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Early Impairment of Gut Function and Gut Flora Supporting a Role for Alteration of Gastrointestinal Mucosa in Human Immunodeficiency Virus Pathogenesis▽

Andrea Gori,[1,2]* Camilla Tincati,[2] Giuliano Rizzardini,[3] Carlo Torti,[4] Tiziana Quirino,[5] Monique Haarman,[6] Kaouther Ben Amor,[6] Jacqueline van Schaik,[6] Aldwin Vriesema,[6] Jan Knol,[6] Giulia Marchetti,[7] Gjalt Welling,[8] and Mario Clerici[9]

Division of Infectious Diseases, Department of Internal Medicine, San Gerardo Hospital, University of Milano-Bicocca, Monza, Italy[1]; Department of Internal Medicine, Clinic of Infectious Diseases, San Paolo Hospital, University of Milan, Milan, Italy[2]; 1st Division of Infectious Diseases, Luigi Sacco Hospital, Milan, Italy[3]; Clinic of Infectious Diseases, University of Brescia, Brescia, Italy[4]; Infectious Diseases Unit, Busto Arsizio Hospital, Busto Arsizio, Italy[5]; Numico Research BV, Wageningen, The Netherlands[6]; Department of Clinical Science, Luigi Sacco Hospital, University of Milan, Milan, Italy[7]; Department of Medical Microbiology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands[8]; and Department of Science Technology Biomedicine, University of Milan, Milan, Italy[9]

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Our results show that impairment of the gastrointestinal tracts in human immunodeficiency virus (HIV)-positive patients is present in the early phases of HIV disease. This impairment is associated with alterations in gut microbiota and intestinal inflammatory parameters. These findings support the hypothesis that alterations at the gastrointestinal-tract level are a key factor in HIV pathogenesis.

The hallmark of human immunodeficiency virus (HIV) infection is the depletion of $CD4^+$ T cells, which occurs throughout the entire disease. Recent data have shown that primary HIV infection is associated with a preferential depletion of $CD4^+$ T cells in the gastrointestinal (GI) tract, where more than 60% of T lymphocytes reside, suggesting a pivotal role for the early impairment of the gut-associated lymphoid tissue in HIV disease (4). However, the precise pathogenic mechanisms underlying the profound $CD4^+$ T-cell loss observed in the various stages of disease are only partially understood.

A characteristic feature of HIV disease is chronic immune activation, which might play a crucial role in the $CD4^+$ T-cell depletion. It has been recently hypothesized that a breakdown of the GI mucosal barrier may contribute to chronic immune activation (4). Thus, the exposure of peripheral immune cells to microbial products after gut injury may result in the abnormal activation of such cells.

The healthy GI tract is colonized by a large variety of commensal microbes that impact on the development of the humoral and cellular mucosal immune system (11, 12). This microbiota is shielded from the immune system via a strong mucosal barrier. Infections and antibiotics are known to alter both the normal GI-tract barrier and the microbiota, which may result in possible immune abnormalities. If HIV infection itself impaired the GI barrier and the composition of the gut microbiota, the breakdown of the GI mucosa would result in the sudden and chronic exposure of peripheral lymphocytes to an abnormal intestinal microbiota (4).

To verify this hypothesis, we studied the effect of HIV infection on the commensal intestinal microbiota and on intestinal inflammation by analyzing the composition of GI tract microbiota and by measuring the levels of fecal calprotectin in 57 healthy, asymptomatic HIV-positive, antiretrovirus-naive individuals (average $CD4^+$ T-lymphocyte count and HIV RNA levels of 520 cells/μl and 28,393 copies/ml, respectively) enrolled in a trial in Italy (COPA study). The results were compared to historical data from the general population not suffering from HIV infection. Informed consent was obtained from all of the patients.

The fecal microbiota of the HIV patients was analyzed by either fluorescence in situ hybridization or quantitative real-time PCR (Q-PCR). Preparation of fixed fecal cells and DNA extraction were performed as described previously (6). For fluorescence in situ hybridization, a set of specific rRNA-targeted oligonucleotides probes were used to quantify *Candida albicans* (Caal [5′-GCCAAGGCTTATACTCGCT-3′]), bifidobacteria (Bif164 [5′-CATCCGGCATTACCA CCC]), lactobacilli (Lab158 [5′-GGTATTAGCAYCTGTTTCCA]), and total bacteria (Eub338 (5′-GCTGCCTCC CGTAGGA GT)) (1, 7, 8, 10). The relative abundance of bacterial groups was determined as the proportion of cells hybridizing with the specific probe to the total cells positive for the Eub338 probe. For the quantification of *Pseudomonas aeruginosa*, a Q-PCR-based method was used as described by Pirnay et al. (14). Calprotectin was measured by using a commercial enzyme-linked immunosorbent assay kit (PhiCal, Eurospital, Italy).

