Am. J. Trop. Med. Hyg., 50(2), 1994, pp. 145–151
Copyright © 1994 by The American Society of Tropical Medicine and Hygiene

ENUMERATION OF SELECTED LEUKOCYTES IN THE SMALL INTESTINE OF BALB/c MICE INFECTED WITH *CRYPTOSPORIDIUM PARVUM*

Y. BOHER, I. PEREZ-SCHAEL, G. CACERES-DITTMAR, G. URBINA, R. GONZALEZ, G. KRAAL, AND F. J. TAPIA

Instituto de Biomedicina, Universidad Central de Venezuela, Caracas, Venezuela; Department of Histology, Free University of Amsterdam, Amsterdam, The Netherlands

Abstract. Neonatal, suckling BALB/c mice inoculated with *Cryptosporidium parvum* produce an infection characterized by continuous shedding of oocysts that spontaneously clears by the time the animals are three weeks of age. Neonatal mice were used to characterize the leukocyte subgroups present in Peyer's patches from the ileum and jejunum of *Cryptosporidium*-infected and healthy mice. After infection, ileal Peyer's patches showed a predominant CD8+ response, with abundant monocytes-macrophages (MOMA-2+) and nonlymphoid dendritic cells (NLDC-145+ cells). In contrast, jejunal Peyer's patches showed more T lymphocytes than ileal patches, with a predominance of CD4+ cells and many dendritic NLDC-145+ cells and MOMA-2+ cells. The present results showed that ileal and jejunal Peyer's patches are functionally different in response to *Cryptosporidium* parasites. These findings suggest a preferential involvement of jejunal Peyer's patches in T cell-dependent immunity against the parasite, whereas ileal patches may be associated with B cell expansion and maturation.

Cryptosporidium is a protozoan parasite that causes diarrheal disease in both nonhuman animals and humans.[1–3] *Cryptosporidium* infection may be present in both immunocompetent and immunodeficient hosts with differences in the outcome of the disease in these two groups.[4–6] In healthy individuals, cryptosporidial infection is self-limited, whereas immunocompromised patients may recover from the infection after restoration of their immune functions.[7,8] These results are highly suggestive that active immunity is necessary for resistance to the parasite.[7] In addition, Ungar and others have shown a T cell involvement in mice infected with *C. parvum*.[8,9]

Antigens that enter the body through the gastrointestinal tract are dealt with by the gut immune system, which includes the Peyer's patches, appendix, tonsils, adenoids, and colonic patches.[10,11] The Peyer's patches are the major sites of antigen sampling and presentation, where intestinal antigens may be directly sampled by membranous (M) cells and intra-epithelial lymphocytes and transported to the T- and B-cell areas of the patch. In these areas, the M cells and the intra-epithelial lymphocytes interact with macrophages, dendritic cells, T cells, and B cells.[11–13] In *Cryptosporidium* infection, parasites have been described within the cytoplasm of M cells and macrophages.[14]

Neonatal, suckling BALB/c mice inoculated with *Cryptosporidium parvum* oocysts develop a patent infection characterized by continuous shedding of oocysts and histologic evidence of parasitism that is spontaneously cleared by the time the animals are three weeks of age.[14–16] The aim of the present study was to characterize the different leukocyte immunophenotypes present in the Peyer's patches of neonatal mice infected with *C. parvum*.

MATERIALS AND METHODS

Cryptosporidium *oocyst preparation*

Cryptosporidium parvum oocysts were purified from the feces of infected children (isolate DC 624). The stool specimens were suspended in 0.1 M phosphate buffered saline (PBS), pH 7.4, and the oocysts were concentrated by centrifugation in Sheather's sucrose solution.[17] The purified oocysts were suspended in 2.5% potassium dichromate containing 0.25 mg/ml of chloramphenicol. Samples were stored at 4°C for up to 40 days before infection of the animals.

