Cytokine Expression in the Colonic Mucosa of Human Immunodeficiency Virus-Infected Individuals before and during 9 Months of Antiretroviral Therapy[▽]

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High-level human immunodeficiency virus (HIV) replication and the rapid breakdown of the mucosal immune system are the hallmarks of HIV infection in the gut. Cytokine dysregulation may be related to both phenomena. Using real-time PCR we quantified the colonic mucosal mRNA expression of selected proinflammatory and regulatory (gamma interferon [IFN-gamma], tumor necrosis factor alpha [TNF- α], and interleukin-2 [IL-2], IL-4, IL-6, and IL-10) and HIV-inhibitory (IL-16, CCL3, and CCL5) cytokines for 10 HIV-infected patients before and during 9 months of highly active antiretroviral therapy (HAART). HIV RNA and T-cell dynamics were measured in the colonic mucosa and the blood. Seven HIV-negative individuals served as controls. The mucosal mRNA expression of TNF- α , IFN-gamma, IL-4, IL-6, and IL-10 was significantly higher in HIV-infected patients than in control patients and remained elevated during 9 months of HAART despite the decline in blood and mucosal HIV RNA levels and an increase in the level of CD4⁺ T lymphocytes. The mRNA levels of CCL3 and CCL5, both of which were elevated before treatment, returned to nearly normal during therapy. Despite reductions in levels of mucosal HIV RNA and the restoration of mucosal CD4⁺ T lymphocytes, antiretroviral therapy failed to restore the normal colonic immunologic environment.

The gut-associated lymphoid tissue represents one of the largest reservoirs of immunocompetent cells in the body. It hosts huge numbers of activated CD4⁺ CCR5⁺ memory T lymphocytes, which makes the gastrointestinal (GI) tract uniquely susceptible to human immunodeficiency virus (HIV) infection (3, 29, 38). Previous studies found reduced numbers of CD4⁺ T lymphocytes in duodenal and rectal mucosa of HIV-infected patients (10, 31, 48). However, unlike circulating CD4⁺ T lymphocytes, mucosal CD4⁺ T cells are almost completely lost in the early days of primary infection (18, 34), without recovery at later stages of HIV disease (48). The GI CD4⁺ T-cell loss is accompanied by high levels of local viral replication (25, 32), with mucosal HIV p24 antigen concentrations exceeding those measured in the blood (16, 44) and the presence of latently and productively infected CD4⁺ T lymphocytes in the mucosa (14, 27, 30, 54).

It has been suggested that changes in levels of mucosal cytokines contribute to the massive destruction of gut-associated lymphoid tissue (33, 40). In fact, there is evidence that high mucosal viral loads may be related to excessive expression of T-helper 1 (Th-1) and Th-2 and proinflammatory cytokines (32), and yet the interplay between mucosal HIV replication and cytokine expression is not understood. Overproduction of

selected cytokines may be the primary event that results in T-cell activation, thereby fueling new rounds of infection and promoting the activation of provirus from latently infected cells. Alternatively, cytokine expression may be a consequence of mucosal HIV replication rather than its cause.

Highly active antiretroviral therapy (HAART) has provided a tool for studying the interplay between HIV replication and cytokine production. If cytokine production were stimulated by the presence of HIV antigen, the cytokine mRNA expression profile should change following the elimination by HAART of the stimulating agent. This study was designed to determine the mucosal viral load, T-cell dynamics, and mRNA expression characteristics of interferon gamma (IFN-gamma), tumor necrosis factor alpha (TNF- α), interleukin-2 (IL-2), IL-4, IL-6, IL-10, and IL-16, and the chemokines CCL3 and CCL5 for HIV-infected patients before and during 9 months of HAART.

MATERIALS AND METHODS

Subjects. This study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the local ethics committee. All participants had given their written informed consent. Ten HIV-1-infected, previously untreated patients who were about to start antiretroviral therapy and seven HIV-negative controls who presented for routine colon cancer screening were enrolled. Subjects with a history of inflammatory bowel disease, colon cancer, diverticulitis, and acute or chronic diarrhea were ineligible.

