Improved thymic function in exclusively breastfed infants is associated with higher interleukin 7 concentrations in their mothers’ breast milk

Pa T Ngom, Andrew C Collinson, Jeffrey Pido-Lopez, Sian M Henson, Andrew M Prentice, and Richard Aspinall

ABSTRACT
Background: In rural Gambians, the season of birth strongly predicts adult mortality. Those born during the harvest season have longer life spans than those born during the hungry season, and the deaths associated with infectious diseases suggest permanent early-life influences on immunity. Thymic measurements showed significantly smaller thymuses in infants born during the hungry season than in those born during the harvest season. The differences were greatest at 8 wk of age, a time when all infants were exclusively breastfed, which suggests the involvement of breast milk factors.

Objective: This study tested whether thymic size differences reflect thymic output and ascertained whether thymic output is associated with breast milk interleukin 7 (IL-7) concentrations.

Design: We studied thymic size and output in a prospective cohort of 138 Gambian infants born in either the hungry or the harvest season by measuring signal-joint T cell receptor–rearrangement excision circles (sjTRECs) at birth and at 8 wk of age. IL-7 concentrations in breast milk were measured by using an enzyme-linked immunosorbent assay.

Results: By age 8 wk, those born in the hungry season had significantly lower sjTREC counts than did those born in the harvest season (0.97 and 2.12 sjTRECs/100 T cells, respectively; \( P = 0.006 \)). At 1 wk postpartum, the breast milk of mothers of infants born in the hungry season had significantly lower IL-7 than did that of mothers of infants born in the harvest season (79 and 100 pg/mL, respectively; \( P = 0.02 \)). The findings were similar at 8 wk postpartum.

Conclusion: These data show a plausible pathway linking external seasonal insults to mothers with thymic development in their infants, which suggests possible implications for long-term programming of immunity.


KEY WORDS Breast milk, thymus, lymphocytes, immune programming, infection, nutritional deprivation, signal-joint T cell receptor–rearrangement excision circles, sjTRECs, interleukin 7

INTRODUCTION
We previously reported that adult mortality in rural Gambian villagers is strongly associated with the season in which they were born (1). This finding emerged from a Kaplan-Meier survival analysis of 1077 deaths in 3102 persons whose date of birth had been accurately recorded with the use of a continuous demographic recording system initiated in 1949. Analysis of harvest-season births versus harvest-season births revealed hazard ratios of 3.7 (\( P = 0.0001 \)) for death at age \( \geq 15 \) y and of 10.3 (\( P = 0.0001 \)) for death at age \( \geq 25 \) y (1, 2). Most of the adult deaths were due to infectious diseases, which leads to the hypothesis that early-life events that are correlated with season of birth permanently program adult immunity. The nature of these early exposures remains to be defined, but it could include fetal growth retardation (3), infection in the mother during pregnancy (eg, malaria; 4), exposure to environmental toxins (eg, aflatoxins; 5), and early postnatal nutritional or infectious insults.

Early nutritional influences represent strong candidates as factors regulating thymic function and immunity because the hungry season is a period of intense farm work that coincides with food shortages as the previous year’s harvest runs out (6). These factors cause a negative energy balance that includes the mobilization of up to 50% of the body fat stores of pregnant and lactating women (7). Intrauterine growth retardation is common at this time of year, when there is a higher prevalence of low-birth-weight infants than during the harvest season (6). The thymus is a likely target for putative programming of immunity because it is both highly sensitive to undernutrition and fundamental to the development of the immune system (8, 9). We used ultrasound to assess the seasonal patterns of thymic growth at 1, 8, 24, and 52 wk of age in a birth cohort of 138 infants recruited prospectively over a 14-mo period. Thymic index was shown to be affected both by the season of birth and by the season in which measurements were taken (10). The differences between thymic index in harvest- and hungry season infants were greatest at 8 wk of age (Table 1), a time when the infants were exclusively breastfed, when clinically evident infections were relatively absent, and when low-birth-weight infants’ growth had caught up to international standards.

The purposes of the current study were twofold: first, to assess whether the differences in thymic size in this cohort were also

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\(^2\)Supported by the Nestlé Foundation, the MRC International Nutrition Group, and the Islamic Development Bank.

