Optimization of methods to assess human mucosal T-cell responses to HIV infection

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Abstract

The majority of HIV-1 infections occur via sexual transmission at mucosal epithelia lining the vagina, cervix or rectum. Mucosal tissues also serve as viral reservoirs. However, our knowledge of human mucosal T-cell responses is limited. There is a need for reliable, sensitive, and reproducible methods for assessing mucosal immunity. Here we report on the collaborative efforts of two laboratories to optimize methods for processing, culturing, and analyzing mucosal lymphocytes. Rectal biopsy tissue was obtained by flexible sigmoidoscopy, which is rapid, minimally invasive, and well tolerated. Of the four methods compared for isolating mucosal mononuclear cells (MMC), collagenase digestion reproducibly yielded the most lymphocytes ($4–7 	imes 10^6$). Furthermore, $0.5–1 	imes 10^6$ MMC could be polyclonally expanded to yield $17 	imes 10^6$ CD8+ T cells allowing mapping of responses to overlapping peptides spanning the HIV-1 genome using IFN-γ enzyme-linked immunospot (ELISpot). Expansion also reduced the spontaneous IFN-γ production normally detected in fresh MMC. Piperacillin–tazobactam and amphotericin B reduced contamination of MMC cultures to 4%. Taken together, these methods will be useful for studies of mucosal immunity to HIV-1 and other pathogens during natural infection and following vaccination.

Keywords: Mucosal immunity; ELISpot; T cells; Tetramer; HIV; GALT

Abbreviations: GALT, gut-associated lymphoid tissue; MMC, mucosal mononuclear cells; ELISpot, enzyme-linked immunospot; APC, antigen-presenting cells; R10, RPMI with 10% fetal calf serum; R15, RPMI with 15% fetal calf serum.

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1. Introduction

There is a substantial body of literature describing antigen-specific T-cell responses to HIV and other viral pathogens in peripheral blood (reviewed in McMichael and Rowland-Jones, 2001; Gandhi and Walker, 2002). However, for many infectious agents the majority of replication occurs in tissue reservoirs such as lymph nodes, urogenital and gastrointestinal mucosa, liver, and lungs. To better understand virus–host interactions in these tissues, it is critical to develop reliable methods for isolating tissue lymphocytes, and to compare antigen-specific T-cell responses in tissues and peripheral blood. In the case of HIV, gut-associated lymphoid tissue (GALT) serves as an important portal of entry as well as a major site of viral replication throughout the disease process (Kotler et al., 1991; Chui and Owen, 1994; Schneider et al., 1995; Fackler et al., 1998; Zeitz et al., 1998). GALT CD4+ T cells have an activated memory phenotype, and express both major HIV coreceptors, CXCR4 and CCR5, rendering them highly sensitive to HIV infection and replication (Lapenta et al., 1999; Anton et al., 2000; Poles et al., 2001b). In Rhesus macaques experimentally infected with simian immunodeficiency virus (SIVmac), rapid and profound CD4+ T-cell depletion occurs in GALT within 2 weeks, regardless of the route of initial infection (Veazey et al., 1998). Active viral replication and CD4+ T-cell depletion have been described throughout the chronic phase of infection (Schneider et al., 1994, 1995; Ullrich et al., 1998). Residual viral replication has been detected in GALT during highly active antiretroviral therapy (HAART), under conditions suppressing replication in peripheral blood (Markowitz et al., 1999; Anton et al., 2001), and viral quasispecies in blood and GALT may differ (Poles et al., 2001a). The gastrointestinal tract is also an important site of opportunistic infections and malignancies during late-stage AIDS (Chui and Owen, 1994). Thus, GALT is an important tissue reservoir for HIV replication at all stages of disease.

The assessment of GALT T-cell responses is also relevant for vaccine development. Because the majority of HIV infections occur via sexual transmission across mucosal epithelia, there is great interest in developing vaccine strategies to induce cell-mediated immune responses in these tissues (Miller and McGhee, 1996). Murine and simian vaccination models have helped to establish the importance of mucosal CD8+ T-cell responses for protection from vaginal and rectal challenge (Klavinskis et al., 1996; Belyakov et al., 1998b; Murphey-Corb et al., 1999).

