T Lymphocytes in Host Defense against Bacterial Translocation from the Gastrointestinal Tract

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Flow cytofluorometric analyses of lymphocytes harvested from the mesenteric lymph node (MLN), mucosal epithelium, and lamina propria of C57BL/6 mice demonstrate that expression of α/β or γ/δ T-cell receptors (TCR) and CD4 or CD8 molecules by T lymphocytes in the intestinal immune system varies depending upon their anatomic location. The MLN contained equivalent numbers of CD4$^+$ and CD8$^+$ T cells, the vast majority of which were α/β TCR positive (α/β TCR$^+$). The lamina propria T cells were predominantly CD4$^+$ and α/β TCR$^+$, while the intestinal intraepithelial lymphocytes consisted of equivalent numbers of α/β and γ/δ T cells, the majority of which were CD8$^+$. There were no significant changes in these T-cell phenotypic profiles when the mice were antibiotic decontaminated or monoassociated with *Escherichia coli*. Mice were depleted of CD4$^+$ T cells and/or CD8$^+$ T cells in vivo by intraperitoneal injections of monoclonal antibody GK 1.5 (rat anti-mouse CD4) and/or monoclonal antibody 2.43 (rat anti-mouse CD8). T-cell depletion was confirmed in the MLN, lamina propria, and the intestinal epithelium by flow cytometry. *E. coli* C25 translocation from the gastrointestinal (GI) tract to the MLN was significantly increased in mice depleted of CD4$^+$ T cells, CD8$^+$ T cells, or both. T-cell-deficient athymic beige/nude mice also exhibited greater levels of *E. coli* C25 translocation to the MLN than beige/het euthymic littermates. *Salmonella typhimurium* translocation also was increased following CD4$^+$ and CD8$^+$ T-cell depletion in mice monoassociated with *S. typhimurium*. Depletion of CD4$^+$ and/or CD8$^+$ T cells also increased the translocation to the MLN of certain indigenous GI flora bacteria. These results confirm that T-cell-mediated immunity is involved in the host defense against bacterial translocation from the GI tract.

Hospitalized patients often succumb to opportunistic infections originating from their own gastrointestinal (GI) tracts. A septic focus is not identified either clinically or at autopsy in greater than 30% of bacteremic patients, even among those dying of septic shock or multiple organ failure syndrome (21, 33). Tancrede and Andremont (66), however, were able to predict the bacterial serotype or biotype most likely to cause septicemia in leukemia patients by identifying in fecal surveillance cultures the numerically predominant serotype or biotype among the indigenous bacterial species. The GI tract has been demonstrated to be a reservoir for bacteria causing life-threatening infections in cancer patients undergoing chemotherapy (15, 20), bone marrow recipients (40), and those with AIDS (69, 70).

Under certain conditions, indigenous bacteria can pass through the epithelial cells lining the intestines, enter the lamina propria, and appear in the mesenteric lymph node (MLN) complex (12). From the MLN, these bacteria may then spread to other sites, such as the liver, spleen, kidneys, and blood. This passage of indigenous bacteria from the GI tract to extraintestinal sites has been termed bacterial translocation (12). It is important to note that the term translocation does not imply any particular mechanism for the movement of the bacteria from the GI tract, only that intestinal bacteria have traveled to extraintestinal sites.

Bacterial translocation, as measured by the appearance of viable bacteria in extraintestinal organs, is promoted by three major mechanisms: (i) physical disruption of the mucosal

barrier (2, 3, 22, 47, 48, 49), (ii) intestinal bacterial overgrowth (9, 10, 14, 65), or (iii) suppression of the host immune defenses (14, 52, 53). Most studies of the mechanisms promoting bacterial translocation have focused on intestinal bacterial overgrowth or physical disruption of the intestinal mucosal barrier. However, since bacterial translocation involves not only the physical passage of bacteria from the GI tract to extraintestinal sites but also the survivability of translocating bacteria in these extraintestinal sites, the host immune defenses are integral components in the bacterial translocation process.

Prior studies implicate a role for T-cell-mediated immunity in the host defense against bacterial translocation from the GI tract. Translocation of indigenous GI bacteria to the MLN, liver, spleen, and kidney occurs spontaneously in athymic (*nu/nu*) nude mice but is inhibited in heterozygous (*nu/+*) mice and in thymus-grafted nude (*nu/nu*) mice (52). Neonatal thymectomy of euthymic (+/+) mice also promotes the translocation of bacteria to these organs (53). However, Maddaus et al. (46) reported that T cells have no role in the host defense against bacterial translocation. Consequently, to further confirm the role of T cells in the host defense against bacterial translocation, we specifically depleted mice in vivo of CD4$^+$ and/or CD8$^+$ T cells by injection of monoclonal antibodies (MAbs) and tested for increased bacterial translocation.

To quantitate the efficacy of the T-cell depletion regimen, it was first necessary to characterize the normal populations of T-cell phenotypes present in the intestinal immune system (intraepithelial, lamina propria, and MLN lymphocytes). It also was necessary to determine whether the proportions of the T-cell phenotypes were affected by the manipulations of the indigenous microflora in the monoassociation model, such as antibiotic decontamination and oral challenge with a single

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microorganism. The results presented here demonstrate that the T-cell phenotypic ratios are not affected by these procedures and that CD4$^+$ and/or CD8$^+$ T-cell depletion promotes bacterial translocation from the GI tract.

MATERIALS AND METHODS

Animals. Specific-pathogen-free, female, C57BL/6NHsd mice between 8 and 12 weeks old were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, Ind.). Male nude (*nu/nu*) mice (retired breeders; Harlan) were used to produce ascites fluid from the MAb-producing hybridomas 2.43 and GK 1.5. The specific-pathogen-free mice were housed under barrier-sustained conditions and fed Purina Lab Chow no. 5001 (Ralston Purina Co., St. Louis, Mo.) and acidified water (0.001 N HCl) ad libitum. Germfree N:NIH(III)S (24, 25) beige/nude (*bg/bg nu/nu*) mice and beige/heterozygous (*bg/bg nu/+*) mice were obtained from the University of Wisconsin Gnotobiote Laboratory, Madison, and housed in Trexler-type vinyl isolators (Standard Safety Supply, Palatine, Ill.). The isolators were sterilized with ABQ sterilant (Alcide Corp., Norwalk, Conn.). The germfree mice were housed in polypropylene cages on autoclaved bedding and fed autoclavable Purina Lab Chow no. 5010 (Ralston Purina). Mice were sacrificed by cervical dislocation. The animals were maintained as described in the recommendations of the *Guide for the Care and Use of Laboratory Animals* (51a), and the experiments were approved by the Louisiana State University Medical Center (LSUMC) Animal Care Committee.

