A CYTOTOXIC T LYMPHOCYTE INHIBITS ACQUIRED IMMUNODEFICIENCY SYNDROME VIRUS REPLICATION IN PERIPHERAL BLOOD LYMPHOCYTES

By HIROSHI TSUBOTA, CAROL I. LORD, DAVID I. WATKINS, CHIKAO MORIMOTO* and NORMAN L. LETVIN

*From the Harvard Medical School, New England Regional Primate Research Center, Southborough, Massachusetts 01772; and the *Dana-Farber Cancer Institute, Boston, Massachusetts 02115*

The cellular immune response to the AIDS virus remains poorly understood. The cell-mediated immune response to virus infection usually involves the generation of antigen-specific $CD8^+$ lymphocyte populations. One subset of these $CD8^+$ cells, the CTL, lyses virus-infected MHC class I-matched target cells (1) using the CD8 molecule (2). Studies have indicated that a $CD3^+CD8^+$ cell in PBL of HIV-1-infected individuals exists that lyses HIV-1 antigen expressing target cells in an MHC class I-restricted fashion (3–5). What role this cell actually plays in controlling an HIV-1 infection is unclear. $CD8^+$ lymphocytes have also been shown to be capable of blocking replication of HIV-1 in PBL of infected individuals (6). These cells could clearly be important in containing the replication of HIV-1 in the infected individual. The functional subset of lymphocytes to which this virus inhibitory cell population belongs and its relationship to CTL have not been defined.

The simian immunodeficiency virus of macaques (SIV_{mac})[1] is homologous in its nucleotide sequence to HIV-1 and HIV-2 (7, 8) and induces AIDS in rhesus monkeys (9). The remarkable similarities in the in vitro and in vivo biological properties of HIV-1 and SIV_{mac} make the rhesus monkey/SIV_{mac} system an important animal model for the study of AIDS (10–13). In developing the rhesus monkey/SIV_{mac} system as a model for AIDS vaccine assessment, we have been studying the role of the $CD8^+$ lymphocyte of the rhesus monkey in the immune response to SIV_{mac}. We have shown that a $CD8^+$ lymphocyte can block SIV_{mac} replication in PBL of infected monkeys (14).

In the present experiments, we have performed parallel studies of the cell-mediated immune response to both SIV_{mac} and HIV-1, characterizing the $CD8^+$ cell that inhibits SIV_{mac} and HIV-1 replication in PBL. These experiments suggest that the cell that inhibits AIDS virus (by this we mean both SIV_{mac} and HIV-1) replication is a CTL.

This work was supported by National Institutes of Health grants AI-20729 and CA-34979, and by Division of Research Resources grant RR-00168. N. L. Letvin is a recipient of an American Cancer Society Faculty Research Award.

[1] *Abbreviations used in this paper:* 1-D IEF, one-dimensional isoelectric focusing; RT, reverse transcriptase; SIV_{mac}, simian immunodeficiency virus of macaques.

Materials and Methods

Lymphocyte Preparation and Cell Cultures. Heparinized blood samples were obtained from rhesus monkeys (*Macaca mulatta*) that had been experimentally infected with SIV_{mac} isolate 251 and clinically healthy HIV-1-infected human volunteers. PBL were isolated from the blood by Ficoll-diatrizoate density gradient centrifugation and activated with 10 μg/ml Con A (Difco Laboratories, Detroit, MI) for 3 d. These cells were then maintained in RPMI 1640 medium supplemented with 10% FCS (Flow Laboratories, McClean, VA), L-glutamine (2 mM), penicillin (50 U/ml), gentamycin (50 μg/ml), and 2 U/ml rIL-2 (provided by E. I. DuPont de Nemours & Co., Wilmington, DE). Cell concentration was regularly adjusted to 1.5 × 10^6/ml.

$CD8^+$ Lymphocyte Depletion. $CD8^+$ cells were depleted from Con A–activated PBL by the panning method. PBL were incubated at a concentration of 1.5 × 10^7/ml for 40 min at 4°C with anti-CD8 mAb (7PT3F9; provided by S. Schlossman, Dana-Farber Cancer Institute, Boston, MA) in ascites form at a dilution of 1:125. The cells were then washed twice with PBS and resuspended in PBS at a concentration of 4 × 10^6 cells/ml. 3 ml of this cell suspension were plated on a 10-cm plastic petri dish coated with 10 μg rabbit anti-mouse Ig (Dako Corp., Santa Barbara, CA) and preincubated with 10 ml of PBS supplemented with 2% FCS (Flow Laboratories). Cells were incubated on these dishes for 70 min at 4°C. The dishes were then swirled and cells in the supernatant were harvested. These cells were used as a $CD8^+$ cell-depleted population. Cells adherent to the dishes were recovered and used as $CD8^+$ lymphocyte-enriched populations.

Anti-CD4 Blocking of Virus Replication in PBL of SIV_{mac} and HIV-1-infected Individuals. $CD8^+$ cell-depleted lymphocytes were prepared from Con A-activated PBL by the panning method. Aliquots of these cells were incubated at various times during the period of culture with a 1:100 dilution of ascites of anti-CD4 mAb (19Thy5D7, S. Schlossman) for 30 min at room temperature at a concentration of 2 × 10^6/ml. Cells were maintained in RPMI 1640 medium supplemented with 10% FCS and IL-2. The anti-CD4 mAb ascites was added to the cultures after this incubation at a final concentration of 1:400 for defined periods of time.

Preparation of $CD8^+2H4^+$ and $CD8^+2H4^-$ Cell Subpopulations. $CD4^+$ cells were eliminated from Con A-activated PBL by the technique of complement-dependent lysis. PBL at a concentration of 10^7/ml were incubated with a 1:200 dilution of anti-CD4 mAb (19Thy5D7) ascites for 30 min at room temperature. Rabbit complement (Pel-Freeze Biologicals, Brown Deer, WI) at a final concentration of 1:8 and 2.5 μg/ml of DNase (Sigma Chemical Co., St. Louis, MO) were then added to the cell suspension. Cells were then incubated on a shaker for 30 min at 37°C. After a total incubation of 1 h, cells were pelleted by centrifugation and supernatant was discarded. The same procedure was repeated two more times and the cells were then washed three times with PBS. The resulting $CD4^+$ cell-depleted PBLs (<3% $CD4^+$ and >90% $CD8^+$ cells as determined by flow cytometric analysis) were separated into $2H4^+$ and $2H4^-$ cell fractions by the panning method using the anti-2H4 mAb (C. Morimoto).

