Digestive Diseases and Sciences, Vol. 47, No. 5 (May 2002), pp. 1147–1157 (© 2002)

Total Parenteral Nutrition-Associated Changes in Mouse Intestinal Intraepithelial Lymphocytes

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Intraepithelial lymphocytes (IEL) play a major role in mucosal defense mechanisms against intraluminal foreign antigens. To address the role luminal nutrients have on the phenotype and function of the IEL, we administered total parenteral nutrition (TPN) to mice, with the absence of enteral intake. We hypothesized that administration of TPN would result in changes in the phenotype and function of the IEL. For this, we utilized a mouse model of TPN. A significant decline in the $CD4^+$ IEL population occurred with TPN. Additionally, the $CD8^+$,$CD44^+$ IEL subset showed a 65% decline ($P < 0.05$), and the $CD4^+$,$CD44^+$ subset declined by 55% with TPN ($P < 0.05$). The $CD8\alpha\beta^+$ population (a marker of thymic-dependence) also declined by 92% ($P < 0.01$) with TPN. IEL in the TPN group showed a significantly lower degree of *in vitro* proliferation. In conclusion, the IEL showed significant phenotypic changes with TPN including the loss of the thymic-derived population. Functionally, the IEL showed a significant decline in proliferation. Such changes demonstrate the important role luminal nutrients have on IEL phenotype and function.

KEY WORDS: intraepithelial lymphocytes; total parenteral nutrition; CD4; CD8; CD8$\alpha\beta$; CD44; CD62L.

Many patients, because of either a lack of gastrointestinal length or gastrointestinal dysfunction, are unable to take feedings via the enteral route. These patients are confined to a prolonged course of total parenteral nutrition (TPN). Total parenteral nutrition is used in over half a million patients each year in the United States, alone (1). Despite being an essential form of therapy, patients receiving TPN have a significantly greater risk of developing septicemia and wound infections compared to patients receiving enteral nutrition (2–4). The etiology of this increased incidence of infections is not clear. It is believed, however, that the intestine may be the source of the organisms responsible for these septic episodes (5, 6). In fact, the intestine of both experimental animals and humans undergoes a number of changes with TPN, including a loss of epithelial height, absorptive capacity, and mucosal barrier function (7–10). Because of the close relation of the intraepithelial lymphocyte (IEL) population and intestinal epithelial cells, we hypothesized that the composition and function of the IEL may rely on normal enteral nutrition and would change with TPN.

Intraepithelial lymphocytes have a complex role. They act as the initial lymphoid defense layer against intraluminal foreign antigens, and IEL, must downregulate immune responses to many enteral antigens in order to prevent an overwhelming immunologic response (11, 12). The IEL possesses a unique phe-

Manuscript received February 5, 2001; accepted June 15, 2001.
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notype of T cells (13). The IEL consists of a predominant subpopulation which is $CD8^+$,$CD4^-$ (70–85%, for mice, single positive), with smaller populations consisting of the following phenotypic distribution: $CD8^-$,$CD4^-$ (5–10%, double negative); $CD8^-$,$CD4^+$ (5–12%, single positive); and $CD8^+$,$CD4^+$ (4–10%, double positive) (13–15). Another important characteristic of the IEL is the large number of TCR$\gamma\delta$ cells; up to 50% of IEL are TCR$\gamma\delta$ compared to less than 2% in peripheral blood lymphocytes of mice (16, 17). Additionally, the derivation of the IEL has been shown to originate from a thymic independent (18), as well as a thymic dependent source (13, 16, 19). Experiments using $RAG2^{-/-}$ knockout mice, injected with bone marrow of nude mice, led to the development of a virtually absent peripheral T-cell population, but an IEL population containing $CD8\alpha\alpha^+$ (homodimeric chains),$CD4^-$ cells with either $\gamma\delta$ or $\alpha\beta$ TCR (20). This extrathymic origin is TCR^+,$CD8\alpha\alpha^+$, whereas TCR^+,$CD8\alpha/\beta^+$ cells are of thymic origin (20).

A considerable amount of work has concentrated on the assessment of IEL function. Investigators have stimulated the IEL under a variety of conditions including alloantigen, plant lectins and, more recently, heat shock proteins and superantigens (21–25). Investigators have shown the IEL to proliferate with many established mediators of lymphocyte stimulation; however, it proliferates at a much lower extent than most other lymphoid populations (18, 21). Because of the large $CD8^+$ population, cytotoxic activity has also been investigated. Mosley *et al* (26) and Ebert (27) demonstrated the IEL to have a large population of cytotoxic T lymphocytes that were able to perform a redirected cytotoxic function without *in vitro* activation. The actual significance of these actions has not been well defined; however, these functions may be critical for the organism to protect itself from harmful intraluminal pathogens.

During early postnatal development, the IEL rapidly develops in the mouse (28). Changes include an expansion of the T-cell receptor (TCR) $\alpha\beta^+$ and $CD4^+$ populations (29). Although it has been previously shown that the presence of luminal bacteria plays a critical role in IEL maturation (29, 30), it is not clear what role luminal nutrients may have on the phenotype or function of the IEL. An interdependence of the IEL and epithelial cells has been well described, including the production of growth factors and cytokines, which support both the composition of the mucosal layer and its function (31). Therefore, the observed changes in epithelial structure and function with administration of TPN may reflect similar changes in the IEL. This study examined phenotypic and functional changes of the IEL in a mouse model of TPN.

MATERIALS AND METHODS

Animals. The studies reported here conformed to the guidelines for the care and use of laboratory animals established by the University Committee on Use and Care of Animals at the University of Michigan and protocols were approved by that committee. Male, 2- to 4-month-old, specific-pathogen-free, C57BL/6 mice (Jackson Lab, Bar Harbor, Mainè, USA) were housed in metabolic cages and maintained in temperature, humidity and light-controlled conditions.