Initially, we focused our attention on two opportunistic pathogens: *P. aeruginosa* and *C. albicans*. The presence of these species was found to be very high in this HIV-positive patient population compared to levels reported for healthy individuals. *P. aeruginosa* was identified in 92% of all fecal HIV samples analyzed. In healthy individuals, *P. aeruginosa* was

* Corresponding author. Mailing address: Division of Infectious Diseases, Department of Internal Medicine, San Gerardo Hospital, University of Milano-Bicocca, Via Solferino 16-20052, Milan, Italy. Phone: 39 039 2333794. Fax: 39 039 2333898. E-mail: andrea.gori@unimib.it.

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found in only 20% of samples (unpublished data) using the same Q-PCR method. Moreover, *P. aeruginosa* accounted for 0.7% ± 0.13% of the total microbiota in our HIV positive group and represented a 10-fold increase compared to the levels in the healthy individuals. Similarly, *C. albicans* was detected in 100% of the feces samples of our HIV-positive group, whereas Bernhardt and Knoke (3) reported that in the general population only 40% are positive carriers of *Candida.* More importantly, the average count of *C. albicans* was almost 10,000-fold higher in the HIV-positive group (ranging from $[9.1 \pm 1.0] \times 10^7$ to $[8.9 \pm 1.0] \times 10^7$ cells/g [wet weight] of feces) than reported for the HIV-negative general population ($\sim 10^4$ cells/g [wet weight] of feces) (3).

Further support of the hypothesis of an impairment of the GI microbiota in HIV-infected patients was revealed by data showing lower levels in other microbial species, e.g., bifidobacteria and lactobacilli, compared to the levels reported for the general population. Both species have been shown to have a positive influence on mucosal immune function and gut health (2, 5, 15, 17). The relative amount of bifidobacteria in the HIV-positive population investigated during our trial was 2.5% (95% confidence interval = 1.4 to 4.2%) of the total fecal bacteria, while the lactobacilli were nearly undetectable at 0.02% (95% confidence interval = 0.009 to 0.05%). It is well documented that in the general population bifidobacteria can vary between 5 and 10% of the total bacterial community, while lactobacilli represent 1 to 2% (9, 13). Our data thus suggest that in HIV-infected individuals the composition of the fecal microbiota is atypical at an early stage of infection.

Levels of fecal calprotectin, a protein secreted by recruited neutrophils in the intestinal lining indicative of intestinal inflammation and used as a marker of mucosal inflammatory activity in patients with inflammatory bowel disease (4, 11, 16, 17), were also analyzed. The cutoff for this marker is at 50 μg/g (wet weight) of feces, with a median value in healthy individuals of 26 μg/g (16). Our results revealed that in our HIV patient group half of the subjects (27 of 53) had increased fecal calprotectin levels (>50 μg/g). Even 34% (18 of 53) of the HIV patients had levels over 100 μg/g (wet weight) of feces, a finding that is clearly indicative of a significant GI inflammation. Since intestinal inflammation is known to reduce the intestinal barrier function, these data confirm the breakdown of the intestinal barrier.

Abnormal immune activation represents one of the main factors associated with HIV disease progression. A better understanding of the mechanisms driving chronic activation of the immune system could shed light onto the mechanism(s) associated with immune cell depletion. The hypothesis that chronic activation stems from a damaged mucosal barrier and stimulation of immune cells by microbial products is therefore fascinating.

The results presented here clearly show that impairment of the GI tract in HIV-positive patients is present already in the early phases of HIV disease. This impairment is associated with elevated levels of intestinal inflammatory parameters and clear alterations in the gut commensal microbiota, confirming a possible correlation between intestinal microbial alteration, GI mucosal damage, and immune activation status. These findings strongly support the recent hypothesis that alterations at the GI-tract level are a key factor in the pathogenesis of chronic HIV infection.

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