Animal infection and tissue processing

Two groups of seven day old BALB/c mice of both sexes were used. The first group con-

sisted of six healthy mice and the second group consisted of six mice that were orally infected with 1.5×10^5 oocysts using a gastric probe. Before inoculation, neonatal mice were checked for the absence of *C. parvum* parasites by serial fecal examinations, using a modified Ziehl-Neelson stain.[18] After inoculation, each experimental group was kept separated. The litters were housed with their dams during the period of evaluation.

Fecal samples from each mouse were examined for *C. parvum* oocysts every other day during the experiment. At 4, 10, and 16 days after the inoculation with *C. parvum*, two mice were killed with ether. The days were selected based on previous studies showing that oocyst shedding begins at day 4, decreases at day 13, and clears at day 21.[14-16] In each mouse using a stereomicroscope, two Peyer's patches from the ileum and two from the jejunum were excised, washed in PBS, cut into pieces, immersed in OCT compound (Miles Laboratories, Elkhart, IN), and frozen in liquid nitrogen. The evaluation of jejunal Peyer's patches was carried out only on the fourth and 16th days after infection.

Monoclonal antibodies

All monoclonal antibodies used were diluted in modified PBS, pH 7.2.[19] These antibodies recognized the following leukocyte markers: CD4 (GK 1.5, T helper-inducer cells at a dilution of 1:50) and CD8 (Lyt-2, T suppressor cytotoxic cells at a dilution of 1:50) (kindly donated by M. Pierres, Centre Immunologie de Marseille, Luminy, France), Thy-1.2 (30H12 at a dilution of 1:50), nonlymphoid dendritic cells (NLDC-145 dendritic cells) at a dilution of 1:50), and monocytes-macrophages (MOMA-2 at a dilution of 1:50).[20,21]

Immunostaining procedure

Frozen sections (7 um) were cut with a cryostat and air-dried overnight before the immunostaining procedure. Sections were immunostained as previously described.[22-24] Briefly, after fixation in fresh acetone for 5 min, the samples were hydrated in PBS and sequentially incubated with primary rat monoclonal antibody for 90 min, biotinylated sheep anti-rat IgG (Vector Laboratories, Burlingame, CA) at a dilution of 1:60 (50 μg/ml) for 45 min, and streptavidin-horseradish peroxidase conjugate (Gibco-BRL, Gaithersburg, MD) at a dilution of 1:300 for 30 min. Five-minute washes with PBS were done between incubations. The reactions were developed for 10 min with 90 μM H_2O_2 and 3-amino-9-ethyl-carbazole (final concentration 0.88 mM), which was dissolved in 50 mM N,N-dimethylformamide in 0.1 M acetate buffer, pH 5.2. The sections were then washed, counterstained with Meyer's hematoxylin, and mounted on glass slides with glycerin gelatin. Controls consisted of sections in which the primary antibody was omitted or the use of a monoclonal antibody of irrelevant specificity at the same protein concentration.

Leukocyte quantification

Cell counting was carried out using a light microscope with a millimeter scale (Zeiss, Wetzlar, Germany) calibrated to determine the number of cell/mm^2 in Peyer's patches. Based on their distribution, dendritic and T cells were counted only in the interfollicular area of the Peyer's patches, whereas macrophages were randomly quantified throughout the patches.

Only cells showing red immunostaining with a visible nucleus were counted as positive. Twenty to thirty fields were examined for each cell marker at a magnification of 1,000×. There were approximately 27,000 cells/mm^2 of Peyer's patches, according to a previous count of the nucleated cells in hematoxylin and eosin–stained sections.

A percentage increment was calculated between the values for healthy and *Cryptosporidium*-infected mice for each particular cell marker.

Statistical analysis

Results are expressed as the mean ± SEM. Comparisons between groups were made with the nonparametric Mann-Whitney test. Any P value less than 0.05 was considered significant.

