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Direct Ex Vivo Simian Immunodeficiency Virus (SIV)-Specific Cytotoxic Activity Detected from Small Intestine Intraepithelial Lymphocytes of SIV-Infected Macaques at an Advanced Stage of Infection

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Human immunodeficiency virus (HIV) induces a profound disorganization of the lymphoid tissues with marked abnormalities of the immune system at the terminal stage of infection. Since the digestive mucosal immune system is by far the largest lymphoid organ of the body, we attempted to evaluate its functional activity in advanced stages of simian immunodeficiency virus (SIV) infection in the SIV-macaque model of HIV infection. Two chronically intravenously SIV-infected macaques, including one at the AIDS stage, were studied. Intestinal intraepithelial lymphocytes (IEL) were isolated, analyzed, and compared to lymphocytes obtained from blood, spleen, and different lymph nodes: IEL were predominantly $CD8^+$ T lymphocytes expressing the αEβ7 integrin and lacking the CD28 coactivatory molecule. A direct ex vivo SIV-specific cytotoxic activity was prominently found in the IEL of both macaques and was weaker or absent in the other sites. To our knowledge, this is the first report of SIV-specific cytotoxic activity from small intestine IEL in SIV-infected macaques. Considering the high similitude of the SIV-macaque model with the HIV infection in humans, these results may be highly important for the pathogenesis of HIV infection and more generally important for the characterization and function of digestive $CD8^+$ IEL population.

Human immunodeficiency virus (HIV) infection induces a profound depletion of peripheral blood $CD4^+$ lymphocytes resulting in AIDS. Over the past few years, HIV infection has been more and more identified as a disease of the lymphoid system, particularly of the lymph nodes (LN) and spleen, in which many pathological changes are observed, from follicular hyperplasia to profound lymphoid depletion with marked terminal disorganization of the lymphoid structure (29, 35). The human mucosal system is by far the largest lymphoid organ of the body (26). In particular, the gut-associated lymphoid tissue is thought to contain 50% of the body's lymphoid mass. Therefore, its involvement during the course of HIV infection is obviously important to determine.

Studies concerning this issue are difficult to perform in humans, and the macaque model of infection with the related simian immunodeficiency virus (SIV) offers an invaluable opportunity to address this question. The SIV-macaque model has long proved to be a very accurate model of human infection by HIV in many respects (21). In particular, it has been shown to be an excellent model of mucosal infection (24). Mucosal transmission remains the predominant route of contamination, although the precise mechanisms of crossing the epithelial barrier are not yet fully understood. A recent study has shed some light on vaginal transmission of SIV in macaques involving dendritic cells as the principal cellular targets of infection and has suggested a regional dissemination of the virus mediated by draining lymphatic ducts (33). After intravenous (i.v.) infection with SIV, several studies have reported the presence of infected macrophages and $CD4^+$ lymphocytes in the digestive mucosa at very early stages of infection and its persistence throughout the course of infection (18, 19). Few studies have evaluated the virus-specific immune response of mucosal lymphocytes in SIV-infected macaques. Recently, Lohman et al. (22) have reported the presence and frequencies of *env*- and *gag*-specific precursors of cytotoxic T lymphocytes (CTLp) within vaginal intraepithelial lymphocytes (IEL) of vaginally infected macaques. Here, we report the presence of SIV-specific direct ex vivo cytotoxic activity in small intestine IEL of two i.v. infected macaques who had been infected for over 2 years by SIV.

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MATERIALS AND METHODS

Animals. Rhesus macaques (*Macaca mulatta*) were housed at the P3 facility of the Commissariat à l'Energie Atomique (Fontenay, France) in accordance with European Economic Community guidelines. Two macaques were infected experimentally with $SIV_{mac}251$. This viral stock, originally obtained from R. Desrosiers, was a generous gift of A.-M. Aubertin (Strasbourg, France). Infection was performed in December 1993 by i.v. inoculation of 10 50% animal infectious doses. Uninfected macaques served as controls.