Production of anti-CD4 MAb and anti-CD8 MAb and in vivo depletion of CD4$^+$ and CD8$^+$ T cells. Nude (*nu/nu*) mice were primed with 0.2 ml of incomplete Freund's adjuvant (Sigma Chemical Co., St. Louis, Mo.) injected once intraperitoneally (i.p.). Seven days later, the mice were injected i.p. with 5 × 10^6 GK 1.5 hybridoma cells (ATCC TIB 207; rat anti-mouse CD4 [23]) or 2.43 hybridoma cells (ATCC TIB 210; rat anti-mouse CD8 [60]). Ascites fluid from each hybridoma type was pooled, delipidfied, and precipitated with ammonium sulfate (50% final concentration). The immunoglobulin-containing pellets were resuspended in phosphate-buffered saline (PBS) and dialyzed overnight at 4°C in PBS. The supernatants also were dialyzed in PBS and saved for injection of control mice. The antibody concentrations were determined by high-performance liquid chromatography (HPLC) over a protein G column. The MAb were adjusted to 1 mg/ml (for MAb 2.43) and 750 μg/ml (for MAb GK 1.5).

Mice were divided into four groups (10 mice per group), and each mouse was injected with either (i) 750 μg of MAb GK 1.5 (CD4$^+$ T-cell-depleted mice), (ii) 1 mg of MAb 2.43 (CD8$^+$ T-cell-depleted mice), (iii) both MAbs GK 1.5 and 2.43 (CD4$^+$ and CD8$^+$ T-cell-depleted mice), or (iv) 1 ml of supernatant from the NH$_4$SO$_4$ precipitation (control mice). The mice received two i.p. injections 1 week apart.

Isolation of MLN and splenic cells. Single-cell suspensions of spleen or MLN were prepared by pressing the organs through a 60-mesh stainless-steel screen and washing the cells by centrifugation in Ca^{2+}- and Mg^{2+}-free modified Hank's balanced salt solution (pH 7.2; HBSS; Sigma). Erythrocytes were removed by treatment with Tris-ammonium chloride (0.02 M Tris base and 0.14 M NH$_4$Cl in distilled H$_2$O) for 5 min in a 37°C water bath. The cells were resuspended in 15 ml of HBSS, and clumps were removed by passing the suspensions through a 5-ml plastic syringe containing loosely packed glass wool. To recover larger volumes of cell suspensions, loosely packed glass wool was used to remove clumps instead of allowing the clumps to settle out. The glass wool procedure

does not affect the composition of B cells in the suspensions (data not shown). After an additional washing, the cells were resuspended to 10^7 cells per ml in HBSS.

Isolation of intestinal intraepithelial lymphocytes and lamina propria lymphocytes. The procedure of Van der Heijden and Stok (67) was utilized in a modified form to harvest cells from the mucosal epithelium and lamina propria. The procedure of Fujihashi et al. (27) employing discontinuous Percoll density gradients was used to enrich each cell suspension for lymphocytes. The entire small intestine from the duodenum to the ileocecal junction was removed and cut into quarters, and each quarter was processed separately. The mesentery and fat were removed from each quarter, and the luminal contents were flushed with HBSS. To prevent contamination of the intestinal epithelial lymphocytes and lamina propria lymphocytes with Peyer's patch lymphocytes, the macroscopically visible Peyer's patches were excised and discarded. The intestinal quarters were sliced longitudinally and then cut into 1-cm pieces. The pieces of intestine were placed into a 50-ml polypropylene centrifuge tube containing 25 ml of HBSS with 5 mM dithiothreitol (DTT; Sigma), 2 mM EDTA (Sigma), 25 mM Tris buffer (Sigma), 100 U of penicillin G (Sigma) per ml, and 100 mg of streptomycin sulfate (Sigma) per ml (pH 7.2; HBSS-DTT-EDTA). The mixture was incubated for 15 min at 37°C in a shaking water bath (110 strokes per min). The supernatant was decanted, fresh HBSS-DTT-EDTA was added, and the incubation procedure was repeated. The supernatants containing the epithelial cells from the two incubations were pooled, and the cells were washed by centrifugation with RPMI 1640 (Sigma). The lamina propria lymphocytes were liberated from the remaining intestinal debris by placing the debris in RPMI 1640 containing 5% (vol/vol) iron-supplemented calf serum (Seru-Max 4; Sigma), 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid) buffer, 0.1 mg of DNase I (Sigma) per ml, 0.15 mg of collagenase type II (Sigma) per ml, 100 U of penicillin G per ml, and 100 mg of streptomycin sulfate (pH 7.2) per ml for 90 min in a 37°C shaking water bath.

The epithelial and lamina propria cell suspensions were each washed twice by centrifugation and subjected to discontinuous gradient centrifugation to enrich for lymphocytes. The cells were resuspended in 2 ml of 75% Percoll. The 75% Percoll was overlaid with 2 ml of 40% Percoll and then finally with 2 ml of 30% Percoll to form a discontinuous gradient. The various dilutions of Percoll were prepared from an isotonic Percoll solution consisting of 9 ml of Percoll (Sigma) and 1 ml of a 10× solution of HBSS (pH 7.2). After centrifugation at 1,500 × *g* for 20 min at 4°C, the interface between the 75 and 40% layers was removed and the cells were washed by centrifugation in 15 ml of RPMI 1640. The epithelial cells and lamina propria cells were then each resuspended in 2 ml of 40% Percoll and centrifuged at 1,500 × *g* for 20 min at 4°C. The cell pellets, enriched for lymphocytes, were collected and washed by centrifugation.

MAb staining and flow cytometry. The lymphocyte preparations from the various organs were placed in 96-well plates (10^6 cells per well) and washed in flow cytometry buffer consisting of PBS containing 2% (vol/vol) iron-supplemented calf serum and 0.1% (wt/vol) sodium azide. The cells were resuspended in 50 μl of flow cytometry buffer, and the following labeled MAbs (PharMingen, San Diego, Calif.) were used at predetermined concentrations: (i) fluorescein isothiocyanate (FITC)-labeled MAb GK 1.5 and FITC-labeled MAb RM4-4 (rat anti-mouse CD4), (ii) FITC-labeled MAb 2.43 (rat anti-mouse CD8), (iii) biotin-labeled MAb H57-597 (hamster anti-mouse α/β T-cell receptor [TCR]), (iv) biotin-labeled MAb GL3 (hamster anti-

mouse γ/δ TCR), (v) FITC-labeled MAb T-24 (rat anti-mouse Thy 1). FITC-labeled streptavidin (Biomeda, Foster City, Calif.) was added to the cells that had been reacted with biotinylated MAbs. To demonstrate that the absence of CD4$^+$ or CD8$^+$ T cells was due to actual physical depletion of T cells rather than masking of the CD4 or CD8 molecules by residual MAb from the depletion regimen, cells in one control well were reacted with FITC-labeled MAR 18.5 (mouse anti-rat κ immunoglobulin light chain [41]). The cells were resuspended in 100 μl of flow cytometry buffer and fixed with 100 μl of a 1% (wt/vol) paraformaldehyde (Sigma)-in-PBS solution (0.5% final concentration) on ice for 20 min. After fixation, the cells were washed and resuspended in 100 μl of flow cytometry buffer, and the plates were wrapped in aluminum foil and stored at 4°C until analyzed by flow cytometry in the LSUMC Core Facility for Flow Cytometry with an EPICS 753 flow cytometer and an MDADS II computer. The percentage of T cells in each preparation was determined by expression of either the α/β TCR or the γ/δ TCR. The percentage of T cells expressing a particular T-cell phenotypic marker, such as CD4 or CD8, also was calculated from the flow cytometric results.