RESULTS

Cryptosporidium parvum *infection in neonatal mice*

Cryptosporidium parvum–infected neonatal mice shedded oocysts during the first 10 days of infection. In addition, histologic analysis of the

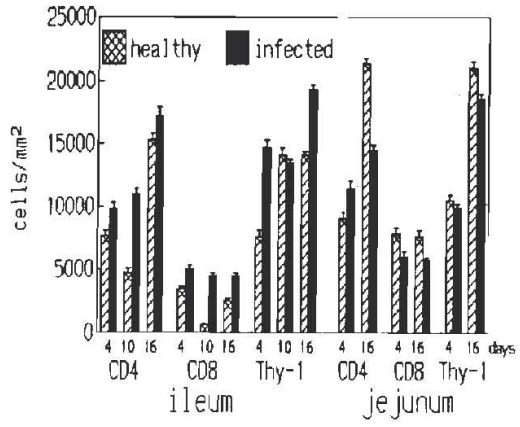

FIGURE 1. Density of lymphocyte immunophenotypes in ileal and jejunal Peyer's patches of healthy and *Cryptosporidium parvum*–infected neonatal mice. Bars show the mean and SEM.

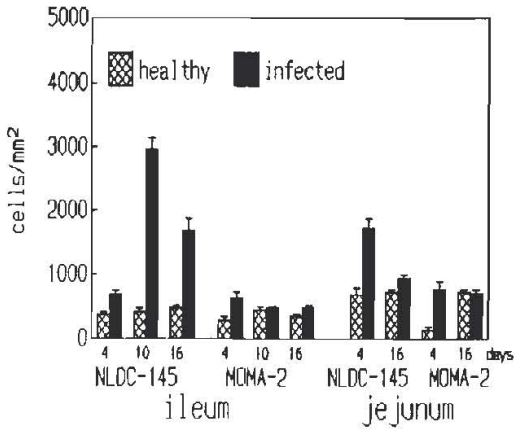

FIGURE 2. Density of nonlymphoid dendritic cells (NLDC-145+) and monocytes-macrophages (MOMA-2+) in ileal and jejunal Peyer's patches of healthy and *Cryptosporidium parvum*–infected neonatal mice. Bars show the mean and SEM.

gut showed various parasite stages in the epithelial cells of the villi, and inside the ileal and jejunal Peyer's patches. Histologically, very few parasites were observed on the 16th day after infection.

Leukocyte immunophenotypes in ileal Peyer's patches of healthy and C. parvum*–infected mice*

The infection of neonatal BALB/c mice with *C. parvum* induced the proliferation of T helper-inducer CD4+ cells, T suppressor-cytotoxic CD8+ cells, Thy-1.2+ cells, dendritic NLDC-145+ cells, and MOMA-2+ cells in the ileal Peyer's patches (Figures 1 and 2, and Table 1). The CD4+, CD8+, Thy-1.2+, and NLDC-145+ cells were abundant in the interfollicular area, and scarce in the dome area and the epithelium. The MOMA-2+ cells were present in the entire Peyer's patches. These cellular increases were significantly higher ($P \leq 0.05$) for the infected mice on days 4, 10, and 16 after infection, except for Thy-1.2+ and MOMA-2+ cells, which showed values similar to those found in healthy neonatal mice on day 10 (Table 1 and Figure 2). The maximal increments in the infected mice were observed for CD8+ T cells and dendritic NLDC-145+ cells on day 10 after infection (Table 1).

In healthy neonatal mice, the density of T helper-inducer CD4+ and T suppressor-cytotoxic CD8+ cells varied significantly ($P \leq 0.05$) on the three experimental days. On day 10, CD4+ cells decreased to half the values on day 4, and showed a two-fold increase on day 16 (Figure 2 and Table 1). The CD8+ values decreased markedly between days 4 and 10, but increased again on day 16 (Figure 1 and Table 1). The Thy-1.2+ cells increased significantly between days 4 and 10, and showed similar values on day 16 (Figure 2 and Table 1). The densities of dendritic NLDC-145+ cells were very similar on the three experimental days, and MOMA-2+ cells increased significantly ($P \leq 0.05$) between days 4 and 10, and showed similar values on day 16 (Figure 2 and Table 1).