Several new technologies have led to a reassessment of the magnitude of CD8+ T-cell responses during viral infection. The use of MHC class I tetramer–peptide complexes (Altman et al., 1996) and enzyme-linked immunospot (ELISpot) (Czerkinsky et al., 1988; Larsen et al., 1999) assays has demonstrated that traditional limiting dilution analysis (LDA) underestimates the frequency of CD8+ T cells by 10–100-fold (Moss et al., 1995; Doherty, 1998; Murali-Krishna et al., 1998; Tan et al., 1999). These new assays have become widely accepted surrogates for assessing cytotoxic T-lymphocyte (CTL) populations (Shacklett, 2002). In addition to their sensitivity and relative technical simplicity, these two methods require smaller numbers of lymphocytes, making them suitable for samples containing fewer than 5 million fresh lymphocytes, as is often the case with primary tissue specimens.

Studies in animal model systems have relied on GALT lymphocytes from relatively large segments of intestinal tissue taken from necropsy or surgical resection (Veazey et al., 1997; Murphey-Corb et al., 1999). However, several reports have demonstrated the feasibility of isolating viable lymphocytes from intestinal biopsies obtained during flexible sigmoidoscopy or upper endoscopy (Anton et al., 2000; Shacklett et al., 2000). For the purposes of pathogenesis studies as well as vaccine trials, there is a need to develop optimized and reproducible procedures that will provide acceptable yields of viable lymphocytes without significant clinical risk and with minimal laboratory manipulation. In this report, we present the results of comparative studies undertaken in two laboratories to optimize the yield and viability of lymphocytes obtained from human rectal biopsy specimens, and to develop assays for detecting antigen-specific T cells in GALT.

2. Methods

2.1. Patient recruitment and biopsy collection

HIV-positive individuals and seronegative, healthy controls were recruited from clinic patients, clinical
trial subjects and local volunteers. All subjects gave informed consent and research protocols were approved by Institutional Review Boards on each campus (UCLA or UCSF/GIVI). Rectosigmoid biopsies were endoscopically acquired using a flexible sigmoidoscope (EC3831L, Pentax Precision Instrument, Orangeburg, NY; Olympus Instruments, Melville, NY) as previously reported (Anton et al., 2000; Olsson et al., 2000). In brief, the rectosigmoid colon was routinely sampled between 10 and 30 cm from the anal verge. Biopsy specimens were obtained by use of large cup endoscopic biopsy forceps (Microvasive Radial Jaw no. 1589; Boston Scientific, Natick, MA) with an outside diameter of 3.3 mm. At each biopsy procedure, a total of 20–25 biopsy samples were obtained. Tissue samples were placed in 15 ml of RPMI 1640 (Gibco BRL, Rockville, MA) with 10% fetal calf serum (designated R10), supplemented with antibiotics (Merck, Whitehouse Station, NJ) and amphotericin B (Gibco BRL). The cells were then used for lymphocyte isolation, flow cytometry, and functional studies.

2.2. Culture antibiotics

Initially, MMC were isolated and cultured in media containing amphotericin B (2.5 μg/ml) and one of the following: penicillin and streptomycin (250 μg/ml each) (Gibco BRL), gentamicin (50 μg/ml) (Gibco), or imipenem–cilastatin (Merck). Due to contamination and toxicity concerns, the current protocol utilizes piperacillin–tazobactam (500 μg/ml) (Lederle Piperacillin, Carolina, Puerto Rico) and amphotericin B (1.25 μg/ml).

2.3. Screen isolation of MMC

Biopsy specimens were placed on a sterile #40 stainless steel mesh (Small Parts, Miami Lakes, FL) inside a 10 × 35 mm Petri dish along with 5.5 ml of RPMI 1640 (Gibco BRL, Rockville, MA) with 10% fetal calf serum (designated R10), supplemented with antibiotics (Merck, Whitehouse Station, NJ) and amphotericin B (Gibco BRL). The cells were then used for lymphocyte isolation, flow cytometry, and functional studies.

2.4. Mechanical isolation of MMC

Mechanical isolation of MMC was performed using a MediMachine (manufactured for Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA) according to the manufacturer’s instructions. Briefly, five biopsy specimens and 800 μl of R10 (containing antibiotics) were placed in the 50 μM sterile Medicon grinder port and homogenized for 40 s. The tissue fragments were visually inspected and if needed, any intact material remaining on the blades was moved onto the screen and ground for an additional 40 s. The media and cells were transferred to a 50-ml centrifuge tube. The Medicon grinder was rinsed by gently pipetting 800 μl of media over the blades and screen, then adding the rinse volume to the previously collected cells. This step was repeated as needed to remove all of the cells. Additional biopsies were processed in groups of five using a new sterile Medicon each time. The single cell suspension was then filtered, spun, and resuspended in 1.3 ml of R10+ antibiotics.