Horseradish peroxidase permeability testing. The procedure of Rhodes and Karnovsky (57) in modified form was used to determine if MAb treatment increased intestinal permeability. Mice that had received MAb GK 1.5, MAb 2.43, both MAbs, or control supernatants were anesthetized with an i.p. injection of 0.1 ml of a 1:5 dilution of sodium pentobarbital (The Butler Co., Columbus, Ohio). The intestines were exposed, and a 2- to 3-cm section of the ileum was ligated, taking care not to disrupt the blood supply to the intestinal section. Horseradish peroxidase (0.1 ml of a 5-mg/ml solution) was injected into the intestinal lumen. After 30 min, 2.4% (wt/vol) glutaraldehyde and 0.8% (wt/vol) paraformaldehyde fixative in 0.1 M phosphate buffer (pH 7.4) were injected into the lumen. The ligated segment was removed, cut longitudinally, pinned flat into a petri dish, covered with fixative, and allowed to incubate for 1 h at room temperature. The segment then was cut into strips (1 by 3 mm) and allowed to incubate for another 3 h in fixative at room temperature. The tissue strips were placed into a 0.05% (wt/vol) solution of 3,3-diaminobenzene tetrachloride, incubated in the dark for 1 h at room temperature, and then placed into fresh 3,3-diaminobenzene tetrachloride containing freshly made 0.01% H_2O_2 and incubated in the dark for 60 min at room temperature. The tissues were washed twice in 0.1 M Tris-HCl buffer (pH 7.6) and embedded in low-viscosity embedding medium (Spurr's Kit; Electron Microscopy Sciences, Ft. Washington, Pa.). The tissue blocks were sliced with a microtome (1-μm thickness), and the slices were examined by light microscopy.

Monoassociation with *Escherichia coli* C25 or *Salmonella typhimurium* and testing for translocation. Mice were injected i.p. twice, 1 week apart, with the appropriate MAbs. Penicillin G (1,500 U/ml) and streptomycin sulfate (2 mg/ml) were placed in the drinking water ad libitum, beginning with the first MAb injection, to eliminate the indigenous GI microflora. Decontamination was confirmed by Gram staining and bacteriologic culture of the feces. At the time of the second MAb injection (7 days later), the antibiotic-decontaminated mice were monoassociated (orally challenged) with *E. coli* C25, a streptomycin-resistant strain, or *S. typhimurium*. Five ml of an overnight stirred culture of *E. coli* C25 or *S. typhimurium* in brain heart infusion (Difco Laboratories, Detroit, Mich.) was placed into the drinking water, and an additional 5 ml of the overnight culture was placed onto the food pellets. Mice monoassociated with *E. coli* C25 continued to receive strepto-

mycin in the drinking water, whereas both antibiotics were discontinued in mice monoassociated with *S. typhimurium*.

After monoassociation for 3 days with *E. coli* C25 or *S. typhimurium*, the mice were sacrificed by cervical dislocation, the MLN complex was aseptically excised, and a portion of each MLN (approximately 0.01 g) was placed in a 15-ml centrifuge tube containing HBSS for MAb staining to determine the efficacy of the T-cell depletion regimen. The remainder of each MLN was placed in a sterile, preweighed grinding tube (Glas-Col Apparatus, Terre Haute, Ind.) containing 0.5 ml of PBS, weighed, and homogenized with a glass-reinforced Teflon grinder (Glas-Col) for testing for bacterial translocation. The homogenates were plated onto two MacConkey agar plates (Difco; 0.2 ml of homogenate per plate) containing an additional 5.0 g of Bacto Agar (Difco) per ml. In the experiments with *E. coli* C25, the MacConkey agar contained 2.0 mg of streptomycin sulfate per ml. After the MLNs were removed, the spleens, livers, and kidneys were removed, placed into preweighed grinding tubes containing 5 ml of PBS, weighed, and homogenized, and 0.2 ml of each was plated onto MacConkey agar. The cecum then was removed and placed in a preweighed grinding tube containing 9.0 ml of PBS and weighed, and serial 100-fold dilutions (0.1 ml of each) were plated onto MacConkey-streptomycin agar. The identities of *E. coli* C25 and *S. typhimurium* were confirmed by the API 20E system (Analytab Products, Plainview, N.Y.).

Translocation of indigenous bacteria. The specific-pathogen-free mice tested for translocation of indigenous GI bacteria received two i.p. injections of the appropriate MAbs 1 week apart as described above but were not antibiotic decontaminated. Seven days after the second injection of MAbs, the mice were sacrificed by cervical dislocation, and the MLNs and ceca were aseptically removed and homogenized as described above. Each of the homogenates was plated (0.1 ml of homogenate per plate) onto two Trypticase soy agar plates containing 5% defibrinated sheep blood (blood agar plates; BBL Microbiology Systems, Cockeysville, Md.) to quantitate gram-positive bacteria and on two MacConkey agar plates (containing no antibiotics) to quantitate gram-negative bacteria. One-half of the plates were incubated aerobically for 24 h at 37°C, and one-half were incubated anaerobically for 48 to 72 h at 37°C in an anaerobic chamber (model 1024 anaerobic system; Forma Scientific, Marietta, Ohio). After the MLN was removed, the cecum was removed and homogenized, and serial 100-fold dilutions were plated on blood agar and MacConkey agar and incubated both aerobically and anaerobically. Gram-negative bacteria were identified by the API 20E system, and the gram-positive bacteria were identified by the LSUMC Clinical Microbiology Laboratory by using standard procedures.

Statistics. The mean numbers of translocating bacteria (CFU per gram of MLN) ± standard errors of the means (SEM), the mean cecal populations of bacteria (CFU per gram of cecum) ± SEM, and the mean numbers of lymphocytes isolated from the various organs ± SEM were compared by Student's *t* test. Probabilities of less than 0.05 were considered significant.

