

Quantitative Analysis of Mononuclear Cells Expressing Human Immunodeficiency Virus Type 1 RNA in Esophageal Mucosa

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Summary

The mucosa of the gastrointestinal tract is presumably an important reservoir for human immunodeficiency virus type 1 (HIV-1), but the level of virus-expressing cells within the mucosa of infected patients is not known. To study this issue, we identified HIV-1 mRNA-expressing (positive) mononuclear cells by in situ hybridization in specimens of esophageal mucosa from eight patients with acquired immune deficiency syndrome (AIDS) and esophageal infections. Such cells were not found in four patients with AIDS and no esophageal disease. Immunocytochemical staining revealed that the mononuclear cells expressing HIV-1 mRNA were lamina propria macrophages. The prevalence of positive cells was measured by triplicate determinations in each of three experiments using an inverse sampling technique. No significant differences in prevalence were found among patients or among experiments. The overall prevalence of HIV-1 mRNA-expressing cells in the esophageal lamina propria was $0.059 \pm 0.01\%$. This prevalence of cells expressing HIV-1 mRNA in the mucosa of patients with mucosal infections may reflect the local abundance of stimuli such as bacterial endotoxin and certain cytokines capable of inducing viral transcription.

The mucosa of the gastrointestinal tract plays a critical role in the immunopathogenesis of HIV-1 infection (1, 2). Among homosexual men, still the largest population of persons at risk for HIV-1 infection in developed countries, the mucosa offers access to host tissues for both virus-infected cells and cell-free virus. As the largest lymphoid organ in the body, the mucosal lamina propria may also serve as a potential reservoir for HIV-1-infected mononuclear cells. Such cells likely contribute, either directly or indirectly, to the reduction in lamina propria helper/inducer (CD4⁺) cells (3), which parallels the reduction in circulating CD4⁺ T cells. As local immunosuppression progresses, the esophagus and intestinal tract become sites of opportunistic infection(s) (4, 5).

Despite involvement of the mucosa in the immunopathogenesis of AIDS, information on the presence of HIV-1 in the gastrointestinal tract mucosa is limited. For example, the level of HIV-1 mRNA-expressing cells in the mucosa has not been determined, yet such cells likely have an important impact on mucosal immune function. Therefore, we used in situ hybridization and immunolabeling to identify HIV-1 RNA-expressing macrophages in the mucosa and then determined the prevalence of these cells in patients with AIDS.

Materials and Methods

Mucosal Specimens. Esophageal biopsy specimens were obtained from eight patients with AIDS who were part of an ongoing study

of gastrointestinal opportunistic infection(s) in AIDS (4, 5). The histologic diagnoses for the eight patients were esophagitis due to *Candida albicans* in six patients, cytomegalovirus in one patient, and *C. albicans* plus herpes simplex virus in one patient. Esophageal mucosa from four patients with AIDS but no esophageal disease served as control tissue.

In Situ Hybridization. Biopsy specimens were evaluated for HIV-1 RNA by in situ hybridization according to our previously described protocol (6, 7). Briefly, esophageal biopsy specimens were fixed in 1.3 M formaldehyde (8), embedded in paraffin, sectioned, and then bonded to silanized microscope slides (3% triethoxy-3-aminopropyl silane in anhydrous acetone followed by washing in distilled water), deparaffinized, and hydrated in graded alcohols. Slides were treated with 0.2 M HCl (30 min) and digested with proteinase K (0.1 mg/ml; Sigma Chemical Co., St. Louis, MO) for 15 min. The slides were derivatized, first with acetic anhydride followed by succinic anhydride (0.1 M) in triethanolamine buffer (0.1 M, pH 8.0) to remove background and to block myelocytic basic protein. After prehybridization, the slides were hybridized overnight in probe mixture (200,000 counts per slide) with an excess of nonsense mRNA (*Escherichia coli*) followed by extensive washing and removal of single-stranded RNA with ribonuclease A and T1 followed by washing. The slides were dipped in Kodak NTB 2 or 3 emulsion (Eastman Kodak Co., Rochester, NY) and exposed for 4 d. Slides were developed in Kodak D19 (1:1, 15°C, 4 min) and stained with hematoxylin and eosin.

