Serum Enhances the Ex Vivo Generation of HIV-Specific Cytotoxic T Cells

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The ex vivo expansion of antigen-specific cytotoxic T lymphocyte (CTL) lines is being developed for immunotherapy of viral infections and cancer and is critically dependent on the precise cell expansion and stimulation conditions. In this article, we investigate medium requirements for the development of HIV-specific CTL in cell lines generated from the peripheral blood of seven asymptomatic HIV-infected individuals. We find that HIV-specific CTL do not readily develop in the serum-free medium AIM V but do develop if the medium is supplemented with 1% plasma or serum. T cell lines with antigen-specific cytolytic activity express more cell-surface CD57 than do cell lines grown in the absence of serum or plasma. Three sources of serum (human autologous, human AB, or fetal calf) are comparable. Human plasma is somewhat less effective than serum from an identical source. © 1996 John Wiley & Sons, Inc.
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INTRODUCTION

Immunotherapy with ex vivo expanded T cells is being developed to treat a wide range of diseases including malignancies and viral infections. Early trials involved the infusion of LAK-like cells stimulated with high concentrations of IL-2, whose function largely did not involve specific TcR engagement.[9,20] However, more recent approaches have sought to target more precisely specific tumor or viral antigens presented by cell surface MHC molecules. The goal of many of these therapies is the infusion of large numbers of antigen-specific cytotoxic T lymphocytes (CTL) which can be triggered via TcR engagement to lyse transformed or virally infected target cells. Treatment with polyclonal T cell lines is likely to minimize the development of escape mutants and to be less labor intensive and costly than development of T cell clones for infusion. The ex vivo development of antigen-specific CTL lines, however, is critically dependent on the precise growth conditions used for expansion.

We have developed methods for the ex vivo expansion of HIV-specific CTL lines and are performing clinical trials with ex vivo expanded CTL lines in HIV-1 infected patients.[14] There is a high frequency of HIV-specific CTL memory cells and activated CTL in HIV-infected individuals before they develop opportunistic infections.[10,19,23] Because of this, it is possible to generate, by polyclonal activation, T cell lines that have readily detectable antiviral CTL activity. In this article, we use polyclonal activation of peripheral blood mononuclear cells (PBMC) from infected patients to compare different media for their ability to support the development of viral-specific CTL.

Serum-free media have been developed and approved for the ex vivo expansion of T cells used for treatment. These media have several potential advantages for clinical use. There is little lot-to-lot variation, often a problem with undefined fetal calf or human sera. They are free of possible infectious agents, especially viruses, that may cause clinical disease, especially in immunosuppressed patients. Because they are not contaminated with multiple ill-defined proteins, they are less likely to induce allergic or antibody responses. Serum-free media also have been reported to reduce nonspecific or background proliferation.[11]

In this article we will show that a widely used serum-free medium, AIM V, does not support the development of HIV-specific CTL. Cells grown in serum-free AIM V proliferate as well as cells grown in serum-containing media. However, they have undetectable HIV-specific CTL activity compared with readily apparent antiviral cytolytic activity when grown in serum-containing media. Another serum-free medium, X-VIVO 20, is also unable to support efficiently the ex vivo generation of HIV-specific CTL. The addition of as little as 1% serum can support antigen-specific CTL generation from the PBMC of HIV-infected donors. Either human or bovine serum can support the development of antiviral CTL activity. Human plasma is less effective than serum at supporting CTL generation.

MATERIALS AND METHODS

Cell Lines

PBMC were obtained by Ficoll–Hypaque density centrifugation from heparinized blood from HIV-1-infected