Depletion of NK Cells from PBL. Complement-dependent lysis was performed to deplete NK cells from Con A–activated PBL. Anti-$NKH1_A$ (provided by J. Ritz, Dana-Farber Cancer Institute) and anti-Leu11b (anti-CD16; Becton-Dickinson & Co., Mountain View, CA) were used for these treatments. An irrelevant mAb (5E5C) and an anti-CD8 mAb (7PT3F9) were also used to prepare control cell populations.

Functional Blocking Experiments with mAbs. Anti-CD8 (21Thy2D3, 7PT4F12, 7PT3F9, 1mono2E7, S. Schlossman; 6F7, 6B7, C. Morimoto; MT122, P. Rieber, University of Munich, Munich, FRG); anti-CD2 (3PT2H9, S. Schlossman); anti-CD11a (2F12, J. Ritz); anti-CD11b (904, S. Schlossman); and an irrelevant mAb (5E5C) were used. Con A–activated PBL were incubated with a 1:100 dilution of ascites of each mAb for 30 min at room temperature at a concentration of 10^7/ml, then maintained in the continuous presence of the same antibody at a dilution of 1:400. Reverse transcriptase (RT) activity of the supernatants was regularly monitored.

Flow Cytometric Analysis. For the two-color analyses, 10^6 cells were incubated with the first mAb (a 1:400 dilution of ascites of anti-S6F1 [C. Morimoto], −2H4 or $-NKH1_A$) for 40 min at 4°C, then washed and incubated with the second antibody (FITC-conjugated goat-anti-mouse IgG [Tago Inc., Burlingame, CA] for anti-S6F1 and anti-2H4; FITC-

conjugated goat anti-mouse IgM [Tago Inc.] for anti-NKH1$_A$) for 40 min at 4°C. Cells were washed with PBS and then incubated with the third antibody (phycoerythrin [PE]-conjugated anti-CD4, OKT4-RD1; PE-conjugated anti-CD8, OKT8-RD1; Ortho Diagnostic Systems, Raritan, NJ; PE-conjugated-anti-4B4, Coulter Immunology, Hialeah, FL). After washing with PBS, cells were resuspended in PBS or 1.0% formalin-PBS (cells from infected individuals were resuspended in 1.0% formalin-PBS and left overnight to inactivate virus). Flow cytometric analyses were performed on an EPICS-CS (Coulter Electronics, Hialeah, FL). Cells were also first reacted with an irrelevant antibody to demonstrate the specificity of the staining shown with the other antibodies.

RT Assay. RT assays were performed as previously described (10).

One-Dimensional Isoelectric Focusing (1-D IEF) Analysis of MHC Class I Glycoproteins. 1-D IEF studies were performed on lectin-activated PBL of rhesus monkeys as previously described (15, 16). In brief, PBL were biosynthetically labeled with [^{35}S]methionine and lysed. Lysates were phase separated, precleared, and MHC class I molecules were immunoprecipitated with the mAb BB7.7 (17). Neuraminidase-treated samples were subjected to 1-D IEF; the pH gradient across the gel was approximately pH 5-7.

Results

CD8$^+$ Lymphocytes Inhibit SIV$_{mac}$ Replication in PBL of Infected Monkeys. We first sought to define the cell population in PBL of SIV$_{mac}$-infected rhesus monkeys which inhibits SIV$_{mac}$ replication. As shown in Fig. 1, Con A-activated PBL of SIV$_{mac}$-infected, clinically healthy monkeys maintained in culture in the presence of IL-2 do not generate RT activity. This indicates that virus replication is not occurring. However, after depletion of CD8$^+$ cells from those lymphocytes, viral replication is easily demonstrated. The addition of autologous CD8$^+$ cells to these CD8$^+$ lymphocyte-depleted PBL inhibits this virus replication. Thus, as described for HIV-1 by Walker et al. (6), SIV$_{mac}$ replication in PBL of rhesus monkeys is inhibited by autologous CD8$^+$ lymphocytes.

In Vitro AIDS Virus Replication in PBL of Infected Humans or Monkeys Is Perpetuated by Cell-to-Cell Spread of Virus. Virus replication in such CD8$^+$ lymphocyte-depleted PBL could result either from uninhibited replication in a large number of previously infected cells or an infection spreading to previously uninfected cells from a limited number of cells. Since anti-CD4 antibody should block the spread of infectious virus from one cell to another, inhibition of replication by addition of anti-

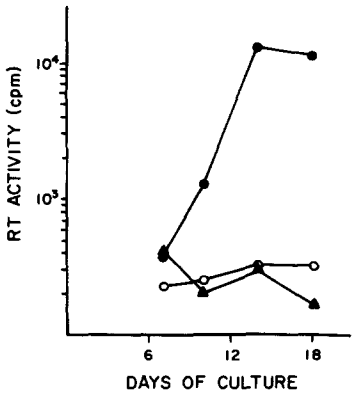

FIGURE 1. Autologous CD8$^+$ lymphocytes inhibit the generation of RT activity in vitro in CD8$^+$ lymphocyte-depleted PBL from SIV$_{mac}$-infected rhesus monkeys. After Con A activation, PBL were left unfractionated (○) or CD8$^+$ lymphocytes were depleted by the technique of panning (●) and cells were then maintained in IL-2-supplemented culture medium. 5×10^5 autologous CD8$^+$ lymphocytes were added back (▲) to 5×10^5 CD8$^+$ lymphocyte-depleted cells in a 2-ml volume. RT activity was regularly assessed in the culture medium as a measure of virus replication.

CD4 would indicate that infection is propagated under these culture conditions by spread from infected cells to uninfected cells. $CD8^+$ lymphocyte-depleted PBL from an SIV_{mac}-infected monkey were Con A activated and then maintained in IL-2-supplemented medium in the absence of mAbs or in the continuous presence of an irrelevant mAb, an anti-CD4 mAb or with the anti-CD4 mAb added on day 3 or day 10 of culture (Fig. 2 *A*). While SIV_{mac} replication occurred in the absence of mAb and in the presence of an irrelevant mAb, all detectable replication was inhibited when anti-CD4 antibody was added on day 3 of culture. Moreover, ongoing replication was blocked by the addition of anti-CD4 mAb to the cells on day 10 of culture. Similar results were seen in parallel studies of $CD8^+$ lymphocyte-depleted PBL of HIV-1-infected humans (Fig. 2 *B*). Not only did the addition of an anti-CD4 mAb on day 12 of culture block ongoing HIV-1 replication, but the removal of the anti-CD4 mAb on day 8 from the cultures continuously exposed to anti-CD4 antibody resulted in a delayed initiation of detectable HIV-1 replication in these PBL. Thus, in vitro replication of both SIV_{mac} and HIV-1 in $CD8^+$ lymphocyte-depleted PBL of infected individuals appears to reflect spread of infectious virus from a limited number of cells to the other IL-2-driven lymphocytes. The $CD8^+$ lymphocyte may, therefore, be capable of inhibiting the cell-to-cell spread of infectious virus.