Peripheral and mucosa-associated tissues. On the day of autopsy, all tissues (spleen, axillary and mesenteric LN, and small and large intestines) and blood were removed under sterile conditions. Individual tissues were transferred to tubes containing Hanks balanced salt solution (Gibco, Cergy-Pontoise, France) supplemented with 10% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 80 μg of gentamicin per ml, 1% glutamine, and 10 mM HEPES buffer (Gibco). This will be further referred to as complete medium. All materials were cooled on ice and processed in our laboratory within 2 h after collection of tissues.

Lymphocyte preparations. Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation of heparinized blood on a Ficoll-Hypaque gradient.

Spleen and LN lymphocytes were obtained by mechanical disruption on sterile nylon mesh and centrifugation on Ficoll-Hypaque. Lymphocytes from the small intestine were isolated by a mechanical and enzymatic dissociation method slightly modified from a previously described procedure (3). Briefly, approximately 20 cm of small intestine was washed extensively in complete medium and then dissected into small fragments in petri dishes. These fragments were incubated in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution with 1 mM dithiothreitol (Sigma, St. Louis, Mo.) for 20 min in an Erlenmeyer flask on an orbital shaker at room temperature and then vortexed for 3 min. After a short sedimentation ($1 \times g$), supernatant was collected and kept on ice. This step was repeated three times. Cells released into these supernatants (epithelial cells and IEL) were washed, resuspended in a 44% Percoll solution (Pharmacia, Paris, France), and carefully layered on top of a 67% Percoll layer in order to form a discontinuous gradient. After centrifugation at room temperature for 20 min at $500 \times g$, the lymphocyte layer at the Percoll interface was collected, washed, and used for further assays. Fragments remaining in the sediment were rinsed twice in RPMI 1640 (Gibco) and then incubated in an Erlenmeyer flask on an orbital shaker at 37°C for 1 h in the same medium containing 10% fetal calf serum, 90 IU of collagenase (Sigma) per ml, and 0.02% DNase (Sigma). The supernatant was then filtered through a 100-μm sterile nylon mesh, and the cells were washed in RPMI 1640. Lamina propria lymphocytes (LPL) were then separated by Ficoll-Hypaque gradient centrifugation, collected from the interface, and washed and resuspended in phosphate-buffered saline or culture medium as appropriate. By these dissociation procedures, greater than 95% of the cells obtained from the intestine were viable as determined by trypan blue exclusion.

Histology. Tissue pieces were fixed in 4% formalin for 24 h and embedded in paraffin. Sections of 5 μm were stained with eosin-hemalun or periodic acid-Schiff stain and mounted with Eukitt.

Phenotypic analysis. Murine anti-human monoclonal antibodies (MAb) known to cross-react with simian antigens were used for phenotypic analysis. Cells isolated from all different tissues were incubated with MAb for 30 min at 4°C, then washed in phosphate-buffered saline, and fixed with 4% formaldehyde. Antibodies utilized were either fluorescein isothiocyanate or phycoerythrin conjugated. The following MAb were used: anti-CD2 (T11; Coulter, Margency, France), anti-CD20 (B1; Coulter), anti-CD4 (OKT4A; Ortho Diagnostic, Roissy, France), anti-CD8 (Leu2a; Becton Dickinson, Le Pont-de-Claix, France), and anti-CD28 (Leu28; Becton Dickinson). The HML-1 MAb, recognizing the αEβ7 integrin/CD103, was a generous gift of N. Cerf-Bensussan. The 525 MAb, a gift from A. Le Bivic, recognizes a basolateral protein on epithelial cells. Cells were incubated first with either of these two unconjugated MAb and then with fluorescein isothiocyanate-conjugated goat anti-mouse antibody (Pasteur Diagnostic, Paris, France) for 30 min at 4°C. Two-color analysis was performed for CD4/CD8 and CD8/CD28 staining. The percentage of positive cells was determined on an Epics Elite flow cytometer (Coulter). Isotype-matched irrelevant antibodies (Coulter) were used as controls.

