Virology **270,** 317–327 (2000)
doi:10.1006/viro.2000.0299, available online at http://www.idealibrary.com on IDEAL®

Characterization of HIV-1-Specific Cytotoxic T Lymphocytes Expressing the Mucosal Lymphocyte Integrin CD103 in Rectal and Duodenal Lymphoid Tissue of HIV-1-Infected Subjects

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Received November 18, 1999; returned to author for revision January 10, 2000; accepted March 6, 2000

Acute HIV-1 infection depletes $CD4^+$ T cells in gut-associated lymphoid tissue (GALT). The failure of containment of local viral replication, and consequent $CD4^+$ T cell depletion, might be due to delayed mobilization of effector $CD8^+$ T cells or absence of functioning HIV-1-specific $CD8^+$ T cell effectors within GALT. No studies have addressed human intestinal HIV-1-specific $CD8^+$ T cell functions. We sought to determine whether functional HIV-1-specific CTL were present in GALT and whether the repertoire differed from HIV-1-specific CTL isolated from peripheral blood mononuclear cells. From three HIV-1-infected subjects, we isolated HIV-1-specific $CD8^+$ T cells expressing the mucosal lymphocyte integrin CD103 from GALT. These antigen-specific effector cells could be expanded *in vitro* and lysed target cells in an MHC class I-restricted manner. HIV-1-specific CTL could be isolated from both duodenal and rectal GALT sites, indicating that $CD8^+$ effectors were widespread through GALT tissue. The breadth and antigenic specificities of GALT CTL appeared to differ from those in peripheral blood in some cases. In summary, we found HIV-1-specific $CD8^+$ effector T cells in GALT, despite HIV-1-induced $CD4^+$ T cell lymphopenia. This suggests that HIV-1-specific CTL in gut tissue can be maintained with limited $CD4^+$ T cell help.

INTRODUCTION

The gut-associated lymphoid tissue (GALT) is the largest lymphoid tissue in the human body and harbors a majority of the body's lymphocytes (Mowat and Viney, 1997). Defining the mucosal immune response to HIV-1 is of critical importance for several reasons. First, diarrhea and wasting syndromes are prevalent in AIDS patients worldwide and particularly in developing countries (Ullrich *et al.,* 1998). Second, the gastrointestinal (GI) tract is a frequent site of AIDS-associated opportunistic infections and malignancies and also serves as a major portal of entry for HIV-1 (Chui and Owen, 1994; Zeitz *et al.,* 1996). Third, several studies have documented active replication of HIV-1 in GALT tissues, as measured by p24 expression (Fackler *et al.,* 1998; Kotler *et al.,* 1991) or viral RNA levels (Markowitz *et al.,* 1999; Smith *et al.,* 1994); therefore, the GI tract serves as an important reservoir for replicating virus.

GALT tissue is both anatomically and immunologically distinct from peripheral lymphoid tissue, and little information is available regarding trafficking of lymphocytes between these two compartments, either in control individuals or in virally infected subjects (reviewed in Mowat and Viney, 1997). Lymphocyte homing to mucosal tissues apparently requires expression of $\beta 7$ integrins (Lefrancois *et al.,* 1999), and tethering of intestinal intraepithelial lymphocytes (IEL) to adjacent epithelial cells may require expression of CD103 ($\alpha^E\beta_7$ integrin) (Cepek *et al.,* 1994). Studies in nude mice have established that a portion of murine intestinal lymphocytes mature extrathymically (reviewed in Lefrancois, 1991), perhaps in specialized areas of the lamina propria designated cryptopatches (Saito *et al.,* 1998). In humans, some studies suggest that extrathymic T cell maturation may take place; however, this issue remains controversial (Howie *et al.,* 1998; Lundqvist *et al.,* 1995; Lynch *et al.,* 1995; Taplin *et al.,* 1996). Nevertheless, because of the anatomical and functional distinction between peripheral and mucosal tissues, and since GALT itself is a site for HIV-1 replication, the possibility exists that mucosal cellular immune responses to HIV-1 will be significantly different from those in peripheral blood.

Studies in rhesus macaques have shown that GALT tissue rapidly becomes infected with simian immunodeficiency virus (SIV), even when animals are infected intravenously (Heise *et al.,* 1994). Potential target cells for

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0042-6822/00 $35.00

HIV-1 and SIV replication in GALT include macrophages and activated T lymphocytes in the lamina propria (Heise *et al.,* 1994; Lapenta *et al.,* 1999), as well as dendritic cells in intestinal lymphoid follicles (Jarry *et al.,* 1990; Pope, 1999). Lamina propria $CD4^+$ T lymphocytes (LPL) were recently demonstrated to be permissive for both CXCR4- and CCR5-utilizing strains of HIV-1 (Lapenta *et al.,* 1999). When virus enters the body directly via mucosal surfaces, rather than parenterally, it must first cross the epithelial layer to reach the underlying lamina propria or lymphoid follicles. Proposed mechanisms have included uptake by epithelial cells via an alternative receptor such as galactosyl ceramide (Fantini *et al.,* 1991) or Fc receptors (Hussain *et al.,* 1991), by transcytosis (Bomsel, 1997), or through uptake by microfolded dome cells (M cells) (Amerongen *et al.,* 1991) overlying the follicles.

In HIV-1-infected individuals, the most dramatic phenotypic alteration in GALT lymphocyte populations is the loss of $CD4^+$ T cells from the lamina propria of both duodenal and colorectal mucosa, concurrent with a rise in the $CD8^+$ T cell population in these areas (Schneider *et al.,* 1994, 1995). In rhesus macaques intravenously inoculated with pathogenic SIV, dramatic declines were also observed in $CD4^+$ intestinal LPL; this occurred as early as 7 days postinoculation (Mattapallil *et al.,* 1998; Veazey *et al.,* 1998). Coincident with the acute decline in mucosal $CD4^+$ cells was an increase in absolute numbers of $CD8^+$ T cells, primarily IEL, in intestinal tissues (Mattapallil *et al.,* 1998; Veazey *et al.,* 1998). These $CD8^+$ cells were found to secrete interferon-γ (IFN-γ) and the chemokine MIP-1β (Mattapallil *et al.,* 1998) and to have SIV Gag- and Env-specific cytotoxic activity in chromium release assays.

