated sIL-2R levels in infants, particularly from 2 to 6 months of age, agrees with previous studies of elevated levels in childhood where individual values were highest before 3 years of age (16–18). A possible criticism of our study is the use of blood from neonates who had physiologic hyperbilirubinemia and therefore may not be completely normal. We feel that this was a reasonable approximation, as sick neonates were excluded, and in any case the differences in sIL-2R levels were even greater when compared to healthy adults. Unfortunately, IFN-γ levels could be measured only using pooled samples due to the small amount of plasma available, and hence these data need to be confirmed, but nevertheless, their elevation does support the hypothesis. We plan to measure IFN-γ levels in individual infant samples in future studies, to confirm the present results.

The question arises whether there was any other reason why immune activity was elevated during infancy? Other environmental antigens and the decline in maternal IgG might be considered to lead to immune reactivity, but these should have been evident in peripheral blood. Immunization was not a cause of elevated sIL-2R and pooled IFN-γ levels, as these were increased at 2 months of age, prior to any immunization having been administered, and blood was subsequently taken approximately 7 to 8 weeks after each preceding immunization. It might be argued that another lymphoid organ such as the spleen (the second largest lymphoid organ after the GALT) might have been the source of this increased immune activity. The spleen is an unlikely source, as it is known to be immature until at least 5 months of age and shows low activity until 2 years (7). This is also supported by our studies in the rat, which showed that the spleen decreased in size and had low expression of IL-2R+ T cells until after weaning (2, 3). Moreover, any lymphoid source would have to have a physiologic dissociation from circulating blood lymphocytes, which has not been described.

We suggest that the GALT is the source of immunologic activity for several reasons. First, it is the largest lymphoid organ (19) and is, therefore, potentially the largest source of sIL-2R. Second, there is a good physiologic description of sequestration of the GALT from the systemic immune system due to the portal circulation through the liver and selective homing properties of mucosal versus systemic lymphocytes (8). The pattern of high sIL-2R but reciprocally low or normal IL-2R+ blood lymphocytes is the same as that observed in celiac disease, which is an inflammatory T-cell disease confined to the GALT (11, 20, 21). This similarity suggests that the immune activity in infancy may also originate from the GALT. Third, there is direct evidence that the GALT and intestinal mucosal tissue are activated in the first few weeks to months of life because HLA-DR and IgA secretory component expression is immunologically induced on intestinal epithelium at this age and fecal IgA appears (22). Fourth, the GALT has the greatest antigenic exposure from food, bacterial, and environmental antigens, which have an obligate association with weaning. Fifth, our studies in the rat have already localized the increased immune activity to the GALT and intestinal mucosa and have confirmed that peripheral immune activity is low (1–3).

We speculate that the timing of immune activation during weaning is explained by the release of immunosuppression associated with breast milk-feeding as well as by exposure to the diversity of antigens associated with weaning (Fig. 3). Breast milk contains high concentrations of the immunosuppressive cytokine, transforming growth factor β (TGFβ), which is responsible for the strong immunosuppressive effect of breast milk *in vitro* (23, 24). This seems to be important *in vivo* as well, as illustrated by the TGFβ knockout mouse, which remains in good health for the first 2 weeks of life during nursing (while receiving maternal TGFβ in breast milk) but subsequently develops inflammation in multiple sites with weaning (25). It is also of interest that the intestinal epithelium, and specifically intraepithelial lymphocytes, produce TGFβ, at least in mice (26), and that the numbers of these intraepithelial lymphocytes have an exponential

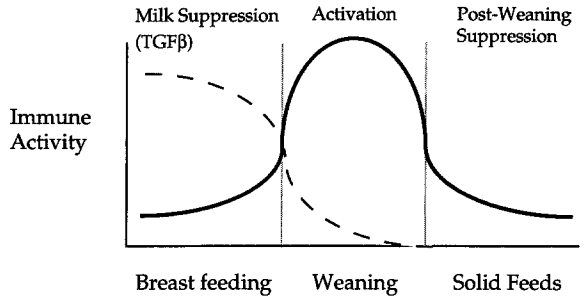

Fig. 3. Possible schema for regulation of immunologic activity during infancy based on the present human study and on previous studies in the rat (1–3). This proposes that immune activity is suppressed during milk feeding, that activation occurs during weaning, and that relative immunosuppression (oral tolerance) stabilizes this activation postweaning.

peak at weaning in rats (2, 3). Thus, it is possible that the decline in immune activity after weaning is mediated by intrinsic production of TGFβ or other suppressor factors. It is already known that weaning is a critical time, at least in mice, in switching immune responses to systemic tolerance to food antigens (27).

We have commenced a study of infants undergoing endoscopy and collecting duodenal biopsies and paired blood samples, although a deficiency of any such study will be that the subjects cannot be regarded as being in good health, as were those that participated in the present study. In the meantime, our study has shown that total immune activity during infancy is increased, which is contrary to the prevailing notion that the human infant has an immature immune system of low activation status. Perhaps the concept of an immature immune system during infancy is not entirely valid. We are not aware of studies that show that infant T cells continue to express immature phenotypic markers. Thus, what is apparent immaturity may be due to such processes as minor functional deficiencies, lack of antigenic stimulation, segregation of the mucosal versus systemic immune systems, and active immunologic suppression. The scheme (Fig. 3) which we have proposed should allow these possibilities to be investigated.

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