Study design. Control patients presented for a single fiberoptically guided flexible colonoscopy. HIV-infected participants underwent colonoscopy before as well as 1, 4, and 9 months after the start of antiretroviral therapy consisting of administration of two nucleoside reverse transcriptase inhibitors and a protease inhibitor. During each biopsy procedure, seven punch biopsy samples were obtained from noninflamed colonic tissue. Two specimens were used for determi-

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nation of levels of IFN- γ , TNF- α , IL-2, IL-4, IL-6, IL-10, IL-16, CCL3, and CCL5 mRNA expression. For HIV-infected subjects, levels of CD4⁺ T lymphocytes and HIV RNA in the blood and the remaining mucosa samples were quantified.

mRNA extraction from colonic mucosa. Total cellular RNA was extracted using TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) according to a modified version of the supplier's protocol as described before (45). Briefly, frozen tissue samples were transferred into clean tubes containing 750 μ l of ice-cold TRIzol reagent. Following ultrasonic tissue homogenization, RNase-free water was added to achieve a final volume of 1,000 μ l. After incubation and phase separation with 200 μ l of chloroform, the aqueous phase containing the RNA was transferred into a clean tube. RNA was precipitated by adding 500 μ l of propanol-2 and 10 μ l of glycogen (20 μ g/ μ l in RNase-free water). The RNA pellet was washed with 75% ethanol and redissolved in RNase-free water. The RNA pellet concentration and purity were determined spectrophotometrically at 260 and 280 nm, respectively. A260/A280 ratios greater than 1.8 were considered an indicator of RNA purity. Extracted RNA was of good quality, with 28S/18S ratios between 1.5 and 1.9 as determined by agarose gel electrophoresis analysis using ethidium bromide staining. The median total RNA recovery from tissue was 98% (range, 90% to 105%).

DNase treatment. To remove DNA contamination, RNA extracts were treated with DNase I and 10 $\times$ DNase I buffer as specified in the manual of the supplier (Gibco BRL, Eggenstein, Germany). Total RNA preparations were tested for DNA contamination by performing real-time PCR in the presence and absence of reverse transcriptase.

DNA transcription. cDNA was transcribed using Omniscript reverse transcriptase (Qiagen GmbH, Hilden, Germany) and oligo-(dT)16 primer (Applied Biosystems, Darmstadt, Germany) according to the protocols of the manufacturers.

Primers and probes. Primers and hybridization probes were designed based on reference sequences available from the National Center for Biotechnology Information. All primer and probe sequences were tested for specificity by submitting them to the National Center for Biotechnology Information BLAST program (www.ncbi.nlm.nih.gov/BLAST). Hybridization probes were synthesized at TIB MOLBIOL GmbH, Berlin, Germany, and carried a 5' LightCycler Red fluorophore (cytokine probes, LC Red 640; GAPDH [glyceraldehyde-3-phosphate dehydrogenase] probe, LC Red 705) and a 3' fluorescein. Sequences of the primers and probes are shown in Table 1.

Calibration standards. Target sequences of cytokines and GAPDH were amplified from patient samples by use of conventional PCR. Following agarose gel purification (QIAquick gel extraction kit; Qiagen GmbH, Hilden, Germany), *Taq* amplification of first-round PCR products resulted in cDNA fragments which were ligated into a pCR 2.1 cloning vector (TOPO TA cloning kit; Invitrogen, Karlsruhe, Germany). Competent *Escherichia coli* bacteria (One-Shot TOP10 chemically competent *E. coli*; Invitrogen, Karlsruhe, Germany) were transformed with the expression vector and propagated overnight. Cells were harvested and lysed. Plasmids were extracted from cell lysates by use of a QIAprep plasmid extraction kit (Qiagen, Hilden, Germany). Purified plasmids were linearized by use of EcoRI, HindIII, or XbaI (GIBCO BRL, Eggenstein, Germany). Inserts were sequenced, and the results were compared to the reference sequences. DNA concentrations were determined photometrically, and the number of copies per microliter was calculated as described before (42). Calibration standards were produced by serial dilution of the plasmids, with final concentrations ranging from 10¹ to 10⁷ copies/reaction.