RESULTS

Phenotypic characterization of T cells from the intestinal epithelium, lamina propria, and MLN. T cells accounted for greater than 95% of the lymphocytes obtained from the intestinal epithelium, while B cells accounted for the remainder of the lymphocytes (data not shown). CD8$^+$ T cells accounted for approximately 80 to 90% of the T cells in the
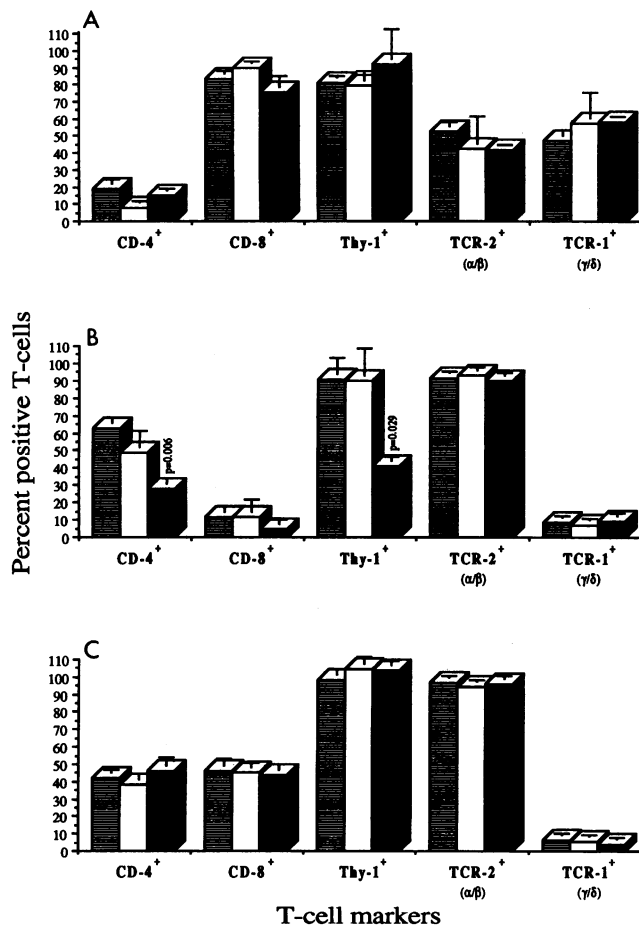
FIG. 1. Phenotypic characterization of intestinal intraepithelial T cells (A), T cells from the lamina propria (B), and T cells from the MLN (C) obtained from the intestinal immune systems of untreated (▤), antibiotic-decontaminated (□), and *E. coli* C25-monoassociated (■) C57BL/6 mice. Results are expressed as means ± SEM.

FIG. 2. Complete depletion of CD4+ (□) and CD8+ (▲) T lymphocytes by MAb GK 1.5 and MAb 2.43 from the intestinal epithelium, lamina propria, MLN, and spleen by day 14.

intestinal epithelium, while the remainder were CD4+ T cells (Fig. 1A). This ratio of CD8+ to CD4+ T cells was not significantly altered by antibiotic decontamination alone or by antibiotic decontamination followed by *E. coli* C25 monoassociation. The T cells in the intestinal epithelia of untreated mice were 52% α/β TCR positive (TCR+) and 47% γ/δ TCR+, whereas those in the intestinal epithelia of antibiotically decontaminated and *E. coli* C25-monoassociated mice were 42% α/β TCR+ and 57% γ/δ TCR+. However, these differences in T-cell phenotypes were not statistically significant.

T cells account for approximately 65% of the lymphocytes in the lamina propria, and B cells account primarily for the remainder (data not shown). Within the lamina propria, 60% of the T cells were CD4+ and 10% were CD8+ (Fig. 1B). Thus, 30% of the T cells in the lamina propria of untreated mice expressed neither CD4 nor CD8. When the mice were antibiotic decontaminated, the percentage of T cells expressing CD4 decreased from 60 to 48% (not statistically significant). However, monoassociation with *E. coli* C25 decreased the percentage of CD4+ T cells from 60 to 28% ($P = 0.006$). The percentage of CD8+ T cells (approximately 10%) in the lamina propria remained basically unchanged between untreated mice and antibiotic-decontaminated plus *E. coli* C25-monoassoci-
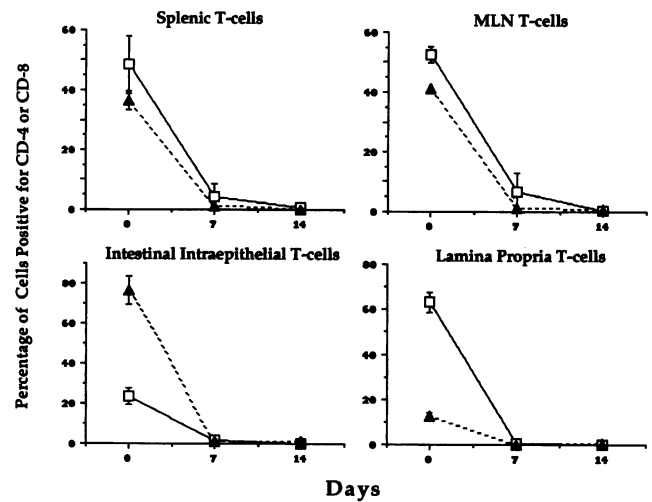
ated mice. There was a significant decrease in T cells from the lamina propria expressing Thy-1 after *E. coli* C25 monoassociation (90 to 41%; $P = 0.03$). However, the proportions of T cells in the intestinal immune system expressing the various T-cell surface markers were not changed when the mice were monoassociated with either *Proteus mirabilis* or *S. typhimurium* (data not shown).

T cells and B cells are found in roughly equivalent numbers within the MLN (data not shown). Equivalent numbers of MLN T cells from untreated mice express CD4 or CD8, but 95% of the T cells were α/β+ versus only 5% expressing γ/δ TCR (Fig. 1C). There were no significant changes in the percentages of any of the MLN T-cell phenotypes when the mice were antibiotic decontaminated or antibiotic decontaminated plus monoassociated with *E. coli* C25. Overall, antibiotic decontamination and bacterial monoassociation appear to have little, if any, effect on the phenotypic profiles of the T cells from the intestinal immune system.

Determination of the efficacy of MAbs in depleting CD4+ and CD8+ T cells from the intestinal immune system. The efficacy of the T-cell depletion regimen was tested 1 week after the first MAb injection and again 1 week after the second injection. The spleen was tested as a positive control because this T-cell depletion regimen was shown previously to deplete splenic T cells (37).

There were no significant decreases in the total number of cells isolated from the intestinal immune system in MAb-treated mice. However, one injection of MAb 2.43 (anti-CD8) completely eliminated CD8+ T cells in the spleen, MLN, lamina propria, and intestinal epithelium (Fig. 2). One injection of GK 1.5 (anti-CD4) completely eliminated CD4+ T cells from the lamina propria and intestinal epithelium and eliminated CD4+ T cells >90% from the MLN and >95% from the spleen. Two injections of MAb GK 1.5 completely eliminated CD4+ T cells in all of the organs tested.