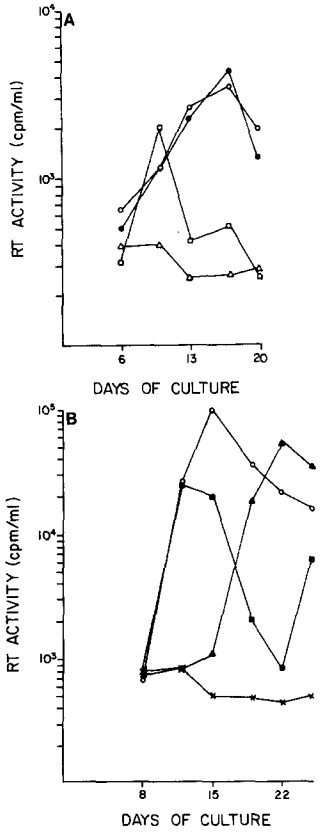

FIGURE 2. (*A*) Anti-CD4 antibody blocks virus replication in $CD8^+$ lymphocyte-depleted PBLs of an SIV_{mac}-infected monkey. (*B*) Anti-CD4 antibody blocks virus replication in $CD8^+$ lymphocyte-depleted PBL of an HIV-1-infected human. PBL were obtained from a SIV_{mac}-infected rhesus monkey (*A*) and HIV-1 infected human (*B*). $CD8^+$ cells were depleted by the panning method. (*A*) Monkey PBL were maintained in the absence of mAb (○), or reacted with and maintained in the presence of an irrelevant mAb during d3-d20 (●), anti-CD4 during days 3-20 (△), or anti-CD4 during days 10-13 (□). (*B*) Human PBLs were maintained in the absence of mAb (○), or reacted with and maintained in the presence of anti-CD4 during d3-d25 (**X**), during d3-d8 (▲), or during d12-d15 (■).

$CD8^+$ Inhibitory Lymphocytes Are Detectable only Weeks after SIV_{mac} Infection. We initially sought to determine whether the $CD8^+$ cells that inhibit AIDS virus replication in PBL of infected individuals have characteristics of antigen-specific lymphocytes. Detection of these cells in PBL of rhesus monkeys immediately after infection with SIV_{mac} would be consistent with their being NK cells. Their detection in monkey PBL only after a period of weeks after virus infection would suggest that they may undergo clonal expansion and might, therefore, be antigen specific. Four normal rhesus monkeys were experimentally inoculated with SIV_{mac} and their $CD8^+$ PBL were assessed for inhibition of SIV_{mac} replication. Such an inhibitory cell population was not detectable in PBL of any of the four animals 1 wk after infection (data not shown). As summarized in Table I, this $CD8^+$ cell population was detectable in one of the four animals at 13 d after infection, in three of the four animals at 30 d after infection and in all of the animals 60 and 90 d after infection. This observation suggests that a clonal expansion of the $CD8^+$ cell population capable of inhibiting viral replication may occur in the initial weeks after infection. Thus, these $CD8^+$ cells may be antigen specific rather than an NK cell population.

The $CD8^+$ Lymphocyte That Inhibits AIDS Virus Replication Is not a NK Cell. The possibility that the cell that inhibits AIDS virus replication in vitro might be a NK cell was then investigated. Virus replication was assessed in NK cell-depleted PBL from SIV_{mac}-infected, clinically healthy rhesus monkeys. These PBL were treated with anti-CD8, anti-NKH1, or anti-CD16 and complement and then maintained in IL-2-supplemented medium. As shown in Table II, SIV_{mac} replication was demonstrated in $CD8^+$ lymphocyte-depleted, but not in $NKH1^+$ or $CD16^+$ lymphocyte-depleted PBL. Similar results were observed in parallel studies of PBL from HIV-1-infected humans (Table III). These studies suggest that the $CD8^+$ cell that inhibits SIV_{mac} and HIV-1 replication in vitro does not express NK cell-associated surface antigens.

TABLE I

Time after Virus Inoculation of Generation of $CD8^+$ AIDS Virus Inhibitory Response

	Days after infection			
Animal	13	30	60	90
Mm129-86	$\frac{101{,}561}{60{,}870}$* (1.7)‡	$\frac{39{,}896}{19{,}091}$ (2.0)	$\frac{16{,}574}{3{,}959}$ (4.2)	$\frac{22{,}992}{2{,}860}$ (8.0)
Mm161-86	$\frac{45{,}970}{2{,}229}$ (20.6)	$\frac{35{,}922}{6{,}772}$ (5.3)	$\frac{14{,}344}{986}$ (14.6)	$\frac{14{,}592}{537}$ (27.2)
Mm179-86	$\frac{34{,}019}{13{,}060}$ (2.6)	$\frac{1{,}352}{255}$ (5.3)	$\frac{1{,}611}{148}$ (10.9)	$\frac{3{,}800}{149}$ (25.5)
Mm244-86	$\frac{6{,}173}{5{,}342}$ (1.2)	$\frac{7{,}154}{504}$ (14.2)	$\frac{6{,}123}{751}$ (8.2)	$\frac{3{,}956}{322}$ (12.3)

* $\frac{\text{Peak RT activity (cpm/ml) of } CD8^+ \text{ cell depleted culture supernatant}}{\text{Peak RT activity (cmp/ml) of unfractionated PBL culture supernatant}}$

‡ Ratio calculated as shown, with larger number indicating more pronounced $CD8^+$ cell inhibitory response.

TABLE II

Virus Replication in PBL of SIV_{mac}-infected Rhesus Monkeys after Elimination of NK Cells

Treatment with complement and	Positive cells*		RT activity		
	Before treatment	After treatment	day 11	day 17	day 24
	%		*cpm/ml*		
Mm 161-86					
Negative Ab‡	2	1	657	455	702
Anti-CD8	54	3	935	1,936	42,293
Anti-NKH1	5	<1	789	475	503
Anti-CD16	4	<1	468	742	231
Mm244-86					
Negative Ab	1	1	559	378	502
Anti-CD8	39	1	15,334	24,757	12,706
Anti-NKH1	4	<1	652	451	242
Anti-CD16	4	1	691	632	362

* Determined by flow cytometric analysis.
‡ Irrelevant mAb.