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individuals. The subjects were all asymptomatic homosexual males with CD4 counts of 230 to 670 cells/mm^3 (CDC Stages A1 and A2). Informed consent was obtained and the study was approved by the New England Medical Center Human Investigation Review Committee. T cell lines were generated by adding 2 μg/mL PHA-P (Difco Laboratories, Detroit, MI) to PBMC obtained by Ficoll–Hypaque density centrifugation from heparinized blood. In some instances, density-separated PBMC were cryopreserved in fetal calf serum (Sigma, St. Louis, MO) supplemented with 5% DMSO using a programmed cell freezer (Cryomed). Fresh or thawed cryopreserved cells were incubated at 5×10^5 cells/mL in T cell media. The media were AIM V (Gibco, Gaithersburg, MD) or X-VIVO 20 (BioWhittaker, Walkersville, MD) supplemented with 600 IU/mL rhuIL-2 (Aldesleukin, Cetus Oncology, Emeryville, CA), 2 m*M* glutamine, 2 m*M* HEPES, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μM β-mercaptoethanol, to which was added nothing or various concentrations of human plasma or serum or fetal calf serum (Sigma). The fetal calf serum was free of endotoxin and was chosen from among six lots for its ability to optimize the growth and cytotoxicity of T cell lines. The human sera tested included autologous serum or plasma or human AB plasma or serum (obtained from Sigma or normal volunteers). Plasma was obtained from heparinized blood, spun at 2500 rpm for 15 min to remove cellular components. Twice a week the cultures were adjusted to 5×10^5/mL with fresh media. Autologous B cell lines were generated for each subject using B95-8 marmoset cell line supernatant by standard methods.[1]

Flow Cytometry Analysis of T Cell Lines

T cell lines (2 to 10×10^5) of >90% viability were harvested and resuspended in 50 μL of cold FACS media (PBS with 1% BSA and 0.02% sodium azide) to which 5 μL of Leu-2a-PE (CD8), and 5 μL of Leu-3a+3b-FITC (CD4) or other conjugated mAb (Leu-2a-FITC [CD8], Leu-4-FITC [CD3], Leu-28-PE [CD28], Leu-17-PE [CD38], anti-HLA-DR-PE, Leu-11a-PE [CD16], Leu-7-FITC [CD57], Becton Dickinson, Mountain View, CA) were added. After incubation for 20 min at 4°C, the cells were washed with 1 mL of FACS media, pelleted, and washed again with 500 μL. Before two-color flow cytometry, the cells were fixed with 1% formaldehyde in FACS media. Previously phenotyped $CD4^+$ and $CD8^+$ clones were stained simultaneously to establish gates and gain settings.

Vaccinia Vectors

Vaccinia vectors encoding lacZ (vSC8), gp160 of the BH8 isolate of HIV-1_{LAI} (vPE16), all but the last 22 residues of RT of subclone HXB.2 (vCF21), and HXB.2 gag (vDK1) were used to screen cell lines for specific cytotoxicity against gp160, RT and gag-expressing targets.[2,3,6] The HIV-1 sequences were inserted downstream from the 7.5 early–late promotor of vaccinia. The vaccinia virus preparations were titered by plaque assay on CV-1 cells.[16]

Chromium-Release Assay

Cytotoxicity assays were performed against target cells that were infected with vaccinia constructs encoding HIV-1 proteins or lacZ control. For infection, 10 pfu/cell of vaccinia virus was added to 5×10^5 exponentially growing B cells in 500 μL in a 24-well plate. The plate was incubated at 37°C over CO_2 with rocking for the first hour. After 16 h the cells were harvested and resuspended in 200 μL of serum-containing media to which 100 μCi of Na_2 ($^{51}CrO_4$) (Du Pont, NEN Research Products, Boston, MA) was added. After incubation for 1 h at 37°C with occasional mixing, the targets were washed three times and resuspended at 10^5/mL. Labeled targets (10^4) were added to triplicate wells of U-bottom microtiter plates. Effector cells were suspended at an effector:target (E:T) ratio of 25:1 in 100 μL and added to target cells and the plates incubated at 37°C over CO_2 for 4 h. For each target, spontaneous release (SR) was determined from wells to which 100 μL media was added and total release (TR) calculated from wells containing 100 μL 1% Nonidet P-40. Supernatants (35 μL) from each well were counted on a Packard Topcount microplate reader after addition of 35 μL 1% Nonidet P-40. Percent specific cytotoxicity was calculated from the average cpm as [average cpm − SR)/(TR − SR)] × 100. Spontaneous release was below 20% of total release. Gp160, gag, or RT-specific cytotoxicity was defined as the difference between the percent specific cytotoxicity against gp160, RT, or gag-expressing targets and that against lacZ-expressing targets.

