American Journal of Pathology, Vol. 142, No. 6, June 1993

Simian Immunodeficiency Virus Infection of the Gastrointestinal Tract of Rhesus Macaques

Functional, Pathological, and Morphological Changes

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Gastrointestinal dysfunction and wasting are frequent complications of human immunodeficiency virus (HIV) infection. Nutrient malabsorption, decreased digestive enzymes and HIV transcripts have been documented in jejunal mucosa of HIV-infected patients; however, the pathogenesis of this enteropathy is not understood. Rhesus macaques infected with simian immunodeficiency virus (SIV) also exhibit diarrhea and weight loss; therefore, we investigated the use of this animal model to study HIV-associated intestinal abnormalities. A retrospective study of intestinal tissues from 15 SIV-infected macaques was performed to determine the cellular targets of the virus and examine the effect of SIV infection on jejunal mucosal morphology and function. Pathological and morphological changes included inflammatory infiltrates, villus blunting, and crypt hyperplasia. SIV-infected cells were detected by in situ hybridization in stomach, duodenum, jejunum, ileum, cecum, and colon. Using combined immunohistochemistry and in situ hybridization, the cellular targets were identified as T lymphocytes and macrophages. The jejunum of SIV-infected animals had depressed digestive enzyme activities and abnormal morphometry, suggestive of a maturational defect in proliferating epithelial cells. Our results suggest that SIV infection of mononuclear inflammatory cells in intestinal mucosa may alter development and function of absorptive epithelial cells and lead to jejunal dysfunction. (Am J Pathol 1993, 142: 1759–1771)

Intestinal malabsorption, diarrhea, and wasting are frequent manifestations of human immunodeficiency virus (HIV) infection and may occur in early stages of the disease.[1–4] Although a wide range of opportunistic enteric pathogens contribute to intestinal dysfunction in HIV infection, opportunists are not identified in all patients.[5,6] HIV enteropathy, proposed as a diagnosis for HIV-infected patients having diarrhea without detectable opportunistic enteropathogens,[1] is associated with the presence of HIV-infected cells in the intestinal mucosa, alterations in cell populations of the mucosal immune system, and changes in the structure and function of mucosal epithelium.[1,3,7,8] Intestinal biopsies from HIV-infected patients showed nonspecific pathological changes, including acute and/or chronic inflammation, crypt hyperplasia, and villus atrophy.[1,3,7] Previous studies of HIV-infected patients suggested that the small intestinal dysfunction is related to the presence of immature enterocytes in jejunal mucosa with depressed mucosal digestive enzyme activities.[3,7] Several investigators have detected HIV infection in jejunal enterocytes, rectal enterochromaffin cells, lamina propria mononuclear cells, and intraepithelial lymphocytes in duodenal, jejunal, and rectal mucosa.[3,7,9–11]

Although the presence of HIV in the intestinal mucosa is now well-established, the relationship of infection to mucosal abnormalities and dysfunction is not defined, and pathogenic mechanisms of HIV enteropathy or wasting are not well understood. Human studies are limited by variations among patient populations in relation to clinical status, the presence of other pathogens, and ethical considerations.

Supported by NIH grants NS27338, NIDDK43183, NIDDK35747, and RR00169.

Accepted for publication November 25, 1992.

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The availability of animal models is crucial for understanding the pathogenesis of AIDS-associated gastroenteropathy.

Simian immunodeficiency virus (SIV) causes an immunodeficiency syndrome in nonhuman primates and is closely related to HIV with respect to pathogenicity, genomic structure, and virology.[12,13] Because the pathobiology of SIV is similar to that of HIV, the simian model is suitable for pathogenesis studies.[14–21] Clinical abnormalities in SIV-infected macaques include generalized lymphadenopathy, reversed T4/T8 ratios, chronic diarrhea, wasting, and opportunistic infections.[22,23]

The objective of this retrospective study was to evaluate the SIV-infected rhesus as a model of AIDS-associated enteropathy. We examined a) the cellular targets and regional distribution of SIV infection throughout the gastrointestinal (GI) tract, b) alterations in the mucosal immune system reflected by quantitative changes in mucosal immune cells, and c) changes in the structure and function of mucosal epithelium by morphometric and digestive enzyme analyses. We found that intestinal pathology and clinical symptoms were associated with the severity of SIV infection in intestinal tissues. In addition, changes observed in intestinal CD4/CD8 ratios, villus/crypt morphology, and digestive enzyme activities are very similar to those reported in HIV-infected patients.

Materials and Methods

Animals and Viral Infection

Twenty-two (15 SIV-infected and seven uninfected) colony-bred, male and female rhesus macaques (*Macaca mulatta*) from the California Primate Research Center were evaluated for the study. All animals were seronegative for STLV-1 and SRV-1 (type D retrovirus) and were housed in accordance with American Association for Accreditation of Laboratory Animal Care standards. Animals were inoculated with SIV from macaques (SIV mac)[24] or sooty mangabeys (SIV sm)[25] grown in human peripheral blood mononuclear cells. Fifteen animals were inoculated with the virus either intravenously or genitally. The disease induced by either route of inoculation is indistinguishable.[26] A 1 ml suspension of viral stock was used for intravenous inoculation of 10 animals, (nine with SIV mac and one with SIV sm). A dose of 2.5×10^{-1} tissue culture infectious dose ($TCID_{50}$) induced persistent viremia within 14 days, and simian acquired immunodeficiency syndrome (SAIDS) occurred in all of the animals 3 to 9 months postinoculation. For genital inoculation of the remaining five animals (three with SIV mac, two with SIV sm), a 1 ml suspension of inoculum (15 to 50 $TCID_{50}$) was infused through a 2.5 mm-outer-diameter (8 French) soft plastic pediatric nasogastric feeding tube (American Pharmaseal, Valencia, CA) into the vaginal vault or the urethral orifice of each immobilized animal. Three to 10 inoculations were given over a 2-week period. This dosage produced persistent viremia 14 to 37 days postinoculation.[26] To evaluate viremia, SIV was isolated from stimulated rhesus peripheral blood mononuclear cells by co-cultivation with fresh human peripheral blood mononuclear cells or a human CD4+ lymphocyte cell line (CEMX174). Each culture supernatant was monitored weekly for viral production by measurement of reverse transcriptase activity.[26]

Animals were killed 3 to 17 months postinoculation. Three of the 15 SIV-infected animals were clinically healthy at the time of necropsy, whereas the others had clinical signs consistent with SAIDS. Medical complications included anemia, anorexia, dehydration, and persistent diarrhea that was unresponsive to antibiotic therapy. Tissue specimens obtained at necropsy were either fixed in 10% formalin and embedded in paraffin, frozen directly in liquid nitrogen, or embedded in optimum cold temperature Compound (Miles Scientific, Naperville, IL) before freezing.