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Received October 1, 2003.

Accepted for publication March 25, 2004.
The Gambia, where they were spun by centrifugation at 800 rpm for 5 min (Mistral 30001; Henderson Biomed Ltd, Beckenham, United Kingdom). Next, the supernatant fluids were decanted off; this step was followed by washing in 1 mL phosphate-buffered saline (PBS), and then the cell pellet was resuspended in 300 μL PBS-1% paraformaldehyde. The samples were then acquired and analyzed by flow cytometry, by gating around the lymphocyte population. CD45 staining was used to determine the purity of the lymphocyte population with data accepted for ≥95% purity. The number of CD3 cells/mL whole blood was calculated by multiplying the percentage of lymphocytes by the absolute WBC count/mL to obtain the absolute number of lymphocytes, which was then multiplied by the number of CD3+ cells as a percentage of the total lymphocyte count in the sample (CD3%) to give the number of CD3 cells/mL whole blood (% lymphocytes × WBCs/mL × CD3% = CD3/mL). CD3% was validated by ensuring that the sum of CD4% and CD8% was approximately equal to CD3% and that CD16/56%, CD19%, and CD3% totaled 100 ± 10% (ie, the T cells, B cells, and natural killer cells equal the total lymphocytes).

Thymic size was assessed at 1, 8, 24, and 52 wk by using sonography as described in detail elsewhere (10). Briefly, infants were scanned in the supine position; a minimum of 3 measurements were taken, and the means of 3 good-quality measures were used. The transverse diameter and sagittal area of the largest lobe were multiplied to obtain a volume-related thymic index. The work described here was based on 99 (of the original 138) mother-infant pairs who were selected because they had, along with matching milk samples, sufficient residual cell samples to allow the sjTREC analysis. The breast milk sampling was standardized as much as possible by manual expression of milk from both breasts into separate containers between 0600 and 0700. This would have been the first feed of the day for most mothers. Ethical approval was granted by the Medical Research Council and Gambian Government Joint Ethical Committee (Reference number SCC 734/705).

**Definitions**

In line with the original mortality analysis (1) and the ultrasound study of thymic size (10), the harvest season was defined as spanning January through June (a period of complete absence of rain), and the hungry season was defined as spanning July through December (a period including the rainy season). As well as being a time of relative food shortage, which is especially acute in August and September, the hungry season is a time of greatly increased infectious load (16).

**TABLE 1**

<table>
<thead>
<tr>
<th>Age (wk)</th>
<th>Harvest season</th>
<th>Hungry season</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20.7 ± 4.2 [75]</td>
<td>19.0 ± 4.2 [62]</td>
<td>0.01</td>
</tr>
<tr>
<td>8</td>
<td>32.5 ± 5.7 [80]</td>
<td>28.7 ± 5.4 [57]</td>
<td>0.0001</td>
</tr>
<tr>
<td>24</td>
<td>37.4 ± 8.3 [61]</td>
<td>34.2 ± 7.2 [74]</td>
<td>0.01</td>
</tr>
<tr>
<td>52</td>
<td>33.5 ± 7.4 [50]</td>
<td>30.8 ± 7.6 [60]</td>
<td>0.05</td>
</tr>
</tbody>
</table>

For all values are \( \bar{x} ± SD; n \) in brackets. The harvest season is from January through June and the hungry season is from July through December. The thymic index measurements were used to generate figures to show percentage changes in thymic index at weeks 1, 8, 24, and 52 (10).

2 Student’s \( t \) test.

reflected in changes in thymocyte output assessed by measuring signal-joint T cell receptor–rearrangement excision circles (sjTRECs) and, second, to test whether postnatal thymic growth might relate to the concentration of IL-7 in the mother’s breast milk. The latter investigation was suggested by both the fact that immunoprotective factors in the milk of Gambian mothers were previously shown to vary substantially by season (11) and the observation that breastfed infants have larger thymuses than do formula-fed infants (12). IL-7 is known to improve thymic output and in human models (13–15) but has not previously been studied in human milk.