In infected neonatal mice, T helper-inducer CD4+ cells increased during the evaluation period, whereas T suppressor-cytotoxic CD8+ cells decreased slightly (Figure 1 and Table 1). The Thy-1.2 values were similar between days 4 and 10, but increased significantly ($P \leq 0.05$) on day 16 (Figure 1 and Table 1). Dendritic NLDC-145+ cells increased significantly ($P \leq 0.05$) between days 4 and 10, and decreased ($P \leq 0.05$) on day 16 (Figure 2 and Table 1). In contrast, the density of MOMA-2+ cells was very similar on the three experimental days (Figure 2 and Table 1).

Leukocyte immunophenotypes in jejunal Peyer's patches of healthy and C. parvum*–infected mice*

The evaluation of the jejunum was carried out only on days 4 and 16. The results showed a

TABLE 1

Immunocompetent cell densities in Peyer's patches from the ileum of healthy and Cryptosporidium parvum*–infected neonatal BALB/c mice**

Phenotype	Day 4			Day 10			Day 16		
	Healthy	Infected	% increment	Healthy	Infected	% increment	Healthy	Infected	% increment
CD4	7,745 ± 357	9,792 ± 502	26	4,687 ± 371	10,937 ± 530	133	15,302 ± 511	17,180 ± 730	12
CD8	3,474 ± 181	5,016 ± 328	44	620 ± 84	4,505 ± 148	627	2,500 ± 186	4,437 ± 278	77
Thy-1.2	7,646 ± 462	14,693 ± 602	92	14,083 ± 629	13,385 ± 314	−5	14,047 ± 338	19,250 ± 433	37
NLDC-145	375 ± 43	677 ± 80	80	417 ± 53	2,948 ± 193	607	484 ± 26	1,694 ± 176	250
MOMA-2	297 ± 47	641 ± 98	116	448 ± 49	474 ± 34	6	359 ± 40	482 ± 40	34

* Density values are the mean ± SEM cells/mm². $P \leq 0.05$ for healthy versus infected mice (except for Thy-1.2 and MOMA-2) on days 4 and 10 and for healthy versus infected mice on day 16. NLDC = nonlymphoi dendritic cells; MOMA = monocytes-macrophages.

different response pattern to the parasite between the ileal and jejunal Peyer's patches. Jejunal Peyer's patches have a higher T cell count than ileal patches. In the jejunum, only T helper-inducer CD4+ cells, dendritic NLDC-145+ cells, and MOMA-2+ cells increased on day 4 after infection (Figures 1 and 2, and Table 2). In contrast, the only cell subgroup that increased on day 16 was the NLDC-145+ cells (Figures 1 and 2, and Table 2). The maximal increment in the infected mice was observed for the MOMA-2+ cells on day 4 (Table 2).

In healthy neonatal mice, T helper-inducer CD4+ cells, Thy-1.2+ cells, and MOMA-2+ cells increased between days 4 and 16 (Figures 1 and 2, and Table 2). The T suppressor-cytotoxic CD8+ cells and dendritic NLDC-145+ cells had similar values on the two days of evaluation (Figures 1 and 2, and Table 2).

In infected neonatal mice, CD4+, CD8+, and Thy-1.2+ cells showed the same increment pattern, except that there were more dendritic NLDC-145+ cells on day 4 than on day 16, and that MOMA-2+ values were very similar on both days (Figures 1 and 2, and Table 2).

Differences in leukocyte immunophenotypes between ileal and jejunal Peyer's patches

The comparison between ileal and jejunal Peyer's patches was carried out on days 4 and 16. In healthy neonatal mice, jejunal Peyer's patches contain more leukocytes than ileal patches. Only MOMA-2+ cells were more abundant in the ileal patches on day 4. These differences were statistically significant ($P \leq 0.05$) for each of the cell subgroups analyzed on both experimental days.

A similar pattern was observed in infected, neonatal BALB/c mice, where most cell subgroups were significantly higher ($P \leq 0.05$) in the jejunal than in the ileal patches. No statistically significant differences were observed between ileal and jejunal CD8+ and MOMA-2+ subsets on day 4. In the ileal patches, only CD4+ and NLDC-145+ cells were present in a higher ($P \leq 0.05$) proportion than in the jejunum on day 16.