Real-time PCR. A LightCycler PCR and detection system (Roche Diagnostics GmbH, Mannheim, Germany) was used for amplification and online quantification. PCR was performed using glass capillaries and a 20- μ l final volume. The reaction mixture was formulated by combining water, 2 μ l of LC FastStart DNA master hybridization probe kit mix (Roche Diagnostics GmbH, Mannheim, Germany), 1 μ l of each primer (10 μ M), 1 μ l of each hybridization probe (4 μ M), 0.8 μ l of MgCl₂ (25 mM) for analysis of GAPDH, IL-4, IL-16, TNF- α , CCL3, and CCL5, and 2.4 μ l of MgCl₂ (25 mM) for analysis of IL-2, IL-6, IL-10, and IFN- γ . A 2- μ l sample volume was added (1- μ l sample volume for analysis of GAPDH).

The PCR program was performed using the following sequence: one cycle of denaturation and activation of *Taq* polymerase at 95°C for 10 min followed by 45 cycles of repeated denaturation at 95°C for 10 s, annealing at 58°C (for GAPDH, IL-16, CCL3, and CCL5) or 60°C (for IL-10) or 62°C (for IFN- γ and TNF- α) for 12 s, extension at 72°C for 10 s, and a final extension at 72°C for 5 min. Each assay included (in duplicate) a four-point standard calibration curve (ranging from 10¹ to 10⁴ template copies/reaction) and a no-template control containing water. Fluorescence data were collected at the end of each annealing step, with a single acquisition of fluorescence for each capillary. The crossing point for each reaction was determined using the second-derivative maximum

algorithm. Crossing-point values were plotted as a function of input template copy number, and a least-squares regression was performed with LightCycler 3.6 software. Patient samples were amplified in duplicate. Copy numbers in patient samples were calculated by interpolation of the experimentally determined crossing-point values into the control standard regression curve. Results were normalized to GAPDH mRNA copy numbers and are expressed as the numbers of cytokine mRNA copies per 1,000 GAPDH mRNA copies.

The lower limit of quantification (LLQ) was 10 template copies per reaction. Owing to different weights of the tissue samples used for RNA extraction, the values per weight obtained for the LLQ were variable. Strong linear correlations ($r^2 > 0.998$) between crossing-point values and transcript copy numbers were obtained over a range of 10¹ to 10⁶ copies per reaction. Standard curves generated from dilution series of standards and patient samples had comparable slopes, indicating similar PCR efficiencies for standards and unknowns. The PCR efficiencies showed very few interassay variabilities, with coefficients of variation ranging between 2% and 14%. Cytokine and GAPDH mRNA transcripts were amplified with similar efficiencies at different concentrations, as expressed by the small variability of cytokine mRNA/GAPDH mRNA ratios for serially diluted patient samples. Intra-assay precision was tested by analyzing replicate serial dilutions of mRNA transcripts. Precision for all analytes was satisfactory, with coefficients of variation of <9% for any template number within the calibration range.

Quantification of HIV RNA. HIV RNA was isolated from tissue samples by following the modified TRIzol protocol described above. Isolation from blood was achieved using an Amplicor kit (Roche Diagnostics, Mannheim, Germany). The HIV-1 Amplicor kit was also used for HIV RNA quantification in accordance with the manufacturer's instructions.

Quantification of CD4⁺ and CD8⁺ T lymphocytes. CD4⁺ and CD8⁺ T lymphocytes from mucosal tissue were isolated and quantified by flow cytometry as described previously (45). Briefly, mucosal biopsy specimens were washed twice in phosphate-buffered saline (GIBCO, Berlin, Germany), blotted dry, and weighed. After mechanical disaggregation (Medimachine, Dako, Hamburg, Germany), the resulting cell suspension was centrifuged at 600 $\times$ g for 10 min in a 30% isotonic Percoll solution (Pharmacia, Uppsala, Sweden). The supernatant containing epithelial cells and debris was discarded. The pellet was resuspended in 50 ml of RPMI 1640 containing 10% fetal calf serum (GIBCO, Berlin, Germany) and centrifuged for 10 min at 600 $\times$ g. Cell counts and viability were determined using a Neubauer chamber with trypan blue dye exclusion. Peripheral blood mononuclear cells were prepared from venous blood by Ficoll density centrifugation. Isolated mucosal and circulating mononuclear cells were stained with fluorescein-conjugated anti-CD4, phycoerythrin-conjugated anti-CD8, and Cy5-conjugated anti-CD3 monoclonal antibodies (Dako, Hamburg, Germany) and analyzed by flow cytometry (FACScan; Becton-Dickinson, Heidelberg, Germany) as described before (48). Cell numbers per milligram of biopsy sample were calculated using the following formula: CD4⁺ T cells per milligram of biopsy sample = [number of isolated viable mononuclear cells counted (Neubauer chamber)/biopsy sample wet weight (in milligrams)] $\times$ [number of CD3-positive CD4-positive events (FACS)/number of events in viable mononuclear cell gate (FACS)], where FACS represents "fluorescence-activated cell sorter." The same equation was used for the quantification of CD8⁺ T lymphocytes. The within-subject variability of both absolute CD4⁺ and CD8⁺ T-cell counts per milligram of biopsy sample was <35%. The number of circulating CD4⁺ T lymphocytes per microliter of blood was calculated using data from simultaneously obtained routine blood counts.