The RNA probes used to detect HIV-1 mRNA were synthesized using ¹²⁵I- or ³⁵S-labeled nucleotide triphosphates from DNA templates of subclones of HIV-1 (strain LAI). Five subclones en-

compassing 90% of the HIV-1 genome were used: (a) long terminal repeat-*gag*, BglII, 2.01 kb; (b) *gag-pol*, HindIII-KpnI, 2.6 kb; (c) *pol*, BglIII-EcoRI, 2.65 kb; (d) VIF, VPR, *Tat*, *Rev*, VPU, KpnI-KpnI, 2.4 kb; and (e) *env*, SalI-BglIII, 2.65 kb. Probes were synthesized in the antisense and sense configuration, each subclone having two promoters, and then sheared by alkaline hydrolysis to yield 200 base RNA fragments. Probe fragments were combined in equal amounts to make a single antisense and a single sense probe with the sensitivities of ~30–300 copies of target RNA per cell. The sensitivity was determined by experiments in which a known number of cell-free viral particles fixed in a fibrin clot and hybridized with the above pooled probes was shown to yield 2.4–3.0 silver grains per viral genome (Fox, C.H., unpublished observations). Four serial sections from each tissue specimen were probed for HIV-1 RNA with the antisense probe.

The evaluation of each tissue specimen included the following controls: (a) sections of known HIV-1-infected and uninfected brain tissue from another patient; (b) HIV-1-infected and uninfected H9 cells; (c) a "nonsense" control probe; and (d) two serial tissue sections from each specimen run with the sense probe. Slides were examined by dark-field and bright-field microscopy. Cells (excluding eosinophils and cells at the section edge) were considered positive when more than 20 white grains (dark-field) or black grains (bright-field) were detected overlying a cell in an area of 200 μm^2 in a typical stellate pattern. This procedure was replicated three times in each of three experiments (nine separate determinations for each of eight patients). In the first experiment, bright-field illumination was used to identify positive cells, and the procedure was unblinded, whereas the second and third experiments were blinded, dark-field illumination was used, and the number of positive cells in the first field with at least one positive cell was counted.

Immunohistochemical Staining. Immunohistochemical staining was performed to identify cells that expressed HIV-1 mRNA. Briefly, formaldehyde-fixed sections of mucosal tissue were stained for 1 h with the following monoclonal antibodies: OPD4 (Dako, Carpinteria, CA), which reacts with helper/inducer T lymphocytes; HAM56 (Dako), which labels fixed tissue macrophages; or CD21 (Dako), which reacts with mature B cells and follicular dendritic cells. After washing, the slides were treated sequentially for 30 min with biotinylated rabbit anti-mIgG and alkaline phosphatase-labeled streptavidin (Dako) and then reacted for 10 min with new fuchsin chromogen substrate (Dako). For double-labeling experiments, slides were first stained by the immunohistochemical procedure and then hybridized with RNA probes to detect HIV-1 mRNA according to the in situ hybridization procedure described above (9, 10).

Statistical Analysis. In each slide, nonoverlapping microscopic fields were examined until a field containing one or more positive cells was found (11). The number of negative fields observed (N) and the number of positive cells in the last field (M) were used in the formula $N + 1/(M + 1)$, which is an estimate of the number of fields per positive cell. ($1/(M + 1)$ is the expected fraction of the last field counted if cells were examined one by one.) The reciprocal, $1/[N + 1/(M + 1)]$, is an estimate of the number of positive cells per field, which, when divided by the average number of cells per field, yields the prevalence (P^+) of HIV-1 mRNA-expressing cells in the esophageal mucosa: $P^+ = 1/[4Q\{N + 1/(M + 1)\}]$ where Q is the average number of cells per quadrant.

If positive cells are assumed to be distributed in the lamina propria according to a spatial Poisson law with a mean of θ cells per field, then N will have a geometric distribution (12) $\exp(-N\theta)[1 - \exp(-\theta)]$, $N = 0, 1, 2, \dots$ and M, a decapitated Poisson distribution (13), $\exp(-\theta)\theta^M/[M! \{1 - \exp(-\theta)\}]$, $M = 1, 2, \dots$. In Tables 1 and 2 are shown the observed numbers of negative fields per experimental replicate (Table 1) and the observed numbers of positive cells per positive field (Table 2). Comparison of observed values with the corresponding expected values based on the assumed dis-

Table 1. Distribution of Microscopic Fields Lacking HIV-1 mRNA Positive Cells from Eight Patients with AIDS

	Number of negative fields per replicate						Total
	0	1	2	3	4	5 or more	
Observed	15	26	20	8	3	0	72
Expected (geometric)*	29.6	17.4	10.3	6.1	3.6	5.1	72.0
Expected (Poisson)*	17.5	24.7	17.5	8.3	2.9	1.1	72.0

* For definition of geometric and Poisson (waiting time) distributions see Statistical Analysis. Goodness-of-fit chi-square: geometric, 26.5, 3 df, $p < 0.001$; Poisson, 1.04, 3 df, $p < 0.50$.