$CD8^+$ Lymphocytes Inhibit SIV_{mac} Replication in Autologous but not in MHC Class I-mismatched Lymphocytes. The interactions of antigen-specific $CD8^+$ lymphocytes are restricted by MHC class I surface molecules. We, therefore, sought to determine whether the interactions of the SIV_{mac} inhibitory $CD8^+$ cell population of the rhesus

TABLE III

Virus Replication in PBL of HIV-1-infected Humans after Elimination of NK Cells

Treatment with complement and	Positive cells*		RT activity		
	Before treatment	After treatment	day 11	day 17	day 21
	%		*cpm/ml*		
Volunteer No. 28					
Negative Ab‡	1	1	413	579	496
Anti-CD8	28	4	1,262	6,288	6,823
Anti-NKH1	17	<1	613	182	144
Anti-CD16	9	<1	463	252	160
Volunteer No. 29					
Negative Ab	ND§	ND	579	1,054	748
Anti-CD8	ND	ND	16,034	3,318	1,323
Anti-NKH1	ND	ND	1,064	2,752	767
Anti-CD16	ND	ND	709	1,257	2,681
Volunteer No. 30					
Negative Ab	1	1	533	600	472
Anti-CD8	38	2	415	5,736	2,131
Anti-NKH1	11	<1	389	343	327
Anti-CD16	9	3	300	154	330

* Determined by flow cytometric analysis.
‡ Irrelevant mAb.
§ Not determined because of insufficient numbers of cells.

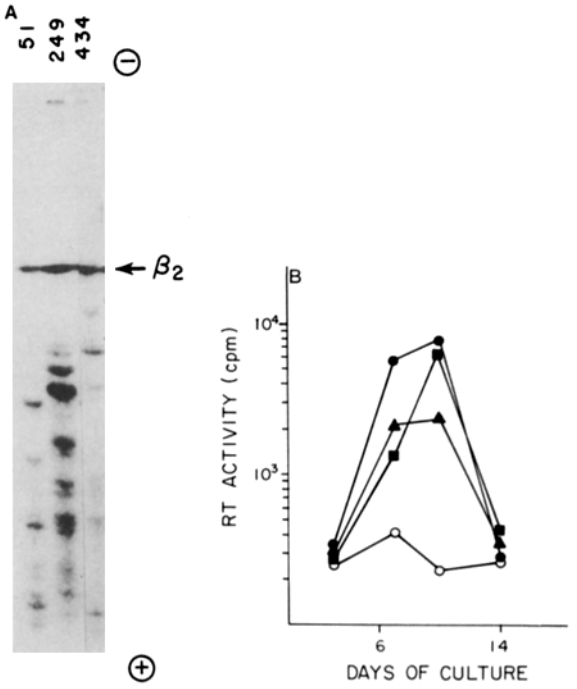

FIGURE 3. (*A*) PBL from animals 51 and 249 express different MHC class I molecules than PBLs of animal 434. Lysates of [^{35}S]methionine-labeled rhesus monkey PBL were phase separated, precleared, and MHC class I molecules were immunoprecipitated. After neuraminidase treatment, samples were subjected to 1-D IEF analysis. The position of β-2 microglobulin (β-2) is indicated. (*B*) Autologous, but not MHC class I mismatched, CD8$^+$ lymphocytes inhibit the generation of RT activity in vitro in CD8$^+$ lymphocyte-depleted PBL from SIV$_{mac}$-infected rhesus monkeys. CD8$^+$ cells from monkey 434 (○), 249 (■) or 51 (▲) were added to CD8$^+$ lymphocyte-depleted PBL of monkey 434 (●) and cultures were maintained in IL-2-containing medium. RT activity was regularly assessed in the culture medium as a measure of virus replication.

monkey are MHC class I restricted. Because MHC typing sera for rhesus monkeys are not widely available and are incomplete in the spectrum of specificities they define, we used 1-D IEF to characterize the MHC class I phenotype of clinically healthy SIV$_{mac}$-infected rhesus monkeys. As shown in Fig. 3 *A*, studies of this type yield a distinct banding pattern on a 1-D IEF gel for each animal, every band representing a different MHC class I allelic product. Although MHC class I-matched, unrelated SIV$_{mac}$-infected monkeys were not available for study, MHC class I-disparate infected animals could be readily defined. Animals 51, 249, and 434 differed in their expression of MHC class I alleles. Con A-activated CD8$^+$ lymphocyte-depleted PBL from rhesus monkey 434 were maintained in culture in the presence of IL-2. As shown in Fig. 3 *B*, virus replication occurred in the absence of reconstitution with CD8$^+$ lymphocytes, and was inhibited with the addition of autologous CD8$^+$ cells. The addition of MHC class I-mismatched populations of CD8$^+$ cells from SIV$_{mac}$-infected animals 249 or 51 failed to inhibit virus replication. These mismatched CD8$^+$ cell populations inhibited SIV$_{mac}$ replication in autologous CD8$^+$ lymphocyte-depleted PBL (data not shown). Therefore, MHC class I-mismatched CD8$^+$ cells do not inhibit the in vitro replication of SIV$_{mac}$ under these culture conditions.

The AIDS Virus Inhibitory Cells Are a Phenotypically Distinct Subpopulation of CD8$^+$ PBL. We further characterized the CD8$^+$ cell that inhibits SIV$_{mac}$ and HIV-1 replication by determining whether the virus inhibitory activity resides in all CD8$^+$ cells or in a subpopulation of phenotypically distinct CD8$^+$ cells. In pursuing these studies, we made use of the observation that recently defined surface antigens are expressed

on functionally distinct subpopulations of human and rhesus monkey $CD8^+$ lymphocytes. The antigens assessed in these studies included 2H4, 4B4, and S6F1. 2H4 is a member of the T200 family of molecules (18), 4B4 is a member of the integrin family of cell surface molecules (19), and S6F1 is an epitope of the cell adhesion molecule LFA-1 (20). As shown in Figure 4 *A*, human $CD8^+$ PBL (and rhesus monkey $CD8^+$ PBL; data not shown) can be fractionated into mutually exclusive $4B4^+2H4^-$ and $4B4^-2H4^+$ subpopulations. Moreover, as shown in Fig. 4 *B*, the $CD8^+4B4^+$ human lymphocytes also express S6F1.