Frequency Analysis of CTL

Target cells, infected with vaccinia virus and labeled with ^{51}Cr, as above, were plated at 8000 cells/well in U-bottom microtiter plates. Effector T cells were added to 30 replicate wells in twofold dilutions from 2500 to 160,000 cells/well and incubated for 16 h over CO_2 at 37°C. The supernatants were counted on a Packard Topcount reader as above. Positive wells were defined as wells with counts >3 SD above the mean cpm of 30 wells to which no effectors were added. Probit analysis was performed as described in Lieberman et al.[14] to determine the LD_{50}, the number of effectors required for half the wells to be positive. The relative frequency of effector cells from cell lines grown with and without serum is inversely proportional to their LD_{50} ratio.

RESULTS

Growth of T Cell Lines Is Not Serum-Dependent

T cell lines were generated in media containing 5% plasma or sera from human or fetal calf sources by a

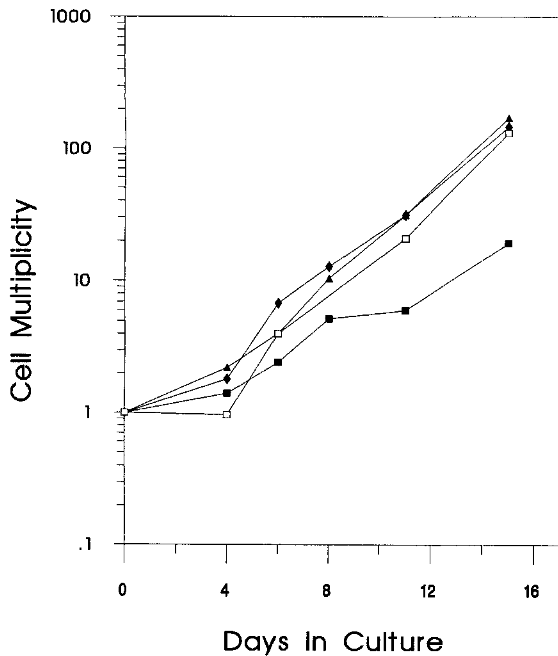
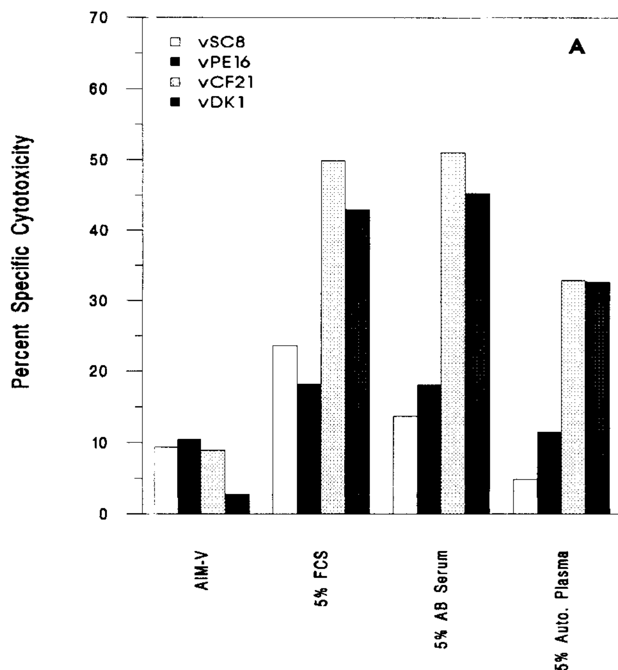


Figure 1. Cell growth is not dependent on serum in AIM V media. Cell growth in a representative cell line (143) was grown in unsupplemented AIM V (□) or in AIM V supplemented with 5% FCS (▲), human AB serum (♦), or autologous human serum (■). The decreased growth in autologous serum was not reproducible.

single stimulation with PHA. Cells were counted biweekly by trypan blue exclusion. The addition of plasma or serum to AIM V medium did not significantly affect the growth of the cell lines. The growth of cell lines from a representative subject is shown in Figure 1.