DISCUSSION

Increasing evidence suggests the importance of the gut immune system in the control of

TABLE 2

Immunocompetent cell densities in Peyer's patches from the jejunum of healthy and Cryptosporidium parvum*–infected neonatal BALB/c mice**

	Day 4			Day 16		
Phenotype	Healthy	Infected	% increment	Healthy	Infected	% increment
CD4	9,078 ± 474	11,469 + 602	26	21,385 + 423	14,437 + 475	33
CD8	7,859 ± 465	5,963 ± 441	−24	7,600 ± 495	5,691 ± 214	−25
Thy-1.2	10,479 ± 490	9,885 ± 344	−6	21,069 ± 492	18,562 ± 394	12
NLDC-145	677 ± 115	1,729 ± 155	155	739 ± 41	947 ± 58	29
MOMA-2	141 ± 40	771 ± 123	450	735 ± 37	711 ± 64	−3

* Density values are the mean + SEM cells/mm². $P < 0.05$ for healthy versus infected mice (except for Thy-1.2) on day 4 and for healthy versus infected mice (except for MOMA-2) on day 16. NLDC = nonlymphoid dendritic cells; MOMA = monocytes macrophages.

Cryptosporidium infection.[8,9] In the present study, we have shown that during murine infection with *C. parvum*, immunocompetent cells from the Peyer's patches proliferate in response to the parasite. On day 4, we observed an increase in the numbers of leukocytes in the ileal Peyer's patches of infected mice. The T suppressor-cytotoxic CD8+ cells and Thy-1.2+ cells showed a greater increase than T helper-inducer CD4+ cells. The numbers of MOMA-2+ cells and dendritic NLDC-145+ cells also increased significantly. On day 10, all cell groups increased except Thy-1.2+ cells, with CD8+ and NLDC-145+ cells undergoing a seven-fold increase. Parasite clearance was observed on day 16, when leukocyte numbers were still high but lower than those observed on day 4. The cell pattern in jejunal Peyer's patches showed a predominance of CD4+, NLDC-145+, and MOMA-2+ cells at day 4. The latter underwent a five-fold increase from control values. The finding that jejunal Peyer's patches have a greater T cell count than ileal patches may suggest a preferential involvement of the former in T cell-dependent immunity.

The present results suggest that ileal and jejunal Peyer's patches are functionally different in response to *Cryptosporidium* parasites. Previous studies, based on morphologic, developmental, and physiologic differences, have suggested that the ileal Peyer's patches are equivalent to the avian bursa of Fabricius, whereas the jejunal Peyer's patches are more involved with generating intestinal immune responses.[24] Our observations suggest that in neonatal mice, the effector phase of the immune response to *Cryptosporidium* parasites may occur in the jejunal Peyer's patches, which are characterized by an abundance of T helper-inducer cells and antigen-presenting cells. The low numbers of CD8+ T cells seen in the jejunal patches are consistent with previous observations for other antigens.[25,26] This may be the result of a selective migration to the mesenteric lymph nodes and thoracic duct, from where they migrate to the lamina propia and intestinal epithelium. The marked increase of CD4+ cells in healthy and infected ileal Peyer's patches at day 16 may indicate an active participation of these cells in inducing B cells to produce antigen-specific antibodies. In *C. parvum*–infected mice, parasite-specific antibodies are also added to the lymphocyte pool. In contrast, ileal Peyer's patches may be the site for the immunostimulatory phase of the immune response, where naive T cells are primed by the large numbers of dendritic NLDC-145+ cells. In the patches, naive T cells become memory T cells capable of migrating to other parts of the gut immune system.