$CD8^+$ lymphocytes were prepared from PBL of an HIV-1-infected, clinically healthy human and fractionated by the panning method into $2H4^+$ and $2H4^-$ subpopulations. These subpopulations of $CD8^+$ cells were then added to $CD8^+$ lymphocyte-depleted autologous PBL and RT activity in the supernatants of these reconstituted cultures was monitored. As shown in Fig. 5 *B*, the unfractionated $CD8^+$ lymphocytes significantly inhibited RT activity in autologous $CD8^+$ lymphocyte-depleted PBL. While the $4B4^-S6F1^-2H4^+$ subpopulation of $CD8^+$ lymphocytes had no HIV-1 inhibitory activity, the $4B4^+S6F1^+2H4^-$ subpopulation totally inhibited HIV-1 replication in vitro. An analogous experiment was done using similarly prepared subpopulations of $CD8^+$ lymphocytes from SIV_{mac}-infected, clinically healthy rhesus monkeys. As shown in Fig. 5 *A*, virtually no inhibitory activity was observed in the $CD8^+$ cell subpopulation that was $4B4^-2H4^+$. However, complete inhibition of RT activity was seen in cultures reconstituted with $CD8^+4B4^+2H4^-$ cells. Thus, both in PBL of HIV-1-infected humans and SIV_{mac}-infected rhesus monkeys, viral replication was inhibited by a subpopulation of $CD8^+$ lymphocytes.

Since 4B4 is a member of the integrin family of molecules, molecules involved in cell adhesion, these findings suggested that cell contact might occur between the virus inhibitory cell and the cell harboring viral replication. Moreover, S6F1 has

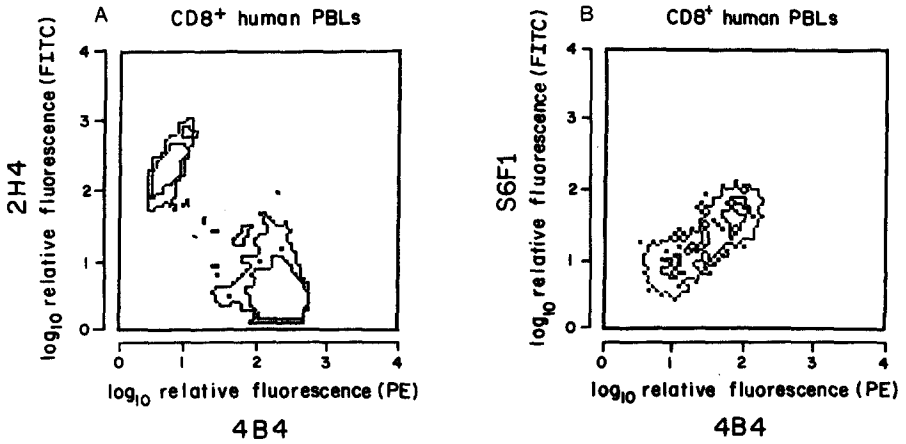

FIGURE 4. (*A*) $CD8^+$ human PBL comprise mutually exclusive $2H4^+4B4^-$ and $2H4^-4B4^+$ subpopulations. (*B*) $CD8^+4B4^+$ human PBL express the surface antigen S6F1. $CD8^+$ cells were enriched by the panning method. These lymphocytes were depleted of residual $CD4^+$ cells 2 d later by antibody and complement lysis. Cells were then reacted with the described antibodies. The horizontal scale indicates red fluorescence and the vertical scale indicates green fluorescence.

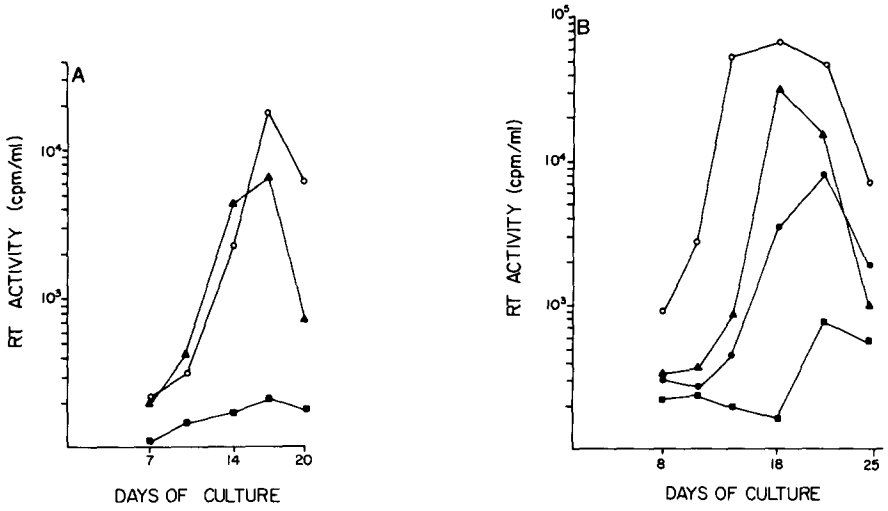

FIGURE 5. CD8$^+$ 4B4$^+$S6F1$^+$2H4$^-$ cells but not CD8$^+$ 4B4$^-$S6F1$^-$2H4$^+$ cells inhibit virus replication in CD8$^+$ lymphocyte-depleted PBL of a rhesus monkey infected with SIV_{mac} (*A*) and a human infected with HIV-1 (*B*). CD8$^+$ lymphocyte-depleted PBL were prepared by the panning method and maintained alone (○), reconstituted with an equal number of unfractionated CD8$^+$ cells (●), CD8$^+$ 4B4$^+$S6F1$^+$2H4$^-$ cells (■) or CD8$^+$ 4B4$^-$S6F1$^-$2H4$^+$ cells (▲).

recently been shown to be expressed by CD8$^+$ cytotoxic effector but not CD8$^+$ suppressor effector lymphocytes (19). This phenotypic characterization of the CD8$^+$ lymphocytes that inhibit AIDS virus replication in vitro suggested that the cell that inhibits viral replication may, in fact, be a CTL that must come into direct physical contact with its target cells in vitro.