Operative Procedures. Mice were anesthetized with sodium pentobarbital (50 mg/kg body weight, intraperitoneally). A silicone rubber catheter (0.012 in. ID × 0.025 in. OD; Konigsberg Instruments, Inc., Pasadena, California, USA) was inserted into the superior vena cava via the left jugular vein. The distal end of the catheter was tunneled subcutaneously. The catheter exited between the scapulae and was attached to a swivel spring, which allowed the mice freedom of movement in their individual cages (Metamount System, Instech, Corp, Plymouth Meeting, Massachusetts, USA). Catheterized mice were connected to an infusion pump (AIM pain provider pump, generously donated by Abbott Laboratories, Abbott Park, Illinois, USA) and saline (dextrose 5% in 0.45NS with 20 meq KCl/liter) was infused at an initial rate of 4 ml/day. After 24 hr, animals were randomized into two groups. The control group received the same intravenous physiologic saline at 7 ml/24 hr, in addition to standard laboratory mouse chow and water *ad libitum*. The TPN group received a standard TPN solution intravenously with no oral intake. The TPN solution contained a balanced mixture of amino acids, lipids, and dextrose in addition to electrolytes, trace elements, and vitamins (32). Caloric delivery was based on previous measurements of caloric intake of control mice and from previous investigators, so that caloric and protein delivery was essentially the same in both groups (32). Mice were killed at seven days using CO_2.

Cell Isolation. IEL was isolated similar to that described by Mosley and Klein (33). After small bowel resection, mesenteric fat and Peyer's patches were removed. The intestine was opened and agitated to remove mucus and fecal material. The intestine was cut into 5-mm pieces, washed three times in an IEL extraction buffer [1 mM EDTA, 1 mM dithiothreitol in phosphate-buffered saline (PBS)], and incubated in the same buffer with continuous brisk stirring at 37°C for 30 min. The supernatant was then filtered rapidly through glass wool, placed in 40% isotonic Percoll (diluted in the IEL extraction buffer; Pharmacia, Piscataway, New Jersey, USA) centrifuged for 20 min at 500*g* at 28°C. Splenocytes, thymocytes, and inguinal and axillary lymph nodes were isolated from the mouse prior to the manipulation of the intestine. Cells were suspended in PBS, filtered through nylon mesh, and for splenocytes and lymph nodes further purified using gradient centrifugation with Lympholyte M (Cedar Lane, Ontario, Canada) for 20

min at 28°C at 500*g*. For proliferation assays, splenic B cells were depleted using standard panning techniques (34).

Bacteriological Cultures. For qualitative analysis, the methods used were similar to those previously described (35). After killing the mice, and using sterile technique, samples of tissue ($N = 8$ per group) from the midportion of the small bowel and cecum were excised. Each tissue sample was incubated in thioglycolate broth for 24 hr at 37°C. Samples were then subcultured aerobically onto either MacConkey media for isolation of gram-negative bacteria, or colistine naladixic acid media (Becton Dickinson, Cockeysville, Maryland, USA) for isolation of gram-positive bacteria. Following an additional 24-hr incubation period, growth on each plate was recorded as either positive or negative.

For quantitative analysis, in a separate group of mice ($N = 6$ per group), using sterile technique, the lumen of the small intestine was flushed with 1 ml of PBS. The effluent was collected and sent for semiquantitative analysis of normal flora and for selected pathogens (Great Smokies Vet Science, Asheville, North Carolina, USA). Because the volume of stool in control and TPN groups varied greatly, with that in control being much greater than TPN mice (at times TPN mice had almost unmeasurable mice amounts of stool), it was impractical to try to determine a precise number of bacteria per small intestine. Therefore, quantification of each organism was expressed on a graded scale from 0 to 4+, and the mean grade was expressed in both groups. A grade of 0 was given for no growth, with progressively higher grades given to larger amounts of growth obtained after 48 hr of culturing the stool.

Flow Cytometry. Except where noted, antibodies were obtained from PharMingen (San Diego, California, USA) and consisted of (clone): CD4 (RM4-5), CD8α (53-6.7), CD8β (53-5.8), CD44 (IM7), CD45RB (16A), T-cell receptor β (Gibco BRL, H57), T-cell receptor γ (Gibco BRL, GL3), and Thy1 (G7). Isotype-matched, irrelevant antibodies were used as negative controls. Samples of IEL and thymocytes were also assayed for cell death with propidium iodide as a marker of cell necrosis and cell surface expression of phosphatidylserine (Annexin V Fluos stain; Boehringer Mannheim, Indianapolis, Indiana, USA), as a marker of apoptosis. Flow cytometry was performed using standard techniques (36) and a Becton-Dickinson FACScan (Becton-Dickinson, Mountainview, California, USA).

Proliferation Assays. Isolated IEL and splenocytes were counted and set to a concentration of 2×10^5 cells/100 μl in tissue culture medium [RPMI 1640, 2 mM glutamine, penicillin and streptomycin, 2-mercaptoethanol (5×10^{-5} M) and 10% heat-inactivated fetal calf serum (all supplied by Gibco BRL, New York, New York, USA)]. Cells were stimulated on anti-CD3 (derived from hybridoma 145-2C11) coated 96-well tissue culture plates (antibody applied at a concentration of 10 μg/ml, and incubated at 4°C overnight), and all assays were performed in triplicate. An additional group of cells were stimulated with exogenously supplied IL-2 (derived from rat-conditioned media) (34, 37) along with bound anti-CD3. Samples were compared to control wells treated with anti-dinitrophenol (clone UC8; kindly supplied by Dr. Jeffery Bluestone, Chicago, Illinois, USA). Samples were incubated at 37°C, with 95% O_2, 5% CO_2 for 72 hr. Proliferation was assessed by the addition of [^{3}H]thymidine (1 μCi/well), followed by harvesting of the cells 18 hr later and counting of radiation. Proliferation was expressed as the mean counts per minute (cpm) of radioactivity.