Two additional studies have documented the presence of SIV-specific cytotoxic effector cells in GALT of rhesus macaques. SIV-specific cytotoxic lymphocytes (CTL) were identified in IEL from the small intestine of two chronically infected rhesus macaques; these cells expressed CD8 and the mucosal lymphocyte integrin CD103 (Couedel-Courteille *et al.,* 1997). More recently, Murphey-Corb *et al.* demonstrated a positive correlation between the presence of SIV Env-specific CTL in jejunal lamina propria and protection from intrarectal challenge with SIV/DeltaB670 (Murphey-Corb *et al.,* 1999). These results suggest that infected individuals mount a significant mucosal cellular immune response to immunodeficiency viruses and that mucosal CTL may be an important correlate of vaccine protection.

Clearly, an understanding of the quantitative and qualitative aspects of the mucosal cellular immune response to SIV and HIV-1 will be important for development of effective immunization strategies. Although studies of the human mucosal immune response to HIV-1 are urgently needed, the technical difficulties inherent in maintaining GALT-derived lymphoid cells in culture have thus far prevented extensive studies in this area. In this report, we demonstrate the isolation and culture of HIV-1-specific CTL from intestinal biopsy tissue obtained by flexible sigmoidoscopy and endoscopy. To our knowledge, this is the first report of HIV-1-specific, MHC class I-restricted CTL obtained from human intestinal biopsy tissue.

RESULTS

Patient clinical information

Three HIV-1-infected patients were studied. Both phenotypic and functional analyses were carried out on intestinal mononuclear cells (MNC) from two patients, CM01 and CM02. GALT-derived MNC from one additional patient, CM03, were analyzed by flow cytometry for the expression of lymphocyte surface markers. Clinical information for the three patients is summarized in Table 1. Patient CM01 was infected with HIV-1 in 1984 and had a history of AIDS-related pathology including non-Hodgkins lymphoma, *Cryptosporidium* infection, and peripheral neuropathy. This patient had suppressed plasma viremia (i.e., fewer than 50 copies/ml) and a peripheral $CD4^+$ count of 531 cells/μl at biopsy. Patient CM02 became infected with HIV-1 in 1993. His viral load was 124,696 copies/ml at the time of biopsy, and his $CD4^+$ count was 26 cells/μl. Patient CM03 was infected with HIV-1 in 1996; he had a history of wasting syndrome, neuropathy, encephalopathy, and severe dementia (resolved) as well as infections with *Candida, Toxoplasma,* and *Mycobacterium avium.* His viral load at biopsy was 237 copies/ml and his $CD4^+$ count was 331 cells/μl. Patients CM01 and CM03 were undergoing highly active antiretroviral therapy (HAART) at the time of biopsy; patient CM02 had undergone HAART but voluntarily discontinued treatment several weeks prior to biopsy.

Isolation and phenotyping of GALT-derived mononuclear cells

Mononuclear cells were obtained by manual disruption of biopsy tissue using sterile forceps. The number of cells obtained by this method was too low ($<2 \times 10^6$ cells) to allow thorough analysis by both flow cytometry and immunologic assays; therefore, we chose to expand mononuclear cells by polyclonal stimulation with anti-CD3 monoclonal antibody (12F6) and allogeneic γ-irradiated feeder cells. MNC cultures were evaluated by flow cytometry approximately 3 to 6 weeks after initiation (Fig. 1; Table 2). Mononuclear cells derived from peripheral blood of the same patients were processed in parallel, using the same polyclonal expansion technique; these results are also shown in Table 2.

It should be noted that the *in vitro* expansion method utilized here may favor outgrowth of specific subpopulations at the expense of others. In most cases, rectal and duodenal cultures contained primarily $CD8^+$ T cells after

TABLE 1

Patient Clinical Information

Patient ID	Sex, age	Virus load[a]	Risk factors	Date HIV+	CD4 cells/μl[b]	AIDS-related illnesses	Antiretroviral therapy
CM01	M, 46	<50	Homosexual contact	1984	531	Non-Hodgkins lymphoma, *Cryptosporidium,* peripheral neuropathy	Saquinavir Nelfinavir Nevirapine
CM02	M, 39	124,696	Heterosexual contact	1993	26	Severe $CD4^+$ cell depletion	D4T 3TC Indinavir (discontinued)
CM03	M, 48	237	Homosexual contact	1996	331	Neuropathy, encephalopathy, severe dementia (resolved), *Mycobacterium avium* complex, *Candida,* CNS toxoplasmosis, wasting syndrome	D4T 3TC Indinavir

[a] Virus load at time of biopsy, expressed as viral RNA copies/ml of plasma. Virus load was determined by Roche RT-PCR, detection limit 50 copies/ml (see text).

[b] $CD4^+$ count in peripheral blood at time of biopsy.

polyclonal expansion (range 60.4 to 94.4% for patients CM01 and CM02) (Table 2). Rectal cultures from patient CM03 were enriched in $CD4^+$ cells (range 44.7 to 63.8%). We did not attempt to segregate IEL and LPL populations during the initial processing of biopsy tissue; therefore, our cultures likely contained lymphocytes from both histologic regions. IEL in uninfected individuals are predominantly $CD8^+$ (Mowat and Viney, 1997). LPL in uninfected individuals have a CD4:CD8 ratio similar to that observed in peripheral blood, but $CD4^+$ LPL are sharply depleted in HIV-1-infected individuals (Mowat and Viney, 1997; Schneider *et al.,* 1994, 1995).