Generation of HIV-Specific Cytotoxicity Is Enhanced by Serum

After 14 to 26 days of culture, T cell lines were screened by ^{51}Cr-release assay for cytolytic activity against autologous B cell lines (B-LCL) infected with vaccinia vectors encoding env_{LAI}, pol, gag, and lacZ control. The cytotoxicity assays for each effector T cell line were repeated at least once for each subject and the amount of HIV-specific cytotoxicity was reproducible and stable over the time of the assays. Significant (>10% over lacZ control) HIV-specific CTL responses were detected in T cell lines from seven patients generated in medium supplemented with fetal calf serum (FCS), normal human AB, or autologous serum or plasma. (Fig. 2, Table I) All patients recognized at least two of the three HIV proteins tested. However, AIM V media alone was not capable of supporting HIV-specific cytotoxicity above background levels in six of the seven patients tested. In patient 219, with the highest level of specific cytotoxicity, there was detectable HIV-specific cytotoxicity in the cell line grown in unsupplemented AIM V, but it was substantially reduced. At an E:T ratio of 25:1, the unsupplemented cell line had 8% gp160-, 12% RT-, and 23% gag-specific cytotoxicity. The corresponding line grown in serum-supplemented medium had 11% gp160-, 64% RT-, and 68% gag-specific cytotoxicity. Results of ^{51}Cr-release assays from two representative patients are shown in Figure 2. For patient 143, RT and gag-expressing autologous targets were lysed by lines generated in autologous plasma, human AB serum, and FCS. No HIV-specific lysis was detected in the line



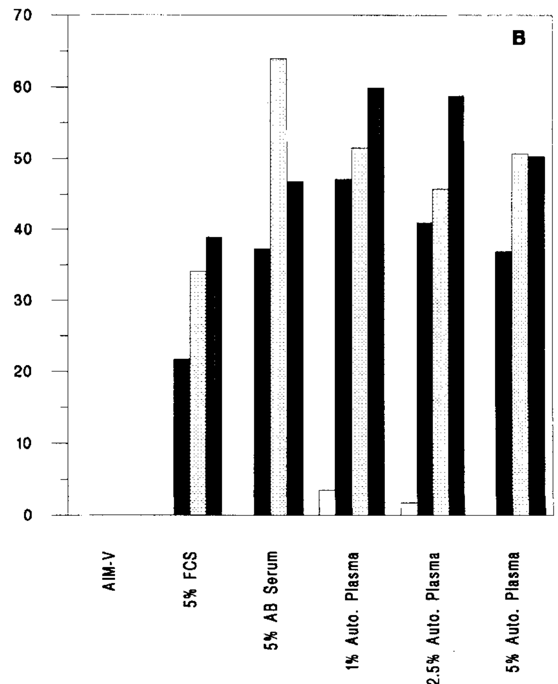


Figure 2. HIV-1-specific CTL derived from representative subjects (A) 143 and (B) 203 in AIM V media supplemented with indicated human or fetal calf sera or plasma. PBMC were stimulated with PHA, cultured for 19 and 16 days, respectively, and assayed for cytotoxicity against autologous B-LCL that expressed gp160 (vPE16), RT (vCF21), gag (vDK1), or lacZ (vSC8) control in a 4-hr ^{51}Cr-release assay.

Table I. Percent specific cytotoxicity of nonspecifically stimulated T cell lines generated from the PBMC of seven HIV-seropositive asymptomatic subjects grown in AIM V media supplemented with 5% added sera or plasma.[a]

