

EVALUATION OF THE ULTRASTRUCTURE OF THE SMALL INTESTINE OF HIV INFECTED CHILDREN BY TRANSMISSION AND SCANNING ELECTRONIC MICROSCOPY

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ABSTRACT - Objectives - To describe HIV children's small intestinal ultrastructural findings. **Methods** - Descriptive, observational study of small intestine biopsies performed between August 1994 and May 1995 at São Paulo, SP, Brazil. This material pertained to 11 HIV infected children and was stored in a laboratory in paraffin blocks. Scanning and transmission electronic microscopy were used to view those intestine samples and ultrastructural findings were described by analyzing digitalized photos of this material. Ethical Committee approval was obtained. **Results** - In most samples scanning microscopy showed various degrees of shortening and decreasing number of microvilli and also completes effacements in some areas. Derangement of the enterocytes was seen frequently and sometimes cells well defined borders limits seemed to be loosened. In some areas a mucous-fibrin like membrane with variable thickness and extension appeared to partially or totally coat the epithelial surface. Fat drops were present in the intestinal lumen in various samples and a bacterium morphologically resembling bacilli was seen in two occasions. Scanning microscopy confirmed transmission microscopy microvilli findings and also showed little "tufts" of those structures. In addition, it showed an increased number of vacuoles and multivesicular bodies inside various enterocytes, an increased presence of intraepithelial lymphocytes, mitochondrial vacuolization and basement membrane enlargement in the majority of samples analyzed. However, some samples exhibited normal aspect. **Conclusions** - Our study showed the common occurrence of various important intestinal ultrastructural alterations with variable degrees among HIV infected children, some of them in our knowledge not described before.

HEADINGS - Microscopy, electron. HIV. Intestine, small. Child.

INTRODUCTION

Diarrhea is one of the most frequent symptoms in Human Immunodeficiency Virus (HIV) infected children. Enteropathy described in those patients may be due to HIV itself or due to common enteropathogenic or opportunistic microorganisms. HIV enteropathy has a negative impact on nutritional status, quality of life, and patient survival. However, diarrhea, intestinal villous atrophy and absorption tests are not always well correlated. Therefore, the mechanism responsible for HIV enteropathy remains unclear.

After a little more than a quarter century of the first descriptions of AIDS cases⁽¹²⁾, there has been increasing resurgence of scientific interest in the role of mucosa in HIV infection. Particular attention has been paid to the gut-associated lymphoid tissue (GALT). Indeed, most HIV transmission occurs

through vaginal or rectal mucosa. This also happens during the vertical transmission of HIV when the virus is inoculated in the upper gastrointestinal tract during the ingestion of infected amniotic fluid in the uterus, ingestion of infected blood and vaginal secretions during delivery or through infected breast milk^(13, 27, 28, 29). In summary, HIV infection is related to villous atrophy of the small bowel mucosa, hyperplasia of crypts, disruption of epithelial barriers with apoptosis of enterocytes, depletion of large numbers of CD4+ T cells and infection of a large proportion of CD4+ T cells by the release of virions, translocation of microbes, and increased electron permeability⁽⁴⁾.

The electron microscope technique stands out for its distinct abilities to assess the relationship of affected organs within the context of cell structure and to allow for the direct observation of structures whose existence could only be inferred by other methods.

Declared conflict of interest of all authors: none

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However, to date, there have been few studies conducted on the ultrastructure of the small intestine in children infected with HIV. Thus, the purpose of this study is to describe the findings of transmission electron microscopy and biopsy specimens from the small intestines of HIV infected children.

METHODS

Materials

We analyzed small intestines biopsies of 11 HIV-infected children between 6-149 months of age (mean age: 35.1 months, six females) classified in 1994 Centers for Diseases Control clinical categories (7) as A (1), B (3) and C (7), as can be seen in Table 1. The samples were obtained by endoscopic biopsy or by using Watson capsule attached to a polyethylene probe and were collected between August 1994 and May 1995 in São Paulo, Brazil.