Immunohistochemistry. Tissue samples were fixed in 4% neutrally buffered formalin and embedded in paraffin. Sections (4 μ m thick) were cut, deparaffinized, and subjected to heat-induced epitope retrieval. Briefly, sections were immersed in sodium citrate buffer solution at pH 6.0 and heated in a high-pressure cooker. The slides were rinsed in cool running water, washed in Tris-buffered saline (pH 7.4), and incubated with primary antibodies according to the instructions of the manufacturers. After being taken from selected specimens, frozen sections were air-dried overnight and fixed in acetone for 10 min. The following antibodies were used: NCL-CD4-IF6 (anti-CD4 antibody; Novocastra) and C8/144B (anti-CD8 antibody; DakoCytomation). Binding of antibodies was visualized by the alkaline-phosphatase-anti-alkaline phosphatase complex method using Fast Red as the capture agent (12). Specificity was tested by submitting samples to the staining procedure without previous incubation with the primary antibodies. T lymphocytes from blinded tissue sections from five patients and five controls were quantified per high-power field (hpf = 0.237 mm²). 0.10 hpf were averaged in each case and results were expressed as cells/mm².

Statistical analysis. For between-group comparisons, the Mann-Whitney U test was used. Paired differences were tested using the Wilcoxon signed-rank test. Correlations between differences in cytokine mRNA and CD4⁺ T-cell numbers

TABLE 1. Primers and probes used for real-time RT-PCR assays

Assay	Primer or probe	Sequence (5' → 3')	Accession no.	Location (bp)
GAPDH	Sense primer	AACAGCGACACCCACTCCTC	M33197	919–938
	Antisense primer	GGAGGGGAGATTCAAGTGTGGT		1176–1156
	FL	CATGGCCTCCAAGGAGTAAGACCCCT		1059–1075
	RED 705	ACCACCAGCCCCAGCAAGAGCA		1078–1099
IL-2	Sense primer	CACAGCTACAACTGGAGCATTAC	U25676	145–168
	Antisense primer	TGCTGATTAAGTCCCTGGGTC		387–358
	FL	TCCCAAACCTCACCAGGATGCTCACA		215–239
	RED 640	TAAGTTTACATGCCCAAGAAGGCCAC		242–268
IL-4	Sense primer	TGCCTCCAAGAACAACAACCTGA	M13982	237–257
	Antisense primer	CCAACGTACTCTGGTTGGCTT		463–443
	FL	CCTTCTCATGGTGGCTGTAGAAGTCC		319–293
	RED 640	AGCACAGTCGCAGCCCTGCAGAAG		290–267
IL-6	Sense primer	CTTTTGGAGTTTGAGGTATACCTAG	M29150	390–414
	Antisense primer	GCTGCGCAGAATGAGATGAGTTGTC		626–602
	FL	AGATGCAATAACCACCCCTGACCCAA		518–543
	RED 640	CACAAATGCCAGCCTGCTGACGAA		545–568
IL-10	Sense primer	TGAGAACCAAGACCCAGACA	M57627	324–343
	Antisense primer	TCATGGCTTTGTAGATGCCT		505–486
	FL	CGGCGCTGTCATCGATTCTTCCCT		400–424
	RED 640	TGAAAACAAGAGCAAGGCCGTGGAGC		426–451
IL-16	Sense primer	GCAAGTCTCTCAAGGGGACC	U82972	1577–1596
	Antisense primer	CCTTCCAGGCTGAAGCCCAGC		1826–1806
	FL	CCCAGCCCTGCCGACATCTTCT		1811–1790
	RED 640	CAGTGTACCCGTGCAGACTGTGGC		1788–1765
IFN- γ	Sense primer	GCATCCAAAAGAGTGTGGAG	X13274	371–390
	Antisense primer	GCAGGCAGGACAACCATTAC		625–606
	FL	TCCAACGCAAAGCAATACATGAATC		491–516
	RED 640	TCCAAGTGATGGCTGAACCTGTCG		518–540
TNF- α	Sense primer	GAGTGACAAGCCTGTAGC	M10988	337–354
	Antisense primer	CCCTTCTCCAGCTGGAAG		699–682
	FL	GCATTGGCCCGGCGGTTT		417–400
	RED 640	CCACTGGAGCTGCCCCCTCAGCT		397–376
CCL3	Sense primer	GCAACCAGTTCTCTGCATCACTT	M23452	136–158
	Antisense primer	CAGCTCCAGGTCGCTGACAT		353–334
	FL	GTGTCATCTTCTTAACCAAGCGAAGCC		265–291
	RED 640	(TT)GTCTGTGCTGACCCAGTGAGGAG ^a		297–320
CCL5	Sense primer	CACGCCTCGCTGTATCCTCA	M21121	43–63
	Antisense primer	TTGGCGGTTCTTTCGGGTGAC		239–219
	FL	GCTGCTTGCCTACATTGCCCGC		124–146
	RED 640	CACTGCCCCGTGCCCACATCAA		148–169