Table 2. Distribution of Positive Cells in Hybridized Esophageal Biopsy Specimens from Eight Patients with AIDS

	Number of positive cells per positive field					Total
	1	2	3	4	5 or more	
Observed	23	12	11	2	0	48
Expected Poisson*	22.5	15.3	7.0	2.4	0.8	48.0

* For definition of (decapitated) Poisson distribution see Statistical Analysis. Goodness-of-fit chi-square = 3.46, 2 df, $p = 0.17$.

tributions shows poor agreement in the data presented in Table 1 (Expected [geometric], $\chi^2 = 26.5$, 3 df, $p < 0.001$) but fairly good agreement in those in Table 2 (Expected [Poisson], $\chi^2 = 3.46$, 2 df, $p = 0.17$). The observed values in Table 1, however, are quite well fitted by a Poisson waiting time distribution ($\chi^2 = 1.04$, 3 df, $p > 0.50$).

A square root transformation (14) was applied to the observed values of $N + 1/(M + 1)$ and a three-way analysis of variance carried out (factors: patient, experiment, replicate order within experiment). Normality of the residuals was checked with a Shapiro-Wilk test (15). No significant main effects were found; however, there was a highly significant patient-experiment interaction due primarily to data collected in the second experiment.

Results

Detection of HIV-1-infected Cells in Esophageal Mucosa. Cells expressing HIV-1 mRNA were identified by in situ hybridization in biopsy specimens of esophageal mucosa from each of eight patients with AIDS and an opportunistic esophageal infection. HIV-1 mRNA-expressing cells occurred as single cells or in groups of up to four positive cells per field (Fig. 1 A). Positive cells were detected only in the lamina propria (Fig. 1 B) and appeared to be mononuclear leukocytes based on size, morphology, and nuclear configuration. In contrast, HIV-1 mRNA-expressing cells were not detected in the control mucosa from AIDS patients without esophageal disease.