TABLE 1. Age, HIV clinical category and report of diarrhea among children with HIV/AIDS

Patient	Age (months)	1994 Clinical Classification for HIV infected children (7)	Diarrhea
1	35	A	No
2	11	C	No
3	6	C	Yes
4	9	C	No
5	14	C	Yes
6	149	C	No
7	12	C	Yes
8	10	B	No
9	10	C	Yes
10	30	B	No
11	100	B	Yes

METHODS

Preparation of intestinal samples for viewing by transmission microscopy

Intestinal fragments were washed in a sodium cacodylate buffer-0.1 M HCl and fixed with Karnovsky solution. Then, they were washed with sodium cacodylate buffer-HCl and fixed with osmium tetroxide 1%. After washing with water, they were placed in a solution of 0.5% sucrose uranyl acetate. Then, they were dehydrated in 70%, 90%, and 100% ethanol and infiltrated with propylene oxide and Araldite® (Joinville, SC, Brazil) resin. After infiltration, fragments were placed in a mixture of pure Araldite® resin and infiltrated under a vacuum in a desiccator. After this they were placed in silicone molds and incubated at 60°C. To prepare each block, ultrathin 90 nm sections were obtained in an MT2 ultramicrotome (SORVALL) with diamond knife (LADD). The sections, collected on copper screens (SEM), were stained with 0.88% lead citrate solution and examined in a transmission electron microscope operating at 80 kV.

Preparation of the intestinal samples for viewing by scanning microscopy

After fixing the fragments of intestine as described above, two further washes with sodium cacodylate buffer-0.1 M HCl were performed. Then, the fragments were transferred to permeable baskets, which were placed in 50% ethanol, and 100% p. a. absolute ethanol (MERCK) for dehydration. Subsequently, fragments were placed in the drying chamber of the apparatus (Balzers CPD 030) using the critical point of carbon dioxide method. With the aid of a stereoscopic microscope, the fragments were oriented with the villi directed upward, placed in a sample to support port scanning, and attached with colloidal silver (SEM) to improve conductivity. After assembly, the fragments were subjected to a metalizer (Balzers - Sputter Coater SCD 050) for the deposition of a thin layer of gold (the “Sputtering” method) to improve conductivity. Observations were made with a scanning electron microscope (JEOL JSM - 5300), operating at 10 kV.

Ethical aspects

The study was approved by the Ethics Committee of Universidade Federal de São Paulo – Escola Paulista de Medicina, and a written consent form was obtained from the parents or legal guardians of all participating children.

RESULTS

Electron transmission microscopy

Some microvilli were preserved in number with apparently normal height (Figure 1). Others showed patterns suggesting

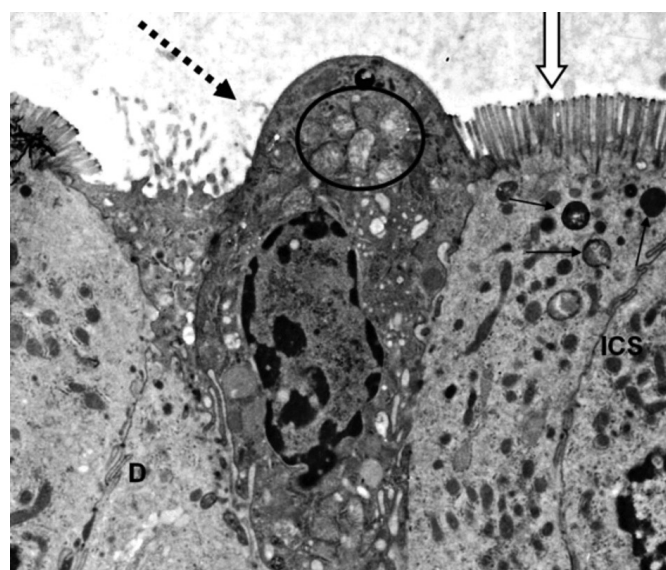


FIGURE 1. Small intestine transmission electron microscopy of an HIV-infected child. Cell extrusion (dashed arrow) exhibiting vacuolated mitochondria (ellipse). Microvilli are preserved in height and number (large arrow). Several multivesicular bodies showing intense endocytic activity (black arrows). Desmosome (D) delineating the cell junctions. Intercellular space is preserved (ICS). 5,000 X



FIGURE 2. Small intestine transmission electron microscopy of an HIV-infected child. Enterocytes with intense vacuolization. Microvilli destruction and tufts formation (black arrows). Vacuolization of mitochondria (ellipse) and of Golgi apparatus (rectangle). Extrusion of enterocytes into the intestinal lumen (dashed arrow). 2,500X