2.5. Collagenase/dispase isolation of MMC

Biopsies were placed in a 10 × 35 mm Petri dish containing Hanks’ buffered saline solution (HBSS) (Gibco BRL) with 1% BSA, 1 mm EDTA, 50 mm 2-mercaptoethanol (Sigma, St. Louis, MO) and teased apart using a pair of 18G needles. The disrupted tissue was transferred to a 50-ml centrifuge tube and incubated at 37 °C for 20 min in a shaking water bath. Following centrifugation, the tissue was digested with a mixture of collagenase and dispase (Boehringer Mannheim, Mannheim, Germany, Cat #269638) (0.1 mg/ml in HBSS/BSA) for 1 h at 37 °C, then further disrupted by passage through a series of needles with decreasing diameters (18–23 gauge). Cells were then resuspended as described above.

2.6. Collagenase type II isolation of MMC

Tissue biopsies were washed once in RPMI medium containing 15% fetal calf serum (FCS) (R15), L-glutamine, and antibiotics, then distributed equally amongst three 50-ml conical centrifuge tubes (5–10 biopsies per tube). Samples were incubated in 20–25
ml RPMI/7.5% FCS containing 0.5 mg/ml collagenase type II-S (sterile filtered) (clostridiopeptidase A from Clostridium histolyticum, Cat #C1764, Sigma-Aldrich, St. Louis, MO) for 30 min in a 37 °C water bath, with intermittent shaking. Tissue fragments were further disrupted by forcing the suspension five to six times through a 30-cm³ disposable syringe attached to a blunt-ended 16-gauge needle (Stem Cell Technologies, Vancouver, BC). The entire suspension was then passed through a sterile plastic strainer (Falcon 2350) to remove free cells and concentrate the remaining tissue fragments. Free cells were immediately washed twice in R-15 medium to remove excess collagenase, and tissue fragments were returned to a 50-ml conical tube. The entire procedure, including 30-min collagenase incubations, was repeated two to three additional times or until tissue fragments were no longer intact.

Mucosal lymphocytes (both intraepithelial and lamina propria lymphocytes, IEL and LPL) were separated from adjacent epithelial cells by centrifuging for 20–25 min on a discontinuous (35%/60%) Percoll gradient (Pharmacia, Uppsala, Sweden). To aid in visualizing the lymphocyte layer, several drops of 0.04% phenol red (in PBS) were added to the 60% Percoll layer. After centrifugation, IEL and LPL, located at the 35%/60% Percoll interface, were harvested, transferred to a 50-ml conical tube, and washed twice in a four- to fivefold excess of PBS or R-15. Cells were then resuspended as described above.

2.7. Polyclonal expansion of CD8+ T cells

CD8+ T cells from fresh PBMC or MMC were polyclonally expanded as previously described (Yang et al., 1997) using a CD3:CD4 bispecific antibody kindly provided by Dr. Johnson Wong (Wong and Colvin, 1987, 1991). This antibody stimulates expansion of CD3+CD8+ T cells and depletion of CD3+CD4+ T cells. Briefly, 50 × 10⁴ to 1 × 10⁶ PBMC or MMC were incubated with 0.1 μg/ml CD3:CD4 bispecific antibody in R10 supplemented with L-glutamine, antibiotics, and 50 U/ml of IL-2. Approximately 7 × 10⁶ autologous PBMC were irradiated with 3000 cGy, and added to the cultures as feeder cells to MMC cultures. After expansion for 14 days, the cultures were determined by flow cytometric phenotyping to contain approximately 90% CD8+ T cells.

2.8. Quantification of lymphocyte subsets

Lymphocyte subsets in whole blood and MMC preparations were quantified by Trucount (BDIS) according to the manufacturer’s instructions. Briefly, 50 μl of whole blood or 50–100 μl of MMC single cell suspensions were each added to two tubes of MultiTest reagent containing either CD3 FITC/CD8 PE/CD45 peridinin chlorophyll protein (PerCP)/CD4-allophycocyanin (APC) or CD3 FITC/CD16 & CD56 PE/CD45 PerCP/CD19 APC. Red blood cells were lysed by the addition of 450 μl of FACS lysing solution (BDIS). Samples were analyzed on a FACSCalibur (BDIS) using CELLQUEST software. Lymphocyte numbers were determined by gating on CD45+ cells and confirmed by back-gating to determine forward and side scatter profiles. The absolute numbers (cells/μl) of CD45+CD3+CD4+, CD45+CD3+CD8+, CD45+CD3−CD16−CD56+, and CD45+CD3−CD19+ lymphocytes were then quantified by dividing the number of positive cellular events by the number of bead events and multiplying the result by the bead concentration [(antibody-positive cells/total bead events) × beads/μl]).