Horseradish peroxidase was utilized as a marker to ensure that the T-cell depletion regimen did not increase the permeability of the intestinal epithelium and consequently promote bacterial translocation because of a loss of the intestinal barrier rather than because of decreased T-cell immunity. None of the groups of mice injected with MAb GK 1.5 and/or

FIG. 3. Horseradish peroxidase permeability of small-intestinal villi. (A) Villus from control mouse receiving NH_4SO_4 supernatant; (B) villus from mouse receiving MAb GK 1.5 ($CD4^+$ T-cell-depleted mouse); (C) villus from mouse receiving MAb 2.43 ($CD8^+$ T-cell-depleted mouse); (D) villus from mouse receiving both MAb GK 1.5 and MAb 2.43 ($CD4^+$ and $CD8^+$ T-cell-depleted mouse). The cells at the tips of the villi that are surrounded by horseradish peroxidase label are epithelial cells that are in the process of sloughing.

MAb 2.43 exhibited an increase in intestinal permeability to horseradish peroxidase (Fig. 3).

E. coli C25 translocation following $CD4^+$ and/or $CD8^+$ T-cell depletion. E. coli C25 translocation increased to the MLN from 208 CFU/g of MLN in control mice to 1,074 CFU/g of MLN in $CD4^+$ T-cell-depleted mice ($P = 0.001$), to 1,417 CFU/g in $CD8^+$ T-cell-depleted mice ($P = 0.001$), and to 1,013 CFU/g of MLN in $CD4^+$ plus $CD8^+$ T-cell-depleted mice ($P = 0.001$; Fig. 4, experiment 1). This experiment was repeated with similar results (Fig. 4, experiment 2). E. coli C25 translocation increased from 441 CFU/g of MLN in the control mice to 1,934 CFU/g of MLN ($P = 0.003$) in $CD4^+$ T-cell-depleted mice, to 2,503 CFU/g of MLN ($P = 0.009$) in $CD8^+$ T-cell-depleted mice, and to 1,479 CFU/g of MLN ($P = 0.005$) in $CD4^+$ and $CD8^+$ T-cell-depleted mice. Thus, $CD4^+$ and/or $CD8^+$ T-cell depletion consistently promoted E. coli C25 translocation.

In further support for the role of T cells in inhibiting translocation, N:NIH(III)S beige/nude (bg/bg nu/nu) mice also exhibited higher levels of E. coli C25 translocation than their bg/bg nu/+ (beige/het) littermates (5,219 CFU/g of MLN versus 622 CFU/g of MLN; $P = 0.001$; Fig. 5). Thus, E. coli C25 translocation is increased in both beige/nude mice and formerly immunocompetent euthymic mice depleted in vivo of T cells.

S. typhimurium translocation following $CD4^+$ plus $CD8^+$ T-cell depletion. The above results demonstrate that T-cell depletion increases the translocation from the GI tract of a relatively nonpathogenic E. coli strain. It also was of interest to determine whether T-cell depletion would increase translocation of an overt pathogen such as S. typhimurium. Mice were depleted of T cells by injection of MAb as described above. Surprisingly, less translocating S. typhimurium was cultured from the MLN of T-cell-depleted mice than from that of untreated mice (Fig. 6; $P = 0.002$). However, greater numbers of S. typhimurium bacteria translocated to the spleens and livers of the T-cell-depleted mice than to those of controls ($P < 0.03$ and $P < 0.02$, respectively).

Indigenous microflora translocation following $CD4^+$ and/or $CD8^+$ T-cell depletion. Specific-pathogen-free mice were de-pleted of $CD4^+$ T cells and/or $CD8^+$ T cells by two i.p. injections of MAb GK 1.5 and/or MAb 2.43 in the manner previously described. At 1 week after the second MAb injection, equal portions of the MLN homogenates were cultured aerobically and anaerobically. $CD4^+$ T-cell depletion promoted translocation of indigenous bacteria that were cultured aerobically or anaerobically on blood agar (Table 1). However, there was not an increase in translocating bacteria cultured aerobically or anaerobically on MacConkey agar (Table 1). The bacterial species that translocated to the MLN also were the predominant bacterial species cultured from the cecum. Hemolysin-negative Staphylococcus sp. and P. mirabilis were the predominant cecal bacteria cultured aerobically on blood agar, and Lactobacillus sp. was the predominant cecal bacterium cultured anaerobically on blood agar. Depletion of $CD8^+$ T cells promoted a smaller, although significant, increase in the translocation of Staphylococcus sp. (aerobic) and Lactobacillus sp. (anaerobic). $CD8^+$ T-cell depletion promoted the translocation of P. mirabilis. Depletion of $CD4^+$ plus $CD8^+$ T cells promoted the translocation of all bacterial species predominant in the cecum and appeared to cause synergistic increases in the translocation of P. mirabilis and Lactobacillus sp. Thus, depletion of $CD4^+$ T cells and/or $CD8^+$ T cells promoted the translocation of the predominant bacterial species of the indigenous intestinal microflora from the GI tract to the MLN.

DISCUSSION

There is substantial interest in how the immune system prevents or at least controls infections from mucosal surfaces which are normally heavily colonized by bacteria. Most attention has focused on overt pathogens such as Salmonella, Shigella, and Yersinia spp. Of particular interest in this era of AIDS and selective immunosuppressive therapies, however, is the role of T cells in the defense against opportunistic bacterial infections by members of the indigenous GI microflora. The massive populations of indigenous bacteria normally coloniz-
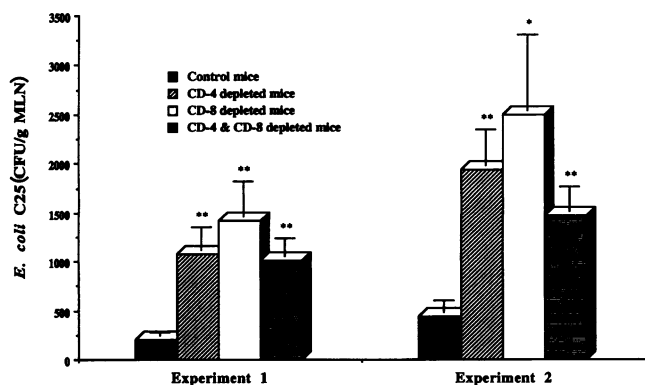

FIG. 4. T-lymphocyte depletion promotes E. coli C25 translocation to the MLN. Results are expressed as means ± SEM. *, $P < 0.01$; **, $P < 0.005$.
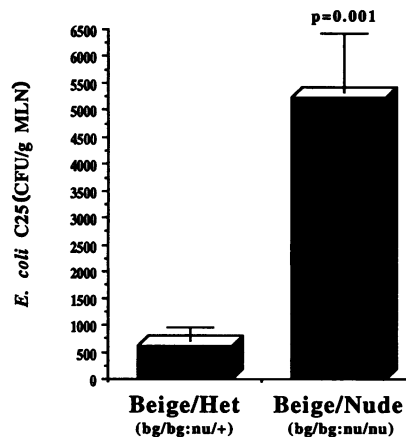

FIG. 5. E. coli C25 translocation to the MLN in gnotobiotic beige/nude mice and their beige/het littermates. Results are expressed as means ± SEM.