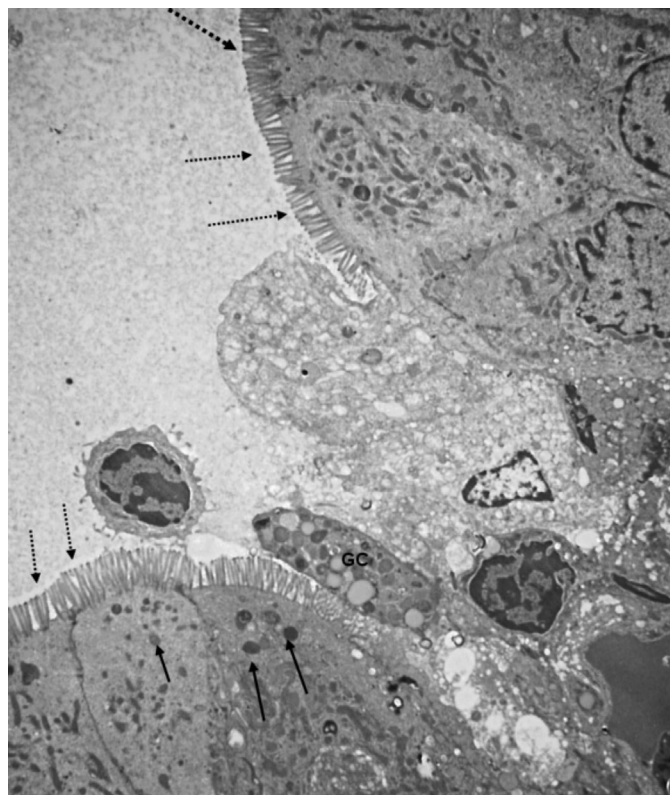


FIGURE 3. Small intestine transmission electron microscopy of an HIV-infected child. Microvilli with differences in height and number showing tufts formation (dashed arrows) Cellular extrusion also including the presence of goblet cells (GC). Multiple multivesicular bodies (black arrows). Intracytoplasmic vacuolization (large arrow). 2,500X

that they had been destroyed and significantly decreased (Figure 2 and Figure 3) with a tendency to form tufts as if they had been compressed at the base, resembling a “bouquet of flowers” (Figure 3). Additionally, we noticed focal areas with microvilli disruption as compared to other areas, which showed reasonably preserved microvilli, but with changes in the number and height of microvilli and the presence of tufts. Differences in the height and number of microvilli were also seen near each other (Figure 3).

Intracytoplasmic vacuoles (Figure 3 and Figure 4) and intense vacuolization of enterocytes were present in some samples (Figure 2).

Multivesicular bodies were frequently identified in large numbers and were present in most specimens (Figure 1).

Mitochondria were preserved in some samples and vacuolated in others (Figure 2).

The structure of the epithelium showed membrane thickening in some samples (Figure 3), and the extrusion of enterocytes into the intestinal lumen was also observed (Figures 1, Figure 2 and Figure 3). The intercellular space was well defined, and desmosomes of normal appearance could be observed (Figure 1).

Intraepithelial lymphocytes of varying frequency and quantity were observed (Figure 4).