Uninfected control animals were euthanized for either trauma, chronic colitis, endometriosis, or liver disease. Of 11 animals used for controls, seven had no gastrointestinal complications. The remaining four had colitis (inflammation associated only with the large intestine) and diarrhea; however, the analyses were limited to the jejunum, which showed no morphological or pathological changes. Measured parameters (digestive enzymes, morphometry, and T lymphocytes) were not different in control animals with colitis when compared to those without. No animals with evidence of small intestinal disease were included as controls in the study.

Detection of Enteric Pathogens

Sequential jejunal tissue sections were treated with Warthin-Starry silver stain for detection of *Campylobacter* and spirochetes, Giemsa stain for protozoa, and Brown and Brenn gram stain for bacteria. Bacterial cultures of intestinal necropsy tissues for *Salmonella*, *Shigella*, *Yersinia*, and *Campylobacter* were performed on four animals with severe diarrhea. In one other animal, *Campylobacter* was detected by rectal culture. Intranuclear inclusions characteristic of cytomegalovirus (CMV) infection

were detected by histopathological examination, and CMV infection was confirmed by *in situ* hybridization with a rhesus CMV DNA probe that contained the immediate early gene. (DNA probe was provided by Dr. Peter Barry and Donald J. Alcendor, University of California, Davis). Other pathogens (*Cryptosporidium*, *Trichomonas*, *Trichuris*, and mycobacteria) were identified by histopathological observation of tissue sections.

In Situ *Hybridization for SIV and CMV Nucleic Acids*

In situ hybridization of SIV and CMV nucleic acids with radiolabeled DNA probes was performed by a modification of the procedure described by Brahic and Haase.[3,27] Formalin-fixed, paraffin-embedded intestinal tissues collected at necropsy were cut in 4- to 5-micron sections and placed on SuperFrost Plusglass slides (Fisher Scientific, Pittsburgh, PA). Sections were deparaffinized and treated for 15 minutes at 37 C with proteinase K (1 μg/ml in 50 mmol/L ethylenediamine tetraacetic acid, and 100 mmol/L Tris-HCl, pH 7.4), rinsed in phosphate-buffered saline, placed in 0.2 mol/L Tris/0.1 mol/L glycine for 15 minutes, dehydrated, and air-dried. The hybridization solution contained 50% deionized formamide, 10% dextran sulfate, 50 mmol/L NaH2PO4, 0.6 mol/L NaCl, 0.5 mmol/L ethylenediamine tetraacetic acid, 1× Denhardt's solution, 75 μg/ml *E. coli* transfer RNA, 100 μg/ml salmon sperm DNA and 20 mmol/L dithiothreitol. ^{35}S-labeled SIV DNA (specific activity of 1×10^8 cpm/μg or greater), obtained either by random-priming or by nick-translation of a 9-kb DNA fragment of SIVmac containing gag, pol, and env regions[28] (or a 9.4-kb DNA fragment of the rhesus CMV immediate early gene region for CMV detection), was added at 20×10^6 cpm/ml of hybridization solution. Slides were placed in a humidification chamber to hybridize overnight at 37 C and were washed with 50% deionized formamide/2× standard saline citrate at 42 C, followed by three washes with 2× standard saline citrate at room temperature. Autoradiography was performed with NTB2 emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with 0.6 mol/L ammonium acetate. The slides were exposed at 4 C for 3 to 14 days, developed with Kodak D-19, and counterstained with hematoxylin. Controls included SIV-infected and uninfected cultured CEM × 174 cells, hybridization of intestinal tissue from uninfected macaques, hybridization with nick-translated probe containing only the pSP64 vector, and RNAse treatment of tissue before hybridization.

Immunohistochemical Detection of T Lymphocytes and Macrophages

To determine the number and distribution of T lymphocytes and CD8+ T cells, immunohistochemistry was performed on cryostat jejunal sections from 10 SIV-infected and seven uninfected macaques. A pan-T cell monoclonal antibody, T11 (Coulter Immunology, Hialeah, FL), which is specific for the CD2 antigen, was used to identify the total T lymphocyte population in the jejunal mucosa. The cytotoxic/suppressor subset of T lymphocytes was detected with anti-Leu-2a (Becton-Dickinson, San Jose, CA), a monoclonal antibody that detects the CD8 cell marker. We were unable to obtain an antibody that specifically recognized only CD4+ helper T lymphocytes in monkey tissue sections by immunohistochemistry. Therefore, to estimate the percentage of CD4+ cells, serial sections were immunostained with T11 or Leu2a antibodies. Total T cells or CD8+ T lymphocytes were counted in the same area of the specimen and the difference assumed to be CD4+ cells. The contribution of a specific T cell subset (CD4+ or CD8+) to the total T cell population (CD2+) was determined by counting the number of immunostained cells in both the villus and crypt lamina propria in areas containing 300 to 600 hematoxylin-stained mononuclear cells. Numbers of T cell subsets were also estimated in jejunal lymphoid follicles. The percentage of a specific T cell subset was calculated from the number of immunostained cells divided by total number of CD2+ T cells. In addition, the relative contribution of T cells to the mononuclear cell population of the jejunum was calculated by dividing the number of CD2+ cells by the total number of mononuclear inflammatory cells counted.

In formalin-fixed, paraffin-embedded tissue sections, macrophages and T cells were identified by HAM56 and anti-CD3 antibodies, respectively (DAKO Corp., Carpenteria, CA). These antibodies were used alone and in combination with *in situ* hybridization. For frozen tissue, cryostat sections were fixed in acetone, washed with Tris-buffered isotonic saline and nonspecific binding was blocked in 10% normal serum before incubation with the primary antibody. Formalin-fixed, paraffin-embedded sections were deparaffinized in xylene and hydrated through graded ethanol before blocking and application of primary antibodies. A biotinylated anti-mouse/anti-rabbit IgG was applied, followed by a peroxidase-conjugated streptavidin complex (DAKO Corp.). Diaminobenzidine (Vector Laboratories, Burlingame, CA) was used as the chromogen, and slides were counterstained with hematoxylin.