**SUBJECTS AND METHODS**

**Subjects**

The 138 mother-infant pairs enrolled in this study were recruited over a 14-mo period from rural subsistence-farming communities in the West Kiang region of The Gambia, a country in West Africa. Detailed anthropometric measurements of the infants were performed at birth and at 4-wk intervals throughout infancy. Active surveillance for infections was carried out fortnightly, and a full clinical examination was performed at monthly intervals. Appropriate treatment for any infection was given. The full Gambian Government program of immunization was administered. Blood was drawn at birth and at 8, 16, and 52 wk of age for analysis of nutritional status, aflatoxin exposure (assessed by plasma aflatoxin adduct concentrations; 5), lymphocyte populations, and vaccine responses. The white blood cell (WBC) count (cells/μL) was estimated by using the Coulter counter (Beckman Coulter, United Kingdom), and the percentage of lymphocytes was determined by using microscopy. Lymphocyte subsets including CD3 cells were evaluated by flow cytometry using the FACS Calibur flow cytometer (Becton Dickinson UK Ltd, Oxford, United Kingdom). In the field, 100 μL of EDTA-containing whole blood was incubated for 10 min at room temperature in the dark with 10 μL anti-CD3 (or other lymphocyte subsets analyzed) monoclonal antibody (Cyto-stat; Beckman Coulter SA, Nyon, Switzerland) conjugated to PC5 fluorescent dye. The red blood cells were then lysed, and the WBCs were fixed and stabilized with the use of the QPrep machine (Beckman Coulter) with accompanying reagents. The stained samples were stored at 4 °C for a few days and then packed in cold boxes and transported by road to the Medical Research Council laboratories in Fajara, The Gambia, where they were spun by centrifugation at 800 rpm.

**Breast milk enzyme-linked immunosorbent assay**

Breast milk samples (2–5 mL) were collected in the early morning by maternal manual expression. Samples were collected at 1 and 8 wk postpartum and frozen at −70 °C. An enzyme-linked immunosorbent assay was developed by using commercially available reagents to measure IL-7 levels in breast milk. Briefly, 96-well Maxisorp plates (VWR International, Poole, United Kingdom) were coated with 100 μL of 5 μg monoclonal mouse anti-human IL-7 antibody (R&D Systems Europe Ltd, Abingdon, United Kingdom)/mL in PBS (Sigma-Aldrich, Guildingham, United Kingdom) and incubated overnight at 4 °C. The plates were washed and blocked with PBS/1% bovine serum albumin (BSA). Doubling dilutions were prepared of recombinant human IL-7 in PBS/0.1% BSA to give a standard range from...
Measurement of sjTRECs

sjTRECs are generated when the T cell receptor (TCR) δ locus gene segments (VDJC) are removed from within the TCRα locus during rearrangement of TCRα gene segments (VJC), a phenomenon observed in 70% of αβ T cells (17). DNA was isolated from the frozen whole-blood samples by using the QIAamp DNA Mini Kit (QIAGEN, Crawley, United Kingdom), according to the manufacturer’s instructions. The sjTREC concentrations were analyzed according to a previously described method (17) by using real-time polymerase chain reaction (PCR) on a light cycler (Roche Diagnostics, Lewes, United Kingdom). sjTRECs were measured by Hot-start real-time PCR in the light cycler with the use of primers GCCACATCCCTTTCAACCATGCTGAC forward and TTGGTCCGGTGCTGTGCTGGCATC (reverse). A standard was prepared by using serial dilutions of a known number of copies of a fragment of the sjTREC gene sequence and included in each light cycler run to generate a standard curve. Using 0.2-mL PCR tubes on ice, we made a master mix of 2 μL SYBRGreen (Roche Diagnostics), which binds double-stranded DNA, and 0.16 μL of anti-Taq antibody (Sigma Aldrich) and incubated the mix for 10 min at room temperature; then 3.2 μL (4 mmol/L) MgCl₂, 0.2 μL (0.4 mmol/L) of each of the primer pairs and 10.64 μL sterile water were combined, and 18 μL of this mixture was pipetted into all tubes followed by 2 μL of standards, samples, or a negative control in corresponding tubes to obtain a 20-μL reaction volume. Real-time PCR was then performed in glass capillaries under the following conditions: 1 cycle of 95 °C for 1 s; 5 preamplification cycles of 95 °C for 5 s, 60 °C for 10 s, 72 °C for 12 s, and 83 °C for 5 s; and 40–60 cycles of second amplification and fluorescence measurement of 95 °C for 1 s, 60 °C for 5 s, 72 °C for 12 s, and 83 °C for 5 s, during which fluorescence measurements were taken. The number of copies of sjTRECs in the original samples was automatically determined by reading off the standard curves generated.