TABLE 1. Effect of T-lymphocyte depletion on the translocation of normal flora organisms from the GI tract to the MLN

Group	n	Cultured aerobically								Cultured anaerobically							
		MLN				Cecum				MLN				Cecum			
		Blood agar[a]		MacConkey agar[b]		Blood agar[c]		MacConkey agar[b]		Blood agar[c]		MacConkey agar[b]		Blood agar[c]		MacConkey agar[b]	
		CFU/g[d]	P[e]	CFU/g	P	CFU/g (10^9)	P	CFU/g (10^7)	P	CFU/g	P	CFU/g	P	CFU/g (10^9)	P	CFU/g (10^7)	P
Control	9	29 ± 14		0 ± 0		5.4 ± 1.4		3.2 ± 0.7		24 ± 15		0 ± 0		4.5 ± 0.5		1.9 ± 0.4	
CD4 depleted	9	1,554 ± 1,196	≤0.05	0 ± 0	NS[f]	2.5 ± 0.8	≤0.05	4.9 ± 1.8	NS	1,615 ± 1,222	≤0.05	0 ± 0	NS	6.7 ± 0.9	≤0.05	5.9 ± 1.5	≤0.01
CD8 depleted	9	489 ± 193	≤0.02	86 ± 32	≤0.01	1.6 ± 0.9	≤0.02	3.6 ± 1.8	NS	375 ± 141	≤0.02	14 ± 14	NS	7.0 ± 1.8	NS	6.3 ± 3.1	NS
CD4 and CD8 depleted	9	2,277 ± 1,279	≤0.05	354 ± 267	≤0.05	7.6 ± 3.7	NS	7.6 ± 3.7	NS	3,234 ± 1,897	≤0.05	50 ± 30	≤0.05	7.1 ± 1.7	NS	6.7 ± 4.2	NS

[a] Organisms cultured aerobically on blood agar were predominantly Staphylococcus species and P. mirabilis.
[b] P. mirabilis was the predominant organism cultured either aerobically or anaerobically on MacConkey agar.
[c] Lactobacillus species were the predominant bacteria cultured anaerobically on blood agar.
[d] Organisms translocated were measured as CFU per gram of organ (mean ± standard error of the mean).
[e] Each group was compared with the control.
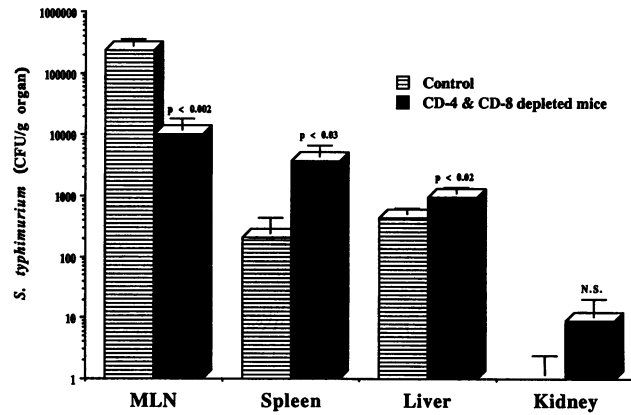[f] NS, not statistically significant.

FIG. 6. CD4+ and CD8+ T-lymphocyte depletion increases S. typhimurium translocation from the GI tract to the spleen and liver, but not the MLN. Results are expressed as means ± SEM. The x axis is a logarithmic scale.

ing the GI tract are the predominant cause of opportunistic infections in debilitated patients (15, 20, 21, 40, 66, 69, 70).

Most studies on the role of T cells in the intestinal immune system have focused on intestinal intraepithelial T cells, especially their phenotypic and in vitro functional characteristics (19, 26, 44, 59, 61). T-cell-mediated immunity is involved in the defense against intracellular pathogens such as Toxoplasma gondii (31, 55), Mycobacterium tuberculosis (38), Mycobacterium leprae (34), Listeria monocytogenes (39, 51), S. typhimurium (56), Salmonella enteritidis (1), and Brucella abortus (63). Our research has focused on the contribution of T cells in what may be the main function of the intestinal immune system, i.e., the defense of the host organism against infection by indigenous organisms normally inhabiting the gut.

Owens and Berg (52, 53) previously reported that congenitally athymic nude (nu/nu) mice exhibit spontaneous translocation of various indigenous GI bacteria from the GI tract to extraintestinal organs, especially the spleen, liver, and kidney, in comparison with their euthymic (nu/+) littermates (52). Grafting thymuses from 1- to 2-day-old nu/+ mice to adult nu/nu mice decreased the incidence of bacterial translocation in the thymus-grafted mice to that of the heterozygous (nu/+) euthymic mice. Thymectomy of neonatal euthymic (+/+) mice also promoted the translocation of indigenous GI bacteria to the MLN, spleen, liver, and kidneys when the mice were tested at 8 weeks of age (53). In the present study, E. coli C25 translocation to the MLN occurred to a greater degree in E. coli-monoassociated gnotobiotic N:NIH(III)S beige/nude (bg/bg nu/nu) mice than in E. coli-monoassociated beige/heterozygous (bg/bg nu/+) mice (Fig. 5).

Maddaus et al. (46), however, did not detect an increase in spontaneous bacterial translocation in nude (nu/nu) mice compared with that in nu/+ mice nor did they find an increase in E. coli translocation in E. coli-monoassociated nu/nu mice compared with that in E. coli-monoassociated nu/+ mice. Furthermore, Maddaus et al. did not detect increased bacterial translocation after i.p. injection of antithymocyte globulin or MAb GK 1.5 to specifically deplete CD4+ T cells. They concluded that T-cell-mediated immunity does not play a role in the host defense against bacterial translocation from the GI tract.

The CD4+ T-cell depletion regimen of Maddaus et al. (46) utilizing MAb GK 1.5 was only 75% effective in depleting T cells from the peripheral lymph nodes. In our studies, CD4+

and CD8$^+$ T cells were completely eliminated from the intestinal epithelium, lamina propria, MLN, and spleen as demonstrated by flow cytometry (Fig. 2). Because of the disparity in our previous results and those of Maddaus et al. (46), we extended our earlier studies to determine whether specific depletion of CD4$^+$ and/or CD8$^+$ T cells would increase bacterial translocation from the GI tract.

To study the effect of T-cell depletion on bacterial translocation, it was first necessary to determine if the T-cell depletion regimen employed could completely deplete T cells from the intestinal immune system (intraepithelial lymphocytes, lamina propria lymphocytes, and MLN lymphocytes). We did not test lymphocytes from the Peyer's patches. While Peyer's patches are thought to be a major sampling site for antigens encountered by the enteric route and the initiation site for immunoglobulin A mucosal immunity, they do not appear to be important in the process of bacterial translocation from the GI tract. Unpublished observations performed previously in our laboratory demonstrate that bacterial translocation from segments of the GI tract containing Peyer's patches is not increased when compared with that from segments of the GI tract without Peyer's patches.