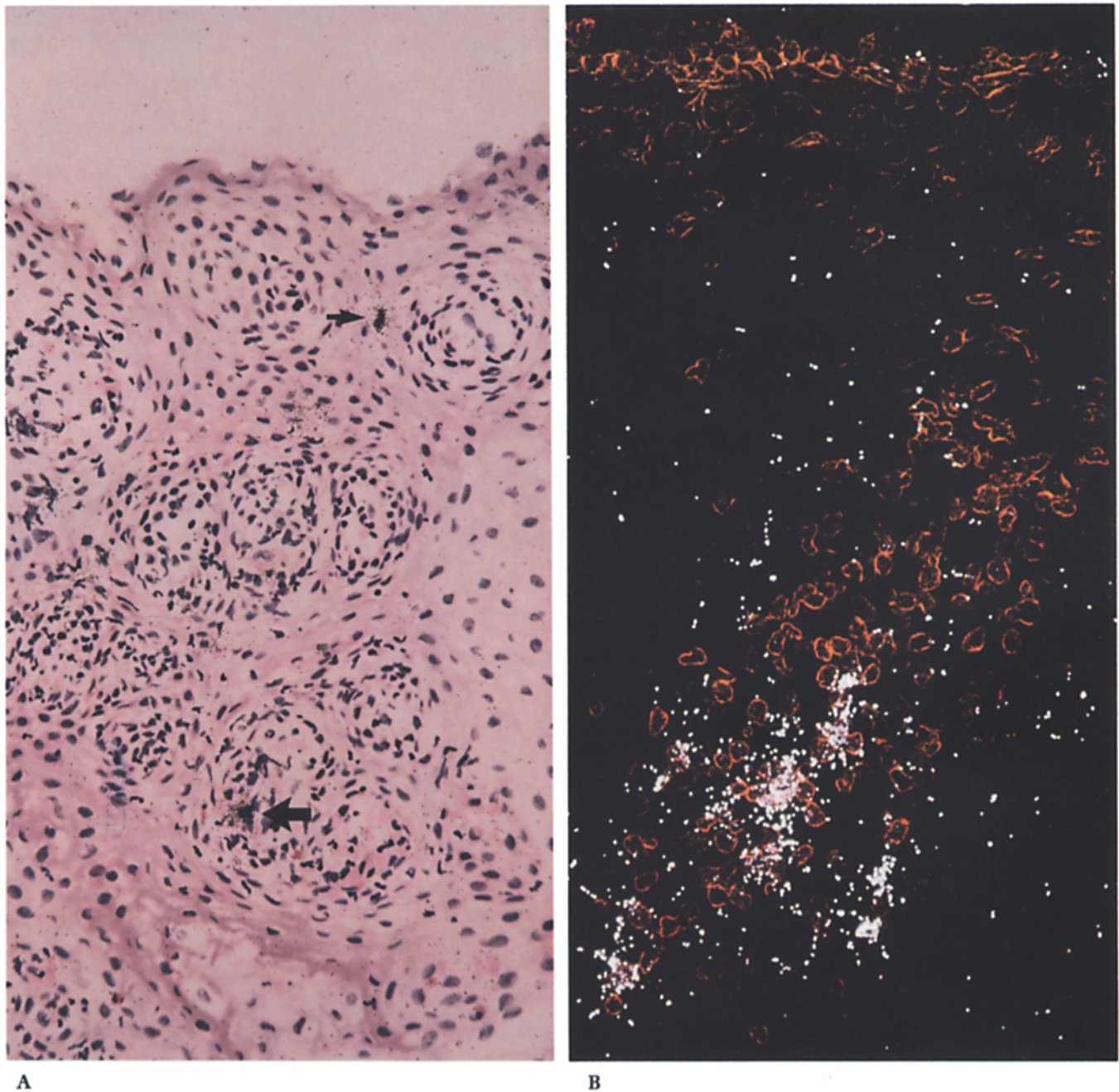


Figure 1. Mucosal biopsy of the esophagus from two AIDS patients probed by in situ hybridization for HIV-1 mRNA. (A) Bright-field illumination shows two HIV-1 mRNA-expressing cells (arrows) within the lamina propria (hematoxylin and eosin, 63 \times). (B) Dark-field illumination shows several HIV-1 mRNA-expressing cells detected by the collections of white dots which represent silver grains in the developed image (400 \times).

Immunohistochemical Identification of HIV-1 mRNA-expressing Cells. Mucosal biopsy specimens from four patients were evaluated for the presence of OPD4⁺ T cells, HAM56⁺ macrophages, and CD21⁺ B or follicular dendritic cells by immunohistochemical staining and for the presence of HIV-1 mRNA-expressing cells by in situ hybridization. Lamina propria macrophages (Fig. 2), but not lymphocytes (data not shown), expressed HIV-1 mRNA by this double-labeling technique. The productively infected cells were present in the lamina propria, but not in the epithelium. These findings corroborated the morphological findings, which showed that

the cells expressing HIV-1 mRNA were mononuclear cells located exclusively within the lamina propria.

Prevalence of Mucosal Mononuclear Cells Expressing HIV-1 mRNA. Tables 1 and 2 summarize the data from the three experiments. The number of cells inspected per replicate was $\sim 2,000$ (on average $4 \times 349 \times 1.416 = 1976.74$), which together with the very small number of positive cells detected, suggested that the number of negative fields read before finding a positive field would follow a Poisson distribution (12). Our data (Table 1, Expected, Poisson distribution) confirmed this supposition: goodness-of-fit chi-square = 1.04, 3 df, $p > 0.50$.