Table 1. *Inoculation Data, Necropsy Time Points, Diarrhea, Weight Loss, Enteric Pathogens, and Necropsy Diagnoses of SIV-Infected Rhesus Macaques*

Animal	SIV Strain* and Inoculation Route	Months Post-Infection	Diarrhea	% Wt Loss, Past 3 Mos	Enteric Pathogens	Diagnoses
21293	sm/genital	3	–	None	None identified	Pneumonia (clinically healthy)
21311	sm/genital	3	–	1	None identified	Pneumonia, lymphoid hyperplasia, gastritis, meningitis, glomerulopathy (clinically healthy)
21891	mac/IV	4	+	15	None identified	Pneumonia, encephalitis, glomerulonephritis, gastritis, CMV neuritis (clinically healthy)
22852	mac/IV	4	–	None	None identified	Encephalomyelitis, gastroenteritis, lymphoid hyperplasia, bone marrow hyperplasia, glomerulonephritis, pneumonia
23455	mac/IV	5	+	22	*Cryptosporidia* (I) *Trichuris* (Co)	Lymphoid depletion, pneumonia, cachexia, CMV neuritis
23201	mac/IV	5.5	–	9	None identified	Lymphoid depletion, pulmonary edema
21943	mac/genital	6	+	17	*Campy* (S, D, J, Ce, Co) Focal trichomoniasis (J)	Gastritis, disseminated CMV, enterocolitis, cachexia, pneumonia, glomerulopathy, lymphadenitis, meningitis
23609	mac/IV	7	+	None	*Campy* (J, Co)	Pneumonia, peritonitis, splenomegaly, enterocolitis
23608	mac/IV	8	+	22	*Campy* (RC)	Pneumonia, lymphadenopathy, splenomegaly, colitis
16260	mac/genital	8.5	–	8	None identified	Anemia, bone marrow hyperplasia, nephritis, enterocolitis
23217	mac/IV	8.5	+	22	*Campy* (D, J, Ce, Co)	Enterocolitis, cachexia, dehydration, pneumonia
23476	mac/IV	11	+	6	*Cryptosporidia* (I, Co) *Trichuris* (Ce)	Jaundice, lymphoid hyperplasia, cachexia, gastroenteritis
22704	sm/IV	12.5	+	25	*Campy* (S, Co)	Gastroenterocolitis, pneumonia, pancreatitis
23597	mac/IV	13	+	None	*Mycobacterium* (D, J, Ce, Co)	Hepatitis, enterocolitis, pneumonia, lymphadenitis, disseminated Mycobacteriosis
23382	mac/genital	17	+	25	*Trichuris* (RC) *Cryptosporidia* (RC)	Enteritis, lymphoid depletion, cachexia, pneumonia

* mac, SIV mac; sm, SIV sooty mangabey. Abbreviations: *Campy*, *Campylobacter*; S, stomach; D, duodenum; J, Jejunum; I, ileum; Ce, cecum; Co, colon; RC, rectal culture.

Controls included stains in which the primary antibody was deleted or in which primary antibodies were replaced with an irrelevant mouse monoclonal antibody of similar isotype.

Combined in Situ *Hybridization and Immunohistochemistry*

The procedure was performed with formalin-fixed, paraffin-embedded intestinal tissue sections. Following deparaffinization and hydration, slides were incubated for 15 minutes at room temperature with 0.25 mg/ml protease XXIV (Sigma Chemical Co., St. Louis, MO), and *in situ* hybridization was performed as described above. Immunohistochemistry was performed as described above, immediately following posthybridization washes, using 3-amino-9-ethylcarbazole (Vector Laboratories) as the chromogen. Slides were briefly rinsed and air-dried before coating with emulsion and then exposed for 1 to 2 weeks at 4 C.

Jejunal Morphometry

Using paraffin-embedded jejunal specimens stained with hematoxylin and eosin, villus height, and crypt depth of at least 10 well-oriented villi were measured using an eyepiece micrometer. The mitotic index was determined in the same sections by counting the number of mitotic figures in the crypts and expressed as the mean number of mitoses per crypt. Jejunal tissues from five uninfected animals without diarrhea were used to establish the range of normal measurements.

Jejunal Enzyme Activities

Sections of jejunum were frozen in liquid nitrogen and stored at −70 C. After thawing, mucosa was scraped and homogenized in phosphate-buffered saline to determine sucrase and lactase activities,[29] expressed as nmole of substrate, and hydrolyzed per minute per mg of protein. Jejunal activities from seven uninfected macaques without small intestinal disease were used for normal ranges.

For *in situ* detection of alkaline phosphatase activity, formalin-fixed, paraffin-embedded jejunal sections were incubated with naphthol phosphate esters and diazonium salts (Vector Laboratories), followed by counterstaining with nuclear fast red.

Statistical Analyses

Student's *t*-test was used to determine differences in morphometric measurements and enzyme activities between SIV-infected animals and controls.