^a Two deviant nucleotides (TT) at positions 295–296 were added for technical reasons.

and viral load were tested using the Spearman's rank correlation test. The *P* values were adjusted using the Bonferroni method.

RESULTS

Study population. The clinical part of the study was conducted from 1997 until 1998. Ten (nine male) HIV-infected subjects were included into the study. Seven (four male) non-HIV-infected individuals served as controls. The median ages for the study group and the control group were 42 years (range, 27 to 53 years) and 56 years (range, 48 to 70 years), respectively. The median level of HIV RNA for the study group at baseline was 4.9 (range, 3.2 to 6.3) log₁₀ copies/ml, and the CD4⁺ T-cell count was 102 (range, 23 to 1,217) cells/ μ l.

HIV-infected subjects were in CDC stages C3 (*n* = 7), B2 (*n* = 1), A3 (*n* = 1), and A1 (*n* = 1). Following the first colonoscopy, all HIV-infected subjects started antiretroviral therapy. Antiretroviral drug regimens consisted of zidovudine, lamivudine, and indinavir (*n* = 5), zidovudine, zalcitabine, and indinavir (*n* = 3), stavudine, didanosine, and nelfinavir (*n* = 1), and zidovudine, zalcitabine, and nelfinavir (*n* = 1) in standard dosages.

Mucosal cytokine expression. The mucosal mRNA expression of the cytokines IFN- γ , TNF- α , IL-4, IL-6, and IL-10 and the HIV-inhibitory chemokines CCL3 and CCL5 was significantly higher in the HIV-infected study group than in the control group. The between-group differences were

FIG. 1. Mucosal cytokine mRNA expression in HIV-negative controls (white boxes), HIV-positive study participants before HAART (light gray boxes), and HIV-positive patients after 9 months of HAART (dark gray boxes). Box plots display the 10th, 25th, 50th (median), 75th, and 90th percentiles. *, statistically significant difference compared to HIV-negative control results at the 0.05 level; ns, not significant; w/o, without; m, months.

moderate (three- to fivefold differences) for IFN- γ , IL-4, and CCL5 and were equal to or greater than 1 order of magnitude for TNF- α , IL-6, IL-10, and CCL3. IL-2 and IL-16 mRNA levels in patients and controls did not differ significantly (Fig. 1).