Reports on the expression of $\alpha^E\beta_7$ integrin (CD103) by GALT MNC have varied, but in healthy individuals this antigen is believed to be expressed on 60–80% of IEL *in situ* and roughly 40% of LPL (Lundqvist *et al.,* 1995). In AIDS patients, expression of CD103 by GALT-derived MNC was reportedly decreased from a mean of 41% in controls (range 31–54%) to 22% (range 16–59%) (Schneider *et al.,* 1994); this reduction was attributed to profound depletion of mucosal $CD4^+$ T cells. In our expanded cultures, expression of CD103 was markedly increased in GALT-derived MNC (5.2 to 43.7%), relative to peripheral blood MNC from the same patients (0.9 to 1.6%) that were expanded and cultured in the same manner (Table 2). A comparison of CD103 surface staining in MNC derived from GALT and peripheral blood of patient CM02 is shown in Fig. 1A. Although enhanced expression of CD103 by peripheral blood MNC has been reported following PHA stimulation (Schieferdecker *et al.,* 1990), in the present study expression of this antigen by PBMC was restricted to fewer than 2% of polyclonally stimulated, cultured cells. Given the bulk of the original tissue samples and the extensive washing procedures used to remove trace amounts of blood prior to culture, we believe that contamination of our GALT MNC cultures with blood-derived MNC was minimal.

GALT-derived MNC in our cultures expressed primarily (generally >90%) the TCR$\alpha\beta$ isotype. Intestinal MNC contained higher percentages of TCR$\gamma\delta$ cells (range 0.7 to 23.1%) than cultures derived from peripheral blood (range 0.2 to 0.6%). In particular, three GALT-derived cultures from patient CM03 contained >10% TCR$\gamma\delta$ cells (Table 2; Fig. 1B). The enhanced expression of TCR$\gamma\delta$ chain by GALT-derived MNC populations further suggested that these cells were not derived from contaminating peripheral blood but were indeed of intestinal origin.

Following stimulation with anti-CD3, flow cytometry analysis revealed greater than 95% $CD3^+$ lymphocytes in most cultures; however, two duodenal cultures from patient CM03 contained 7–11% $CD3^-$ cells (Table 2). These cultures also contained >5% MNC coexpressing natural killer (NK) cell markers CD56 (neural cell adhesion molecule) and CD16 (FcγRIII) (Table 2). Coexpression of these markers in most other cultures was less than 1%. Expression of CD57 was detected in expanded cultures of both mucosal and peripheral blood MNC, frequently on cells also expressing CD3 and CD8 (not shown). Classical NK subsets expressing CD56 and CD16 or CD57 in the absence of CD3 are reportedly reduced in blood and mucosal tissues of HIV-1-infected individuals (Hu *et al.,* 1995; Margolick *et al.,* 1991; Schneider *et al.,* 1994). However, a subset of cells expressing both CD8 and CD57 is expanded in the peripheral blood of AIDS patients (Lewis *et al.,* 1985) and may inhibit cytotoxicity via a soluble factor (Sadat-Sowti *et al.,* 1994). Cells expressing $CD8^{+\,low}/CD57^+$ may function as NK cells in immune surveillance of cytomegalovirus infections,

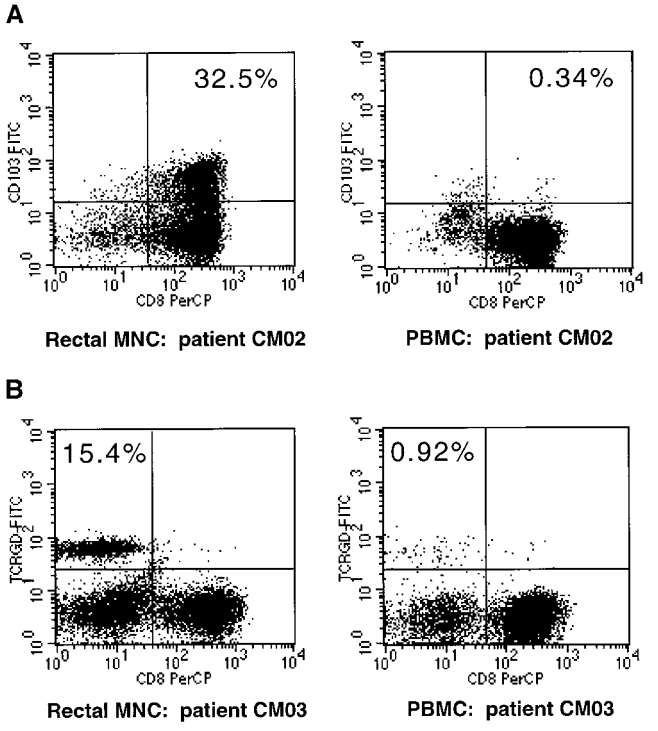

FIG. 1. Expression of CD103 and T cell receptor $\gamma\delta$ chain by cultured MNC. (A and B) Results of four-color flow cytometry experiments performed on a FACSCalibur instrument (Becton–Dickinson). (A) Polyclonally expanded MNC cultures from peripheral blood (right) and GALT (left) of patient CM02 were analyzed for expression of T cell markers CD3, CD4, and CD8 and mucosal adhesion/retention marker CD103 ($\alpha^E\beta_7$ integrin). In the experiment shown, cells were gated on forward scatter versus side scatter and assessed for expression of CD8 and CD103. Numbers shown in the upper right quadrant indicate the percentage of cells expressing both CD8 and CD103 in these populations. (B) Polyclonally expanded MNC cultures from blood (right) and GALT (left) of patient CM03 were assessed for expression of CD3, CD4, and CD8 and T cell $\gamma\delta$ receptor. Most $\gamma\delta$ cells were found among $CD4^-/CD8^-$ double-negative T cells. The numbers shown in the upper left quadrant indicate the percentage of CD8-negative cells expressing TCR$\gamma\delta$. Quadrant markers were established based on staining with appropriate isotype controls (not shown).

which are common in AIDS patients (Wang and Borysiewicz, 1995).