Statistical analysis

Data were tested for normal distribution, and nonnormally distributed data were log transformed to achieve normalization. Student’s t test was used to compare means, and the 95% CI was calculated. P values of < 0.05 were considered statistically significant. For the breast milk IL-7 enzyme-linked immunosorbent and sjTREC assays, each sample was analyzed in triplicate and observed low CVs; outliers were easily identified and excluded from the analysis.

RESULTS

Variation in sjTREC concentrations by season of birth

To ascertain whether the observed difference in thymic size (Table 1) would be reflected by similar differences in sjTREC concentrations, we analyzed blood drawn from infants at age 8 wk. The results revealed that, despite considerable monthly variations (Figure 1A), the harvest-season infants had significantly higher geometric mean (95% CI) sjTREC concentrations than did the hungry-season infants for the aggregated monthly values of the 2 seasons (Table 2). Similar results were seen for absolute sjTREC counts expressed per milliliter of whole blood (Figure 1B, Table 2). Further analysis of the monthly pattern of variation in measurements at age 8 wk revealed sustained higher monthly sjTREC concentrations during the harvest season (January–June) but a declining trend during the hungry season (July–December). The uncharacteristically low geometric mean of sjTRECs/100 T cells during the harvest season, seen in May, could have been due to the small sample size (n = 2).
To explore the possibility that the observed differences in sjTREC concentrations at age 8 wk may already have existed at birth, we analyzed cord blood sjTRECs from the harvest and hungry seasons. T cell counts were considered unreliable for the cord blood samples analyzed for sjTRECs, and therefore we expressed sjTRECs/\mu g DNA. Cord blood red blood cells are known to be particularly difficult to lyse (18), and some cord blood–extracted DNA may contain high concentrations of components that are inhibitory to PCR (19); as a result, it was not possible to obtain adequately pure amounts of DNA from all the samples for the cord blood sjTREC analysis. The results of the cord blood sjTREC analysis showed that there was no significant seasonal difference in cord blood sjTRECs/\mu g total DNA (data not shown).

**Variation in lymphocyte counts and T cell numbers by season of birth**

To assess possible relations between T cell numbers and sjTRECs, the total lymphocyte and T cell counts were analyzed for all 99 infants included in the sjTREC analysis at age 8 wk. There was some variation in the T cell counts obtained during the harvest- and hungry-season months (Figure 2), but this difference was not statistically significant for the percentage of CD3+ cells (Table 2). There was also no significant difference in absolute numbers of CD3+ cells (Table 2). Total lymphocyte count was, however, significantly higher in the hungry season than in the harvest season (Table 2).

**Variation in mother’s breast milk IL-7 by season of infant’s birth**

Frozen whole breast milk from samples taken in week 1 from the mothers of 59 harvest-season and 64 hungry-season infants and in week 8 from the mothers of 75 harvest-season and 52 hungry-season infants were analyzed for IL-7 concentrations. Despite considerable monthly variation, breast milk from the mothers of harvest-season infants contained significantly (P = 0.02) higher IL-7 concentrations than did that of the mothers of hungry-season infants at 1 wk postpartum (Figure 3). At 8 wk postpartum, however, the IL-7 concentrations were still higher in the mothers of harvest-season infants than in the mothers of hungry-season infants, but the difference was not significant (data not shown).