Table 1. Cells Isolated from Various Lymphoid Populations

Cell Population	*Cells ($\times 10^6$/isolated tissue, mean ± SD)**	
	Control	*TPN*
IEL	8.1 ± 5.6	10.2 ± 6.6
Intestinal epithelial cells	10.6 ± 6.9	21.7 ± 18.5
Splenocytes	44 ± 1.6	41.9 ± 3.1
Thymocytes	50.2 ± 9.0	34.5 ± 4.7

*Cell counts isolated from each lymphoid population in the control and TPN groups ($N = 6$).

Data Analysis. Statistical analysis was carried out using paired *t* tests for the flow cytometry and proliferation data, with SPSS software (SPSS Inc., Chicago, Illinois, USA). $P < 0.05$ was considered significant. Unless otherwise indicated results are expressed as the mean ± SD. For analysis of semiquantitative cultures, a Wilcoxon Signed Ranks test was used.

RESULTS

General Description of Mice. Total amount of administered intravenous fluid was a mean of 7.3 ± 1.4 ml/day for the TPN group and 7.0 ± 1.8 ml/day for the control group ($P > 0.05$). Mean caloric intake for the TPN mice was 9.0 ± 1.7 kcal/day. Body weights were recorded before the initiation of the study and after mice were killed. Body weights at the beginning and end the study were 25.6 ± 1.4 g and 24.2 ± 1.3 g for the TPN group and 25.0 ± 1.7 g and 24.8 ± 1.4 g for the control group, respectively ($P > 0.05$, between and within groups).

Number of Isolated Cells. Cell counts isolated from each population are shown in Table 1. No significant differences were noted in IEL, epithelial cells, or splenocytes between TPN and control groups. The number of thymocytes in the TPN group was lower than the control group; ($P < 0.05$). It was interesting to note that the yield in the number of IEL and intestinal epithelial cells rose in the TPN group. Because of fairly large standard deviations, the differences did not approach significance.

Bowel Colonization and Cultures. Small intestinal bacterial colonization results were similar for the TPN and control groups. Specifically, gram-positive bacteria were cultured in 11 of 13 mice in the TPN group and 8 of 11 control mice. Gram-negative bacteria were identified in 10 of 13 TPN mice and 7 of 11 controls mice ($p > 0.05$).

TABLE 2. SEMIQUANTITATIVE, AEROBIC STOOL CULTURES FROM SMALL BOWEL*

Organism	*Control group (range)*	*TPN group (range)*
Lactobacillus[a]	0	1.0 (0–4)
Bifidobacteria[b]	0.2 (0–1)	2.3 (0–4)
Enterobacter sp.[a]	2.0 (0–4)	0.67 (0–4)
Gamma strep	3 (1–4)	2.67 (0–4)
Klebsiella sp.	0.5 (0–3)	0.33 (0–1)
Pseudomonas sp.	0.8 (0–4)	0.17 (0–1)
Staphyloccocus sp.	0.7 (0–4)	1.3 (0–4)
Proteus mirabilis	0	0.7 (0–4)

*Results are given from mice in either the control or TPN study groups ($N = 6$/group). Cultures are performed on the selected, prominent aerobic species and common pathogens. Quantification is given as the mean amount for each group of bacterial species with grades ranging from 0 (None identified) to 4+ (maximal growth). Comparisons were made between control and TPN groups by using a Wilcoxon signed ranks test.
[a]$P < 0.05$, [b]$P < 0.01$; remaining comparisons were not significant.

Semiquantitative small bowel cultures ($N = 6$ in each group) are shown in Table 2. Most bacterial strains were similar between the study groups. Some differences, however, were noted. It was interesting to note that both *Bifidobacterium* sp. ($P < 0.01$) and *Lactobacillus* sp. ($P < 0.05$) were isolated more frequently and in higher amounts in the TPN group. *Enterobacter* sp. were found more frequently and in higher numbers in the control group, and this difference approached significance ($P = 0.1$). This suggests that the short-term removal of enteral nutrition has relatively little influence on bacteria in the small intestine and may not be the driving for behind the observed changes in the IEL. Nevertheless, *Bifidobacterium* and *Lactobacillus* have been shown to influence immune responses in the intestine and may have contributed to some of the observed changes.

Phenotypic Results. Regarding phenotypic results in IEL subpopulations; flow cytometric analysis showed several unique differences between the TPN and control groups (Table 3 and Figures 1–4). Administration of TPN was associated with a statistically significant decrease in the number of $CD4^+$ IEL. This was noted for both the single positive $CD4^+$,$CD8^-$ (87% decline, $P < 0.01$) and the double positive $CD4^+$,$CD8^+$ (80% decline, $P < 0.05$) IEL subpopulations (Figure 1). The $CD4^-$,$CD8^+$ and $CD4^-$, $CD8^-$ populations did not appreciably change between the two groups ($P > 0.05$). CD44 was used as a marker of lymphocyte maturation and is manifested by increased expression. The $CD44^+$ IEL subset declined in the TPN group compared to control animals (Figure 2). The $CD8^+$,$CD44^+$ subset in the TPN group showed a 65% decline ($P < 0.05$), and the $CD4^+$,$CD44^+$ subset showed a 55% decline ($P < 0.05$) with TPN. Other markers of T-cell maturity showed variable changes. The CD62L population, which is normally not expressed in mouse small intestine IEL, rose from less than 2% in the control group to 8% in the TPN group. The $CD45RB^+$ population was relatively unchanged between the two groups.