Bandeira et al. (4) reported that germfree BALB/c mice exhibit a different proportion of γ/δ^+ T cells to α/β^+ T cells in the intestinal epithelium than conventional BALB/c mice. Lillehoj and Bacon (44) also found significant changes in the composition of T-cell subpopulations in the duodenums of chickens after infection with the intestinal protozoan *Eimeria acervulina*. These studies suggest that the state of intestinal bacterial colonization could affect the phenotypic characteristics of T cells in the intestinal immune system and introduce confounding variables in our bacterial overgrowth model. Consequently, we compared the phenotypic profiles of the T cells in the intestinal epithelium, lamina propria, and MLN in untreated mice with the T-cell profiles following oral administration of penicillin G and streptomycin sulfate and also after monoassociation of antibiotic-decontaminated mice with *E. coli* C25. In agreement with other reports in the literature (5, 16, 32, 43, 68, 71, 72), the intestinal intraepithelial lymphocytes in untreated mice were primarily CD8$^+$ T cells bearing equivalent numbers of α/β or γ/δ TCRs (Fig. 1A). Lamina propria lymphocytes were predominantly CD4$^+$ and α/β^+ T cells (Fig. 1B). The MLN lymphocytes were approximately 50% CD4$^+$ and 50% CD8$^+$ T cells bearing primarily the α/β TCR (Fig. 1C). The T-cell phenotypic profiles of untreated mice, antibiotic-decontaminated mice, and antibiotic-decontaminated mice monoassociated with *E. coli* C25, *P. mirabilis* (data not shown), and *S. typhimurium* (data not shown) were similar, indicating that manipulations of the GI microflora minimally alter the T-cell phenotypic profile in the intestinal epithelium, lamina propria, and MLN.

The T-cell depletion regimen utilized in our experiments has been demonstrated previously to deplete CD4$^+$ and/or CD8$^+$ T cells from the spleen and peripheral lymph nodes (37). However, the effect of this regimen on T-cell depletion in the MLN, lamina propria, and intestinal epithelium has not been tested. Flow cytometric analysis demonstrated complete CD4$^+$ and CD8$^+$ T-cell depletion (100%) in the intestinal epithelium, lamina propria, MLN, and spleen after two i.p. injections of MAb GK 1.5 (rat anti-mouse CD4) or MAb 2.43 (rat anti-mouse CD8). More importantly, flow cytometric analyses of the T-cell-depleted mice at the time the mice were sacrificed and tested for bacterial translocation demonstrated complete T-cell depletion in these important anatomic regions throughout the time course of the experiments.

It also was important to demonstrate that the increased bacterial translocation after T-cell depletion was not due to physical damage to the intestinal mucosa by the anti-T-cell MAbs rather than the elimination of T cells. Intestinal permeability to horseradish peroxidase was not increased following T-cell depletion, demonstrating that the anti-T-cell MAbs did not physically alter the permeability of the mucosal barrier (Fig. 3).

Depletion of CD4$^+$ T cells, CD8$^+$ T cells, or both CD4$^+$ and CD8$^+$ T cells significantly increased *E. coli* C25 translocation to the MLN (Fig. 4). The difference between the groups depleted of either CD4$^+$ or CD8$^+$ T cells alone and the groups depleted of both CD4$^+$ and CD8$^+$ T cells is not statistically significant. An important point is that depletion of both CD4$^+$ and CD8$^+$ T cells does not increase *E. coli* translocation synergistically when compared with depletion of CD4$^+$ or CD8$^+$ T cells alone. This suggests that both CD4$^+$ and CD8$^+$ T cells could interact to play a role in the host defense against *E. coli* translocation. Our results confirm that T-cell-mediated immunity is important in the host defense against *E. coli* C25 translocation from the GI tract to the MLN.

In further support of the notion that T cells are involved in the immune defense against *E. coli* C25 translocation from the GI tract, we found that N:NIH(III)S beige/nude mice monoassociated with *E. coli* C25 exhibited greater numbers of translocating *E. coli* C25 in the MLNs than gnotobiotic beige/het mice (Fig. 5). T-cell-mediated immunity has been demonstrated to be responsible for the acquired resistance to systemic candidiasis after mucosal colonization in gnotobiotic beige/nude mice (17).

Translocating *E. coli* C25 in the T-cell-depleted mice and the congenitally athymic mice did not spread systematically from the MLN to post-MLN sites such as the spleen, liver, kidney, and blood. This may be due to the fact that *E. coli* C25 is relatively nonpathogenic and perhaps the remaining components of the immune system, such as polymorphonuclear leukocytes and macrophages, appear to control *E. coli* C25 spread to post-MLN sites.

Surprisingly, T-cell depletion increased *S. typhimurium* translocation to the spleen and liver but not to the MLN. In contrast, T-cell depletion increased translocation of *E. coli* to the MLN but did not promote the spread of translocating *E. coli* from the MLN to the spleen and liver. *S. typhimurium*, an overt pathogen, is one of the most efficient translocating bacteria that we have studied to date and translocates even in immunocompetent mice (45). T-cell depletion does not cause an increase of *S. typhimurium* in the MLN, although there are higher numbers in the spleen, liver, and other sites when compared with the numbers in immunocompetent mice. The apparent decrease in *S. typhimurium* in the MLN of T-cell-depleted mice may be due to more rapid transit through the MLN. *E. coli* C25, on the other hand, is relatively nonpathogenic, and T-cell depletion allows *E. coli* C25 to translocate to the MLN but it is not pathogenic enough to spread systematically to the spleen and liver, as is *S. typhimurium*. In any case, it is interesting that T-cell depletion has different effects on the translocation of *S. typhimurium*, an intracellular pathogen, than on the translocation of *E. coli* C25, an opportunistically pathogenic microorganism.

T-cell depletion also promoted the translocation of certain members of the indigenous GI microflora from the GI tract to the MLN. The species of the indigenous flora which translocated in this study were (i) coagulase-negative *Staphylococcus* sp. (cultured aerobically on blood agar), (ii) *P. mirabilis* (cultured aerobically on MacConkey agar), and (iii) *Lactobacillus* sp. (cultured anaerobically on blood agar). These species were also the species predominantly cultured from the cecum.

Staphylococcus sp. and *Lactobacillus* sp. were present at greater than 10^9 CFU/g of cecum, and *P. mirabilis* was present at greater than 10^7 CFU/g of cecum. These bacterial species are commonly reported to translocate from the GI tract in other immunosuppression models (11–13, 52, 53).