Cell-to-Cell Contact Is Required for CD8$^+$ Lymphocyte-mediated Inhibition of AIDS Virus Replication. We then examined the cell interactions leading to the inhibition of viral replication, assessing the possible importance of cell contact in these interactions. Anti-LFA-1 antibodies have been shown to inhibit killer effector-target adhesion (21). Lectin-activated PBL from a clinically healthy HIV-1-infected human were placed in culture in IL-2-supplemented medium in the presence of two irrelevant mAbs or in the presence of an anti-LFA-1α mAb (Fig. 6). While no virus replication was seen in the absence of a mAb or in the presence of irrelevant mAbs, viral replication was readily demonstrated in the culture maintained in the presence of the antiLFA-1α mAb. (These studies were not done on PBL from SIV_{mac}-infected monkeys because this anti-LFA-1α mAb does not react with rhesus monkey cells.) This finding suggested that direct cell contact is required between the CD8$^+$ lymphocyte and its target cell in vitro for inhibition of viral replication to occur.

We further characterized the lymphocyte that inhibits SIV_{mac} replication by examining the role of the CD8 molecule in this inhibitory process. Anti-CD8 mAbs have been shown to block the effector function of both allo-specific and virus-specific CTL (2, 22). If the CD8 molecule were specifically involved in the inhibition of virus replication, mAbs directed against critical epitopes of the CD8 structure might be expected to inhibit the ability of the CD8$^+$ lymphocytes to subserve their inhibi-

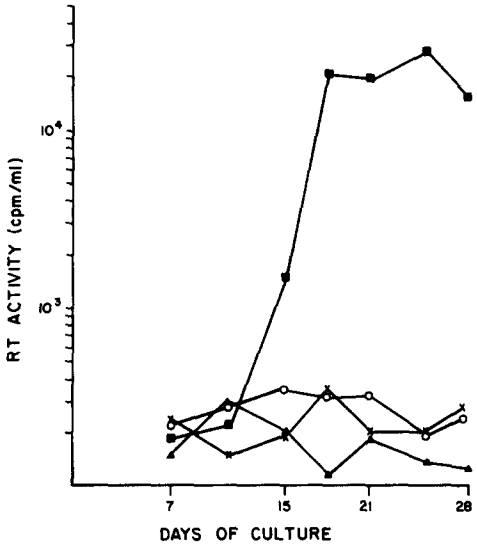

FIGURE 6. Virus replication is facilitated in PBL of an HIV-1-infected human by an anti-LFA-1 antibody. Con A-activated PBL from an HIV-1 infected human were maintained in culture without mAb (O), with anti-CD11a (LFA-1α; ■) anti-CD11b (Mol; ▲), and anti-CD2 antibody (X). RT activity was regularly assessed in the culture medium as a measure of virus replication.

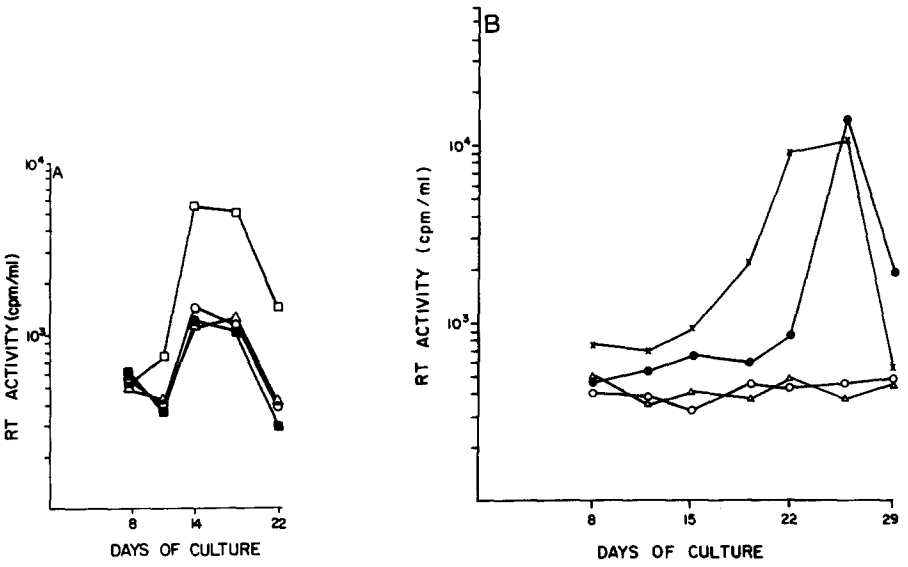

FIGURE 7. Virus replication is facilitated in PBL of a SIV_{mac}-infected rhesus monkey (*A*) or HIV-1-infected human (*B*) by anti-CD8 mAb. Con A-activated PBL from a SIV_{mac}-infected monkey or HIV-1-infected human were maintained in IL-2 containing medium. (*A*) Monkey PBL were maintained in the presence of an irrelevant control mAb (O), and anti-CD2 mAb (3PT2H9) (■) or one of two anti-CD8 monoclonal antibodies, 7PT4F12 (□) or 6F7 (Δ). Monkey PBL maintained in the presence of one other anti-CD8 antibody (7PT3F9) did not generate RT activity (data not shown) (*B*) Human PBL were maintained in the absence of a mAb (O), in the presence of an irrelevant control monoclonal antibody (Δ), or one of two anti-CD8 mAbs: 21Thy2D3 (●), or MT122 (X). RT activity was regularly assessed in the culture medium as a measure of virus replication. Human PBL maintained in the presence of three other anti-CD8 antibodies (7PT4F12, 6F7, 1Mono2E7) did not generate RT activity (data not shown).

tory function. As shown in Fig. 7 *A*, while Con A-activated unfractionated PBL from SIV_{mac}-infected monkeys did not generate significant RT activity when maintained in culture with an irrelevant or an anti-CD2 mAb, virus replication occurred if these cultured PBL were maintained in the presence of an anti-CD8 mAb. Thus, the addition of certain anti-CD8 mAbs to PBL from SIV_{mac}-infected monkeys in vitro blocked the inhibitory function of the $CD8^+$ lymphocytes.

We then sought to determine the relevance of this observation for the control of HIV-1 replication in human PBL. As previously shown in the SIV_{mac} system, virus replication was not detected in Con A-activated PBL of clinically healthy, HIV-1-infected humans. HIV-1 replication was, however, detected after $CD8^+$ lymphocyte depletion (data not shown). Importantly, when the function of the CD8 molecule was blocked by certain anti-CD8 mAbs, these $CD8^+$ T lymphocytes were no longer capable of inhibiting virus replication (Fig. 7 *B*). Thus, the CD8 molecule appears to play a crucial role in the cell-cell interactions that result in the inhibition of both SIV_{mac} and HIV-1 replication in vitro.