The $CD8\alpha\beta$ subpopulation (ie, $CD8\alpha$ heterodimeric; thymic-dependent subpopulation) significantly declined ($P < 0.01$) in the TPN group (91% decrease). The $CD8\alpha\alpha$ subpopulation (eg, $CD8\alpha$ homodimeric) did not change significantly with TPN (Figure 3). The $Thy1^+$ population (Table 3) was

TABLE 3. PHENOTYPIC DESCRIPTION OF IEL IN CONTROL AND TPN MICE*

Cell population	*Description*	*Control [mean % (range)]*	*TPN [mean % (range)]*
$CD4^+$,$CD8^-$	Single positive	4.7 (3.9–6.1)	0.6 (0.2–0.7)**†
$CD4^+$,$CD8^+$	Double positive	3.0 (2.5–4.3)	0.6 (0.1–1.0)*‡
$CD4^-$,$CD8^+$	Single positive	65 (58–70)	64 (37–79)
$CD4^-$,$CD8^-$	Double negative	28 (23.2–34)	35 (19.3–64)
$\alpha\beta^+$	T-cell receptor	39 (23–33)	22 (12–31)
$\gamma\delta^+$	T-cell receptor	62 (33–53)	70 (37–56)
$CD8\alpha\beta^+$	Thymic-dependent	4.8 (3–6.5)	0.4 (0.1–.08)**†
$CD8\alpha\alpha^+$	Thymic-independent	64 (59–71)	52 (40–63)
$CD4^+CD44^+$	Mature T-cell	4.4 (1.9–5.4)	2.0 (0.4–6.6)
$CD8^+CD44^+$	Mature T-cell	29 (28.4–37.2)	10 (1.0–18.0)‡
$Thy1^+CD8^+$	Thymic dependent	23 (18–38)	10 (7–20)‡
$CD4^+CD25^+$	IL-2 receptor positive	0.3 (0–0.9)	0.4 (0–1.8)
$CD4^+$,$CD25^-$	IL-2 receptor negative	5.7 (4.4–6.6)	1.9 (0.9–2.5)*‡
$CD8^+$,$CD25^+$	IL-2 receptor positive	3.8 (0–7.7)	8 (1.2–14.7)
$CD8^+$,$CD25^-$	IL-2 receptor negative	58 (48.5–64.8)	59 (48.7–69.8)

*Results are expressed as the mean percent of gated cells (based on forward and side scatter characteristics) of the IEL and represents an $N = 8$ for each group.
†$P < 0.01$.
‡$P < 0.05$.

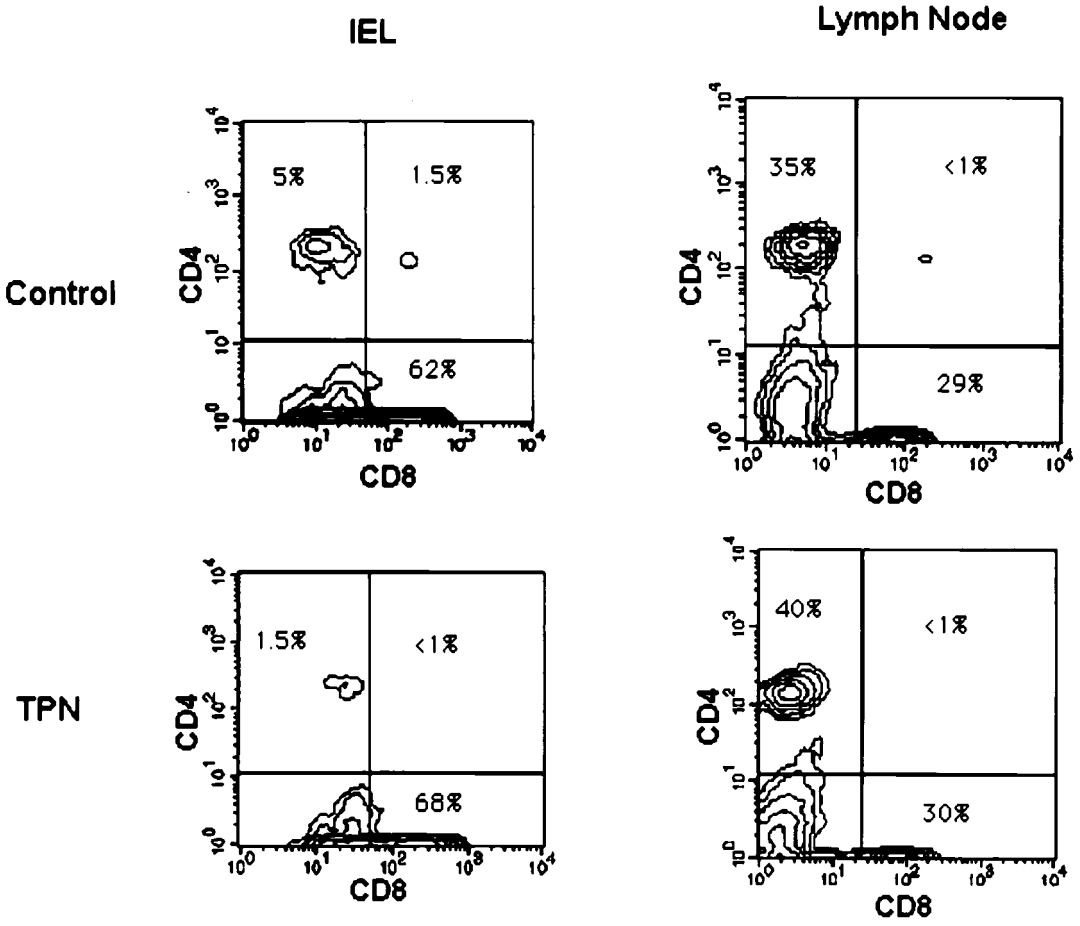

Fig 1. Distribution of $CD4^+$ and $CD8^+$ intraepithelial lymphocytes (IEL) (left column) and inguinal lymph nodes (right column) of the same mouse for both a representative control (top row) and TPN (bottom row) mouse. Gated populations are expressed as the percent with each phenotypic marker based on isotype matched control antibodies. Control group received intravenous saline and *ad libitum* chow; TPN group received intravenous nutrition and no enteral nutrition or water. Note the loss of $CD4^+$ cells in the TPN IEL compared to stable $CD4^+$ populations in the lymph node of the same animal.