Rectal MNC from patient CM01 contained HIV-1-specific CTL

Approximately 3 weeks after *in vitro* stimulation, expanded MNC from GALT tissue of patient CM01 were assessed for cytotoxic activity in standard ^{51}Cr release assays (Coligan *et al.,* 1999). Preliminary testing of MNC derived from rectal tissue against a panel of allogeneic HLA-matched and -mismatched B lymphoblastoid cell lines (B-LCL) showed mild to moderate killing (10 to 35% specific lysis at E:T ratios from 10:1 to 30:1) of targets expressing HIV-1 Gag, Pol, and Nef (not shown). Additional experiments using a series of B cell lines sharing individual MHC class I alleles with donor CM01 were used to define the MHC restriction of the observed CTL activity. Figures 2A and 2C show the results of two such assays. For these experiments, target B-LCL shared either HLA-A*0201 or HLA-Cw*08 with donor CM01. HLA-A*0201-restricted cytotoxic activity was directed toward target cells expressing either Pol or Nef, and HLA-Cw*08-restricted killing was directed toward B-LCL expressing Nef, Gag, or Pol. For the experiments shown in Fig. 2, lysis of control vaccinia-infected target cells ranged from 0 to 7%; results are expressed as percentage specific lysis after subtracting lysis of control cells. For all ^{51}Cr release assays, lysis was considered significant when specific killing of target cells expressing HIV-1 antigens (after subtracting control lysis) was $\geq$10%. No specific killing was observed against target cell lines matched at HLA-A*32 or HLA-B*14, the other MHC class I alleles expressed by donor CM01 (not shown). In parallel assays, blood-derived MNC from patient CM01 exhibited HLA-A*0201-restricted killing of target cells expressing HIV-1 Pol, Gag, and Nef (Fig. 2B) and HLA-Cw*08-restricted killing of target cells expressing HIV-1 Gag (Fig. 2D). Lysis of MHC class I-mismatched target cells expressing HIV-1 antigens was less than 10%.

Duodenal MNC from patient CM02 contained HIV-1-specific CTL

Blood and GALT-derived MNC from patient CM02 were assessed for cytotoxic activity against a panel of six allogeneic HLA-matched B-LCL. Of the GALT-derived cultures, two duodenal populations demonstrated reproducible, moderately strong lysis of B-LCL expressing HIV-1 antigens. Duodenal culture D1 (Fig. 3A) showed greater than 30% specific lysis of target cells expressing HIV-1 Pol at an effector:target ratio of 50:1. Although single-allele-matched B-LCL were not available for patient CM02, assessment of culture D1 effector cells versus a panel of four B-LCL suggested that the Pol-specific cytotoxic response was restricted by HLA-B*45 (Fig. 3B). Another duodenal culture, designated D4, showed 41% specific lysis of HIV-1 Gag-expressing targets at an E:T ratio of 30:1 (Fig. 3C); lysis of control cells was 4%. Assessment of D4 effector cells against a panel of three HLA-matched target cells suggested restriction of this response by the HLA-B*15 allele. To confirm these results, $CD8^+$ effector cells were purified from culture D4 using magnetic beads linked to anti-CD8 antibody. The CD8-enriched population was used at an E:T ratio of 25:1 in a ^{51}Cr release assay (not shown). Specific lysis of HIV-1 Gag-expressing target cells in this assay was 17%, confirming that $CD8^+$ effector cells played a significant role in the observed Gag-specific killing. The reduction in specific lysis from 41 to 17% suggested that other cytotoxic effector cell populations were also present in this

TABLE 2

Phenotyping of GALT and Blood-Derived MNC Cultures after Stimulation with Anti-CD3

		CD3 positive lymphocyte gate				Lymphocyte gate (FSC vs SSC)						
Sample	Biopsy site	$CD4^+$	$CD8^+$	$CD4^+/8^+$	$CD4^-/8^-$	$CD3^+$	$CD103^+$	$TCR\alpha\beta^+$	$TCR\gamma\delta^+$	$CD56^+$	$56^+/16^+$	$CD57^+$
CM01.R1	Rectum	9.5	87.2	2.5	0.8	99.9	43.7	97.6	2.4	81.7	0.6	nd
CM01.R2	Rectum	4.2	94.4	1.1	0.4	99.9	13.0	98.0	2.0	57.1	0.2	nd
CM01 blood	Blood	16.2	60.4	2.5	20.9	97.7	1.1	97.3	0.4	nd	nd	19.0
CM02.D4	Duodenum	1.2	69.2	0.5	29.1	99.5	10.1	93.4	4.9	15.7	0.3	26.5
CM02.R1	Rectum	1.6	79.7	1.7	17.0	94.7	5.2	90.0	3.1	10.7	0.1	31.8
CM02.R4	Rectum	0.6	87.7	0.5	11.2	99.7	23.6	96.5	1.7	7.8	0.3	28.2
CM02.R5	Rectum	1.0	92.0	1.0	5.3	99.7	34.2	95.1	2.4	12.1	0.7	18.1
CM02 blood	Blood	0.3	91.8	0.6	7.3	98.8	0.9	96.1	0.6	23.2	1.7	34.6
CM03.D1	Duodenum	13.9	75.7	2.3	8.1	92.3	17.6	60.4	23.1	13.7	6.2	32.4
CM03.D2	Duodenum	17.2	66.4	0.6	15.7	89.4	29.5	67.6	14.5	10.1	6.1	22.8
CM03.R1	Rectum	63.8	27.7	0.3	8.1	99.4	14.8	93.5	0.7	0.9	0.2	24.9
CM03.R2	Rectum	44.7	48.0	0.8	6.5	99.7	7.6	78.7	16.8	0.9	0.9	42.1
CM03 blood	Blood	8.6	86.7	1.3	3.4	93.0	1.6	84.4	0.2	2.4	2.4	16.6

Note. Values shown are from three- and four-color flow cytometry experiments performed as described in the text.

culture. Additional studies will be required to identify these populations.

Blood-derived MNC from patient CM02 showed no specific lysis of any of the six HLA-matched B-LCL in ^{51}Cr release assays (not shown). Lysis of control vaccinia-infected cells in these ^{51}Cr release assays was unusually high (20–50% or higher, depending upon E:T ratios), suggesting the presence of CTL directed toward vaccinia or Epstein–Barr virus antigens or possibly natural killer cell activity.