HIV RNA and CD4⁺ T-cell counts. Nine months after the initiation of HAART, the median reduction of plasma viral load was greater than 2.1 (range, 0.6 to 3.7) log₁₀ copies/ml ($P < 0.05$). Eight out of 10 patients had plasma viral loads below the LLQ of 400 copies/ml (Fig. 2). Virus replication in the mucosa as expressed by the number of HIV RNA copies relative to the number of GAPDH mRNA copies also decreased significantly by 2.5 (range, -0.3 to 5.1) log₁₀ copies/ml ($P < 0.05$). Yet 9 out of 10 subjects continued to have detectable mucosal HIV RNA throughout the study (Fig. 2).

During 9 months of antiretroviral treatment we saw a median blood CD4⁺ T-lymphocyte gain of 90 (range, -3 to 340) cells/ μ l ($P < 0.05$). In the same period, the CD4⁺ T-cell count

FIG. 2. Viral loads and CD4⁺ and CD8⁺ T-lymphocyte counts in the blood (light gray boxes) and the mucosa (dark gray boxes) before antiretroviral treatment (0) and 1, 4, and 9 months after the start of therapy. Box plots display the 10th, 25th, 50th (median), 75th, and 90th percentiles. Note that the mucosal viral load is reported relative to GAPDH mRNA levels; therefore, the LLQ depends on the amount of GAPDH mRNA copies/sample and an LLQ of 10 cytokine mRNA copies/sample, the median LLQ for the ratio would be 0.00017.

in the mucosa increased by 1,071 (range, 632 to 4,719) cells/mg of biopsy tissue ($P < 0.05$). This considerable rise in absolute numbers translated into an only moderate increase in the percentage of mucosal CD4⁺ T lymphocytes per total lymphocytes from 0.5% (range, 0.0 to 22.8) before HAART to 15.3% (range, 5.7 to 21.1) after 9 months of HAART.

Lymphocyte dynamics were also studied using immunohistochemistry: T-cell numbers in multiple stained tissue sections from five HIV-infected patients were counted before and after 9 months of HAART. Mucosal specimens taken before HAART were depleted of CD4⁺ T cells, with a CD4⁺ T-lymphocyte density of 72 (range, 46 to 118) cells/mm², and repopulated after 9 months of HAART with a CD4⁺ T-lymphocyte density of 489 (range, 194 to 920) cells/mm² ($P < 0.05$). The latter level significantly exceeded the mucosal CD4⁺ T-cell numbers measured in samples obtained from five seronegative control patients (173 [range, 122 to 367] cells/mm²) ($P < 0.05$). CD8⁺ T-lymphocyte counts in the blood and the mucosa as determined by FACS analysis did not change significantly during the study (Fig. 2). Again, immunohistochemistry found similar patterns of lymphocyte dynamics, with a median mucosal CD8⁺ T-lymphocyte density of 1,156 (range, 743 to 1,747) cells/mm² before HAART and 785 (range, 456 to 1,232) cells/mm² after 9 months of HAART. At both time

FIG. 3. Immunohistochemical analyses of CD4⁺ (arrows) and CD8⁺ T-cell distribution in representative colonic mucosal tissues of HIV-infected patients before HAART and after 9 months of HAART. Note the increased cellularity in the lamina propria in the colonic biopsy samples after 9 months of HAART compared to that seen in the biopsy samples before HAART, which consisted predominantly of lymphocytes and plasma cells, indicating persistent chronic inflammation.

points, mucosal CD8⁺ T-cell densities were significantly higher than the levels seen in control samples (76 [range, 55 to 160] cells/mm²) ($P < 0.05$). Figure 3 shows representative mucosal tissue sections, illustrating the changes in CD4⁺ T-cell numbers during 9 months of HAART.

Mucosal cytokine mRNA expression before and after 9 months of HAART. All study subjects were included in the analysis irrespective of virologic response to antiretroviral therapy. The response to HAART in terms of mucosal cytokine mRNA expression was highly variable: after 9 months of HAART, some patients presented differences in expression levels compared to baseline mRNA expression of up to 2 orders of magnitude (Fig. 4). Nevertheless, the overall change from baseline was not significant, as both increases and decreases occurred. Pairwise analysis showed that changes in cytokine mRNA expression at 9 months compared to baseline levels were not associated with changes in cytokine mRNA expression levels or changes in viral loads or CD4⁺ T-cell counts in the mucosa or blood (Table 2). Although not statistically significant, the high correlation coefficient between changes in viral load and IFN- γ levels should be examined in further studies. Exclusion of those individuals with HIV plasma RNA levels of >400 copies/ml or with undetectable mucosal viral loads did not affect the findings with respect to mucosal cytokine expression.