studied as an additional marker of thymic dependence. $Thy1^+$ IEL declined with TPN from 23% of all $CD8^+$ cells in the control group to 10% of $CD8^+$ cells in the TPN group, although the difference was not significant. The TCR $\alpha\beta$ subpopulation of the IEL declined from 39% to 22%; however, the difference was not statistically significant. The $\gamma\delta$ subpopulation in the IEL was not statistically different between the two study groups (62% vs 70%, for the control and TPN groups, respectively; Table 3).

Regarding results in systemic lymphoid populations the numbers of splenocytes were not significantly different between study groups (Table 1). The phenotypic changes in the IEL did not appear to reflect systemic changes. Analysis of $CD4^+$ and $CD8^+$ populations in peripheral lymph nodes (Figure 1) and splenocytes (not shown) showed no significant differences between TPN and control groups. Splenocytes showed the following mean percent distribution: $CD4^+$ 70% vs 63%; $CD8^+$ 30% vs 37%; $CD44^+$ 70% vs 63%; and $CD8\alpha\beta^+$ 93% vs 94%, for the TPN and control groups, respectively.

Because of the loss of the IEL thymic-dependent population, thymic subpopulations were also evaluated. The total number of thymocytes was statistically lower in the TPN Group (Table 1). Analysis of thymocyte subpopulations showed a significant decline ($P < 0.05$) in the $CD4^+$,$CD8^+$ double-positive population (from a mean of 74% in the control group to 42% in the TPN group). Along with this was a subsequent rise in CD4 (mean of 8% to 30%, control and TPN, respectively) and CD8 (mean of 5% to 18%)

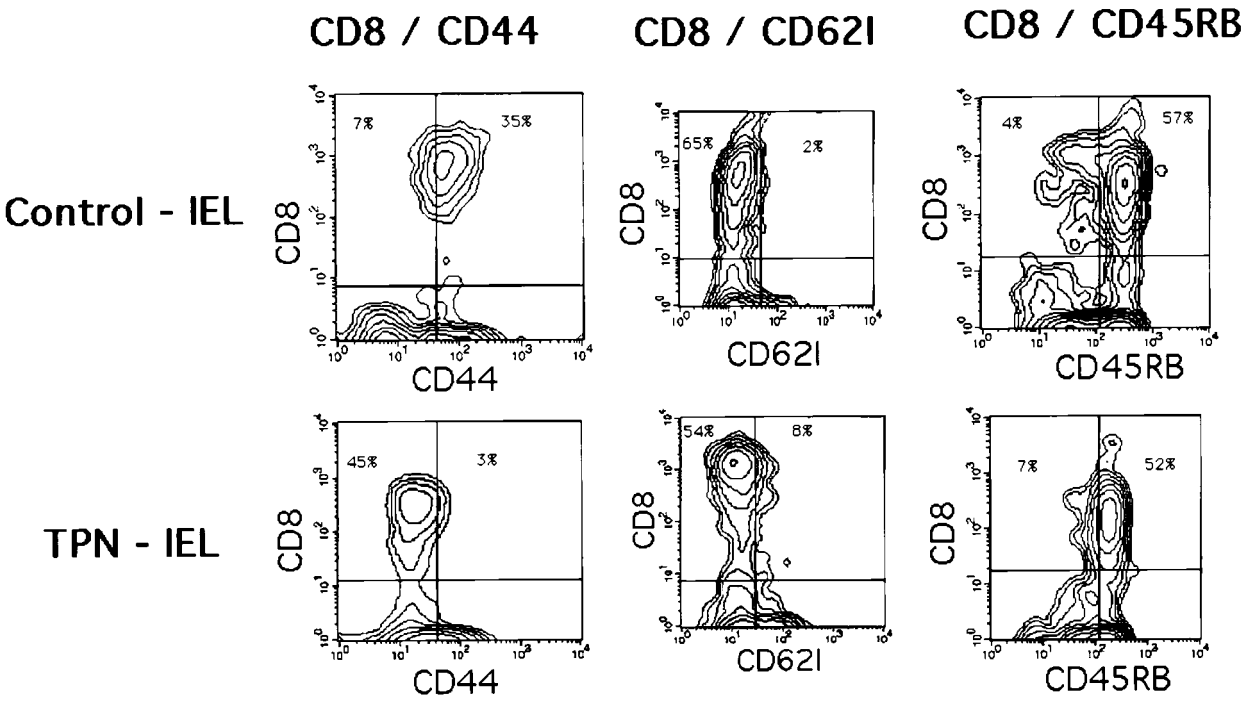

Fig 2. Distribution of phenotypic markers for T-cell maturation. $CD8^+$ cells are shown as well as CD44 (left column), CD62L (middle column), and CD45RB (right column) of the same mouse for both a representative control (top row) and TPN (bottom row) mouse. Note the decline of $CD44^+$ cells in the TPN group, as well as a slight increase in the $CD62L^+$ cells and little to no change in $CD45RB^+$ cells.

single positive cells (Figure 4). It is uncertain whether this represents a loss of the more immature thymocytes or an accelerated maturation into single positive cells.