Duodenal MNC from patient CM02 secreted IFN-γ in response to antigenic stimulation

To confirm and further characterize the CTL activity detected in duodenal cultures from CM02, we performed an IFN-γ ELISPOT assay. Mononuclear cell cultures derived from blood and duodenum (culture D4) were infected with control (tk^-) vaccinia virus or recombinant vaccinia expressing HIV-1 Gag (Larsson *et al.,* 1999). Cells producing IFN-γ were detected by monoclonal antibodies specific for human IFN-γ, linked to horseradish peroxidase. Results (Fig. 3D) demonstrated that duodenal MNC from CM02 contained a high frequency (greater than 1000 spot-forming cells (SFC) per million MNC after subtracting background spots) of Gag-specific IFN-γ SFC, while blood-derived MNC from the same patient showed almost no IFN-γ production. For comparison, blood-derived MNC from patient CM01 yielded 730 Gag-specific SFC per 10^6 MNC in the same assay (not shown). This result demonstrates that, in addition to performing MHC class I-restricted cell killing, antigen-reactive T cells present in culture D4 could secrete IFN-γ in response to HIV-1 antigen-specific stimuli.

DISCUSSION

The major finding of this paper is that HIV-1-specific cytotoxic lymphocytes can be successfully cultured from rectal and duodenal biopsies of AIDS patients. In the past, functional characterization of mucosal T cells has been limited by the technical difficulties associated with their long-term *in vitro* propagation. Here, we have used a simple polyclonal stimulation method to expand these cells *in vitro,* allowing assessment of MHC class I-restricted cytotoxic activity and antigen-specific IFN-γ production. To our knowledge, this is the first functional description of anti-HIV-1 CTL activity in human intestinal biopsy samples from HIV-1-infected individuals.

The specificities of CTL from patient CM01 were similar in blood and GALT (Table 3). Both MNC populations exhibited HLA-A*0201-restricted killing of Pol- and Nef-expressing target cells, as well as HLA-Cw*08-restricted killing of Gag-expressing targets. In the absence of CTL clones from both blood and GALT it was not possible to compare the fine specificities of these cultures. However, the observation of shared antigenic specificity and MHC restriction suggested that some degree of epitope specificity was common to CTL from both blood and GALT of patient CM01, assuming minimal contamination of GALT cultures with blood-derived MNC. This shared CTL specificity also suggests that similar viral quasispecies may have been present in blood and GALT of this patient. Extensive trafficking of antigen-specific T cells between blood and GALT is not believed to occur. Antigen-specific T cells primed in GALT inductive sites are thought to enter peripheral circulation via the thoracic duct and eventually localize or "home" to mucosal tissues upon expression of $\alpha^4\beta_7$ and $\alpha^E\beta_7$ integrins (Brandtzaeg *et al.,* 1999; Mowat and Viney, 1997). However, antigen-

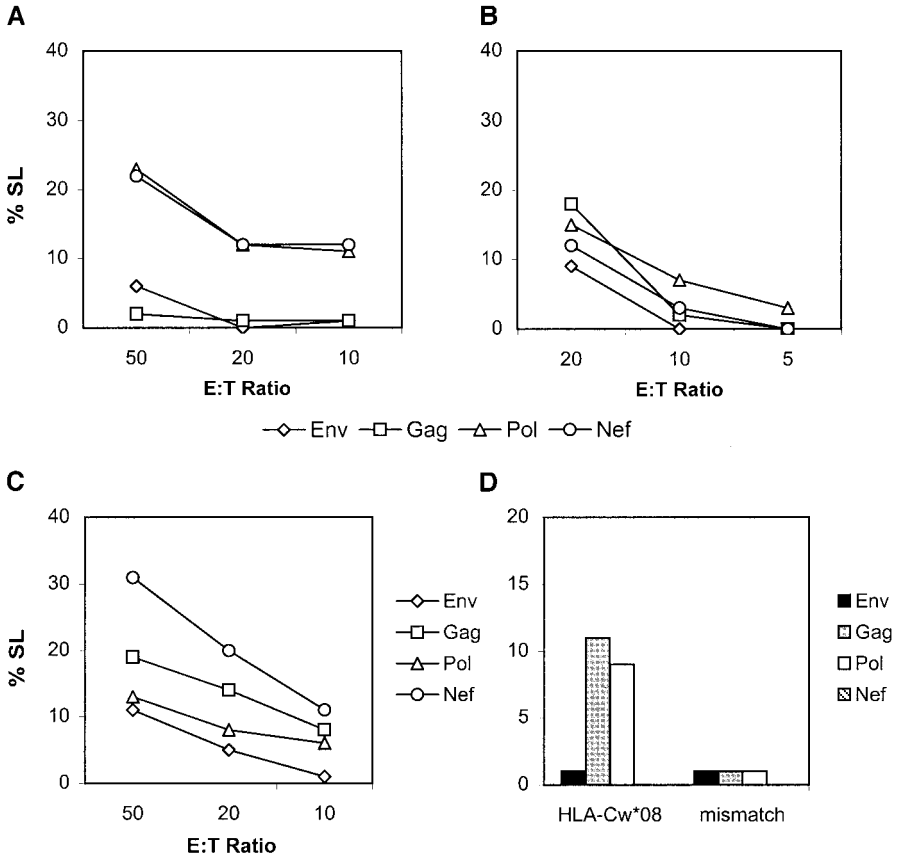

FIG. 2. HIV-1-specific CTL in blood and GALT MNC cultures from patient CM01. Results of ^{51}Cr release assays performed with rectal- (A and C) or blood-derived (B and D) MNC as effectors and allogeneic B-LCL matched at HLA-A*0201 (A and B) or HLA-Cw*08 (C and D) as targets. The full HLA types for B-LCL used in these experiments were (A) HLA-A*01, A*0201, B*62, B*35, Cw*04, Cw*03; (B) HLA-A*0201, A*26, B*35, B*38, Cw*04, Cw*12; and (C and D) HLA-A*24, A*68, B*07, Cw*07, Cw*08; mismatched targets were A*29, A*26, B*38, Cw*12. Mean values are expressed as percentage specific lysis (*y* axis) after subtracting percentage lysis of control target cells (control lysis ranged from 0 to 7% in these assays). Effector:target cell ratios are shown on the *x* axis. For the experiment shown in (D), the E:T ratio was 25:1.

specific T cells primed in peripheral lymphoid tissues are not believed to localize to mucosal surfaces (Brandtzaeg *et al.,* 1999; Mowat and Viney, 1997).