2.9. ELISpot analysis for HIV-specific IFN-γ release

Enzyme-linked immunospot (ELISpot) assay for IFN-γ was performed using the protocols and reagents previously described (Tary-Lehmann et al., 1998). Briefly, CD8+ T cells were isolated either by polyclonal expansion as described in Section 2.7, or separated from PBMC by Rosette Sep™ CD8+ T-cell enrichment cocktail (Stem Cell Technologies). CD8+ T cells (2 × 10⁵) were plated in triplicate with 5 μg/ml of peptide (see Section 2.10), for 24 h at 37 °C. For positive control wells, antibodies to CD2/2R and CD28 (Musgrave et al., 2003; Azuma et al., 1992) (BDIS) were added at 10 and 20 μg/ml, respectively. Culture medium served as the negative control. All controls were plated in triplicate. After color development, the plates were air-dried and read on an ImmunoSpot Analyzer (Cellular Technology, Cleveland, OH). The number of peptide-specific CD8+ T cells was quantified as spot forming cells (SFC)/10⁶.
cells after subtracting the value obtained in the negative control wells.

2.10. Determination of epitope specificity

Epitope specificities of CD8+ T cells were determined using peptides (15-mers overlapping by 11 residues) corresponding to Clade B HIV sequences as follows: nef, pol, vpu, vpr, vif, tat, and rev from the HIV-1 Clade B consensus sequence; gag from HIV-1 HXB2, and the env from HIV-1 MN (all from the NIH AIDS Research and Reference Reagent Program, Rockville, MD). Peptides were combined to create a total of 53 pools, each containing 15–16 peptides, such that the entire genome was represented in each assay. Positive wells were defined as satisfying two criteria: (1) containing a minimum of 50 SFC/106 cells, and (2) exceeding the mean +2 S.D. of the negative control wells (Mwau et al., 2002).

2.11. Tetramer staining

Peripheral blood and GALT were obtained from seven HIV-infected patients previously determined to be HLA-A*0201-positive by serological typing. Blood and GALT were tested for the presence of HIV-specific CD8+ T cells using MHC class I tetramer reagents directed towards epitopes in Gag (aa 77–85, SLYNTVATL) and Pol (aa 476–484, ILKEPVHG). Tetramer staining was performed by incubating cells with antibodies to CD3, CD8 (BDIS), and MHC class I tetramer for 30 min at 4 °C. Cells were then washed twice in PBS/2% FCS, fixed in 1% paraformaldehyde and assessed by flow cytometry, collecting 100,000 events in the live lymphocyte gate whenever possible. For analysis, cells were gated on CD3+/CD8+ lymphocytes and plotted as CD8+ vs. recently activated, CD69-positive cells. Responses were considered positive if a distinct population corresponding to >0.03% of CD8+ T cells bound tetramer. As controls for the specificity of tetramer staining, PBMC and rectal tissue were obtained from controls who were HIV-positive and HLA-A*0201-negative, or HIV-negative and HLA-A*0201-positive.

2.12. Intracellular cytokine staining

To assess the production of IFN-γ by antigen-specific CD8+ T cells, fresh mononuclear cells from blood and GALT were stimulated with HIV Gag peptide (SLYNTVATL, aa 77–85, 10 μg/ml) or media alone for 6 h at 37 °C, 5% CO2, as previously described (Donahoe et al., 2000). As a positive control, cells were stimulated with staphylococcal enterotoxin B (SEB) (5 μg/ml) (Sigma). To provide costimulation, antibody to CD28 (clone L293, BDIS) was added to each sample (1 μg/tube). Brefeldin A (10 μg/ml) was added 1 h after the stimulating antigens to block vesicular transport and cytokine release. Six hours later, the cells were washed, fixed in 4% paraformaldehyde (Sigma), and stored at 4 °C. The following day, cells were washed, permeabilized (FACSPerm, Becton-Dickinson), and stained with MAb specific for CD3, CD8, CD69, and IFN-γ. Samples were analyzed by four-color flow cytometry on a FACSCalibur (BDIS), collecting 100,000 events whenever possible. For analysis, samples were gated on CD3+/CD8+ lymphocytes, then plotted as IFN-γ-producing cells vs. recently activated, CD69-positive cells. The percentage of CD8+ T cells responding to peptide stimulation was determined by subtracting the IFN-γ response detected in cells stimulated with media alone. To establish background levels, PBMC and GALT from HIV-negative individuals were tested with the same peptides. To be considered significant, the net antigen-specific IFN-γ production was required to exceed 0.05% of CD8+ T cells, and to exceed background levels of IFN-γ production (in media controls) by at least twofold.