**DISCUSSION**

Our previous observation—that early-life factors, in correlation with the season of birth, have a profound impact on mortality...
that is due to infectious diseases in young adulthood—requires a biological explanation. The wide variety of seasonal exposures in the community studied, combined with the complexity of the human immune system, means that no single study will be able to identify the causal mechanism or mechanisms. For this reason, we have initiated several studies in different age groups and using different investigative tools (especially vaccine challenges) in this and other seasonally affected populations. Cell-mediated immunity is a likely candidate as the explanation for the biological mechanisms involved in the effects of season of birth on mortality, and the study of early thymocyte development makes a logical target in view of the known sensitivity of the thymus to nutritional, infectious, and stress-related insults (8, 20).

There could be several explanations for the observed increase in thymic size during the harvest season in infants in this community. For instance, it could have been due to increased thymopoiesis, which is shown to result in increased numbers of CD4<sup>+</sup>CD8<sup>+</sup> (double-positive) thymocytes in the thymic cortex during reversal of starvation-induced thymic involution in ob/ob mice (21), to increased numbers of fat cells (22), or to the possible entry of lymphocytes from the periphery (23). The current finding that the increased thymic size was also associated with increased sjTREC concentrations suggests an association between an improved thymic output and harvest-season births. Although there are other possible explanations for the seasonal differences in sjTREC concentrations, they could plausibly be linked to maternal nutritional status. Improved maternal nutrition during the harvest season may improve the infants’ thymic activity through an increase in a factor or factors in breast milk that enhance the generation of thymocytes—hence, the observed increase in sjTREC concentrations. We have not used purified peripheral blood mononuclear cells for our sjTREC assay because only frozen whole blood was available, but the use of whole blood was unlikely to introduce errors to the assay because we took care to extract and purify DNA from whole blood by using the QIAamp blood purification kit, which is reliable for the generation of pure PCR-amplifiable DNA from such samples. Because of their restriction to the αβ T cell lineage, it is not necessary to purify CD3<sup>+</sup> T cells before their analysis (24). The calculation of sjTREC concentrations per T cell is considered to be an appropriate indicator of changes in thymic function, although its use as a marker for absolute thymic function is controversial, partly because of the influence that T cell proliferation (usually in disease situations) may have on sjTREC concentrations independent of thymic output (25). During an infection, activation of T cells and their expansion to produce effector cells lead to a dilution of the episomal sjTRECs that do not divide with the cell. Therefore, the extent of T cell proliferation must be taken into account in interpreting sjTRECs as a marker of thymic function. The CD3<sup>+</sup> T cell count does not support any significant dilution of sjTRECs in our analysis, and other peripheral WBCs, such as monocytes, granulocytes, and B cells, are negative for sjTRECs because they are not known to express the TCR; therefore their proliferation is irrelevant to the concentration of sjTRECs per T cell. The analysis of the number of sjTRECs/mL whole blood, which reflects the absolute sjTREC numbers, has also confirmed significantly greater average sjTREC content during the harvest season. Nonetheless, the characteristic rise in infections during the hungry season increases the risk of productive infection and the related possibility of increased T cell proliferation that would result in sjTREC dilution. Although these possibilities may exist for many persons in the study community, they were considered unlikely for those at the specific age of 8 wk, when exclusive breastfeeding was practiced, the infants had good weight and diminished prevalence of infections, and they appeared healthy. Taken together, our data imply that the higher sjTREC counts of the harvest season were a likely reflection of the greater number of recent thymic emigrants in the harvest-season infants than in the hungry-season infants.

The crude division of the year into the harvest and hungry seasons was limited by sample size considerations, and thus inevitable overlaps may have diluted some of the statistical power in the correlations of the immunologic values measured with thymic size. A closer look at the months of September, October, and November, which fall within or immediately after the peak of the rainy season with its high prevalence of malaria and other infections, found that they coincided with the observed increases in lymphocyte counts. The slightly higher CD3<sup>+</sup> T cell count of these months (Figure 2) could be attributed to the relative increases in proliferation of these cells that is driven by the antigenic load from the endemicity of various infections at this time (16). The higher total lymphocyte counts at this time may be due to expansions in the B lymphocyte population that are often associated with malaria infection and associated diseases (26, 27). This period is also the height of the hungry season, during which more external nutritional deprivation is expected to further suppress the thymus and lead to additional lowering of thymic output. That is evidenced by the occurrence of the lowest sjTREC concentrations during these months, which is consistent with findings that nutritional rehabilitation of malnourished children resulted in greater thymic size and improved immunologic function (28) and that the reverse was true during dietary restriction (29).