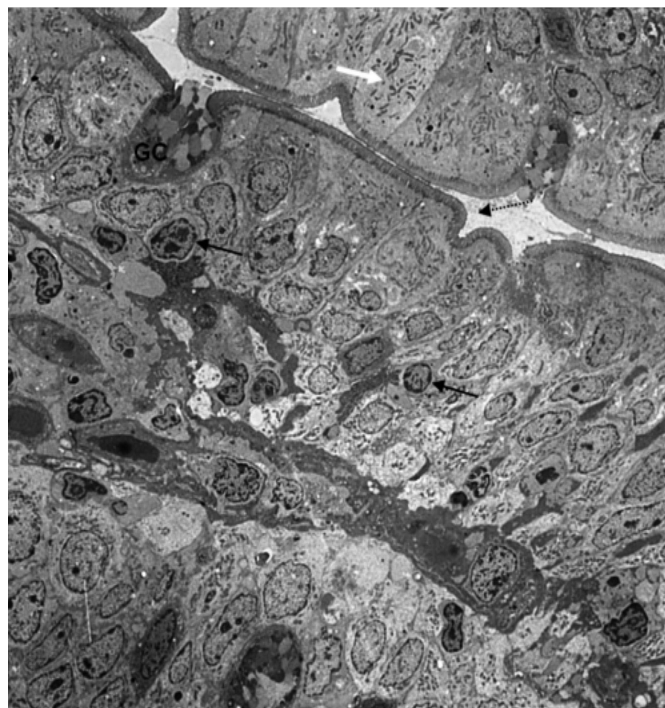


FIGURE 4. Small intestine transmission electron microscopy of an HIV-infected child. Microvilli with preserved structure although reduced in height (dashed arrow). Intraepithelial lymphocytes (black arrow). Intracytoplasmic vacuolization. Preserved mitochondria (white arrow). Goblet cell (GC) eliminating mucus to the lumen. Basement membrane thickening. 1,000X

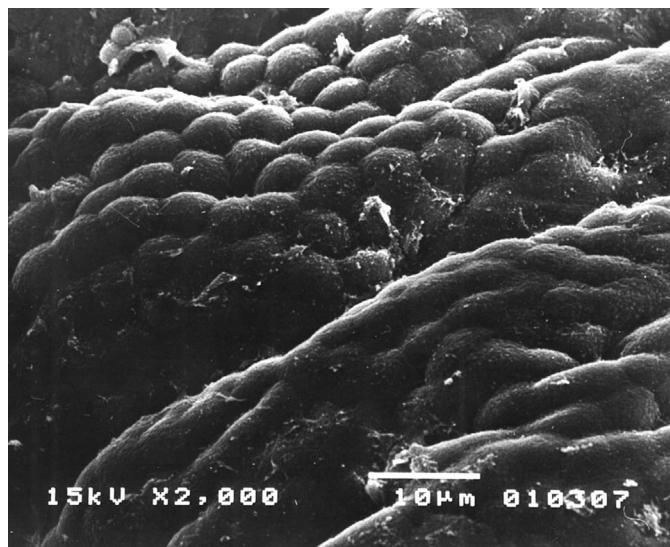


FIGURE 5. Small intestine scanning electronic microscopy of an HIV-infected child. Microvilli are decreased in number and height and showing total effacement in some areas. Focal areas with enterocytes showing villous architecture disruption. 2,000X

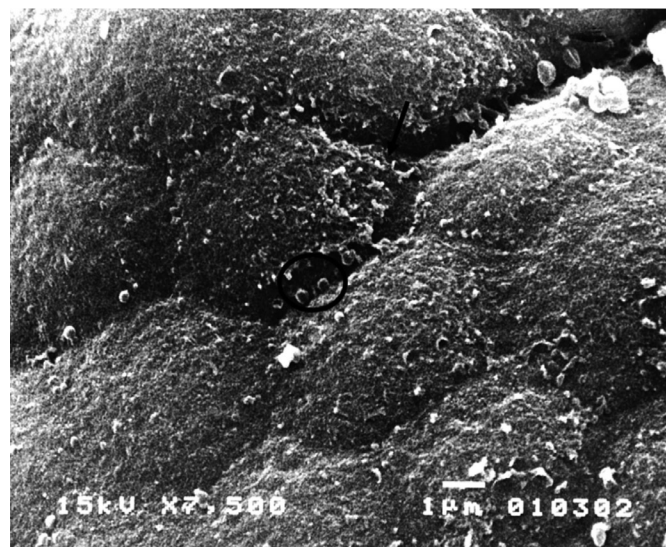


FIGURE 7. Small intestine scanning electronic microscopy of an HIV-infected child. Mucosa covered with fibrin-crust (arrow) and presence of fat drops. 7,500X