The present study supports the increasing evidence that cell-mediated immunity participates in the control of *Cryptosporidium* infection. The findings of Ungar and others have clarified the role of CD4+ and CD8+ T cells in protective immunity against the parasite.[8,9] These studies have also shown that CD4+ T cells are very important during the initial phases of gut maturation, but once they are fully developed, other cell groups may be involved in protection. These conclusions were drawn from the observations that adult mice previously infected with *C. parvum* and treated with anti-CD4 resulted in mild infections, but previously infected athymic adult mice and previously infected neonatal mice treated with anti-CD4 generated severe infections.

Previous investigations have shown striking

differences in the local microenviroment of the ileum and the jejunum.[26] Similarly, variations in the microenviroment may explain the differences in cell patterns observed in the present study in healthy neonatal mice. A parallel study has shown a predominance of anaerobic bacteria in the ileum, which may contribute to the development of a particular type of immune response (Boher Y and others, unpublished data). Recent evidence has shown the influence of the indigenous microflora on the development of intestinal immune responses.[27, 28]

Further studies will be required to clarify the role of intestinal bacteria in modulating the gut immune system. Gnotobiotic mice will be good models in which to carry out experiments with selected species of bacteria, and will allow the evaluation of their effect during the immune response. In addition, the participation of B cells and other cell groups from the Peyer's patches in the immunoregulatory mechanisms associated with *Cryptosporidium* infection need to be elucidated.

Acknowledgment: We are grateful to Marian Ulrich for reading and commenting on the manuscript.

Financial support: This research was supported by Consejo Nacional de Investigaciones Cientificas y Tecnologicas grant S1-2107 to I. Perez-Schael. Part of this study was presented in Paris at the VII International Congress of Parasitology, August 1990, with financial support from Consejo de Desarrollo Cientifico y Humanistico-Universidad Central de Venezuela, Asociacion Civil para el Estudio de Enfermedades de la Infancia, and Fundacion Polar to F. J. Tapia.

Authors' addresses: Y. Boher, I. Perez-Schael, G. Caceres-Dittmar, G. Urbina, R. Gonzalez, and F. J. Tapia, Laboratorio de Biologia Molecular, Instituto de Biomedicina, Universidad Central de Venezuela, Apartado 4043, Caracas 1010A, Venezuela. G. Kraal, Department of Histology, Free University of Amsterdam, Amsterdam, The Netherlands.