Cytotoxicity assays. Target cells were autologous B-lymphoblastoid cell lines (B-LCL), obtained by transforming PBMC with herpesvirus papio (produced by the S594 cell line). These B-LCL were infected with different recombinant vaccinia viruses containing sequences for the SIV genes *env*, *gag*, and *nef* (Transgène, Strasbourg, France). The wild-type vaccinia virus (Copenhagen strain) served as a control. Infection was carried out for 18 h with the recombinant viruses at a multiplicity of infection of 20 PFU per cell. The targets were then washed and labelled with 100 μCi of $Na_2{}^{51}CrO_4$ (Amersham, Buckinghamshire, United Kingdom) for 1 h. Direct cytolytic activity was measured in V-bottom 96-well microtiter plates by mixing 5×10^3 ^{51}Cr-labelled target cells with effector cells at various ratios in a final volume of 200 μl/well. All experimental points were performed in duplicate. Plates were incubated for 4 h at 37°C, and 100 μl of supernatant was harvested and analyzed in a gamma counter. Spontaneous release was determined after incubation of target cells with medium alone and never exceeded 20% of the total ^{51}Cr incorporation. Results were expressed as specific chromium release: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

RESULTS AND DISCUSSION

The two SIV-infected animals were sacrificed 28 months postinfection. Macaque 86757 was still asymptomatic, and no pathologic changes were found, but the blood CD4 cell count was reduced (417 versus 923/μl before infection), reflecting a chronic stage of infection. In contrast, macaque 40504 showed signs of advanced disease with a major wasting syndrome (40% weight loss) and dramatically low blood cell counts: absolute numbers of circulating CD4 and CD8 cells were 43 and 390/μl, respectively. Histologic evaluation of this macaque revealed lymphoid follicular hyperplasia in LN and spleen and marked acute colitis with infiltration of erythrocytes and polymorphonuclear leukocytes within the lamina propria. Conversely, the small intestine was normal.

TABLE 1. Phenotypes of T lymphocytes recovered from different tissues

Macaque(s)	Tissue	% of positive lymphocytes			CD8/CD28[a]
		CD2	CD4	CD8	
86757	Blood	76	24	40	ND
	PLN[b]	83	29	52	44
	MLN[c]	85	36	51	40
40504	Blood	65	6	55	24
	Spleen	40	6	36	6
	PLN	42	17	28	26
	MLN	54	11	44	29
Uninfected[d]	Blood	89 ± 9	34 ± 8	48 ± 4	55 ± 6
	PLN	74 ± 4	33 ± 8	32 ± 6	86 ± 7
	MLN	82 ± 7	37 ± 7	25 ± 1	87 ± 6

[a] Data are expressed as percentages of $CD28^+$ cells within the $CD8^+$ cell population. ND, not done.
[b] PLN, peripheral LN.
[c] MLN, mesenteric LN.
[d] Data are expressed as mean ± SD of phenotypes observed on six uninfected healthy macaques.

Table 1 displays the phenotypes of T-cell subpopulations in different tissues. The decreased CD4/CD8 ratios observed in blood and all tissues for both macaques accounted for the long duration of their SIV infection, especially for macaque 40504. Recently, several reports underlined the importance of the $CD8^+$ $CD28^-$ population in HIV-infected individuals (10, 11). In this regard, we investigated the expression of the CD28 molecule. This antigen is known to be an accessory molecule involved in transducing a correct activation signal and to interact with the B7.1/B7.2 molecule expressed by antigen-presenting cells. In PBMC from uninfected macaques, we observed that around 50% of $CD8^+$ cells were $CD28^+$, as in humans. In healthy macaques, we found a higher proportion of $CD8^+$ $CD28^+$ cells in LN than in blood. In both infected macaques, the percentages of $CD8^+$ $CD28^+$ cells were markedly decreased in blood and LN. The low proportion of these cells is likely due to an increase in $CD8^+$ $CD28^-$ cells as reported in HIV-infected individuals (32).