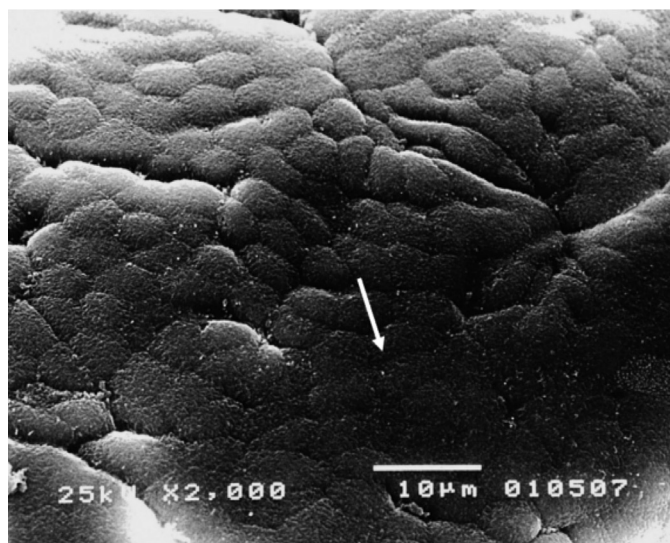


FIGURE 6. Small intestine scanning electronic microscopy of an HIV-infected child. Villi flattening and loss of cellular structures delineation. Some areas showing more severe changes in epithelial surface (arrow). 2,000X

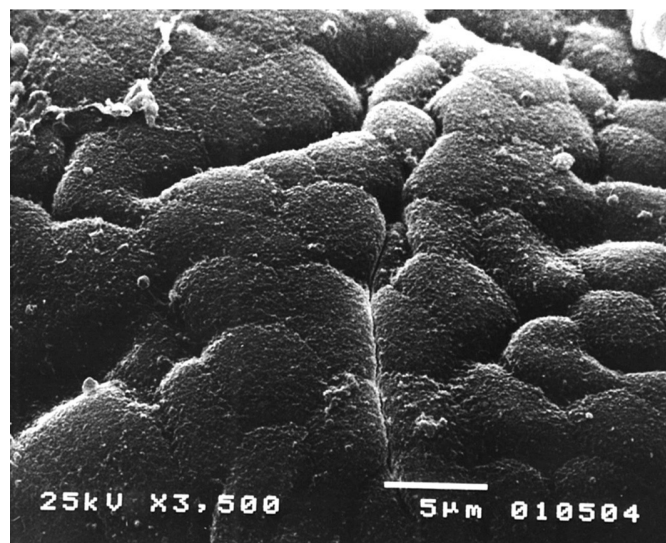


FIGURE 8. Small intestine scanning electronic microscopy of an HIV-infected child. Denudated epithelium showing loss of cellular architecture, villi flattening and no mucus. 3,500X

Electron scanning microscopy

The architecture of the villi showed significant breakdown (Figure 5) in some samples, and flattening of these structures was observed in others (Figure 6). A fibrin-mucoid crust of variable thickness (Figure 7) often partially or completely obscured the enterocytes. In addition, fat droplets were observed at a variable quantity in the lumen (Figure 7).

Enterocytes were observed both with well-defined contours and architecture, as well as with disorganized architecture (Figure 5 and Figure 8); in some samples, partial or

complete loss of boundaries between the cells was observed (Figure 9).

The microvilli showed great height variability, sometimes resulting in its complete disappearance (Figure 5). Furthermore, microvilli varied in number. In the same sample, we also observed “pieces” of enterocytes showing microvilli and in some areas, enterocytes completely denuded of microvilli (Figure 8). We also observed the formation of tufts of microvilli (Figure 9) and variable disorganization of these structures.

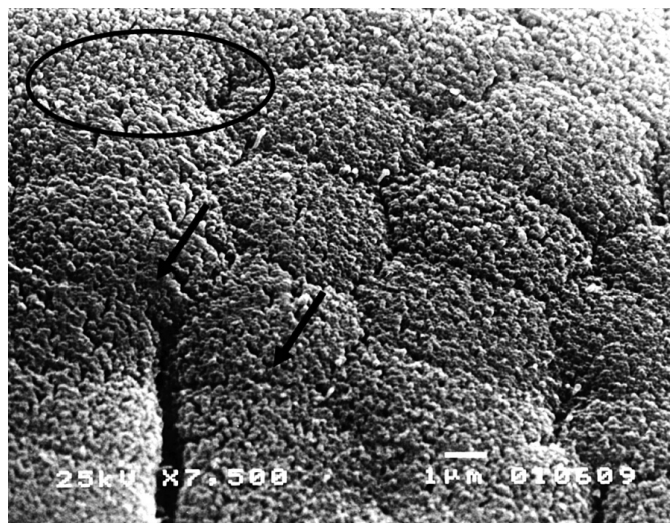


FIGURE 9. Small intestine scanning electronic microscopy of an HIV-infected child. Areas denuded of microvilli (arrows). Others areas with presence of microvilli showing differences in height and tendency to form tufts (ellipse). 7,500X