		Percent specific cytotoxicity of T cells grown in AIM V supplemented with:					
Patient	Target	—	Autologous plasma	Autologous serum	Human AB plasma	Human AB serum	Fetal calf serum
143	vSC8	10.7	3.0	N.D.	N.D.	21.1	28.0
	vPE16	10.2	9.7	N.D.	N.D.	23.4	26.8
	vCF21	9.2	22.0	N.D.	N.D.	54.7	52.5
	vDK1	8.5	23.7	N.D.	N.D.	49.3	51.1
169	vSC8	0	7.9	10.5	10.4	12.4	N.D.
	vPE16	N.D.	11.6	28.9	13.2	23.4	N.D.
	vCF21	N.D.	26.8	35.7	23.2	33.3	N.D.
	vDK1	0	35.3	44.6	30.6	41.8	N.D.
203	vSC8	0	0	N.D.	N.D.	0	0
	vPE16	0	36.9	N.D.	N.D.	37.3	21.7
	vCF21	0	50.7	N.D.	N.D.	63.9	34.1
	vDK1	0	50.3	N.D.	N.D.	46.8	38.9
219	vSC8	0.4	N.D.	N.D.	N.D.	15.0	N.D.
	vPE16	8.7	N.D.	N.D.	N.D.	35.8	N.D.
	vCF21	12.1	N.D.	N.D.	N.D.	78.8	N.D.
	vDK1	23.3	N.D.	N.D.	N.D.	82.8	N.D.
221	vSC8	0	N.D.	N.D.	N.D.	0	N.D.
	vPE16	10	N.D.	N.D.	N.D.	19.9	N.D.
	vCF21	3.4	N.D.	N.D.	N.D.	6.3	N.D.
	vDK1	9.0	N.D.	N.D.	N.D.	29.8	N.D.
307	vSC8	0.2	0	4.5	N.D.	8.0	9.4
	vPE16	0	14.5	43.3	N.D.	33.7	30.9
	vCF21	0	12.8	29.3	N.D.	12.5	16.1
	vDK1	7.5	12.1	25.6	N.D.	31.5	29.9
322	vSC8	1.1	N.D.	N.D.	N.D.	1.1	N.D.
	vPE16	6.2	N.D.	N.D.	N.D.	37.3	N.D.
	vCF21	2.8	N.D.	N.D.	N.D.	37.3	N.D.
	vDK1	0	N.D.	N.D.	N.D.	53.8	N.D.

[a]Cytotoxicity assays were performed for 4 h at an E:T ratio of 25:1. Representative experiments of duplicate experiments performed after 16 to 26 days of culture are shown. Autologous targets were infected with recombinant vaccinia virus expressing HIV-1 $gp160_{LAI}$ (vPE16), RT (vCF21), gag (vDK1), or a lacZ control (vSC8). N.D. = not done.

grown in AIM V only (Fig. 2A). For patient 203 (Fig. 2B), three concentrations of autologous plasma (1%, 2.5%, and 5%) were tested and compared to 5% FCS and 5% human AB serum (Fig. 2B). All three HIV targets were lysed to a comparable extent by all the lines on day 16 except for the cells grown in AIM V alone which exhibited no cytolytic activity at all. As little as 1% plasma was able to restore full cytotoxic function to cells grown in AIM V media. The reduced killing in Figure 2B by the FCS line from patient 203 on day 16 was not seen when retested on day 22 (data not shown). In addition, when the T cell line originally grown in AIM V alone was supplemented with 5% FCS on day 18 of culture, no HIV-specific cytotoxicity developed (data not shown).

Background lysis of lacZ-expression autologous targets was higher by plasma- or serum-supplemented lines compared to unsupplemented AIM V lines in three of five patients. There was also somewhat less background lysis by cell lines grown in the presence of autologous plasma compared to those grown in human AB or FCS.

Cell lines for donor 221 were also grown in another serum-free medium, X-VIVO 20. In this medium, as well as AIM V, cell lines grown in the absence of serum had no HIV-specific cytolytic activity above background but clearly did in the presence of 5% human AB serum (Fig. 3).

Frequency Analysis of HIV-Specific CTL Grown in the Presence and Absence of Serum

To verify that the lack of HIV-specific cytotoxicity is due to a reduced frequency of HIV-specific CTL in the serum-free cultures, and not to their suppression, we performed a frequency analysis of gag-specific CTL in cell lines generated from donor 221. Because the assumptions of limiting dilution are not valid for a CTL assay (namely a single effector cell cannot be detected) we have developed a relative frequency analysis using probit analysis which provides reliable estimates of the relative frequency of specific CTL in cultures.[14] Using this method, we find that there are 7.5 times as many gag-specific CTL in the cell line grown in the presence of serum as in the line grown in AIM V in the absence of serum (Fig. 4).