Because this study involved long-term cultures from only two patients it is difficult to draw broad conclusions concerning CTL repertoire overlap between peripheral and mucosal T cell populations. In contrast to patient CM01, patient CM02 had CTL of limited specificity in GALT-derived MNC cultures, but no detectable HIV-1-specific CTL in peripheral blood. Duodenal MNC from this patient showed lytic activity in ^{51}Cr release assays against B-LCL expressing HIV-1 Pol and Gag and strong IFN-γ spot-forming activity in ELISPOT assays. Blood-derived MNC were negative in both assays. In contrast to the findings for patient CM01, these results suggested limited trafficking between mucosal and peripheral compartments and/or an absence of shared specificity in the respective CTL repertoires. Several explanations could be proposed to account for this observation. First, the *in vitro* expansion methods utilized may favor outgrowth of specific populations at the expense of others; in addition, cultures were derived from a small number of lymphocytes and therefore may not provide a complete, representative sampling of the CTL repertoire in intestinal mucosa. If we accept that HIV-1-specific CTL were present in intestinal mucosa but absent from peripheral blood of patient CM02, possible explanations might include a difference in viral quasispecies present in GALT versus peripheral blood (Lapenta *et al.,* 1999), locally enhanced viral replication, or antigen "trapping" in mucosal tissues (Frankel *et al.,* 1996).

The differences observed between patients CM01 and CM02 might also be related to differences in HIV-1 viral load and/or CD4$^+$ T cell counts in these individuals. Only patient CM01 had detectable HIV-1-specific CTL in blood (Tables 1 and 3). At the time of sampling, patient CM01 was undergoing HAART therapy and had undetectable plasma viremia and >500 CD4$^+$ T cells/μl in peripheral blood. Patient CM02, who had voluntarily discontinued HAART at the time of biopsy, had >100,000 HIV-1 viral RNA copies/ml of plasma and 26 CD4$^+$ T cells/μl. As CD8$^+$ CTL responses rely upon CD4$^+$ T cell help, the absence of HIV-1-specific CTL in peripheral blood from patient CM02 might be related to profound CD4$^+$ T cell

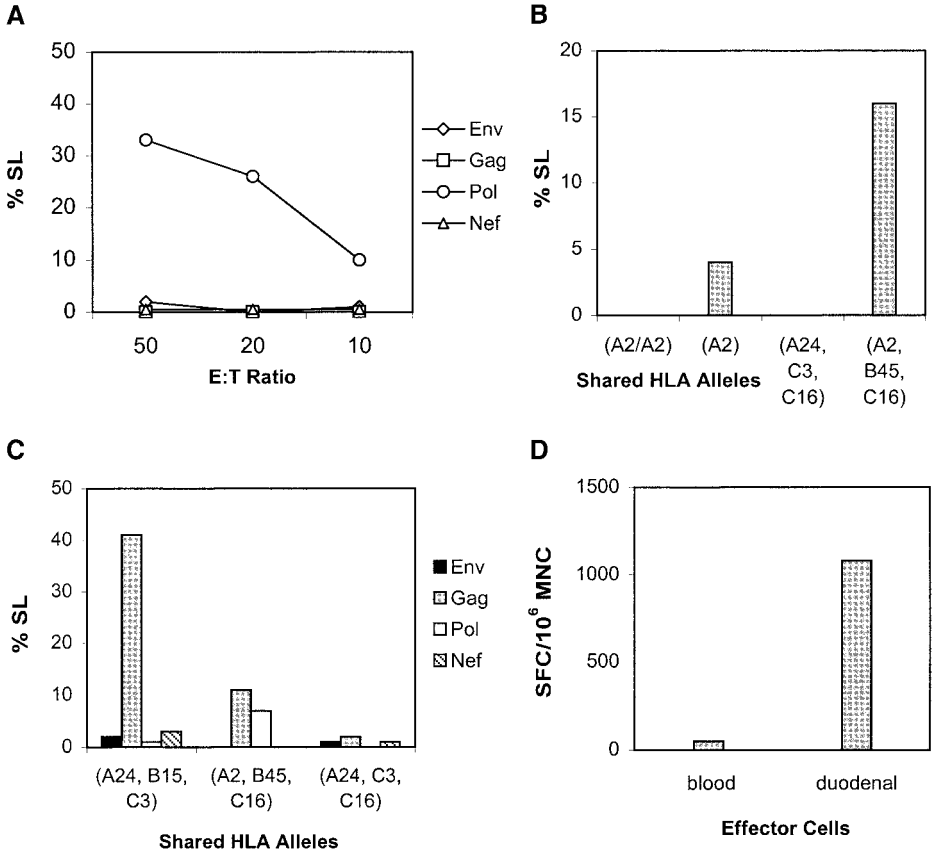

FIG. 3. HIV-1-specific CTL in duodenal MNC from patient CM02. (A) Results of a ^{51}Cr release assay performed with duodenal-derived MNC (culture D1) as effectors and allogeneic B-LCL matched at HLA-A*0201, HLA-B*45, and HLA-Cw*16 as targets. Mean values for ^{51}Cr release are expressed as percentage specific lysis (*y* axis) after subtracting lysis of control target cells. Effector:target cell ratios are shown on the *x* axis. (B) Results of an experiment to determine the HLA restriction of the Pol-specific response shown in (A), using multiple HLA-matched B-LCL. The E:T ratio for this experiment was 50:1. (C) Results of a ^{51}Cr release assay using duodenal culture D4 as effectors and a panel of HLA-matched B-LCL as targets. The E:T ratio for this experiment was 30:1, and lysis of control cells was 4%. (D) IFN-γ ELISPOT assay comparing blood and duodenal (D4) cultures. The number of HIV-1 Gag-specific IFN-γ spot-forming cells (SFC) per 10^6 MNC is shown on the *y* axis. Numbers were obtained by counting the number of Gag-specific spots per well in duplicate wells (each containing 5×10^4 cells), subtracting the average number of spots in control wells (range 0–20 SFC per well), and adjusting to SFC per 1×10^6 cells.

depletion (Bennett *et al.,* 1998; Doherty *et al.,* 1997; Kalams and Walker, 1998; Matloubian *et al.,* 1994). Under conditions of CD4$^+$ depletion, the priming of CTL responses in mucosal tissues might proceed by an alternate mechanism, perhaps involving direct contact of CD8$^+$ CTL with antigen-presenting cells expressing CD40 (Ridge *et al.,* 1998; Schoenberger *et al.,* 1998; Spiegel *et al.,* 2000). Such a mechanism might explain the CTL activity observed in duodenal lymphoid tissue from patient CM02. Additional studies will be required to test this hypothesis.