In comparison to control results, mucosal mRNA expression of IFN- γ , TNF- α , IL-6, IL-4, and IL-10 remained significantly elevated even after 9 months of successful antiretroviral therapy. In contrast, CCL3 and CCL5 mRNA expression in HIV-infected patients returned to nearly normal levels (Fig. 1).

DISCUSSION

Mucosal cytokines have been studied using *in situ* hybridization (40), enzyme-linked immunosorbent assays (50), and conventional PCR (33, 49). Due to the limitations of these techniques, available data are rather qualitative or semiquantitative, making direct comparisons difficult. Real-time reverse transcription (RT)-PCR was applied in a single cross-sectional analysis that

FIG. 4. Intraindividual ratios of mucosal cytokine expression at 1, 4, and 9 months to cytokine expression before treatment (0). A ratio of 1 means no change. Box plots display the 10th, 25th, 50th (median), 75th, and 90th percentiles of the calculated ratios. Note that due to technical reasons, only four samples were available for the measurement of IL-4 at this time point (1 month after start of HAART).

characterized cytokine profiles of HIV-infected patients and healthy controls (32). In the present study, we used real-time RT-PCR to prospectively investigate the dynamics of cytokine mRNA expression following the initiation of antiretroviral treatment. Although there is evidence that cytokine expression is also controlled at the posttranscriptional level (13, 19–22, 26, 36, 39, 41, 43) and that HIV infection itself may alter message translation or interfere with protein synthesis, real-time PCR is currently the most sensitive method for obtaining data on cytokine production by use of limited tissue samples.

High levels of HIV replication and profound CD4⁺ T-cell depletion are the hallmarks of colonic mucosal HIV infection. Our report shows that these go along with an excess of mucosal mRNA expression of IFN- γ , TNF- α , IL-4, IL-6, and IL-10. Our data are consistent with those of a previous report of increased levels of IFN- γ , TNF- α , IL-6, and IL-10 mRNA expression in the rectosigmoidal mucosa of viremic HIV-infected patients (32) and confirm findings revealing similar mucosal IL-2 mRNA levels in untreated HIV-infected and -seronegative individuals (32, 33, 40). The pattern of cytokine expression found in our patient results does not conform to the findings associated with a predominant Th-1 response (characterized by IL-2 and IFN- γ levels) or with a Th-2 response (characterized by IL-4, IL-6, and IL-10 levels) but favors the concept of generalized mucosal immune activation proposed by McGowan and coworkers (32).

The strong mucosal mRNA expression of the natural HIV inhibitors CCL3 and CCL5 (11, 17) apparently does not protect the mucosa from HIV replication and its devastating effect

TABLE 2. Correlations between changes in mucosal cytokine mRNA expression levels and changes in CD4⁺ T-cell numbers and virus loads during 9 months of HAART

Sample category	Spearman's <i>rho</i> correlation coefficient ^a								
	IL-2	IL-4	IL-6	IL-10	IL-16	IFN- γ	TNF- α	CCL3	CCL5
CD4 ⁺ T cells (mucosa)	0.143	0.314	−0.029	−0.429	−0.200	−0.429	−0.036	0.657	−0.071
CD4 ⁺ T cells (blood)	0.050	0.150	0.100	0.333	0.300	0.452	0.248	0.000	0.624
Viral load (mucosa)	0.283	0.150	0.667	0.786	0.367	0.905	0.152	0.310	−0.261
Viral load (blood)	0.000	0.283	0.017	0.333	−0.483	−0.167	0.309	0.667	0.079

^a Spearman's *rho* correlation coefficients for the correlation between changes in cytokine mRNA expression and changes in CD4⁺ T-lymphocyte numbers (mucosa and blood) and changes in viral loads (mucosa and blood) during 9 months of HAART.

on the mucosal CD4⁺ T-cell population. In addition, production of the antivirally active IL-16 (6, 23, 53) is not upregulated during HIV infection, suggesting that endogenous defense mechanisms are ineffective in controlling mucosal HIV replication.