Phenotypic analysis of IEL from the small intestine, shown in Fig. 1, was performed on a gate targeted on mucosal lym-

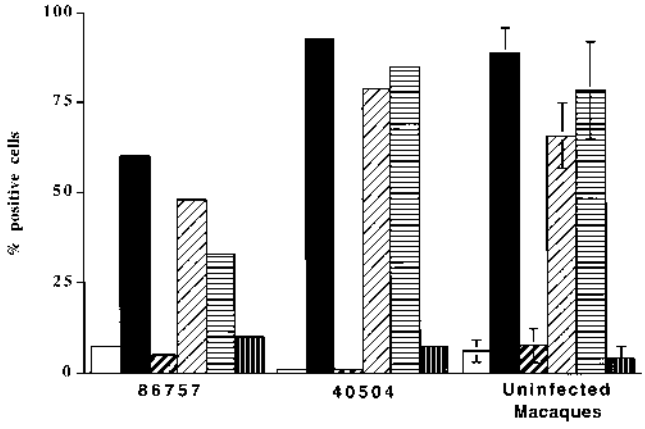

FIG. 1. Phenotypic analysis of IEL. Expression of CD20 (□), CD2 (■), CD4 (▨), CD8 (▨), CD103 (▤), and CD28 (▥) on IEL extracted from the small intestine of macaques 86757 and 40504 or uninfected macaques (mean ± SD for six animals). Data are expressed as percentages of positive lymphocytes except for $CD28^+$, where data represent the $CD28^+$ lymphocytes within the $CD8^+$ population.