REFERERNCES

1. Fayer R, Ungar LP, 1986. Cryptosporidium spp. and Cryptosporidiosis. *Microbiol Rev 50:* 458–483.
2. Current WL, Bick PH, 1989. Immunobiology of Cryptosporidium spp. *Pathol Immunopathol Res 8:* 141–160.
3. Casemore DP, 1990. Epidemiological aspects of human Cryptosporidiosis. *Epidemiol Infect 104:* 1–28.
4. Perez-Schael I, Boher Y, Mata L, Perez M, Tapia FJ, 1985. Cryptosporidiosis in Venezuelan children with acute diarrhea. *Am J Trop Med Hyg 34:* 721–722.
5. Ungar BLP, Soare R, Fayer R, Nash TE, 1986. Enzyme immunoassay detection of immunoglobulin M and G antibodies to Cryptosporidium in immunocompetent and immunocompromised persons. *J Infect Dis 153:* 570–578.
6. Ungar BLP, Gilman RM, Lanata CF, Perez-Schael I, 1988. Seroepidemiology of Cryptosporidium infection in two Latin American populations. *J Infect Dis 157:* 551–556.
7. Miller RA, Holmberg RE, Clausen CR, 1983. Life-threatening diarrhea caused by Cryptosporidium in a child undergoing therapy for acute lymphocytic leukemic. *J Pediatr 103:* 256–259.
8. Ungar BLP, Burris JA, Quinn CA, Finkelman FD, 1990. New mouse models for chronic Cryptosporidium infection in immunodefficient hosts. *Infect Immun 58:* 961–969.
9. Ungar BLP, Kao T-C, Burris JA, Finkelman FO, 1991. Cryptosporidium infection in an adult mouse model. Independent roles for IFN-γ and CD4+ T lymphocytes in protective immunity. *J Immunol 147:* 1014–1022.
10. Clancy J Jr, Klein R, 1989. Development of the gastrointestinal tract cellular immunity. Lebenthal, E. ed. *Human Gastrointestinal Development.* New York: Raven Press, 699–708.
11. Owen RL, Ermak TH, 1990. Structural specializations for antigen uptake and processing in the digestive tract. *Springer Semin Immunopathol 12:* 139–152.
12. Owen RL, Jones AL, 1974. Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. *Gastroenterology 65:* 189–203.
13. Marcial MA, Madara JL, 1986. Cryptosporidium: cellular localization, structural analysis of absorptive cell-parasite membrane-membrane interactions in guinea pigs, and suggestion of protozoan transport by M cells. *Gastroenterology 90:* 583–594.
14. Heine JH, Moon W, Woodmansee DB, 1984. Persistent Cryptosporidium infection in congenitally athymic (nude) mice. *Infect Immun 43:* 856–859.
15. Current WL, Reese NC, 1986. A comparison of endogenous development of three isolates of Cryptosporidium in suckling mice. *J Protozool 33:* 98–108.
16. Ernst JA, Blagburn BL, Lindsay DS, 1986. Infection dynamics of Cryptosporidium parvum in neonatal mice. *J Parasitol 72:* 796–798.
17. Current WL, Reese NC, Ernst JV, Bailey WB, Heyman MB, Weinstein, WM, 1983. Human cryptosporidiosis in immunocompetent and immunodeficient persons. *N Engl J Med 308:* 1252–1257.
18. Henriksen SA, Pohlenz JF, 1981. Staining of cryptosporidia by a modified Ziehl-Neelson technique. *Acta Vet Scand 22:* 594–596.
19. Hofman FM, Billing RJ, Parker JW, Taylor CR, 1982. Cytoplasmic as opposed to surface Ia antigens expressed on human peripheral blood lymphocytes and monocytes. *Clin Exp Immunol 49:* 355–363.

20. Kraal G, Breel M, Janse M, Bruin G, 1986. Langerhans cells, veiled cells and interdigitating cells in the mouse recognized by a monoclonal antibody. *J Exp Med 163:* 981–997.

21. Kraal G, Rep M, Janse M, 1987. Macrophages in T and B cell compartments and other tissue macrophages recognized in monoclonal antibody MOMA-2. *Scand J Immunol 26:* 653–661.

22. Tapia FJ, Rojas E, Kraal G, Mosca W, Convit J, 1988. Immunocytochemical analysis of Langerhans cells in murine cutaneous leishmaniasis. Thivolet J, Schmit D, eds. *The Langerhans Cell.* London: Colloque INSERM/John Libbey Eurotex Ltd., 479–490.

23. Gross A, Weiss E, Tapia FJ, Aranzazu N, Gallinoto ME, Convit J, 1988. Leukocyte subsets in the granulomatous response produced after inoculation with Mycobacterium leprae-BCG in lepromatous patients. *Am J Trop Med Hyg 38:* 608–612.

24. Reynolds JD, 1987. Maturation of the mitotic rate in Peyer's patches of the fetal sheep and in the bursa of Fabricius of the chick embryo. *Eur J Immunol 17:* 503–506.

25. Klein JR, Lefrancois L, Kagnoff MF, 1985. A murine cytotoxic T lymphocyte clone from the intestinal mucosa that is antigen specific for proliferation and displays broadly reactive inducible cytotoxic activity. *J Immunol 135:* 3697–3703.

26. Reynolds JD, Kirk D, 1989. Two types of sheep Peyer's patches: location along gut does not influence involution. *Immunology 66:* 308–311.

27. Van der Waaij D, 1988. Evidence of immunoregulation of the composition of intestinal microflora and its practical consequences. *Eur J Clin Microbiol 7:* 103–106.

28. Roszkowski Ko HL, Beuth J, Ohshima Y, Roszkowski W, Jeljaszewicz J, Pulverer G, 1988. Intestinal microflora of BALB/c mice and function of local immune cells. *Zentralbl Bakt Hyg 270:* 270–279.