3. Results

3.1. Isolation of MMC

To determine the optimal method of recovering MMC, we compared four different techniques: manual disruption with a steel mesh screen, mechanical
isolation in a tissue homogenizer (MediMachine), enzymatic digestion using a mixture of collagenase and dispase, and digestion with collagenase type II alone. These methods were tested in parallel using tissue biopsies obtained from each of seven individuals. Cell counts and subset distributions were determined by Trucount. Viability was determined by trypan blue dye exclusion. While the MediMachine proved to be the easiest and fastest method, the collagenase type II method clearly yielded the most lymphocytes (Fig. 1). Using this method, viable lymphocyte and CD3+ T-cell recoveries were 4- and 5.6-fold higher, respectively, than obtained with the MediMachine, and ranged from ~4 to 7 × 10^6 lymphocytes and ~3 to 6 × 10^6 CD3+ T cells per 20 biopsy samples. Due to the high yield of cells, the collagenase type II method was used for all subsequent studies.

3.2. MHC class I tetramer staining

To determine the frequency of HIV-specific CD8+ T cells in blood and GALT, samples were obtained from seven HIV-infected, HLA-A*0201-positive individuals. Mononuclear cells isolated from blood and GALT were stained with MHC class I tetramers specific for HLA-A*0201-restricted epitopes in Gag (aa 77–85, SLYNTVATL) (SL9) and Pol (aa 476–484, ILKEPVHGIV) (IV9). Of the seven HIV-infected, HLA-A*0201-positive patients tested, five had detectable populations of CD8+ T cells specific for Gag SL9 in peripheral blood, and one of these also recognized Pol IV9 (Fig. 2). In PBMC, the frequency of Gag-specific CD8+ T cells ranged from 0.17% to 1.53% of CD8+ T cells. In GALT, frequencies ranged from 0.55% to 2.60% of CD8+ T cells. Staining of peripheral blood and GALT from HIV-negative, HLA-A*0201-positive and HIV-infected, HLA-A*0201-negative patients revealed no specific staining (data not shown).

3.3. Cytokine flow cytometry

Tetramer staining allows enumeration of epitope-specific cells without assessing function. To quantify functional virus specific T cells, mononuclear cells may be stimulated with synthetic peptides, proteins, or vaccinia viruses expressing foreign antigens and assessed for intracellular cytokine production by flow cytometry (Picker et al., 1995; Komanduri et al., 2001). An advantage of this method is the ability to obtain phenotypic information about the cytokine-producing population (Maino and Picker, 1998). Peripheral blood and GALT samples from three HIV-infected, HLA-A*0201-positive patients and two HIV controls were stimulated with minimal epitope peptides specific for HLA-A*0201-restricted SL9 or with the superantigen staphylococcal entero-toxin B (SEB). All three HIV-infected patients had SL9-specific CD8+ T cells in peripheral blood and GALT as determined by tetramer staining (not shown). Samples were treated with brefeldin A to inhibit vesicular transport, then permeabilized and stained as previously described (Donahoe et al., 2000).

Results for two HIV-infected patients are shown in Fig. 3. Unlike PBMC, most GALT CD8+ T cells expressed the early activation marker CD69. This has been previously reported and is believed to be associated with the unusual, partially activated status of GALT memory T cells (Wang et al., 2002). Perhaps as a result of this partial activation, background levels of IFN-γ production in GALT T cells were higher than
in PBMC (mean 0.086 vs. 0.009). Nevertheless, Gag-specific IFN-γ production was detected at levels greater than threefold above background in GALT samples from all three HIV-infected individuals, but not in samples from two healthy negative controls (not shown).

3.4. Adaptation of the ELISpot assay for GALT tissue

Both tetramer and intracellular cytokine assays allow phenotypic analysis of peptide specific cells; however, both assays require high numbers of cells, making it difficult to assess responses to multiple antigens. ELISpot analysis requires only 1–3 × 10^5 cells/well, making this a particularly attractive assay for measuring HIV-1-specific CD8+ T-cell responses in mucosal specimens containing limited numbers of lymphocytes. To adapt the ELISpot assay for assessing HIV-specific CD8+ T-cell responses in MMC and PBMC, we created pools of overlapping peptides (15mers overlapping by 11) spanning the entire HIV-1 genome as described in Section 2.