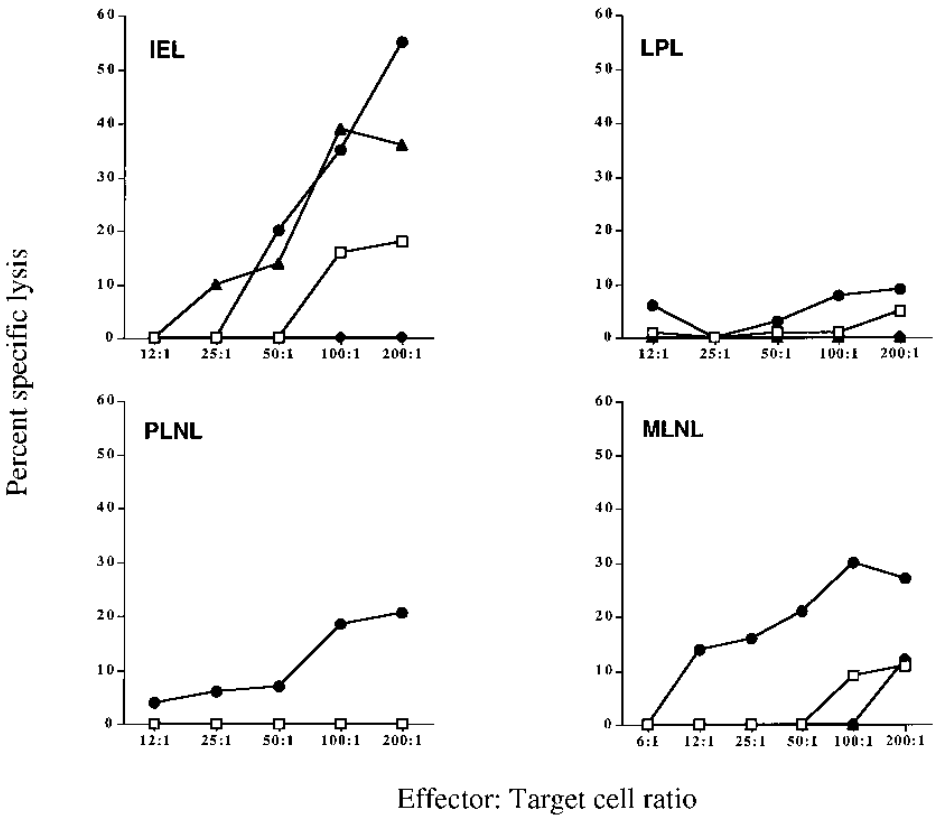

FIG. 2. Ex vivo cytotoxicity of lymphocytes of macaque 86757 from different sites. Target cells were autologous B-LCL infected with *env* (◆), *gag* (●), or *nef* (▲) recombinant vaccinia viruses. The control was autologous B-LCL infected with wild-type vaccinia virus (□). Mean values for ^{51}Cr release from target cells are expressed as percent specific lysis, as described in Materials and Methods. Each graph represents the data observed with a particular effector cell type (PLNL, peripheral LN lymphocytes; MLNL, mesenteric LN lymphocytes). Effector/target cell ratios are shown on the *x* axis.

phocytes taking into account the slightly larger size of these cells compared to PBMC. Lymphocytes represented 50 to 60% of cells recovered with the Percoll gradient in both macaques. The remaining cells were epithelial cells as determined by staining with a MAb recognizing a basolateral protein on epithelial cells. The very low proportion of $CD20^+$ cells (<10%) within the lymphocyte population indicates that contamination of IEL by lymphoid follicle lymphocytes was insignificant. As already described (7), the large majority of IEL belonged to the T-cell phenotype, and most of these were $CD8^+$ cells.

The majority of cells within the LPL population were also $CD2^+$ cells (70 and 92% in macaques 86757 and 40504, respectively). In contrast to humans, where LPL are predominantly $CD4^+$ (20), LPL T lymphocytes from healthy macaques usually contain lower percentages of $CD4^+$ cells (25% ± 10%; mean ± standard deviation [SD]), reflecting what is observed in normal simian blood (data not shown). In these two macaques, we observed a marked decrease in $CD4^+$ LPL (8 and 1% in macaques 86757 and 40504, respectively), confirming the general decrease in $CD4^+$ cells.

Expression of αEβ7 is particularly high on mucosal T cells. In humans, studies have shown that this integrin is expressed on 80 to 90% of IEL and on 30 to 40% of LPL (8). We observed a similar distribution in healthy macaques (79% ± 13% on IEL, as shown in Fig. 1; 45% ± 5% on LPL). The role of this molecule is not entirely understood. It has been described as a ligand of the E-cadherin expressed by epithelial cells (6) and is therefore thought to be important in the anchoring of T cells to epithelial cells. In the two SIV-infected macaques, the expression of this molecule on IEL was close to what is observed in healthy macaques, as shown in Fig. 1. Its expression on LPL was normal in macaque 86757 (30%), but an abnormally high percentage of LPL expressed it in macaque 40504 (95%). In all cases, αEβ7 expression on PBMC, LN, and spleen cells was very low (<5%), similar to what we have found in healthy macaques.

Interestingly, the vast majority of $CD8^+$ IEL did not express CD28 either in uninfected (5 to 10%) or in infected macaques (Fig. 1). This low percentage of $CD8^+$ $CD28^-$ cells was comparable to that observed in the LPL fraction in the infected macaques as well as in the uninfected macaques. To our knowledge, there are no reported data on the expression of CD28 on human mucosal $CD8^+$ cells. In mice, some authors found that a majority of $CD8^+$ cells of the small intestine are $CD28^-$ (13), while others found that the expression of CD28 is dependent on the αβ or αα structure of the CD8 molecule expressed (27). In the lung, another mucosal site, a low proportion of $CD8^+$ $CD28^+$ cells has already been described, with even lower values in HIV infection (32).