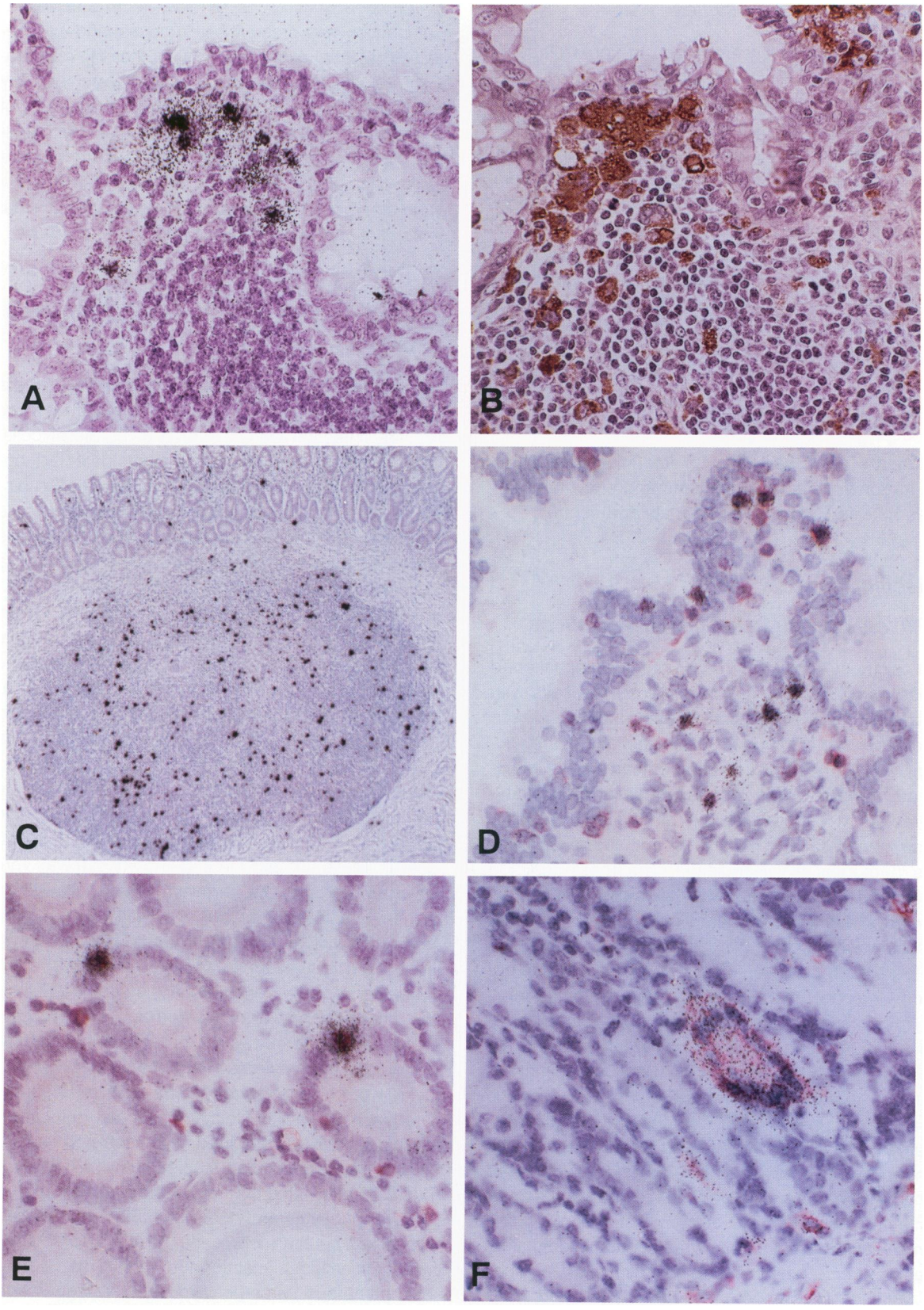
Results

Clinical and Pathological Observations

At necropsy, 10 of the 15 SIV-infected animals had diarrhea (Table 1). Nine of these 10 monkeys with diarrhea had documented opportunistic enteric pathogens, including four with infections of the jejunal mucosa. Weight loss of more than 15% of preinfection weight was documented in seven SIV-infected macaques. Severe diarrhea, depression, and dehydration necessitated euthanasia as early as 5 months postinoculation. The most common pathological finding at necropsy in clinically healthy animals was SIV-associated pneumonia, often with multinucleated giant cell infiltration. As the disease progressed, complications secondary to SIV infection were observed in many organ systems (Table 1). Four animals were positive for *Campylobacter* by culture of intestinal contents obtained at necropsy and one by stool culture. One animal exhibited a *Mycobacterium avium-intracellulare* infiltrative enteritis. The jejunum of one animal contained a focally extensive lesion associated with invasive trichomoniasis, but adjacent sections in this animal lacked evidence of infection. CMV-infected cells were detected by histopathology and *in situ* hybridization in multifocal acute inflammatory lesions in the fundic gastric mucosa of one animal. CMV infection was not present in SIV-infected areas of the intestine of any of the other animals; however, moderate to severe CMV neuritis affected multiple nerve bundles in the mesentery of one animal. Cryptosporidiosis of the terminal ileum and ileocecal junction was present in one animal and was detected by fecal examination in another. No opportunistic agents were detected by special stains in any other areas of the GI tract. In contrast to the rarity of bacterial or protozoal organisms in the small intestine, spirochetosis, trichomoniasis, and trichuriasis were often found in the colon, and *Helicobacter* were common in the glands of the stomach. Minimal inflammation was associated with these infections.

Cellular Targets and Intestinal Distribution of SIV

SIV-infected T lymphocytes and macrophages were detected in the mucosa at all levels of the GI tract. No viral infection was observed in epithelial cells. There was an apparent association detected between the levels of SIV nucleic acids, clinical disease, and intestinal pathology. SIV-infected cells were found in the intestinal mucosa of all 10 animals with diarrhea but were rare or absent in the mucosa of clinically healthy animals. In most animals, virus-infected cells were located mainly in gut associated lymphoid tissues (GALT), mesenteric lymph nodes, and crypt lamina propria. The viral load in these lymphoid tissues was much higher than in the adjacent lamina propria. In Peyer's patches, the infected cells were primarily T lymphocytes located at the periphery of the lymphoid follicles, but SIV-infected macrophages were frequently located below the dome epithelium covering the lymphoid nodule (Figure 1, A and B). The frequency of SIV-infected cells in the lamina propria and submucosa was variable between animals. Terminally ill animals with diarrhea often had virus-positive mononuclear cells throughout the lamina propria and GALT, and infiltrating the epithelium at all levels of the GI tract. However, regional variations in the viral load within the lamina propria were marked and infected cells were most numerous within large intestinal mucosa (Figure 1C). SIV-infected intraepithelial T lymphocytes were also present in some animals (Figure 1, D and E), but viral nucleic acid was not detected in epithelial cells. Combined immunohistochemistry and *in situ* hybridization studies showed that virus-positive cells within the lamina propria were primarily T lymphocytes, but occasional SIV-infected macrophages were also present. In one animal, there was typical T cell infection throughout the GI tract, but numerous virus-positive macrophages were found in a focal area of inflammation associated with invasive trichomoniasis. In contrast, three animals exhibited SIV infection almost exclusively in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large macrophage-derived multinucleated syncytial cells (Figure 1F). The multinucleated giant cells were scattered throughout the lamina propria, submucosa, tunica muscularis, and subserosa of the intestine and in many other tissues. No opportunistic pathogens were associated with these infected cells, indicating that this may be a primary SIV lesion. However, we cannot rule out the possibility that *Microsporidia*, which is difficult to detect without electron microscopy, or other undetected pathogens could have elicited the giant cell response.