Discussion

These studies characterize a PBL population from AIDS virus-infected individuals capable of inhibiting AIDS virus replication. These cells were shown to be $CD8^+$, undergo clonal expansion in vivo after AIDS virus infection of the individual, and block replication of virus in autologous but not MHC class I-mismatched PBL. These cells express membrane structures that are involved in cell adhesion and their ability to inhibit virus replication can be blocked with mAbs that interfere with physical contact between the $CD8^+$ lymphocytes and their target cells.

Several lines of evidence establish that the AIDS virus inhibitory $CD8^+$ lymphocyte in PBL of infected individuals is not a NK cell. Depletion from PBL of cells expressing the NK-associated antigens NKH1 and CD16 did not facilitate virus replication in cultures. The inhibition of AIDS virus replication was also blocked by anti-CD8 mAbs. In other experimental systems, anti-CD8 mAbs, while inhibiting CTL effector function, do not inhibit NK activity (2, 23). Finally, in the current studies, the $CD8^+$ lymphocyte-depleted cells that support virus replication, even at the time of peak AIDS virus replication, contain a substantial number of $NKH1^+$ cells (data not shown).

Since these virus inhibitory $CD8^+$ lymphocytes are not NK cells, they are likely to be antigen specific. We have previously shown that $CD8^+$ cells capable of inhibiting AIDS virus replication are present in PBL of infected but not uninfected individuals (14). We have now demonstrated that a period of weeks is required after infection for a maturation of the $CD8^+$ lymphocyte-mediated inhibitory response. We also have previously shown that cell lines that exhibit this inhibitory function can be established in vitro by stimulating PBL from SIV_{mac}-infected monkeys with autologous SIV_{mac} antigen-expressing cells in the presence of IL-2 (14). It will, however, be necessary to show that the $CD8^+$ cells that inhibit virus replication can be generated by specific antigen priming or that such cells isolated from infected individuals have specific proliferative activity after AIDS virus antigen stimulation to establish conclusively that they are indeed antigen specific.

Antigen-specific $CD8^+$ lymphocytes should be MHC class I restricted in their cell-cell interactions. We were able to demonstrate that $CD8^+$ lymphocytes were

incapable of inhibiting viral replication in PBL of MHC class I-mismatched SIV_{mac}-infected monkeys. However, MHC class I-matched unrelated SIV_{mac}-infected monkeys were not available for study. Thus, we could not formally prove that MHC class I matching between the $CD8^+$ cell population and the $CD8^+$ lymphocyte-depleted PBL was sufficient to facilitate an inhibitory interaction. However, these findings are consistent with our previous observation that a long-term T lymphocyte line, generated from rhesus monkey PBL by repeated in vitro stimulation with autologous SIV_{mac}-infected cells in the presence of IL-2, inhibited SIV_{mac} replication in $CD8^+$ lymphocyte-depleted autologous lymphocytes, but not in $CD8^+$ lymphocyte-depleted cells from a MHC class I-mismatched animal (14). These experiments together demonstrate, within the limitations of our experimental systems, that the interactions of this $CD8^+$ lymphocyte population may be MHC class I restricted.

Thus, these studies provide evidence that the $CD8^+$ inhibitory cells may be antigen specific and MHC class I restricted. These findings, coupled with the observation that the inhibitory cells are $S6F1^+$ and the demonstration that this inhibitory function can be blocked both with anti-LFA-1 and anti-CD8 mAbs, provide compelling evidence that this $CD8^+$ cell is, in fact, a CTL.

The observation that the inhibitory $CD8^+$ cell is phenotypically a CTL makes the studies with the mAbs anti-2H4 and anti-4B4 particularly interesting. While previous work with these mAbs indicates that $CD4^+$ cells can be divided into phenotypically and functionally distinct groups that subserve either helper function or inducer of suppression function (24, 25), little work has been done with these antibodies in the study of $CD8^+$ lymphocytes. The current experiments suggest that functional virus-specific cytotoxic cells may be contained only within the $4B4^+2H4^-$ subpopulation of $CD8^+$ lymphocytes.

While these studies indicate that the cell which inhibits HIV-1 and SIV_{mac} replication in PBL is phenotypically a CTL, the mechanism by which this $CD8^+$ lymphocyte inhibits the replication of virus remains to be elucidated. Cell contact is required between the $CD8^+$ lymphocyte and cells harboring replicating virus since this inhibitory process can be blocked by anti-LFA-1 and anti-CD8 mAbs. It is not clear, however, whether this cell contact is necessary only for the triggering of factor secretion by these $CD8^+$ cells, which in turn inhibits virus replication, or whether this $CD8^+$ cell, in fact, lyses the AIDS virus-expressing target cell. It is interesting to speculate that this AIDS virus-inhibiting cell might represent the same subset of lymphocytes in PBL capable of lysing autologous AIDS virus-expressing target cells (3-5).

Summary

$CD8^+$ (suppressor/cytotoxic) lymphocytes block replication of HIV-1 or the simian immunodeficiency virus of macaques (SIV_{mac}) in PBL of infected individuals. We now show that these $CD8^+$ lymphocytes undergo clonal expansion in vivo after AIDS virus infection of the individual, suggesting they may be antigen-specific T cells. These $CD8^+$ cells block replication of virus in autologous but not MHC class I-mismatched PBL. The inhibitory lymphocytes express the integrin family molecule 4B4 and the CTL-associated S6F1 epitope of LFA-1. Finally, physical contact is required for the $CD8^+$ lymphocyte-mediated inhibition of AIDS virus repli-

cation, since this inhibitory function is blocked by anti-LFA-1 and anti-CD8 mAbs. These studies suggest that the cell that inhibits AIDS virus replication in PBL of infected individuals is a CTL.

We thank M. Hirsch and J. Sullivan for providing blood samples from HIV-1-infected patients and D. Brosseau for preparation of this manuscript.

Received for publication 23 November 1988 and in revised form 29 December 1988.