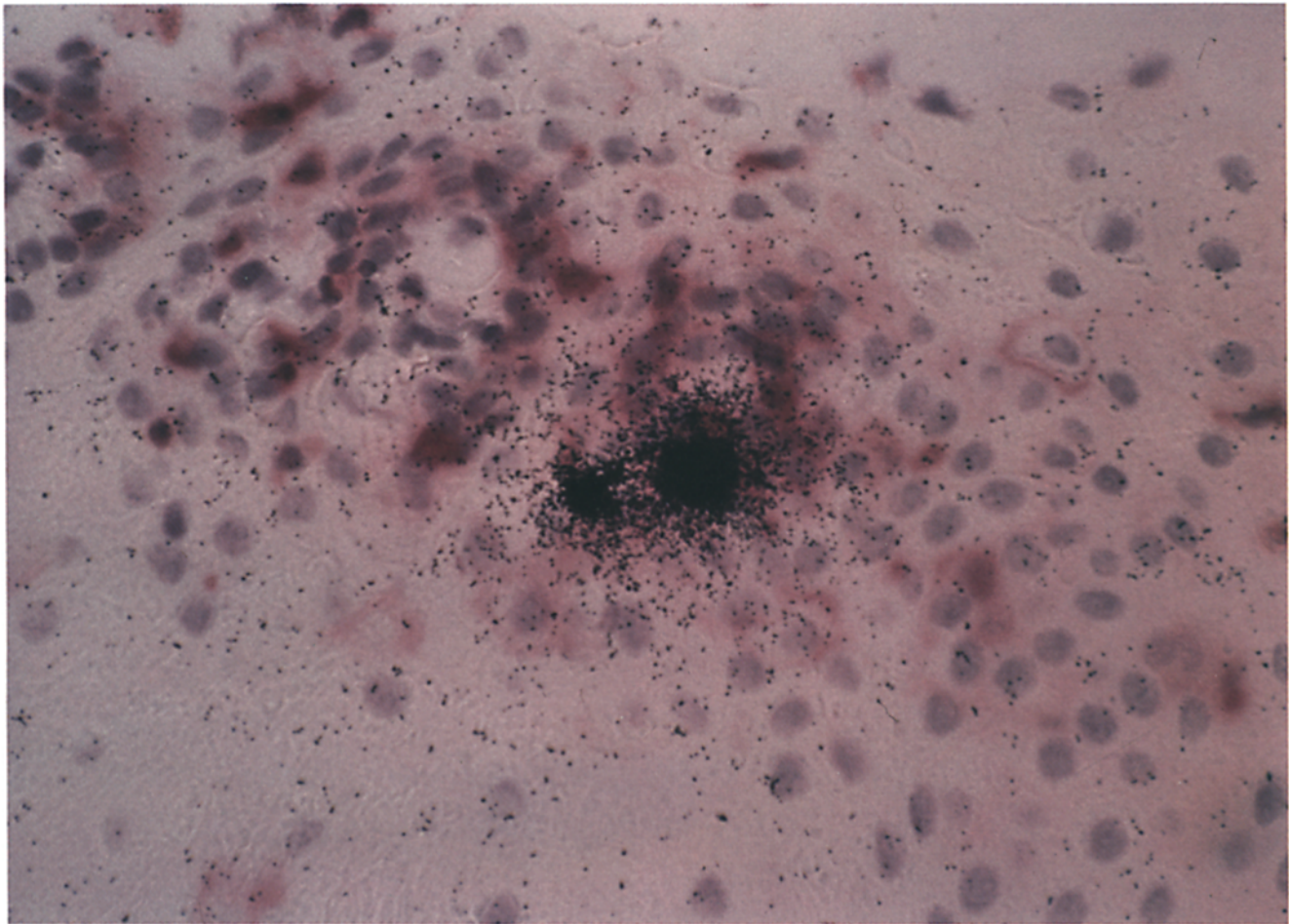


Figure 2. Protease-digested section of esophageal mucosa from an AIDS patient hybridized with HIV-1 ³⁵S-labeled RNA antisense probe and stained with HAM56. Localization of HIV-1 mRNA signal over a HAM56⁺ mononuclear cell in the lamina propria indicates that a lamina propria macrophage is productively infected with HIV-1.

Table 3. Prevalence of Cells Expressing HIV-1 RNA in Esophageal Mucosa among Eight Patients with AIDS

Patient	Average no. cells/ quadrant (\bar{Q})	Average no. negative fields counted (\bar{N})	Prevalence ($\times 10^4$) [*] (P^+)
1	167	1.333	8.88
2	171	1.222	9.28
3	293	1.556	4.47
4	336	1.333	4.41
5	326	.667	7.52
6	180	1.444	7.72
7	650	2.222	1.49
8	429	1.555	3.05
Mean \pm SEM	349 \pm 5	1.416 \pm .152	5.86 \pm 1.02

^{*} $P^+ = 1/[4\bar{Q}\bar{N} + 1/(\bar{M} + 1)]$ where \bar{Q} is the average number of cells per quadrant and \bar{N} , the average number of negative fields counted per patient; \bar{M} is the overall average number of positive cells per positive field ($\bar{M} = 88/48 = 1.833$). See Statistical Analysis for derivation of formula.

There was, nonetheless, evidence that positive cells tended to form small aggregates since, compared with the corresponding numbers of fields expected with a random distribution of cells (see Table 1, Expected, geometric distribution), there was a deficit of fields free of such cells (15 vs. 29.6) but an excess of fields with two to four positive cells (31 vs. 19.9).