As the size of the peptides theoretically allows for epitope recognition by both CD4 and CD8+ T cells (Betts et al., 2001), CD8+ T cells were separated from fresh PBMC by Rosette Sep prior to plating. To enhance antigen presentation to CD8+ T cells, we then added autologous EBV-transformed B cells (BLCL) to PBMC from nine different donors (data not shown). Unfortunately, the addition of BLCL to fresh, autologous T cells resulted in extremely high background even in the absence of peptides or other external stimuli, confirming previous reports (Altfeld et al., 2000; Goulder et al.,
Thus, the use of autologous BLCL as antigen-presenting cells in ELISpot assays gave unsatisfactory results.

We then attempted ELISpot assays using fresh PBMC and MMC in the absence of autologous BLCL. In these assays, freshly isolated MMC exhibited substantial spontaneous IFN-γ secretion that could easily mask detection of epitope-specific responses (Fig. 4). This background was somewhat diminished, but not eliminated, when CD4+ T cells were removed prior to plating. High background levels of IFN-γ secretion were not observed in PBMC and may reflect the high activation status of MMC (Wang et al., 2002). In some cases, background could be reduced by culturing MMC overnight before beginning the ELISpot assay. However, this approach gave inconsistent results, and was therefore not considered an optimal solution.

3.5. Polyclonal expansion of mucosal and blood-derived CD8+ T cells

In our attempts to map HIV-1-specific CD8+ T-cell responses in MMC, we encountered two technical problems. First, given the limited amount of tissue provided by pinch biopsies, not all MMC samples yield sufficient cells to map responses to the full complement of HIV peptides. Second, fresh MMC exhibit spontaneous IFN-γ secretion, often contributing to unacceptably high backgrounds in the ELISpot assay. To circumvent these difficulties, we utilized a previously described method for polyclonal expansion of CD8+ T
cells (Wong and Colvin, 1987, 1991; Yang et al., 1997). Using bispecific antibodies recognizing CD3 and CD4, we selectively expanded CD8+ T cells from 19 PBMC and 18 MMC samples. Within 14 days, 0.5–1 × 10⁶ PBMC or MMC were expanded to an average of 13 × 10⁶ (± 1 × 10⁶, range 5–19 × 10⁶), or 17 × 10⁶ (± 2 × 10⁶, range 10–35 × 10⁶) CD8+ T cells, respectively. The purity of expanded CD8+ T-cell cultures ranged from 91% to 99% (95.0 ± 0.6%) for PBMC and 78% to 99% (90.6 ± 1.9%) for MMC. Contaminants were primarily CD4+ T cells (data not shown). Expanded CD8+ T cells from MMC exhibited substantially lower spontaneous IFN-γ secretion in ELISpot assays compared to fresh MMC. For 10 samples of expanded MMC, the mean background was 100 SFC/10⁶ CD8+ T cells. This background IFN-γ production, corresponding to 20 spots/well, was low enough to be subtracted from antigen specific responses. Thus, polyclonal expansion resulted in sufficient cell numbers for thorough HIV-1 epitope mapping, while decreasing spontaneous IFN-γ release.

3.6. Bacterial contamination of expanded cultures

Expansion of MMC presented specific technical challenges due to microbial contamination from intestinal flora. To overcome this problem, we tested several antibiotic preparations, including gentamicin, penicillin–streptomycin, imipenem–cilastatin (Primaxin, a broad-spectrum synthetic mono-lactam) and piperacillin–tazobactam (Zosyn, a combination of the extended-spectrum penicillin piperacillin and the beta-lactamase inhibitor tazobactam). Cultures containing piperacillin–tazobactam had the highest success rate, presumably due to the favorable spectrum of bowel flora coverage and in vitro stability of the drug. In 29 MMC cultures in which Zosyn was not used, the contamination rate was 55%. However, contamination was observed in only 1 of 25 MMC cultures utilizing piperacillin–tazobactam. Our current protocol for MMC expansion uses piperacillin–tazobactam (500 µg/ml) in combination with amphotericin B (1.25 µg/ml). Growth of expanded cells under these conditions is comparable to that of PBMC in penicillin–streptomycin (Data not shown).