HAART therapy has a well-documented suppressive effect on antiviral CTL responses in peripheral blood, which is thought to be related to the decline in viral antigen brought about by suppression of HIV-1 replication (Ogg *et al.,* 1999). However, the effects of HAART on CTL responses in mucosal tissues have not been studied nor have the effects of HAART on HIV-1 viral load in mucosae been thoroughly explored. HIV-1 viral load may have an uneven distribution in intestinal tissues (Ullrich *et al.,* 1998), with the highest concentrations in ileum and rectum (Kotler *et al.,* 1991). In one study, initiation of HAART within 90 days of onset of symptoms resulted in suppression of viral replication (as measured by multiply spliced mRNA) in GALT biopsies from 10 patients, despite persistence of unspliced mRNA and proviral DNA in these tissues (Markowitz *et al.,* 1999). However, the possibility exists that mucosal and peripheral compartments may respond unequally to HAART, resulting in differences in viral load and CTL populations between circulating peripheral blood and GALT.

Little is known about the induction and maintenance of mucosal antiviral CTL responses in simian or human AIDS or in other primate viral infections. Intraepithelial lymphocytes with CTL function have been identified in mice experimentally infected with reovirus (London *et al.,* 1990) and with lymphocytic choriomeningitis virus (Sydora *et al.,* 1996). Murine vaccination models have also helped to establish the importance of induction of mucosal CTL for protection from mucosal challenge.

TABLE 3

Summary of Results

	HLA type			MHC class I-restricted CTL activity		
Patient ID	HLA-A	HLA-B	HLA-C	Tissue	MHC restriction	HIV antigens
CM01	02, 32	14	08	Blood	HLA-A*0201	Pol, Gag, Nef
					HLA-Cw*08	Gag
				Rectal	HLA-A*0201	Pol, Nef
					HLA-Cw*08	Pol, Gag, Nef
CM02	02, 24	15, 45	03, 16	Blood	None	None
				Rectal	None	None
				Duodenal	HLA-B*45	Pol
					HLA-B*15	Gag
CM03	02, 03	27, 52	02, 12		na	na

Note. na, not analyzed.

Intrarectal immunization of mice with a recombinant vaccinia virus expressing HIV-1 Env resulted in protection from mucosal transmission; this protection was dependent on the presence of $CD8^+$ CTL at the site of challenge (Belyakov *et al.,* 1998). Recently, Murphey-Corb *et al.* demonstrated protection from intracolonic challenge in rhesus macaques rectally immunized with SIV. Protection was correlated with SIV Env-specific CTL in the intestinal lamina propria (Murphey-Corb *et al.,* 1999). In another study, targeted immunization of rhesus macaques with simian immunodeficiency virus immunogens via iliac lymph nodes was shown to elicit SIV-specific CTL in rectal and cervicovaginal mucosa (Klavinskis *et al.,* 1996). Taken together, these studies underscore the relevance of the induction of mucosal CTL to HIV-1 vaccine development. The methods developed in the present report will be useful in assessing mucosal immune responses in AIDS patients undergoing a variety of therapeutic regimes. Information derived from such studies will provide important information that should contribute to the development of more effective vaccine strategies.

MATERIALS AND METHODS

Biopsy and blood samples

Three HIV-1-positive individuals with chronic diarrhea were studied. After an initial examination, patients were enrolled in a study designed to assess the effects of thalidomide therapy on HIV-1-related diarrhea. Informed consent was obtained from all patients, and the study protocol was approved by the Institutional Review Boards of participating institutions. Patients CM01 and CM02 were biopsied prior to initiation of thalidomide treatment, while patient CM03 was biopsied at the conclusion of therapy.

Rectal biopsy tissue was obtained by flexible sigmoidoscopy from sites located in the rectum at 10 cm from the anal verge. A flexible sigmoidoscope with a biopsy channel (ES-3830, Pentax Corp.) was used with jumbo biopsy forceps (Olympus FB-50U-1) for rectal biopsies. Duodenal biopsy tissue was obtained by upper endoscopy from sites located in the second portion of the duodenum. Upper endoscopes were Pentax Models EG-2901 or EG-2731, with jumbo biopsy forceps (Olympus FB-50K-1). Two to five tissue samples were taken at each location.

Blood samples were obtained at the time of biopsy. Plasma was stored at −80°C for virus load determination. Viral RNA was quantified using an ultrasensitive RT-PCR assay (Roche Diagnostics), with a detection limit of 50 copies/ml plasma (Mulder *et al.,* 1994). Class I HLA typing (A, B, and C alleles) was determined by polymerase chain reaction in a 96-well plate format using commercial HLA typing kits (Pel-Freez, Brown Deer, WI).

Mononuclear cell preparation

Tissue biopsies were washed five times in RPMI medium containing 15% fetal calf serum (FCS), L-glutamine, and antibiotics (designated R-15 medium). Each individual tissue sample was gently minced with forceps and blunt scissors and washed five additional times with R-15. Coarse cell suspensions obtained in this manner were placed in R-15 containing 100 U/ml rIL-2 (Hoffmann–LaRoche, Nutley, NJ) and polyclonally stimulated with 0.1 μg/ml monoclonal anti-CD3 antibody (12F6, obtained from Dr. Johnson Wong, Massachusetts General Hospital, Boston, MA) and allogeneic γ-irradiated feeder cells. Several days after stimulation, MNC were enriched by density centrifugation on a Ficoll–Hypaque solution (Pharmacia, Uppsala, Sweden). MNC were transferred sequentially from 96-well to 24-well plates and finally to 25-ml flasks when sufficiently expanded. Cultures were designated "R" (rectal-derived) or "D" (duodenal-derived). When necessary, some cultures were treated with amphotericin B to inhibit the growth of fungi and yeast.