Table 3 shows the average number of cells enumerated per quadrant (\bar{Q}) for each patient and the corresponding average number of fields counted until a field having at least one positive cell was found (\bar{N}). Individual patient prevalences were calculated by introducing the estimates \bar{Q} and \bar{N} into the formula at the foot of Table 3. The overall prevalence of mononuclear cells expressing HIV-1 mRNA in esophageal mucosa of patients with AIDS was estimated by taking the arithmetic mean of the individual patient prevalences: mean $P^+ = 5.86 \times 10^{-4}$. The corresponding standard error was 1.02×10^{-4} . A 95% confidence interval for mean P^+ is $(3.45 - 8.26) \times 10^{-4}$.

Discussion

Using in situ hybridization, we identified mononuclear cells expressing HIV-1 mRNA in the lamina propria of esophageal mucosa from eight AIDS patients with esophageal opportunistic infections. Double-labeling experiments showed that the productively infected cells were macrophages. The depletion of mucosal CD4⁺ lymphocytes, which parallels the depletion of circulating CD4⁺ lymphocytes in patients with AIDS (3), may have contributed to our inability to detect HIV-1 mRNA expressing T lymphocytes in the lamina propria. Quantitative analysis of the hybridized slides revealed that on the average, one HIV-1-infected cell per ~2,000 lamina propria mononuclear cells (0.059%) actively expressed viral RNA at levels detectable by in situ hybridization. We recognize, however, that this percentage may actually underestimate the prevalence of mucosal cells infected with HIV-1, since our technique detected cells expressing viral RNA and not cells containing only integrated or extrachromosomal proviral DNA, which ranges between 0.1% and 13.6% of mononuclear cells in blood (16, 17).

Although HIV-1 has been identified in an esophageal ulcer (18) and in intestinal mucosa (4, 6, 7, 19–23), this is the first quantitative assay of the viral burden in mucosal tissue from patients with AIDS. Moreover, we believe that our evaluation is to date the most reliable estimate of the prevalence of cells expressing HIV-1 transcripts in a body tissue or fluid. The number of negative fields counted during inverse sampling of the hybridized tissue sections was shown to follow a Poisson distribution, and an analysis of variance showed

no significant differences in counts of negative fields among the eight patients or three experiments. This method of estimating the prevalence of mRNA positive cells obviates counting the total number of cells in each field. Instead, the average total number is estimated independently, thereby saving appreciable time and effort.

The 0.059% prevalence of HIV-1 mRNA-expressing cells in esophageal mucosa reported here is 6–60 times higher than the apparent frequency (0.001–0.01%) of cells expressing viral RNA in lymph nodes from patients with AIDS (24). In the latter study, however, Harper et al. (24) used a different hybridization protocol and did not quantitate viral burden (number of infected cells) by the inverse sampling technique described above. Ideally, the same in situ hybridization protocol, probes, and quantitative analysis should be used to compare frequencies of HIV-1 mRNA-expressing cells in different organs.

Recently, we used protease-digested tissue in hybridization experiments to show that lymph nodes are important reservoirs of free virus during early HIV-1 infection and that this reservoir disappears as the germinal centers involute with advancing disease (9). More recently, Embretson et al. (25) extended these observations by combining in situ hybridization and polymerase chain reaction to show that high numbers of CD4⁺ cells in the germinal centers and follicular mantle of lymph nodes from patients with early HIV-1 infection were latently infected with HIV-1 DNA. In contrast to these studies, we determined the frequency of productively infected (HIV-1 mRNA-expressing) cells among all mucosal cells and performed these studies on tissues from patients with late HIV-1 infection (AIDS and opportunistic infections). Thus, during late HIV-1 disease the expression of HIV-1 by mononuclear cells in gastrointestinal mucosa may be different from that of lymph nodes where productively infected cells are no longer present (9, 10, 25).

The frequency of mucosal cells productively infected with HIV-1 reported here may reflect the local availability of extracellular stimuli capable of activating the cellular transcription factors that regulate viral replication. Stimuli that may be present in the gastrointestinal tract mucosa that are capable of activating lymphocytes and macrophages for HIV-1 expression include bacterial lipopolysaccharide (26, 27), although this is still controversial (28, 29), and tumor necrosis factor α (30, 31), which is abundantly expressed by mucosal macrophages in some patients (7). Elucidating the local events that promote HIV-1 expression by mononuclear cells in the mucosa will be critical to understanding the role of the gastrointestinal tract as a reservoir for HIV-1.

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