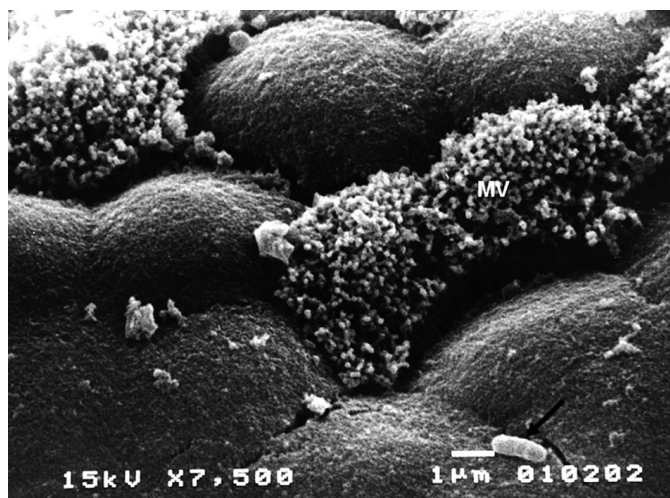


FIGURE 10. Focal epithelial lesion with areas denuded of microvilli (MV). Bacillus's like microorganism (arrow). 7,500X

Microorganisms with structures that were morphologically similar to bacilli were occasionally identified in the intestinal lumen (Figure 10).

DISCUSSION

It is important to emphasize that the intestine samples examined in this study were obtained in the period preceding the introduction of highly active antiretroviral therapy (HAART). Therefore, this is a unique study that almost reflects the natural history of the disease. Moreover, the current research is one of the exceedingly rare studies that

use transmission electron microscopy to analyze the small intestine of HIV infected children.

We observed significant effects on microvilli, including complete denudation (Figure 2), shortening (Figures 3 and Figure 4) and the formation of small “tufts” (Figure 3) that resembled a “bouquet of flowers”. These findings are similar to those described by other authors who have also examined microvilli in similar patients^(3, 10, 11, 25). The repetition of these patterns in different samples appears to be compatible that they are related to HIV itself, rather than a result of a co-occurring infection. This hypothesis was first suggested by Kotler et al.⁽¹⁶⁾. However, one should consider the limitations of this methodology. Intestinal samples were obtained by biopsy and represent tiny fragments of tissue; thus, they may represent only focal alterations.

Another prominent finding was the increase of intracytoplasmic vacuoles in the enterocytes in many of the fragments (Figures 3 and Figure 4). In some samples, this change was so significant that it promoted a structural derangement of the involved enterocytes (Figure 2). It was previously reported^(17, 21, 24, 25) and may be indicative of cellular degeneration or inflammatory activity, particularly when irregularly shaped and located in the periphery of the cytoplasm. Like Kotler et al.⁽¹⁷⁾ we did not detect any intracellular or extracellular viral particles in the enterocytes with increased vacuoles⁽¹⁷⁾. This may suggest that if HIV was involved in this process, it may have occurred through molecular signaling and not by a direct cytopathic effect.

Our observation of the thickening of the basal membrane (Figure 3) constituted a unique and very important change that, to our knowledge, has not been previously described. This component of the epithelia, in addition to promoting the adhesion of epithelial cells to underlying connective tissue, plays a role in the migration, proliferation, and differentiation of cells⁽¹⁵⁾. Thus, that finding could be the result of ongoing inflammation in the gut or a facilitator of detachment of cells to the intestinal lumen, as observed in some samples (Figures 1, 2, 3).

We often observed intraepithelial lymphocytes (Figure 4), which were also previously described in a study that used light microscopy to examine the intestinal mucosa⁽¹⁸⁾. Along with the lymphocytes of the lamina propria, intraepithelial lymphocytes are part of the effector arm of the GALT immune response. They are the first immune cells to recognize pathogens that have invaded the epithelial surface and are comprised of a large population of oligoclonal resting cells able to display phenotypic and functional cytolytic T cells when activated⁽¹⁾. The increased presence of intraepithelial lymphocytes is well established in celiac disease, a condition usually followed by villous atrophy⁽²⁰⁾ due to excessive apoptosis of enterocytes⁽²³⁾. Considering that several histological changes observed in the intestinal mucosa during HIV infection are similar to those seen in celiac disease, it is reasonable