Jejunal T Lymphocyte Populations

The T lymphocyte population was determined in the jejunum of 10 SIV-infected animals and seven uninfected macaques. The GALT of SIV-infected animals contained a higher percentage of CD8+ cells than the control macaques, resulting in a reversal of the CD4/CD8 ratio in the jejunal lymphoid tissue of five of the 10 SIV-infected animals. No differences were observed in T cell populations of the villus or crypt lamina propria. The numbers of intraepithelial T lymphocytes were similar in both infected and uninfected animals, and greater than 85% of the intraepithelial T lymphocytes were CD8+ in both groups. A decrease was seen in the percentage of putative CD4+ cells in the jejunum of SIV-infected animals. When T cell subsets from the GALT and lamina propria were summed, there was a tendency for reversal of the CD4/CD8 ratio in the SIV-infected animals.

Jejunal Morphology

There was often a mild to moderate lymphohistiocytic infiltrate in the lamina propria, accompanied by variable degrees of atrophy and fusion of villi (Figure 2). Jejunal villus height to crypt depth ratios were decreased in SIV animals compared to uninfected controls (2.3 ± 1.4 versus 4.3 ± 1.1; $P < 0.05$; mean ± SD, Figure 3). Villus heights of SIV-infected animals were all in the low normal range. Intestinal crypts were frequently elongated, hyperplastic, and irregular. There was often increased mitotic activity within the crypts, with tightly packed, elongated, basophilic enterocytes extending up to and covering the bases of the shortened villi. There were no statistically significant differences in these parameters between SIV-infected animals with and without jejunal pathogens; however, a more severe crypt hyperplasia and increased mitotic figures were observed in animals with opportunistic infections.

Jejunal Enzyme Activities

Sucrase activity was below the normal range in 11 of 13 SIV-infected animals compared to the controls (32 ± 20 versus 125 ± 56 nmol/min/mg protein; $P = 0.0001$; mean ± SD). Lactase activity was depressed in all of the infected animals (6 ± 3 vs. 23 ± 10 nmol/min/mg protein; $P = 0.0001$, Figure 4). SIV-infected animals with jejunal pathogens had significantly lower sucrase activities than those without pathogens (15 ± 7 versus 39 ± 18 nmol/min/mg protein; mean ± SEM). Nevertheless, the difference between SIV-infected and controls remained statistically significant even when animals with jejunal opportunists were removed from the analyses. Among all SIV-infected animals, there was a significant negative correlation between sucrase activity and crypt depth ($r = -0.81$, $P < 0.01$). There were no differences in lactase activity between the SIV-infected animals with or without secondary pathogens. Variations in tissue processing precluded staining for alkaline phosphatase activity in all jejunal sections. However, dramatic differences in enzyme activity were seen in jejunal specimens from one animal. Enzyme activity was virtually absent in areas heavily infected with SIV, whereas the alkaline phosphatase activity was relatively normal in areas of low infection (Figure 5).

Discussion

Our studies indicate that the intestinal abnormalities observed in SIV-infected rhesus macaques are very similar to those described in HIV infection.[1,3,7,8] The abnormalities include alterations in jejunal morphology, depressed digestive enzyme activity, and abnormal mucosal T lymphocyte populations. The cell-

Figure 1. *Distribution and cellular targets of SIV in the gastrointestinal tract.* **A:** *SIV nucleic acids in cells subjacent to colonic follicle-associated epithelium (site of M cell localization), as indicated by silver grains overlying the cells (*in situ *hybridization with hematoxylin counterstain, ×330).* **B:** *Immunohistochemical stain demonstrates macrophage infiltration subjacent to dome epithelium in colonic specimen shown in Figure 1A (DAB with hematoxylin counterstain, ×330).* **C:** *SIV-infected cells in colonic mucosa and lymphoid nodule (*in situ *hybridization with hematoxylin counterstain, ×41).* **D** *and* **E:** *Immunohistochemistry for T lymphocytes (red stain) combined with* in situ *hybridization for SIV (black grains) demonstrates SIV-infected intraepithelial T lymphocytes in* **(D)** *jejunal villus and* **(E)** *gastric glands. (AEC with hematoxylin counterstain, ×330).* **F:** *Immunohistochemical detection of macrophage (red stain) combined with* in situ *hybridization for SIV (black grains) demonstrates SIV-infected macrophage-derived multinucleated giant cell in mucosa of large intestine (AEC with hematoxylin counterstain, ×330).*