References

1. Zinkernagel, R. M., and P. C. Doherty. 1974. Restriction of *in vitro* T cell-mediated cytotoxicity in lymphocytic choriomeningitis within a syngeneic or semiallogeneic system. *Nature (Lond.).* 248:701.
2. Gotch, F. M., N. Carter, S. Ellis, P. McShane, J. Heyman, M. Palmer, D. W. Dongworth, A. Bewick, P. J. Morris, and A. J. McMichael. 1986. Binding and functional analysis of the workshop T cell specific set of monoclonal antibodies. *In* Leukocyte Typing II. Vol. 1. E. Reinherz, B. Haynes, L. Nadler, and I. Bernstein, editors. Springer-Verlag, New York. 33–44.
3. Walker, B. D., S. Chakrabarti, B. Moss, T. J. Paradis, T. Flynn, A. G. Durno, R. S. Blumberg, J. C. Kaplan, M. S. Hirsch, and R. T. Schooley. 1987. HIV-specific cytotoxic T lymphocytes in seropositive individuals. *Nature (Lond.).* 328:345.
4. Walker, B. D., C. Flexner, T. J. Paradis, T. C. Fuller, M. S. Hirsch, R. T. Schooley, and B. Moss. 1988. HIV-1 reverse transcriptase is a target for cytotoxic T lymphocytes in infected individuals. *Science (Wash. DC).* 240:64.
5. Plata, F., B. Autran, L. P. Martins, S. Wain-Hobson, M. Raphael, C. Mayaud, M. Denis, J. Guillon, and P. Debre. 1987. AIDS virus-specific cytotoxic T lymphocytes in lung disorders. *Nature (Lond.).* 328:348.
6. Walker, C. M., D. J. Moody, D. P. Stites, and J. A. Levy. 1986. $CD8^+$ lymphocytes can control HIV infection *in vitro* by suppressing virus replication. *Science (Wash. DC).* 234:1563.
7. Chakrabarti, L., M. Guyader, M. Alizon, M. D. Daniel, R. C. Desrosiers, P. Tiollais, and P. Sonigo. 1987. Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses. *Nature (Lond.).* 328:543.
8. Franchini, G., C. Gurgo, H. G. Guo, R. C. Gallo, E. Collalti, K. A. Fargnoli, L. F. Hall, F. Wong-Staal, and M. S. Reitz, Jr. 1987. Sequence of simian immunodeficiency virus and its relationship to the human immunodeficiency viruses. *Nature (Lond.).* 328:539.
9. Letvin, N. L., M. D. Daniel, P. K. Sehgal, R. C. Desrosiers, R. D. Hunt, L. M. Waldron, J. J. MacKey, D. K. Schmidt, L. V. Chalifoux, and N. W. King. 1985. Induction of AIDS-like disease in macaque monkeys with T-cell tropic retrovirus STLV-III. *Science (Wash. DC).* 230:71.
10. Kannagi, M., J. M. Yetz, and N. L. Letvin. 1985. *In vitro* growth characteristics of simian T lymphotropic virus type III. *Proc. Natl. Acad. Sci. USA.* 82:7053.
11. Chalifoux, L. V., D. J. Ringler, N. W. King, P. K. Sehgal, R. C. Desrosiers, M. D. Daniel, and N. L. Letvin. 1987. Lymphadenopathy in macaques experimentally infected with the simian immunodeficiency virus (SIV). *Am. J. Pathol.* 128:104.
12. Kannagi, M., M. Kiyotaki, R. C. Desrosiers, K. A. Reimann, N. W. King, L. M. Waldron, and N. L. Letvin. 1986. Humoral immune responses to T cell tropic retrovirus simian T lymphotropic virus type III in monkeys with experimentally induced acquired immunodeficiency-like syndrome. *J. Clin. Invest.* 78:1229.
13. Letvin, N. L., M. D. Daniel, N. W. King, M. Kiyotaki, M. Kannagi, L. V. Chalifoux, P. K. Sehgal, R. C. Desrosiers, L. O. Arthur, and A. C. Allison. 1987. AIDS-like disease in macaque monkeys induced by simian immunodeficiency virus: a vaccine trial. *In* Vac-

cines 87, Chanock, R., Lerner, R., Brown, F., Ginsburg, H., editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 209–213.

14. Kannagi, M., L. V. Chalifoux, C. I. Lord, and N. L. Letvin. 1988. Suppression of simian immunodeficiency virus replication *in vitro* by $CD8^+$ lymphocytes. *J. Immunol.* 140:2237.
15. Neefjes, J. J., B. S. Bruer-Vriesendorp, G. A. van Seventer, P. Ivanyi, and H. L. Pleogh. 1986. An improved biochemical method for the analysis of HLA-class I antigens. Definition of new HLA-class I subtypes. *Human Immunol.* 16:169.
16. Watkins, D. I., M. Kannagi, M. E. Stone, and N. L. Letvin. 1988. Major histocompatibility complex class I molecules of nonhuman primates. *Eur. J. Immunol.* 18:1425.
17. Brodsky, F. M., P. Parham, C. J. Barnstable, M. J. Crimpton, and W. F. Bodmer. 1979. Monoclonal antibodies for analysis of HLA system. *Immunol. Rev.* 47:3.
18. Rudd, C. E., C. Morimoto, L. L. Wong, and S. F. Schlossman. 1987. The subdivision of the T4 (CD4) subset on the basis of the differential expression of L-C/T200 antigens. *J. Exp. Med.* 166:1758.
19. Sanders, M. E., M. W. Makgoba, and S. Shaw. 1988. Human naive and memory T cells. *Immunol. Today.* 9:195.
20. Morimoto, C., C. E. Rudd, N. L. Letvin, and S. F. Schlossman. 1987. A novel epitope of the LFA-1 antigen which can distinguish killer effector and suppressor cells in human CD8 cells. *Nature (Lond.).* 330:479.
21. Springer, T. A., M. L. Dustin, T. Kishimoto, and S. D. Markin. 1987. The lymphocyte function associated LFA-1, CD2, and LFA-3 molecules: Cell adhesion receptors of the immune system. *Annu. Rev. Immunol.* 5:223.
22. Meuer, S. C., S. F. Schlossman, and E. L. Reinherz. 1982. Clonal analysis of human cytotoxic T lymphocytes: $T4^+$ and $T8^+$ effector T cells recognize products of different major histocompatibility complex regions. *Proc. Natl. Acad. Sci. USA.* 79:4395.
23. Ortaldo, J. R., and D. L. Longo. 1988. Human natural lymphocyte effector cells: Definition, analysis of activity and clinical effectiveness. *J. Natl. Cancer Inst.* 80:999.
24. Morimoto, C., N. L. Letvin, J. A. Distaso, W. R. Aldrich, and S. F. Schlossman. 1985. The isolation and characterization of the human suppressor inducer T cell subset. *J. Immunol.* 134:1508.
25. Morimoto, C., N. L. Letvin, A. W. Boyd, M. Hagan, H. M. Brown, M. M. Kornacki, and S. F. Schlossman. 1985. The isolation and characterization of the human helper inducer T cell subset. *J. Immunol.* 134:3762.