We next addressed whether the loss of cells in the IEL and thymus was due to an apoptotic process or cell necrosis (Table 4). IEL showed a 79% increase in apoptosis in the TPN group. Similarly, the thymus showed a 40% increase. Neither of these increases, however, were statistically significant. Other changes between groups for both the IEL and thymus were not statistically significant.

Proliferation Studies. Overall the IEL in TPN mice had lower levels of proliferation compared to controls (Figure 5). IEL proliferation with immobilized anti-CD3 alone resulted in low levels of proliferation compared to splenocytes from the same mouse. The addition of IL-2 significantly ($P < 0.05$) increased IEL proliferation. Although the control IEL population increased proliferation by over 28-fold (310 vs 8820 cpm, with and without IL-2, respectively), the TPN group increased proliferation by only threefold (384 vs 1132 cpm). For the splenocyte population, anti-CD3 stimulated proliferation was low in the TPN group (13,245 vs 48,819 cpm; TPN and control groups, respectively). Splenocyte proliferation improved with addition of IL-2, so that the degree of increased proliferation was actually higher in the TPN group (62%) compared to the control group (38%).

To examine the influence of IL-2 on the proliferative changes, CD25 expression (IL-2 receptor) was studied (see Table 3 for percent changes). There was a significant decline in the IEL $CD4^+$,$CD25^-$ population with TPN, although this most likely represented an overall decline in $CD4^+$ cells. The $CD8^+$,$CD25^+$ population doubled in the TPN group, although the difference was not significant.

DISCUSSION

Our study demonstrated a significant change in both the phenotypic and functional characteristics of the IEL in mice maintained on TPN. This finding is significant in that administration of TPN is associated with a markedly increased risk of septicemia in both a clinical setting and in experimental animal models (3, 38). The intestine has been implicated as the source of this sepsis by many investigators, in that the majority of organisms isolated are of enteric origin (5, 39). It has been hypothesized that a loss of mucosal

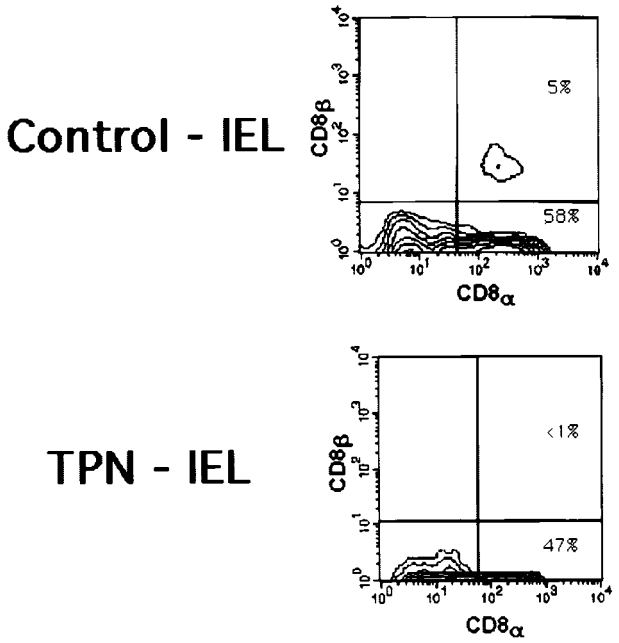

Fig 3. Distribution of CD8α^+ and CD8β^+ IEL in a representative control (top row) and TPN (bottom row) mouse. Note the almost complete loss of CD8$\alpha\beta^+$ cells in the TPN IEL.

immune function may be the cause of this increased risk of sepsis. In a study by Kudsk et al, changes in the IEL during TPN administration to mice were directly correlated with an increased risk of pulmonary infections (40). This study documented a significant decrease in the number of IEL associated with the administration of TPN. In a study by Guihot, et al, rats enterally fed parenteral nutrition (elemental diet) also developed changes in IEL (41). Similar to the Kudsk study, a decline in the number of $CD4^+$ and $CD8^+$ IEL was noted. However, in neither of these studies was a detailed analysis of many of the unique characteristics of the IEL performed. Additionally, it is difficult to know if the administration of an enterally fed elemental diet, as performed in the study by Guihot study, can adequately mimic the changes that occur with TPN. In a more recent study on the immunologic changes associated with TPN, Ganessunker et al described the changes seen within the lamina propria of neonatal piglets receiving TPN (42). In their study, a threefold increase in the number of T lymphocytes (both CD4 and CD8) was observed in those mice receiving TPN. In fact, there was a reverse relationship between the decline in villus height and the rise in T lymphocytes.

Table 4. Analysis of Cell Necrosis and Apoptosis Using Flow Cytometric Analysis

	TPN	*Control*
IEL		
Cell necrosis	4.2 ± 3.1	10.1 ± 9.2
Apoptosis	9.9 ± 10.6	2.1 ± 2.1
Thymus		
Cell necrosis	1.9 ± 2.6	2.9 ± 2.8
Apoptosis	9.3 ± 8.7	5.6 ± 2.4

*Results are expressed as the mean percent of population ± SD, *N* was a minimum of 5 per group. No differences were statistically significant between the two groups.

Intraepithelial lymphocytes are the population of cells located between intestinal epithelial cells (13). These lymphocytes are one of the first immunologic populations to be exposed to luminal antigens and pathogens and thus appear to play a role in mucosal immune defense mechanisms. Their precise function is complex, in that their ability to proliferate against exogenous stimuli or alloantigens is significantly lower than other lymphoid populations (14, 21, 23, 24, 43, 44). The IEL has a unique phenotype bearing both CD4,CD8 double-positive and double-negative T lymphocytes (33, 45). Another unique phenotype of this cell population in the mouse is the large (30–60%) percentage of TCR$\gamma\delta^+$ lymphocytes (46). Further, the origin of the IEL has been shown to arise from both a thymic-dependent and thymic-independent source (20, 47, 48).