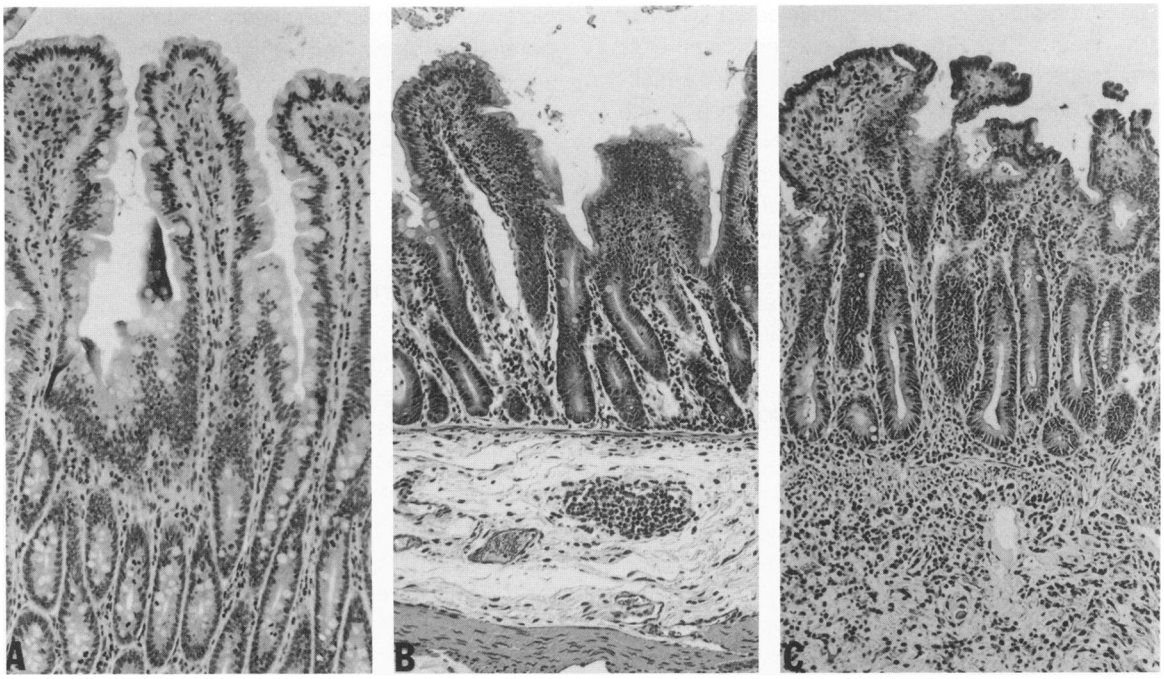

Figure 2. *Jejunal morphology in rhesus macaques (hematoxylin and eosin stain, ×83).* **A:** *Jejunum from SIV-negative animal has normal villi and crypt morphology and no inflammatory infiltrate.* **B:** *Crypt hyperplasia, partial villus atrophy, and chronic inflammation in jejunum from SIV-infected animal with diarrhea and wasting; 5 months postinoculation. No opportunistic pathogens were detected in jejunum of this animal.* **C:** *Severe crypt hyperplasia, villus atrophy and fusion, and inflammation in jejunum of SIV-infected animal with focal invasive trichomoniasis,* Campylobacter, *and SIV infection; 5 months postinoculation.*

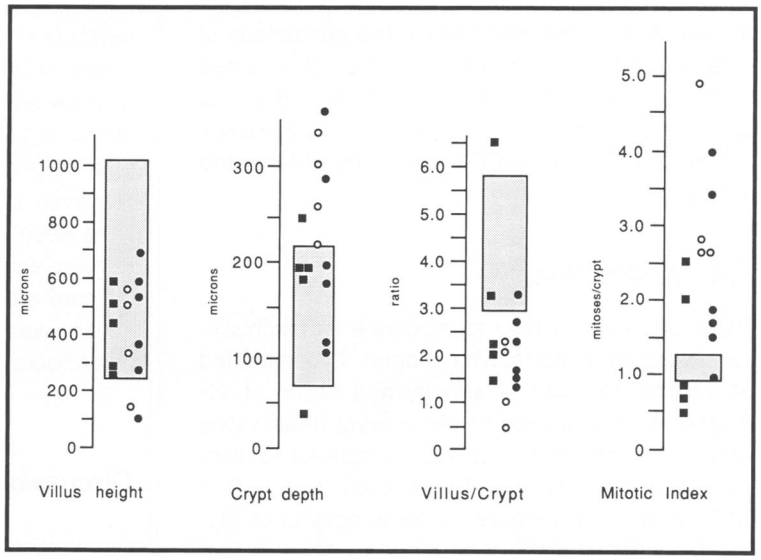

Figure 3. *Villus height, crypt depth, villus height to crypt depth ratios, and mitotic indices of jejunal necropsy specimens from SIV-infected rhesus macaques. The normal ranges (*shaded bars*) represent average measurements from five SIV-negative macaques without diarrhea. SIV-infected animals without diarrhea or jejunal pathogens (■); with diarrhea and without jejunal pathogens (●); with diarrhea and jejunal pathogens (○).*

ular targets of SIV within the intestine were T lymphocytes and macrophages. Opportunistic enteropathogens contributed to the diarrhea seen in most of the SIV-infected animals in this study, but a primary SIV enteropathy seems to be involved in some cases. Decreased sucrase and lactase activities, as well as morphological changes, were observed as early as 3 months postinoculation. There seemed to be an association between levels of SIV transcripts, clinical symptoms, and intestinal pathology, indicating that intestinal mucosal infection may influence the development and function of enterocytes. Human studies have not been able to document a correlation between the presence of HIV in the intestine and clinical symptoms or stage of disease,[3,7,11,30] probably due to the small quantity of tissue available for analyses. The availability of necropsy tissue from animals in various stages of infection allowed an investigation of the effect of SIV infection on the morphology, pathology, and virallocalization, as well as functional analysis as determined by digestive enzyme activities.

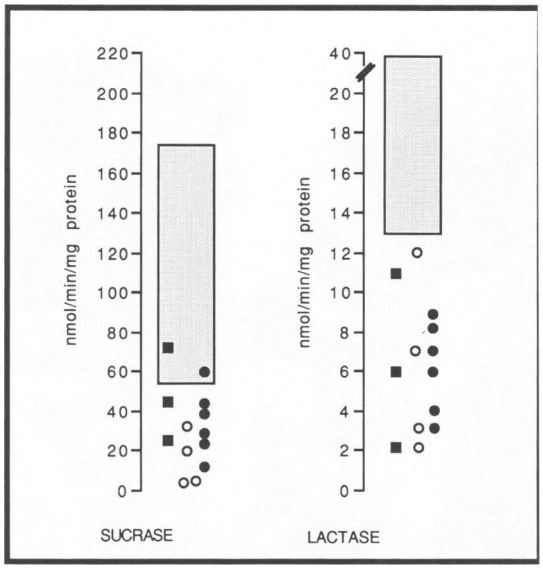

Figure 4. *Sucrase and lactase activities of jejunal mucosal homogenates from SIV-infected rhesus macaques. The normal ranges (*shaded bars*) were based on results from seven SIV-negative macaques without small intestinal disease. SIV-infected animals without diarrhea or jejunal pathogens (■); with diarrhea and without jejunal pathogens (●); with diarrhea and jejunal pathogens (○).*