Peripheral blood mononuclear cells were separated from whole blood using Ficoll–Hypaque separation. Bulk

cultures were obtained by polyclonal simulation with 0.1 μg/ml monoclonal anti-CD3 antibody (12F6), as described above. Cultured T cells were maintained for up to 4 months by restimulating every 3 to 4 weeks with anti-CD3 antibody (without additional feeder cells) and feeding twice weekly with R-15/IL-2.

Chromium release assays for CTL activity

GALT and peripheral blood-derived MNC were tested in chromium release assays for CTL activity (Coligan *et al.,* 1999). For CTL assays, HLA-A, B, or C matched or mismatched B-LCL were infected with recombinant vaccinia viruses expressing HIV-1 (IIIB) antigens (vAbT 299 Env, vAbT 141 Gag, vAbT 204 Pol, vT23 Nef), obtained from Therion Biologicals (Cambridge, MA), or control. Control vaccinia virus was a tk(−) strain with insertional inactivation of the thymidine kinase gene. Cells were infected at a multiplicity of infection (m.o.i.) of 5:1 for 1 h at 37°C, 5% CO_2. Target cells were then labeled overnight at 37°C, 5% CO_2 with 100 μCi of $Na_2\,^{51}CrO_4$ (New England Nuclear, Boston, MA). Standard ^{51}Cr release assays were performed by mixing labeled target cells (3000 to 5000 per well) with effector cells at ratios varying from 50:1 to 5:1, in duplicate or triplicate wells of a 96-well microtiter plate. Assays were incubated for 4 to 5 h, at which time 30 μl of supernatant was transferred to the corresponding wells of a Lumaplate (Packard Instruments, Meriden, CT) using an automated Bio-Mek 2000 workstation (Beckman Instruments, Fullerton, CA). Plates were air-dried and counted on a TopCount scintillation counter (Packard Instruments). The percentage specific ^{51}Cr release was calculated according to the following formula: % specific release = [(experimental release − spontaneous release)/(maximal release − spontaneous release)] × 100. Spontaneous release was determined from wells containing labeled target cells and medium, and maximal release was calculated from wells containing target cells and 1% Triton X-100. CTL responses were considered significant under the following conditions: (1) the spontaneous release was less than 25% and (2) the percentage specific release of target cells expressing HIV-1 antigens was ≥10% above the percentage specific release of target cells infected with control vaccinia virus. Specific release for control target cells was generally below 10%, except where otherwise indicated.

CD8-expressing cells were positively selected by adherence to anti-CD8 magnetic beads following the manufacturer's protocol (Dynabeads; Dynal, Great Neck, NY). The magnetic beads were subsequently removed using Detach-a-Bead (Dynal). CD8-enriched cells were then washed and used as effectors in standard ^{51}Cr release assays.

ELISPOT assay

An ELISPOT assay was used to measure IFN-γ release by cytotoxic $CD8^+$ T cells (Larsson *et al.,* 1999). Ninety-six-well nitrocellulose-bottom microtiter plates (Multiscreen-HA; Millipore, Molsheim, France) were coated overnight with a monoclonal antibody specific to human IFN-γ (Mab 1-D1K; Mabtech, Stockholm, Sweden). Mononuclear cell cultures were infected with recombinant vaccinia viruses expressing HIV-1 antigens (Env, Gag, Pol, Nef) or control vaccinia virus. Infections were carried out at an m.o.i. of 2:1 for 1 h at 37°C in RPMI + 1% FCS. After infection, cells were washed twice, recounted, and added to duplicate or triplicate wells in medium containing 5% pooled human serum and incubated for 14 to 20 h at 37°C, 5% CO_2. Depending upon cell numbers, 5×10^4 or 1×10^5 cells per well were seeded; identical numbers were added to all wells involving the same effector cell populations. Plates were developed using a secondary antibody to IFN-γ (Mabtech) coupled to biotin and an avidin–peroxidase complex (Vectastain ABC; Vector Laboratories, Burlingame, CA). Spot-forming cells were enumerated on a dissecting microscope and reported as SFC per 10^6 mononuclear cells (Larsson *et al.,* 1999).

Surface antigen staining and phenotypic analysis

Expanded MNC cultures were analyzed for expression of T cell markers CD3, CD4, and CD8; T-cell receptor α and γ chains; NK-associated markers CD16, CD56, and CD57; and mucosal adhesion/retention marker CD103 ($\alpha^E\beta_7$ integrin). Monoclonal antibodies used were as follows: CD3–FITC, CD8–PerCP, CD56–FITC, TCRαβ–FITC, TCRγδ–FITC (Becton–Dickinson, San Jose, CA); CD57–FITC (Pharmingen Becton–Dickinson); CD103–FITC (Caltag Laboratories, Burlingame, CA); CD16-PE (Coulter Immunotech, Hialeah, FL); and CD4-APC (ExAlpha, Boston, MA) (abbreviations for fluorochromes: FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein; APC, allophycocyanin).

Stained MNC populations were analyzed for expression of cell surface markers by flow cytometry on a FACSCalibur instrument (Becton–Dickinson). Lymphocyte populations were gated based on forward and side scatter and in some cases based on expression of the CD3 surface antigen. Appropriate isotype controls (generally mouse IgG1) were used to set quadrant markers. For three- and four-color analysis, electronic compensation for spectral overlap was set using MNC samples stained with single-color reagents.

ACKNOWLEDGMENTS

We thank Simon Monard and Jeremy Segal for assistance with flow cytometry, Fang Fang and Linqi Zhang for performing virus load quantitations, Marie Larsson for help with ELISPOT assays, and the members of the Nixon lab for technical advice and useful discussions. We

also thank Maryanne Small for secretarial assistance. We are indebted to the patients who participated in this study. This research was supported by NIH Grant DK-55858 to D.F.N. D.F.N. is an Elizabeth Glaser Scientist supported by the Elizabeth Glaser Pediatric AIDS Foundation.

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