Direct ex vivo (i.e., without any in vitro stimulation) SIV-specific cytotoxic activities were found in IEL of macaque 86757 (Fig. 2). These activities were directed against *gag*- and *nef*-expressing autologous target cells, while no activity was observed against the *env* gene products. It is noteworthy that these activities were quite high for cytotoxic assays performed with ex vivo unstimulated cells. Conversely, no activity was observed within the LPL population. Cytotoxic activity di-

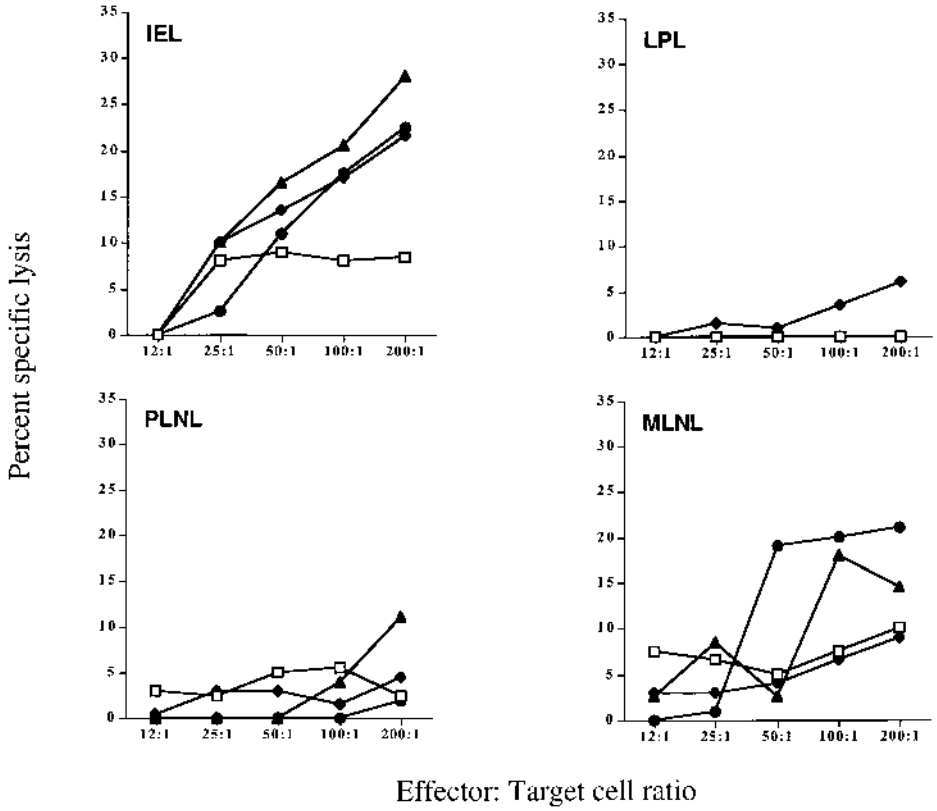

FIG. 3. Ex vivo cytotoxicity of lymphocytes of macaque 40504 from different sites. Target cells were autologous B-LCL infected with *env* (◆), *gag* (●), or *nef* (▲) recombinant vaccinia viruses. The control was autologous B-LCL infected with wild-type vaccinia virus (□). Mean values for ^{51}Cr release from target cells are expressed as percent specific lysis, as described in Materials and Methods. Each graph represents the data observed with a particular effector cell type (PLNL, peripheral LN lymphocytes; MLNL, mesenteric LN lymphocytes). Effector/target cell ratios are shown on the *x* axis.

rected against Gag was also observed in axillary and mesenteric LN, although at lower levels of specific lysis.