SIV-infected mononuclear cells were widespread throughout the intestine. The primary intestinal cell targets of SIV were T lymphocytes. SIV-infected macrophages were observed less frequently and usually associated with focal inflammation or multinucleated giant cells. SIV infection was not observed in epithelial cells. The majority of SIV-infected cells were located in GALT, especially in Peyer's patches of jejunum and ileum and lymphoid follicles of the large intestine. Previous studies have reported intestinal cell targets of HIV to include lamina propria mononuclear cells and intraepithelial lymphocytes in duodenal, jejunal, and rectal mucosa, duodenal dendritic reticulum cells, jejunal enterocytes, and rectal enterochromaffin cells.[3,7,9–11,30] Morphological identification, rather than techniques involving double-labeling of cells, was used in these studies to determine infected cell types. Therefore, primary targets of HIV within the intestine have not been unequivocally identified, and it is not clear if cell tropism is dependent upon stage or severity of the disease or virus strain. Using combined immunohistochemistry/*in situ* hybridization, we observed SIV infection of T lymphocytes at all stages of disease, whereas macrophage infection was associated with more advanced stages of SAIDS. Infection of rhesus macaques with a pathogenic clone demonstrated that macrophage-tropic variants can develop in some animals during the final stages of disease and are associated with primary viral lesions.[31] In the present study, we detected SIV-infected macrophages in both primary viral enteropathy and in granulomatous inflammation secondary to opportunistic pathogens. Thus, it seems that in the final stages of disease, SIV infects macrophages involved in both primary viral lesions and in lesions due to opportunistic infections.

SIV-infected intraepithelial T lymphocytes were frequently identified; however, viral nucleic acid was not detected in epithelial cells. Colon carcinoma epithelial cell lines and primary cell cultures of normal ileal and colonic epithelial cells have been shown to be susceptible to infection by HIV and support active viral production.[32–35] Electron microscopy of HT29 cells with productive HIV infection has demonstrated structural abnormalities suggestive of defects in brush border assembly and differentiation.[32] The clinical significance of these findings are not clear because epithelial cell infection *in vivo* may be a rare occurrence.

This group of rhesus macaques were chronically infected, and presumably the virus gained access to the intestinal tract via the bloodstream. However, the presence of SIV-infected macrophages directly beneath the follicle-associated epithelium in lower colonic and rectal lymphoid nodules suggests a route of viral uptake from the intestinal lumen into the host. It is known that antigens penetrate the intestinal mucosal barrier via membranous epithelial (M) cells that overlie the subepithelial lymphoid follicles.[36] Thus, M cells may provide a portal of entry for HIV transmitted rectally. Transepithelial transport of HIV-1 by M cells has recently been demonstrated using mucosal explants.[37] *In vivo* infections by reovirus,[38] poliovirus,[39] and a number of other pathogens have also been shown to occur via this route. SIV-infected macrophages beneath the dome epithelium were observed even in animals where the majority of infected cells were T lymphocytes.

Despite the absence of enterocyte infection, the presence of SIV-infected immune cells in the intestine seems to affect epithelial cell morphology and function. Our findings of decreased digestive enzyme activities and increased crypt cell proliferation suggest a defect in jejunal epithelial cell maturation in SIV-infected macaques. Depressed lactase and sucrase activities were found in HIV-infected patients independent of intestinal pathogens.[3,7] Nutrient malabsorption is frequently observed as a component of HIV enteropathy and could be due to functional immaturity of the enterocytes caused by rapid turnover, reduced villus surface area, or functional defects secondary to the virus. Previous studies have reported partial villus atrophy and crypt hyperplasia in small intestinal biopsies of HIV-positive patients with diarrhea[3,40]; however, normal crypt length with increased mitotic figures[41] or decreased