There appeared to be a synergistic increase in the translocation of *P. mirabilis* and *Lactobacillus* sp. when the mice were depleted of both CD4$^+$ and CD8$^+$ T cells versus when mice were depleted of either CD4$^+$ T cells or CD8$^+$ T cells alone. CD4$^+$ and CD8$^+$ T cells most likely work in conjunction to inhibit bacterial translocation from the GI tract. Adoptive transfer of T-cell subsets will further delineate the roles of individual subpopulations of T cells in the immune defense against bacterial translocation.

It is not surprising that depletion of CD4$^+$ T cells alone increases bacterial translocation since CD4$^+$ T cells are well known for providing help to phagocytic cells by secreting gamma interferon (50). Somewhat more surprisingly, CD8$^+$ T-cell depletion alone also increased bacterial translocation. These results suggest that the distinct T-cell subpopulations in the different anatomic regions of the intestinal immune system may have different roles at these sites in the host defense against microorganisms exiting from the gut.

Since the majority of the T cells in the intestinal epithelium are CD8$^+$, they may be particularly important in the defense against the initial phase of bacterial translocation, i.e., during the passage of bacteria from the intestinal lumen across the mucosal epithelium to the lamina propria. Hershberg et al. (35) demonstrated that intestinal epithelial cells express the thymus leukemia antigen, a class I major histocompatibility complex-like molecule, proposed to present antigen to γ/δ T cells. Therefore, presentation of antigen by the intestinal columnar epithelial cells in a class I major histocompatibility complex-like manner might explain the predominance of CD8$^+$ T cells in the intestinal epithelium.

CD8$^+$ T cells were originally defined on the basis of their cytotoxic capabilities (62). Subsequent investigators, however, have shown that CD8$^+$ T cells can mediate other nonlytic effector functions such as secretion of tumor necrosis factor, granulocyte/macrophage colony-stimulating factor, interleukin-3, and gamma interferon (6). The secretion of one of these factors or some currently unknown factor by CD8$^+$ intraepithelial lymphocytes could possibly prevent an epithelial cell from transporting a translocating bacterium from the lumen of the intestine to the lamina propria. Indeed, Yamamoto et al. (72) have demonstrated that some γ/δ intraepithelial lymphocytes secrete gamma interferon and are not cytotoxic in response to challenge with *L. monocytogenes*. These results suggest that γ/δ intraepithelial lymphocytes perform helper rather than cytolytic functions in response to bacterial infections arising from the GI tract. Our results demonstrating that depletion of CD8$^+$ T cells promotes bacterial translocation are consistent with the results of Yamamoto et al. (72). However, the actual role of the intraepithelial lymphocytes in the host immune defense against bacterial translocation from the GI tract is not fully understood.

The intestinal lamina propria contains B cells, plasma cells, T cells, macrophages, eosinophils, and mast cells interspersed in a vascularly and lymphatically rich connective tissue (7). The majority of T cells in the lamina propria express CD4 (19, 36, 61) (Fig. 1B). CD4$^+$ T cells in the lamina propria also may aid in the immune defense against translocating bacteria by producing either interleukin-2 or -4 to help B cells produce antibody (50). Alterations in the distribution and function of the lamina propria lymphoid cells have been reported in association with intestinal diseases such as Crohn's disease

(36), AIDS, or pre-AIDS lymphadenopathy syndrome (58). We did not observe significant changes in the ratios of CD4$^+$ T cells to CD8$^+$ T cells among untreated, antibiotic-decontamined, or antibiotic-decontaminated and monoassociated mice.

The MLN is a central organ in the bacterial translocation process as well as a major organ in the intestinal immune system. The MLN is not one single lymph node but a series of lymph nodes that is more accurately called the MLN complex. Different segments of the MLN complex drain lymph from different anatomic regions of the intestine (18). Bacteria populate these different regions of the intestine at different levels. Consequently, we have demonstrated that certain indigenous bacteria translocate to the MLN in levels proportional to their regional intestinal population levels (30).

There are no differences in the phenotypic profiles of T cells isolated from the different segments of the MLN complex (unpublished observations). The lymphocytes of the MLN are about 75 to 80% T cells and 20 to 25% B cells. There are equivalent numbers of CD4$^+$ T cells and CD8$^+$ T cells, the vast majority of which bear the α/β TCR (Fig. 1C). Like CD4$^+$ T cells in the lamina propria, CD4$^+$ T cells in the MLN may provide cytokines to help B cells produce antibody (50). CD8$^+$ T cells, which constitute 50% of the T cells in the MLN, produce gamma interferon and granulocyte/macrophage colony-stimulating factor, which activate phagocytic cells (6, 50).

The mechanisms whereby indigenous bacteria such as *E. coli* or nonindigenous bacteria such as *S. typhimurium* translocate from the GI tract are not fully understood. The MLN is the first organ that can be cultured reliably in the pathway of bacteria translocating from the GI lumen to extraintestinal sites. Consequently, survival of translocating bacteria en route to the MLN and survival in situ in the MLN are all part of the translocation process. In fact, very low numbers of various species of indigenous bacteria are most likely continuously translocating to the MLN in the normal mouse, but the intact host immune system is able to kill these low numbers of translocating bacteria, and MLN cultures remain sterile.

A direct relationship between the population levels of indigenous bacteria in the ileum and cecum and their translocation rate to the MLN has been established (64). In fact, translocation ceases within 24 h after the cecal population levels of *E. coli* are reduced to below 10^8 CFU/g (8). Therefore, indigenous bacteria, such as *E. coli*, must reach a population level that enables them to come in close association with the intestinal epithelium and then be engulfed by the intestinal epithelial cells, which are nonprofessional phagocytes. Therefore, the translocation process, at least for opportunistically pathogenic bacteria, may be a passive process on the part of the bacterium. In fact, nonviable particles, such as latex, also are able to translocate from the GI lumen to extraintestinal sites (42, 54).

Since T cells do not kill bacteria by themselves, the ultimate effector cells are most likely phagocytic cells such as macrophages. Previously, we found that *E. coli*, *P. mirabilis*, and *Enterobacter cloacae* translocation to the MLN is reduced in mice injected with formalin-killed *Propionibacterium acnes*, a nonspecific macrophage activator (28). *E. coli* C25 translocation also is reduced in mice adoptively transferred with *P. acnes*-stimulated macrophages (29). CD4$^+$ T cells mediate their effector functions by secreting lymphokines, while CD8$^+$ T cells mediate their effector functions by either direct cell-to-cell contact or by secreting lymphokines. Quantitation of the types of lymphokines produced by CD4$^+$ or CD8$^+$ T cells from the intestinal epithelium, lamina propria, and MLN will provide additional insights into the types of effector mechanisms

used by the immune system to control bacterial translocation. A better understanding of the role of T lymphocytes in the defense against bacterial translocation is necessary to devise immunotherapeutic strategies to control systemic infection by opportunistic indigenous GI microflora.

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