In our study, the $CD4^+$ IEL population declined by over 80% with TPN. This loss included a decline in the $CD4^+$,$CD8^-$ and the $CD4^+$,$CD8^+$ subpopulations. The implications of this loss are difficult to interpret. It is known that the proliferative activity of the IEL is predominately confined to the $CD4^+$ population (13, 49). It is possible that the observed loss of proliferation in our study may well be attributed to this decline in the $CD4^+$ population.

The majority of IEL appear to have a phenotype consistent with a mature and activated cell population. This is reflected in the relatively large number of $CD44^+$ cells (50–52). Our finding of a decline in the CD44 population suggests that the IEL became less mature with TPN. The finding of large expression of CD45RB has been previously reported (53), and little change was noted in $CD45RB^+$ expression with TPN. The high expression of CD45RB is somewhat in conflict with the expression of CD44. Although CD45RB is conventionally used as a marker of maturation for peripheral CD4 cells (54, 55), it may not represent maturation in $CD8^+$ cells, particularly those in the IEL. It was interesting to note that CD62L expression was almost completely absent in the control group, but rose to 8% of the $CD8^+$ cells in the TPN group. This finding is unusual in that CD62L is typically

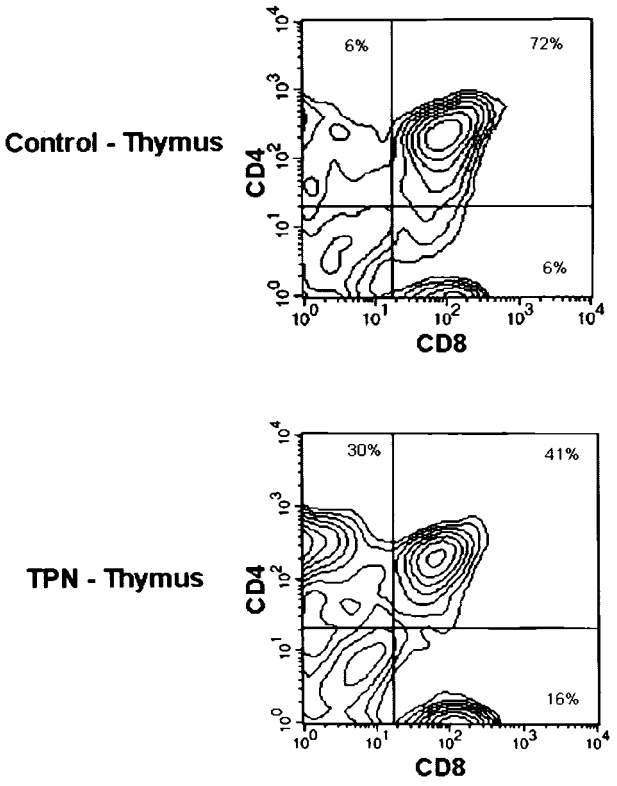

Fig 4. Distribution of $CD4^+$ and $CD8^+$ cells in the thymus of a representative control (top row) and TPN (bottom row) mouse. Note the marked decline in the number of double-positive thymocytes in the TPN group and rise in the single-positive populations.

described in peripheral lymphoid populations and is not found in the IEL. However, a similar rise in CD62L expression was recently noted by Seibold *et al* when IEL were isolated and cultured for several days *in vitro* (50). The expression of CD62L in these cultures was reversed by adding phorbol myristate acetate. This suggests that removing intraluminal factors, possibly those derived from nutrients, may remove a source of stimulation for IEL that drives them into a mature state. The decline in $CD44^+$ cells with TPN may also have other functional implications. CD44 expression is needed for activated T-cell migration, via rolling, into an inflammatory site (56). Therefore, the decline in $CD44^+$ cells with TPN may also represent a decreased requirement for T-cell recruitment into an intestine that is lacking a normal number of foreign luminal antigens. Changes in the functional characteristics of mucosal immunocytes were also seen in a report by Ganessunker et al (42). In this study, neonatal piglets showed an increase in MHC II expression, possibly an indicator of intestinal inflammation, when given TPN.

A number of factors are known to be responsible for the development and maintenance of the IEL. These include the introduction of enteric organisms into the gastrointestinal tract. A direct correlation is noted between the bacterial colonization of the intestine and the maturation of the IEL (35). Germ-free mice will fail to develop a normal IEL population (57). Introduction of bacteria leads to a rapid increase in total cell number and an expansion of the $CD4^+$ and TCR$\alpha\beta$ populations (29, 58). A second mechanism by which the IEL matures is the introduction of nutrients. The IEL has been shown to change significantly with the transition from breast milk to standard rodent chow (59). A number of studies have shown that bacterial organisms can influence IEL development (25, 29, 60). It is possible that these changes in bacterial flora may be one of the mechanisms responsible for IEL changes noted in our study. However, it appears less likely that such TPN-associated changes in the IEL are due to alterations in the microbial environment of the intestine, because colonization of the small and large bowel was not significantly different between study groups. Semiquantitative cultures, however, showed some small but interesting differences between the two groups. Interestingly, the number of *Bifidobacterium* sp. and *Lactobacillus* sp. increased in the TPN group, and the number of *Enterobacter* sp. were higher in the control group. This change was surprising to us because we did not anticipate the number of "beneficial" organism such as *Bifidobacterium* and *Lactobacillus* would increase without enteral nutrition. In fact, it has been shown that several species of *Lactobacillus* can inhibit lymphocyte proliferation, which may explain the observed loss of IEL proliferation with TPN (61). Additionally, *Lactobacillus* has been shown to increase the expression of $CD25^+$ cells in the intestine (62). Such an action may have contributed to the increase in $CD8^+$,$CD25^+$ cells seen in our TPN mice.