Although maternal immune factors and cytokines may affect the fetus via the intrauterine route (30), detection in the harvest season of higher concentrations of breast milk IL-7 at 1 wk postpartum and of evidence of improved thymic activity in exclusively breastfed infants at age 8 wk suggested that the composition of the food originating from the mother and possibly reflecting her own nutritional status may be linked to the enhancement of the infant’s developing immune system. IL-7 is essential for the proliferation and survival of precursor T cells, which show elevated sensitivity to IL-7 during the earliest stages of thymocyte development (31, 32). Once these precursors, which are committed to the T cell lineage, mature and exit the thymus as recent thymic emigrants, IL-7 also induces their proliferation and survival, which maintain the T cell repertoire (33). It was shown recently that homeostatic control of the memory CD4<sup>+</sup> T cell pool is also regulated by IL-7 and does not require IL-15, unlike that of memory CD8<sup>+</sup> T cells (34). These findings emphasize the importance of IL-7 in all stages of T cell development, with particular relevance to the maturing immune system in early life. Although breast milk is known to contain a number of cytokines and growth factors (35), this report is the first that it contains IL-7 and that there are variations in the IL-7 concentrations. The presence of IL-7 in breast milk was not necessarily predictable because IL-7 was previously reported to be produced by stromal cells of the thymus (36), bone marrow (37), and dendritic cells (38). However, active secretion of other cytokines, including IL-18 and transforming growth factor β, in breast milk has been reported (35, 39, 40), and it has been suggested that cytokines made elsewhere in the body may home to the mammary gland (41).
A role for breast milk in the improvement of thymic function has been implicated in findings that breastfeeding is associated with greater thymic size in infancy than is feeding with formula milk (42). Although transfer of the IL-7 from the breast milk across the gut and then onward to the infant’s thymus may seem difficult, it has been shown that breast milk fat globules contain proteins known to resist the infant’s gut digestive enzymes and hence to remain functionally active (43). Furthermore, soluble receptors of cytokines known to bind their ligands may then act as carrier proteins, which results in more stable receptor-cytokine complexes with an extended half-life (44). Preliminary analysis of our results indicates that breast milk IL-7Rα concentrations followed a pattern similar to that of the cytokine, which may further favor successful transfer of viable IL-7 to the infant via breast milk. Receptors for cytokines are known to exist in the gut (45), and cryptopatches, which are part of the murine intestinal immune compartment, express the IL-7 receptor (46); this suggests that IL-7 receptors may also exist in the human gut and that breast milk IL-7 may cross the gut epithelium into the circulation via these receptors. Immune compartments such as Peyer’s patches (47, 48) and M cells (48–50) are known transfer routes for gut proteins. The presence in the thymus of IL-7 receptors and the high density of cells with increased sensitivity to IL-7 (51) make it possible that any IL-7 crossing into the infant’s circulation will affect these thymocytes.

Although by no means conclusive, this study suggests a possible mechanistic route through which external factors (including maternal nutritional restriction) during the hungry season may affect IL-7 concentrations (or related trophic factors) in breast milk, which in turn may influence thymic size and thymopoiesis in early infancy. We showed elsewhere that infants have a characteristic thymic size that tracks through infancy, even after adjustment for body weight (10). It is plausible that such early variations may represent a permanent programming of thymic development, which in turn could influence adult T cell function through differences in T cell pool size, repertoire, or both. The accumulated effects of these events is predicted to precipitate premature immune senescence by inducing accelerated T cell division with age as a response to the disproportionate pressures on an immune system that is fundamentally inhibited by early-life events. We are currently investigating these possibilities in a cohort of older subjects from the same community.

We are grateful to the villagers in the community, in particular the mothers and infants who participated in this study, and to the field, laboratory, clinical, administrative, and other support staff of the MRC laboratories in The Gambia, especially the nutrition group based in Keneba.

PTN, JP-L, and SMH carried out the assays; ACC organized the fieldwork and sample collection; and AMP and RA designed the study. None of the authors had any conflict of interest with the study.

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