More surprisingly, an almost similar profile was observed in macaque 40504 (Fig. 3), who presented a very advanced stage of the disease. In this macaque, IEL expressed significant specific cytotoxicity against *env*-, *gag*-, and *nef*-expressing targets. As for macaque 86757, no cytotoxic activity was found within the LPL population. A *gag*-specific cytotoxic activity was detected in the draining mesenteric LN, contrasting with a lack of detectable activity in axillary LN and spleen. This particular profile was largely unexpected in this latter macaque, since spontaneous ex vivo cytotoxic activity, which is rarely found in viral infections but commonly described in HIV infection (38), is known to disappear with evolution and is normally not observed at an advanced stage of the disease (28).

We report here the first description of an ex vivo cytotoxic antiviral activity from IEL of SIV-infected macaques. Lohman et al. have recently described the presence of T-cell receptor $\alpha\beta^+$ ($TCR\alpha\beta^+$) CTLp in the vaginal epithelium of locally infected macaques (22). This cytotoxic activity was found after in vitro polyclonal activation in limiting-dilution analysis. Similar to our findings, they did not observe any activity from vaginal LPL. In addition, it is interesting that they found a higher frequency of CTLp in chronically infected macaques than in primary infection.

The exact nature of the effector cells displaying the cytotoxic activity remains to be determined. The high proportion of $CD8^+$ $CD28^-$ cells suggests that these cells are responsible for the cytotoxic activity. Indeed, we have shown that this subpopulation is responsible for the spontaneous cytotoxic activity in unstimulated PBMC of HIV-infected individuals (11). It would be interesting to further define the nature of the effector cells, in particular the $\alpha\beta$ or $\alpha\alpha$ structure of the CD8 molecule of the cells involved in this activity. Our anti-CD8 MAb recognizes the α chain, and unfortunately, we have not found a MAb cross-reacting with the β chain of the macaque CD8 antigen. It is worthwhile noting, however, that human CD8 IEL are more prominently $\alpha\beta$ than murine IEL (7). We have not determined the $\alpha\beta$ or $\gamma\delta$ structure of the TCR presented by cytolytic cells. It has to be noted that more than 80% of human IEL express the $TCR\alpha\beta$ (7). It will also be interesting to define the precise mechanisms involved in this cytotoxic activity, i.e., mediated by perforin-granzyme or Fas (APO1/CD95)-FasL. Both mechanisms have been described for murine IEL, depending on the activation status and/or the stimuli used (12).

Cytotoxic activities were evaluated only on autologous target cells, but preliminary results in an acutely infected macaque suggest that allogeneic targets are not lysed. Finally, NK cells are unlikely to be responsible for the observed lysis, since (i) the K562 cell line was not lysed in these experiments, although it may not be susceptible to mucosal NK activity (the DLD-1 cell line might be a more relevant target); (ii) NK activity is usually absent in advanced stage of infection; (iii) $CD16^+$ cells are not found in the intestinal mucosa of chronically infected macaques (10a); and (iv) cytotoxic activities were observed only on targets expressing SIV gene products, suggesting antigenic specificity.

The mechanisms leading to the presence of activated cytotoxic IEL within the epithelium are a major issue, especially in late stages after i.v. infection. The distribution of lymphocytes is dependent on the presence or absence of homing or adhesion molecules that are diversely expressed according to their naive or memory status and to the site of lymphocyte induction and activation (4). Upon activation, peripheral lymphocytes lose the CD62/L-selectin usually responsible for their homing to peripheral (axillary) LN (PLN) (4). In addition, they usually express $\alpha4\beta1$, which induces attachment to VCAM-1 expressed by inflamed endothelium (5). Antigen-induced activation increases the adhesion of $\alpha4\beta7$ to MAd-CAM-1 present on high endothelium veinules (2). This phenomenon induces a homing of peripheral activated cytotoxic cells to the lamina propria and may facilitate their passage to the epithelium. Similar mechanisms may be responsible for the infiltration of $CD8^+$ cytotoxic lymphocytes within the lung frequently observed in HIV-infected individuals (31). The mucosal integrin $\alpha E\beta7$ is not described as a homing molecule (17) and is not detected on peripheral lymphocytes. However, its expression can be detected on PBMC after polyclonal activation partially through the effect of transforming growth factor β (30). Moreover, the SIV PBj14 isolate has been shown to increase $\alpha E\beta7$ expression on PBMC (15). It is noteworthy that this isolate is responsible for a polyclonal activation of $V\beta7$ and $V\beta14$ T lymphocytes, probably through a superantigen effect, and induces a major lymphocytic infiltration of the digestive tract responsible for an acute lethal diarrhea (9). It would be interesting to evaluate the activity of IEL in this acute model of infection. Finally, we have noticed that up to 60% of cells in SIV-specific cytotoxic T-lymphocyte cell lines derived from PBMC of infected macaques and stimulated in vitro by SIV antigens express $\alpha E\beta7$. These findings suggest that $\alpha E\beta7$ has a role other than just anchoring IEL within the epithelium.