The thymic-dependent and thymic-independent portions of mouse IEL have been differentiated by the presence of CD8$\alpha\beta^+$ cells in the thymic-dependent and CD8$\alpha\alpha^+$ cells in the thymic-independent population (20). Each of these populations may posses different functional characteristics (12, 63, 64). One of the most intriguing results of our study was the loss of the CD8$\alpha\beta^+$ population with the removal of enteral nutrition, whereas the CD8$\alpha\alpha^+$ population (thymic-independent) did not significantly change. The precise etiology of these changes is uncertain, but it may have significant functional implications. This is particularly important as the thymic-dependent por-

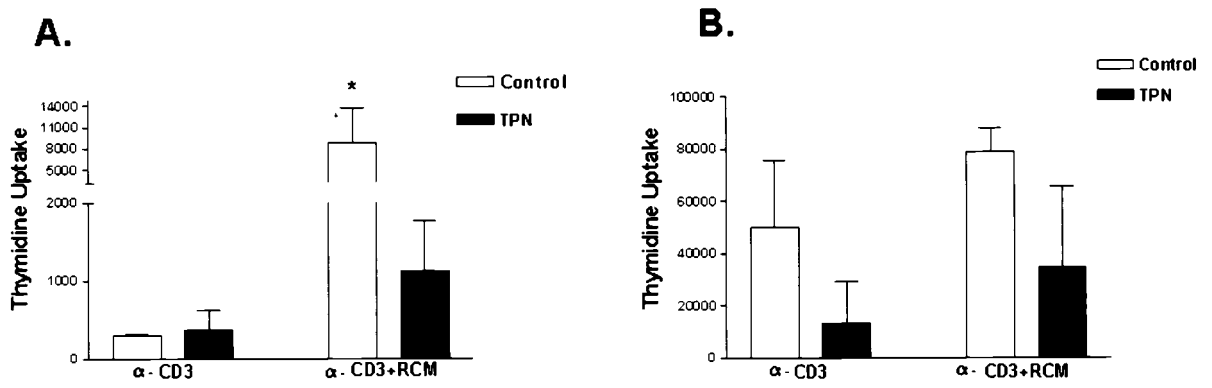

Fig 5. Proliferation results are shown. Proliferation is expressed as thymidine uptake into lymphocytes in counts per minute. Cells were stimulated with either bound anti-CD3 alone or with the addition of rat conditioned media (RCM) as a source of interleukin-2. (A) IEL; (B) splenocytes. $^*P < 0.05$ comparing proliferation before and after the addition of RCM, as well as between control and IEL groups.

tion of the IEL has been shown to be the predominant population contributing to the bulk of IEL proliferative activity (65). The thymic-independent population has been suggested to be a mediator of transforming growth factor expression and may serve to down-regulate immune responsiveness (12). Thus, this loss of the thymic-dependent population may help explain the observed decline in the proliferative response of the IEL. It was interesting to note that the Thy1$^+$ IEL population declined much less than the CD8$\alpha\beta$. Although previously thought to be a marker of thymic derivation, more recently, it appears that Thy-1 does not truly reflect the thymic-dependent portion of the IEL (66). Our finding of a loss of the thymic-dependent subpopulation is similar to the findings of Guihot, et al. In their study, they noted a decline in CD5$^+$ cells, a population that has been considered to be thymic-dependent by some investigators (67). The potential mechanisms by which these changes in the IEL population occur with TPN have not been determined. It is possible that alterations within the thymus during TPN administration might be responsible for many of these observed changes. The number of thymocytes in the TPN group significantly declined. Additionally, the large decline in the CD4,CD8 double positive thymocytes may well have a role in the loss of the thymic-derived portion of the IEL. Other factors, including failure in homing of these lymphocytes to the bowel or a loss of these cells once they have arrived in the epithelium, have not been excluded. Additionally, the fact that there was an increased incidence of septicemia in mice on TPN (68) suggested that this may lead to thymic atrophy via an apoptotic process. Ayala et al have shown that such an apoptotic process may occur during episodes of sepsis (69, 70). Our studies, however, showed that apoptosis was seen in a relatively small percentage of thymocytes (5.5–9%), and this percent did not significantly change with administration of TPN. A recent study has shown that the formation of the CD8$\alpha\beta^+$ population of the IEL requires the presence of major histocompatibility complex proteins K-b and D-b in H-2(b) mice (71). It is possible that the loss of enteral nutrition may result in an alteration in MHC expression or stimulation, which could result in a loss of this thymic-dependent population.

The marked decrease in IEL proliferation with TPN suggests that the IEL may not be as responsive to intraluminal pathogens during TPN administration. This may help explain why patients lacking enteral nutrition are at increased risk for developing systemic septicemia. It was interesting to note that the TPN group was less responsive to IL-2 stimulation compared to controls, despite an increase in IL-2 receptor expression in the CD8 population. It is possible that the concomitant loss of CD4$^+$ cells, and their known ability to proliferate greater than CD8$^+$ cells, was the predominant reason for the loss of IEL proliferation with TPN.

To truly understand the role enteral nutrition has on the development of the IEL, the complete removal of all nutrients from the enteric tract is necessary. Our current study clearly demonstrates the need for such nutrients to maintain normal IEL phenotype and function. Administration of TPN in a mouse model resulted in significant alterations in the phenotypic subpopulations of the IEL and a decline in the proliferative response. Changes in the phenotypic expression appeared to reflect a loss of the thymic-dependent population and a shift to a more immature

group of cells. Such changes may explain why patients on TPN have a higher incidence of septicemia. Potential interventions to reverse these changes may be efficacious in reducing the complications of sepsis during administration of TPN.

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