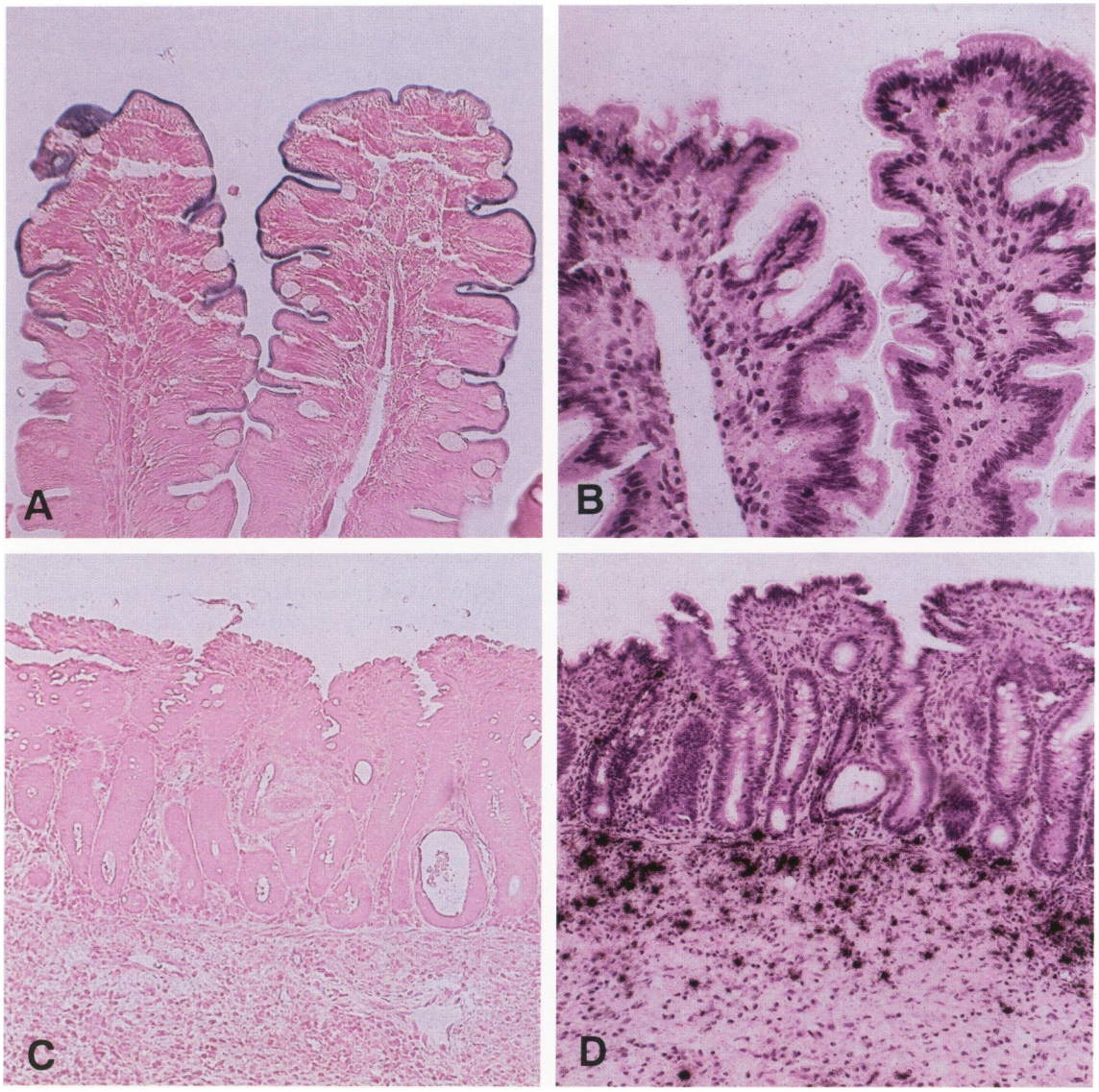

Figure 5. *Detection of alkaline phosphatase activity and SIV infection in two different areas of jejunum from the same animal. The samples from these two regions of jejunum were processed together and embedded in the same paraffin block.* **A:** *Normal alkaline phosphatase activity is represented by a blue reaction product lining the jejunal brush border of villi in an area unaffected by SIV or opportunistic infection (nuclear fast red counterstain, ×208).* **B:** *Absence of SIV-infected cells in specimen shown in Figure 5A* (in situ *hybridization, hematoxylin counterstain, ×208).* **C:** *Absence of alkaline phosphatase activity indicated by lack of blue stain along luminal surface of villi in an area of jejunum with focal trichomoniasis and SIV infection (nuclear fast red counterstain, ×83).* **D:** *SIV-infected cells throughout mucosa and submucosa of jejunal specimen shown in Figure 5C* (in situ *hybridization, hematoxylin counterstain, ×83).*

mitoses[7] have also been observed. The altered intestinal morphology could be mediated by cytokines secreted by virally infected immune cells. Crypt cell proliferation in fetal small intestinal organ culture was shown to be increased twofold within 18 hours of T cell activation, with villus atrophy and increased crypt depth observed by day 3 in culture.[42] Administration of an antibody to tumor necrosis factor prevented villus atrophy, crypt elongation, crypt cell proliferation, and inhibited inflammatory infiltrate in experimental graft versus host disease in mice.[43] Further evidence of cytokine-mediated damage to mucosa was demonstrated by the killing of HT29 colonic epithelial cells by supernatants from stimulated colonic lamina propria lymphocytes. Cytotoxicity was due to the synergistic effects of tumor necrosis factor α and interferon γ.[44] Intestinal lamina propria lymphocytes from normal nonhuman primates have been shown to have high levels of expression of genes associated with T-cell activation,[45] although to our knowledge this has not been studied in SIV-infected macaques. We found SIV-infected T cells throughout the GI tract, and these cells may have altered cytokine production that induces epithelial cell dysfunction and/or mucosal damage or contribute to the wasting syndrome. Me-

diators of the systemic metabolic alterations and wasting observed in HIV infection have not been identified; however, the cytokine system has been proposed as an important component.[46]

In addition to an indirect local immune mechanism due to SIV infection, changes in mucosal immune cell populations may also play a role in intestinal dysfunction. As is observed with HIV, SIV exhibits a primary cell tropism for cells bearing the CD4 molecule, and progressive viral replication results in depletion of CD4+ lymphocytes.[22,47] Increased numbers of CD8+ T lymphocytes and decreased numbers of CD8− T lymphocytes (presumably CD4+) were documented in the jejunal mucosa of this group of SIV-infected macaques. Values for percentages of T cell subsets in our normal animals were lower than a previous report using fluorescence-activated cell sorting; however, CD4/CD8 ratios were similar.[48] Phenotypic studies of T lymphocyte populations in small intestinal mucosa of AIDS patients have shown reduced CD4+, increased CD8+, and inversed CD4/CD8 ratios, similar to findings of circulating T lymphocytes in AIDS.[8,30,49–51] Reversed CD4/CD8 ratios in intestinal mucosa implies altered mucosal immunity, perhaps increasing the susceptibility to intestinal pathogens, including SIV or HIV. *Campylobacter*, *Cryptosporidium*, and *Mycobacterium avium-intracellulare* were detected throughout the small and large bowel of eight of the 10 SIV-infected animals with diarrhea. These intestinal pathogens are frequently identified in HIV-infected patients with diarrhea and malabsorption.[5,52] It has also been proposed that a low-grade bacterial overgrowth could disturb the cytokine balance and play a role in the chronic mucosal inflammation and enteropathy.[53] Five animals with no detected jejunal pathogens exhibited an enteropathy associated with wasting and diarrhea. In these five animals, *Campylobacter*, *Cryptosporidia*, and *Trichuris* were detected in the large bowel and probably contributed to the diarrhea.

The interactions between intestinal mononuclear cells and the epithelium seem to be crucial to intestinal integrity and function. A histopathological lesion in patients with inflammatory bowel disease is a chronic inflammatory infiltrate, and there is increasing evidence that altered intestinal immunoregulation may be an important factor in this disorder. Normal human intestinal lymphocytes express activation antigens and, thus, are in an *in vivo* activated state, in agreement with studies on nonhuman primates.[45] Lamina propria T and B lymphocytes from inflammatory bowel disease patients show an increased expression of interleukin-2 and transferrin receptors.[54] Further evidence of altered intestinal immunity in inflammatory bowel disease is a decrease in lamina propria and intraepithelial T lymphocytes bearing the γ/δ receptor.[55] These T cells may be involved in protection of the integrity of epithelial cells.[56] The presence of activated mononuclear cells and a decrease in γ/δ+ T cells may potentiate the dissemination of lentiviral infection in intestinal mucosa and contribute to enteropathy.

In summary, intestinal disease and dysfunction is an important cause of morbidity in SIV-infected rhesus macaques. The intestinal tract is an early target for SIV infection. SIV-infected mononuclear cells could influence epithelial cell maturation, leading to intestinal dysfunction in early stages of infection before advanced immunodeficiency. These data emphasize the value of the SIV model for understanding the pathogenesis and the role of cytokines in the intestinal dysfunction and wasting associated with HIV infection.

Acknowledgments

The authors would like to dedicate this paper to the memory of Dale J. Martfeld, whose technical contributions were invaluable to the study. We also acknowledge Robert J. Munn for photography.

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