The other issue concerns the potential role of these cytotoxic cells within the epithelium since the macaques were infected by the i.v. route. HIV-infected cells have been found in mucosal sites of men sexually infected by HIV, the virus being present in about 50% of biopsy specimens from throughout the digestive tract (14). Moreover, in macaques infected i.v. by SIV, it has been shown that the virus was recovered within the digestive mucosa throughout infection and massively at early and late time points (18). In this case, the virus was found in macrophages and $CD4^+$ cells within the lamina propria. It has to be noticed that activated macrophages express the $\alpha4\beta7$ integrin (36), which may explain the homing and the presence of infected macrophages in mucosal sites after i.v. infection. In this context, it was unexpected that LPL did not show any cytotoxic activity. However we cannot exclude the possibility that the collagenase treatment has a deleterious effect on the expression of surface components implicated in cytotoxicity, thus preventing the detection of such activity within this LPL fraction. Conversely, although data indicate that epithelial cells might be infected in vitro (1), there is no current evidence for infection of these cells in vivo. In this regard, it is peculiar to detect anti-SIV effector cytotoxic cells predominantly in the IEL subset, since there is no evidence of infected cells within the epithelium. However, it is possible that virus-specific cytotoxic cells migrate at potential sites of infection such as the epithelium in order to protect against putative new viral infection. Such a distribution may also explain the high frequency of HIV-specific cytotoxic T-lymphocytes observed in the lung (31). In this context, it has been shown that activated peripheral lymphoblasts rapidly migrate to mucosal sites (16).

Alternatively, if IEL indeed play no role in the clearance of virus-infected cells, the epithelium may represent a site of accumulation of chronically activated cells (34). The $CD28^-$ phenotype suggests that these cells have reached the terminal differentiation state defined as replicative senescence (25). Cell death may occur through activation-induced cell apoptosis, and in addition, anchoring of IEL to epithelial cells through $\alpha E\beta7$ may contribute to the elimination of such activated cells within the process of epithelium regeneration.

Conversely, the persistence of these cells in late stages of infection by failure of apoptosis-induced mechanisms could also contribute to the impairment of local and general normal lymphocyte homeostasis, which is known to be altered in HIV infection (23).

Finally, whatever their role is in the defense against infection, these cytotoxic cells may also be involved in immunopathologic mechanisms and contribute to the enteropathies not related to opportunistic infections described in HIV-infected individuals and SIV-infected macaques (18, 37). These mechanisms may be, at least in part, mediated by cytokine production, in particular gamma interferon or tumor necrosis factor alpha produced by cytotoxic cells.

Further studies on a larger number of macaques are needed to determine the function of these cytotoxic IEL and their relevance in this site at that stage of infection. In particular, we need to determine whether they are beneficial or deleterious. These studies need also to be performed on rectally infected macaques. The possibilities of easily studying mucosal tissues in the SIV macaque model at various stages of infection should greatly contribute to a better understanding of these important issues.

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