Antiretroviral treatment results in normalization of elevated plasma concentrations of TNF- α , IL-6, and IL-10 (2). Treatment-induced decreases in plasma IL-4, IL-10, and TNF- α levels at the mRNA level have also been observed (52). In the colonic mucosa, however, the excess mucosal cytokine mRNA expression persists, suggesting that the effect of HAART on mucosal HIV production is too small for tissue inflammation to subside.

It is a well-known fact that most patients with plasma viral loads below 50 copies/ml continue to exhibit low-level replication in blood (37, 55), in intestinal mucosa (4, 5, 37), and in other sites (55). Because residual replication may have serious consequences for long-term therapy and the evolution of drug resistance, it seems important to determine to what extent mucosal HIV replication can be tolerated and when to seek to suppress it further.

The evolution of plasma viral load assays has taught us that the threshold of fewer than 400 copies/ml, once a marker indicating satisfactory antiretroviral treatment, was too high. Today, achieving a plasma HIV RNA level of fewer than 50 copies/ml is considered the most important prerequisite for sustained viral suppression. In contrast, measurements of viral load in the mucosa still lack standardization; more studies are needed to investigate the role of mucosal HIV replication in treatment failure. McGowan and coworkers, however, have given us an idea of what might characterize successful treatment in the gut. Their cross-sectional study results show that patients that had been selected with mucosal HIV viral loads of <50 copies/ μ g had normal or even decreased levels of mucosal mRNA expression of TNF- α , IFN- γ , IL-2, IL-10, and IL-12, indicating that mucosal cytokine levels may be a marker for effective mucosal antiviral treatment (32). None of our patients experienced a normalization of their mucosal TNF- α , IFN- γ , and IL-10 mRNA expression levels, and 9 out of 10 patients had detectable mucosal HIV RNA during HAART; such a result, in our opinion, qualifies them as mucosal treatment failures. Why did HAART fail in these patients? Currently there are no data concerning mucosal HIV RNA and cytokine expression for patients on long-lasting modern treatment regimens, so one might speculate that the high failure rate may be attributable to the rather ancient drug combinations used in this trial. In addition, the duration of

treatment may have been too short for the viral load to drop sufficiently, as the elimination of HIV may take longer in tissue reservoirs than in the blood, owing to different cell populations and sources of virus replication. Yet looking at the interplay between mucosal cytokines and HIV replication from a different angle may provide an alternative explanation: persistent mucosal virus production under conditions of HAART may be a consequence of cytokine secretion rather than its cause (24). In this scenario, the force that drives mucosal cytokine production could be the impairment of the epithelial integrity initially induced by HIV itself and maintained by the inflammatory response (1, 46, 47) or by the proper antiretroviral drugs (8).

A number of reports suggest that repopulation of the GI tract with CD4⁺ T cells is delayed despite years of otherwise successful HAART (7, 9, 18, 28, 34, 51). We used both immunohistology and cytofluorometric analysis of isolated cells to examine effects of HAART on mucosal CD4⁺ T cells. Numbers of CD4⁺ T cells determined by flow cytometry were lower than those determined by histology, which was probably due to incomplete recovery of cells by our isolation procedure. However, both methods revealed a slight increase in the mucosal CD4⁺ T-cell percentage to levels far below normal and a rapid rise in the absolute numbers of mucosal CD4⁺ T lymphocytes that clearly exceeded the rise seen in the blood. Similar results had been obtained in a study that looked at duodenal samples (15). These data suggest that CD4⁺ T lymphocytes do repopulate the colonic mucosa, possibly attracted by highly expressed chemokines and cell adhesion molecules (35), but fail to restore the normal immunologic environment.

In summary, this report shows that HIV infection of the GI tract is characterized by high-level mucosal viral replication, profound mucosal CD4⁺ T-cell depletion, and increased mucosal mRNA expression of the cytokines IFN- γ , TNF- α , IL-4, IL-6, and IL-10 and the chemokines CCL3 and CCL5. Following the initiation of HAART, both the plasma and mucosal viral loads decrease but viral replication in the mucosa continues at low levels. Nevertheless, numerous CD4⁺ T lymphocytes return to the GI tract without restoring the normal immunological environment. The general activation of the cytokine network persists, which may be related to the persistence of mucosal HIV replication.

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