3.7. Phenotype of expanded CD8+ T cells

To determine the effects of polyclonal expansion on CD8+ T-cell subset distribution, we assessed the memory/effector phenotype of CD8+ T cells obtained from the peripheral blood (Champagne et al., 2001). These individuals were HLA-A*0201-positive and recognized the HIV-1 SL9 tetramer. It was therefore possible to assess the frequency and phenotype of epitope-specific CD8+ T cells before and after expansion. Overall, the number of SL9-specific CD8+ T cells in 10 samples of expanded MMC, the mean background was 100 SFC/10⁶ ± 229. This background IFN-γ production, corresponding to 20 spots/well, was low enough to be subtracted from antigen specific responses. Thus, polyclonal expansion resulted in sufficient cell numbers for thorough HIV-1 epitope mapping, while decreasing spontaneous IFN-γ release.

Fig. 4. Spontaneous IFN-γ production by freshly isolated MMC. To assess background levels of IFN-γ production by freshly isolated lymphocytes, PBMC or MMC from two participants were plated in an IFN-γ ELISpot assay with culture media alone, in the absence of peptide or polyclonal stimuli. PBMC and MMC from the same individuals were also depleted of CD4+ cells and plated in parallel. Each symbol represents the data obtained from one well, normalized to SFC/10⁶ CD8+ T cells.

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3.8. Epitope specificity of fresh vs. expanded PBMC-derived CD8+ T cells

To determine whether polyclonal expansion altered our ability to detect epitope-specific CD8+ T cells, we mapped HIV-1 epitope recognition by expanded and fresh CD8+ T cells obtained at two time points from three individuals chronically infected with HIV-1. As shown for one representative individual in Fig. 6, we were able to detect CD8+ T-cell responses to epitopes in both structural and accessory genes using either fresh or expanded CD8+ T cells. The only gene product that did not elicit an IFN-γ response from any of the three individuals was Rev. Using the data obtained from fresh PBMC, we calculated the total number of CD8+ T cells responding to HIV-1 antigens in each participant and found that between 1/300 (0.33%) and 1/100 (1%) of CD8+ T cells were HIV-1-specific. This is likely to be an underestimate, as the Clade B consensus peptides are unlikely to represent all of the epitopes presented by primary viruses within an individual.

While responses of greater magnitude were observed in fresh CD8+ T cells than in expanded cells, the pattern of epitope recognition by expanded cells was not independent of that observed for fresh cells (p < 0.001). When fresh CD8+ T cells responded to a particular peptide pool, the probability of polyclonally expanded cultures responding to the same pool was 78%. When fresh CD8+ T cells did not respond to a peptide pool, the probability of a negative response by expanded CD8+ T cells was 83%. While the results obtained from fresh and expanded CD8+ T cells did not correlate perfectly in specificity or sensitivity, these data suggest that fresh and polyclonally expanded cells function similarly in an ELISpot assay. Accordingly, with careful interpretation, polyclonal expansion may be used to obtain reliable data from clinical samples with limited cell numbers.

3.9. Comparison of epitope recognition by CD8+ T cells derived from MMC vs. PBMC

To investigate whether epitope recognition is similar in the gastrointestinal tract and peripheral blood of chronically infected individuals, we mapped CD8+ T-cell responses from fresh PBMC, expanded PBMC, and expanded MMC. Two individuals were tested, using PBMC and MMC obtained at a single time point; the results for one individual are shown in Fig. 6. The pattern of peptide recognition by expanded CD8+ T cells from MMC was similar to that recognized by fresh CD8+ T cells from PBMC (p < 0.001). When no response to a peptide pool was detected using CD8+ T cells from fresh PBMC, the probability of a negative response by expanded MMC was 87%. Conversely, when responses were detected using fresh PBMC, the probability of a positive response by expanded MMC was 79%. When both expanded MMC and fresh PBMC responded to the same peptide pool, the peptides identified by matrix analysis were identical in both cellular compartments. These findings suggest that, at least in these two individuals, the pattern of HIV epitope recognition was similar in both anatomical compartments. These differences may also be attributed, in part, to the comparison between expanded vs. fresh T-cell populations. However, these studies should be extended to larger groups of patients.
at different stages of HIV infection in order to fully explore the relationship between T-cell responses in blood and mucosal tissues.