to assume that the intraepithelial lymphocytes may have a cytolytic effect on the enterocytes of patients with HIV/AIDS. Weber Jr. and Dobbins III⁽³⁰⁾ have suggested it comparing the ultrastructural characteristics of these lymphocytes in both diseases and demonstrating their relative scarcity in healthy patients⁽³⁰⁾. The present paper is novel in its demonstration of the presence of intraepithelial lymphocytes in the intestinal mucosa of young HIV patients through electron microscopy. Currently, it is known that intraepithelial lymphocytes are represented predominantly by CD8+ T cells and cells-T γ/δ ⁽¹⁴⁾. More recently, CD8+ T cells have garnered interest because some of them have demonstrated the capacity to control the replication of HIV in the bloodstream and gastrointestinal tracts and preserve CD4+ T cells given the capacity of various effector cytokines such as interferon- γ , macrophage inflammatory protein 1 β , TNF- α , and interleukin-2 (IL-2). These “multifunctional” CD8+ T cells are specific for HIV and are rarely found among individuals who present progression of HIV infection⁽²⁾. Considering that our patients were progressors, they probably would be better categorized as having a “mono-functional” immune response profile with ineffective CD8+ T cells unable to control viral replication at the level of intestinal mucosa⁽⁶⁾. This information is relevant because it suggests that an effective HIV vaccine should be able to induce a response in CD8+ T cells specific for the virus that fit the “multifunctional” profile in the gastrointestinal tract. Such a vaccine would be capable of inhibiting the entry of HIV into the mucosa and controlling viral replication thereby ultimately, preserving immune function⁽⁴⁾.

Other structures observed frequently in our samples were the multivesicular bodies (Figure 1), intracytoplasmic organelles that are rich in the cholesterol, formed during endocytosis. The increased presence of these structures could be indicative that the intestinal changes occurring in these patients were large such to promote absorption of potentially antigenic macromolecules. This could provoke local chronic inflammatory processes and the consequent recruitment of CD4+ targets of HIV infection. These processes could contribute to the systemic spread of the virus from the gastrointestinal tract, which acts as a major viral reservoir. It is interesting to note the similarity of our findings with those previously described in children with chronic diarrhea due to milk protein intolerance⁽⁸⁾. We hypothesize that it may represent the occurrence of similar pathogenic mechanisms or, alternatively, that different form of injury to the gut could culminate in similar pathological responses. Moreover, it seems reasonable to think that children with HIV/AIDS are at an increased risk of developing intolerance to various components of their diet. This could happen as a result of increased intestinal permeability caused by HIV infection, by co-infection with other pathogens or even by early exposure to cow's milk protein due to breastfeeding contraindication.

It is also estimated that the multivesicular bodies may play an important role in virus entry from their presence in the intestinal lumen, as occurs in HIV transmission. Like other retroviruses, HIV gag proteins contain small binding sites that mediate the interactions between multivesicular bodies and other components related to endocytosis, suggesting that these viral proteins are directly involved in this process⁽¹⁹⁾.

The mitochondria were normal in appearance in most cases, as shown in Figure 4. This differs from the findings of Oktedalen et al.⁽²⁴⁾, who noted a reduction in the number of these organelles. According to these authors, this reduction could cause possible enterocyte dysfunction and could explain the occurrence of pronounced changes in the intestinal absorption of D-xylose in HIV patients as in celiac disease. Nevertheless, in our study, we also observed the vacuolization of mitochondria and Golgi complex (Figure 2), which could be indicative of enterocyte dysfunction.

Analyses using scanning electron microscopy also recognized of several abnormalities on the epithelial surface of the small intestine. Thus, in most images, we could clearly see the impressive extent and variable intensity of microvilli shortening and could often view extensive areas of complete destruction of these structures (Figure 5). Furthermore, tufts of microvilli was also observed in some samples (Figure 9), as were areas showing microvilli derangement. Although these changes were prominent, other areas of mucosa remained essentially unchanged. Thus, our findings revealed a pattern of variable epithelial changes, which differs from the more diffuse pattern of changes and uniformity characteristic of celiac disease⁽²⁶⁾.

In several samples, it was possible to observe a crust of mucus and fibrin, which varied in thickness and in extent, covering the epithelial surface of the intestine (Figure 7). This crust was often sufficiently large to completely prevent the viewing of enterocytes. Apparently, this finding has not been described previously in relation to ultrastructural changes of the intestine in patients with HIV/AIDS. However, there is a close resemblance between this finding and the description given by Fagundes-Neto et al.⁽⁹⁾ of children with persistent diarrhea. Given the frequency and the extent of this phenomenon, it is feasible to postulate that this crust results in diminished intestinal absorption through the mechanical blockage of nutrients. Under these circumstances, the presence of unabsorbed solutes in the intestinal lumen could trigger osmotic diarrhea.

We also found frequent presence of fat droplets in the intestinal lumen (Figure 7). Considering the recommended fasting time before the intestinal biopsies, the presence of these fat droplets is most likely explained by lipid malabsorption since this would only be an expected finding after a meal. The mechanical barrier hypothesis outlined above could be one of the possible explanations for this poor fat absorption.

Alternatively, fat solubilization could be impaired due to primary bile salts disconjugation and 7- α -dehydroxilation secondary to bacterial overgrowth in the intestine. Such a process has been described in environmental enteropathy⁽²²⁾, or as a result of pancreatic dysfunction due to malnutrition⁽⁷⁾.

Sometimes we observed the presence of bacteria with bacilli-like morphology in the intestinal lumen (Figure 10). This may be similar to the occurrence of bacterial overgrowth in the small intestine that occurs in environmental enteropathy, which shares functional and ultrastructural similarities with the pathology observed in our study⁽²²⁾.

CONCLUSION

Significant ultrastructural changes varying in intensity and frequency were found through scanning and transmission electron microscopy in the intestine of HIV infected children. Some of these changes, to our knowledge, had not yet been described. Given their magnitude and the frequency with which recurred in the analyzed samples, they could intensely impact intestinal function and permeability and could therefore profoundly affect children's nutritional status, quality of life and life expectancy.

Leite CAC, Fagundes-Neto U, Haapalainen EF. Avaliação da ultra-estrutura do intestino delgado de crianças infectadas pelo HIV por microscopia eletrônica de transmissão e varredura. *Arq Gastroenterol.* 2013,50(1):70-7.

RESUMO - Objetivos - Descrever achados ultra-estruturais do intestino delgado de crianças infectadas pelo HIV. **Métodos** - Estudo descritivo, observacional de biopsias do intestino delgado, realizada entre agosto de 1994 e maio de 1995 em São Paulo - Brasil. Este material pertencia a 11 crianças infectadas pelo HIV e foi armazenado em um laboratório em blocos de parafina. As amostras de intestino delgado foram analisadas por microscopia eletrônica de transmissão e de varredura e achados os achados ultra-estruturais foram descritos por meio da análise de fotos digitalizadas desse material. Foi obtida aprovação pelo Comitê de Ética. **Resultados** - Na maioria das amostras a microscopia de varredura mostrou vários graus de encurtamento e diminuição do número das microvilosidades e até o completo apagamento dessas estruturas em algumas áreas. O desarranjo dos enterócitos foi visto com frequência e, por vezes, os limites celulares estavam imprecisos. Em algumas áreas uma membrana fibrino-mucosa com espessura e extensão variáveis aparentava revestir parcial ou totalmente a superfície epitelial. Gotas de gordura no lúmen intestinal estavam presentes em várias amostras e bactérias morfológicamente semelhantes a bacilos foram observadas em duas amostras. A microscopia eletrônica de varredura confirmou as observações constatadas nas microvilosidades através da microscopia de transmissão e também mostrou pequenos "tufo" dessas estruturas. Além disso, mostrou aumento do número de vacúolos e de formações multivesiculares dentro de vários enterócitos, aumento da presença de linfócitos intraepiteliais, vacuolização mitocondrial e alargamento da membrana basal na maioria das amostras analisadas. No entanto, algumas amostras apresentavam-se com aspecto normal. **Conclusões** - Nosso estudo mostrou a ocorrência comum de várias alterações ultra-estruturais intestinais importantes e de intensidade variável entre crianças infectadas pelo HIV, algumas delas, ao nosso conhecimento, não descritas anteriormente.

DESCRIPTORIOS - Microscopia eletrônica. HIV. Intestino delgado. Criança.

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