4. Discussion

The methods described in this paper demonstrate the utility of mucosal biopsy tissue for assessing human cellular immune responses to HIV and other viruses. We compared four methods for isolating MMC from biopsy tissue: manual disruption using forceps and screen, mechanical isolation with the aid of a commercial tissue homogenizer, and enzymatic digestion with either collagenase/dispsase or collagenase II alone. These studies demonstrated that treatment with collagenase II resulted in a high yield of viable MMC without compromising cell surface antigens. Fresh MMC obtained in this manner may be assessed for antigen-specific T cells by a variety of methods, including multiparameter flow cytometry, MHC class I tetramer staining, intracellular cytokine flow cytometry, and ELISpot assay.

MHC class I tetramer staining has proven to be a powerful method for obtaining quantitative and phenotypic information about antigen-specific CD8+ T cells.
Advantages of this method are its rapidity, sensitivity, and specificity. However, tetramer staining also has obvious limitations; these include the need to acquire a large number (50–100,000) of events to quantify low-frequency populations, and the reliance on a limited number of predetermined epitopes. Cytokine flow cytometry, like tetramer staining, requires a relatively large number of cells (typically 3–500,000 per antigen) but yields both functional and phenotypic information about cytokine-producing cells. Finally, the ELISpot assay provides quantification of cytokine production and requires a relatively low number of cells (1–200,000 per antigen) to test individual peptides.

Despite the low number of cells required for ELISpot, we found that not all biopsy specimens contained sufficient MMC to test responses to 53 pools of HIV-1-derived peptides spanning the entire genome. To circumvent this problem, as well as the high spontaneous IFN-γ secretion observed in freshly isolated MMC, we polyclonally expanded CD8+ T cells using a CD3:CD4 bispecific antibody, which has been shown to stimulate CD3+/CD8+ T cell proliferation while suppressing growth of CD3+/CD4+ T cells. Given the relatively small numbers of starting CD8+ T cells, we found this to be the most economical method of expansion, avoiding the loss in yield associated with positive or negative immunoselection strategies. Although polyclonal expansion might be predicted to introduce bias through nonuniform proliferation of individual T cell clones, our findings suggested a good correlation between HIV-specific responses in fresh and expanded mononuclear cells. This method therefore provides a reasonable qualitative, and perhaps semi-quantitative readout of CD8+ T cell responses in the mucosal compartment. It should be noted that due to changes in subset composition and frequency, polyclonally expanded cells cannot be used to determine the frequency of epitope-specific cells in a population. In addition, phenotypic information derived from expanded cell populations should be interpreted with some caution.

Another major technical challenge was presented by the extremely high concentrations of bowel microbial flora on mucosal surfaces. Despite vigorous washing of isolated cells and culture in penicillin—streptomycin—amphotericin B, culture contamination rates still exceeded 50%. We successfully addressed this problem by manipulating the tissue culture medium antibiotics to target the bowel flora. We utilized the combination penicillin derivative/beta-lactamase inhibitor, piperacillin—tazobactam. This antibiotic is widely used in clinical practice to treat intra-abdominal infections due to its reliable activity against bowel flora that include enterococci, aerobic gram-negative, and anaerobic gram-negative bacteria. This property, in conjunction with stability comparable to that of penicillin, made this agent an ideal substitute for penicillin—streptomycin.

The methods described in this report will provide the capability of assessing responses to HIV and other pathogens during natural infection and following experimental vaccination. Rectal biopsy tissue can be a particularly useful source of tissue for studies of mucosal immunity. Unlike biopsy procedures involving other mucosal sites, in particular the cervix and tonsil, sigmoidoscopic biopsy of the rectosigmoid colon is well tolerated and nearly painless, with a low incidence of adverse events and high compliance (>97%) (Anton et al., 2000, 2003). The procedure is rapid and may be performed without anesthesia, with patients experiencing only a mild pinching sensation. Bleeding is generally minimal and subsides within hours of the procedure, and can be repeatedly every 2 weeks (Mitsuyasu et al., 2000). As previously described, mucosal biopsy tissue may also be used to provide reliable quantification of tissue viral load (Anton et al., 2001).

Successful immunization against mucosally acquired pathogens will likely require the induction of strong antigen-specific responses at mucosal surfaces (Miller and McGhee, 1996). Although strategies to induce mucosal antibodies and antigen-specific T cells have been assessed in rodents and nonhuman primates (Lehner et al., 1996; Belyakov et al., 1998a, 2001), comparable studies in humans are lacking. The methods we have outlined in this report should facilitate the evaluation of candidate HIV vaccines in mucosal tissues and contribute to our understanding of the role of mucosal effector cells in antiviral immunity during natural infection.

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