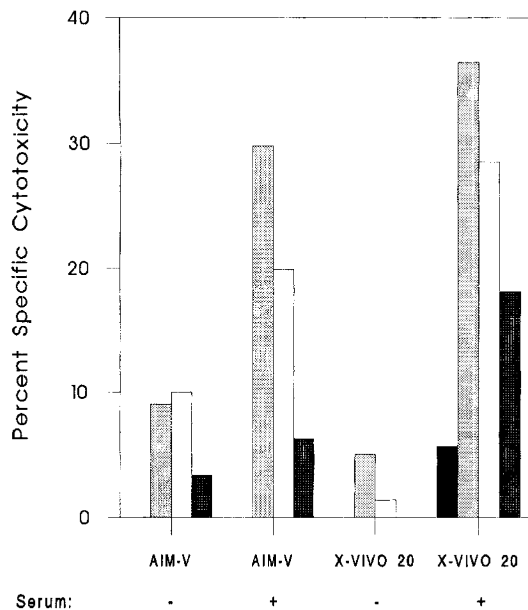


Figure 3. HIV-specific CTL activity is enhanced in the presence of 5% human AB serum in cell lines derived from donor 221 in either AIM V or X-VIVO 20 medium. Percent specific cytotoxicity was measured in a 4-h assay at an E:T ratio of 25:1 against autologous targets expressing lacZ control (black bars), gp160 (stippled bars), RT (open bars), or gag (boxed bars).

CTL Grown in Serum Express More Cell Surface CD57 and CD38

To identify whether the differences in antiviral cytolytic activity correlated with expansion of different subpopulations of T cells, analysis of cell-surface phenotype by flow cytometry was performed. Each cell line was analyzed by flow cytometry at two time points between 8 and 26 days of culture. When high concentrations of rhuIL-2 (600 IU/mL) are used to expand PHA-stimulated T cell lines, the cells are predominantly $CD8^+$, whether derived from HIV-seronegative or -seropositive individuals.[13,18] The percent $CD8^+$ cells in most lines in this study after 2 weeks in culture was usually between 80% and 90%.

The phenotype of $CD8^+$ T cells in each cell line was examined by flow cytometry to correlate functional differences with surface protein expression. The percentage of $CD8^+$ cells staining positively for CD28, CD38, HLA-DR, and CD57 was determined for cell lines from each subject grown in AIM V and in AIM V supplemented with 5% human AB serum. The surface protein whose expression seemed to correlate best with HIV-specific cytotoxicity was CD57 with correlation coefficient $r = 0.65$ (Fig. 5). Cell lines generated in AIM V medium alone have little or no CD57 expressed on their surface (mean 6.2 ± 4.4%), whereas a substantial fraction of the $CD8^+$ cells generated in media supplemented with serum express CD57 (mean 33.5 ± 13.0%) (Fig. 6). The difference in CD57 expression is significant ($P < 0.004$). There was no consistent effect of serum exposure on CD28 expression, which varied considerably among the cell lines from different patients (mean 60.4 ± 37.2% amd 48.2 ± 13.7% $CD28^+$ cells grown in the absence and presence of 5% human AB serum, respectively). There was considerable heterogeneity in the expression of CD38 and DR among the unsupplemented AIM V cell lines; in cell lines exposed to serum, the overwhelming majority of $CD8^+$ cells expressed both CD38 and DR. CD38 expression increased from a mean

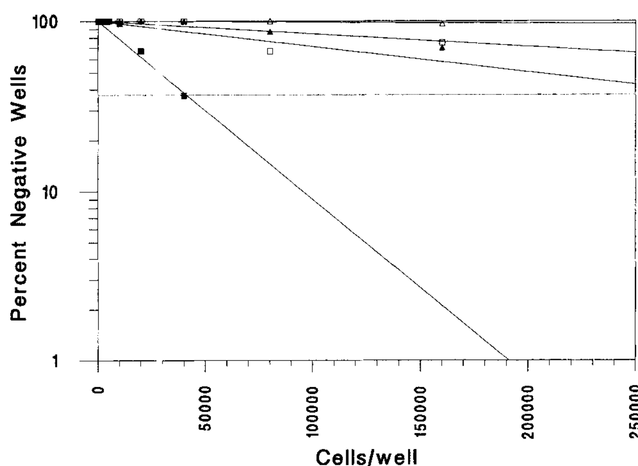


Figure 4. Frequency analysis of gag-specific (□) and lacZ-specific (Δ) CTL in cell lines from donor 221 grown in AIM V (open symbols) or in AIM V supplemented with 5% human AB serum (shaded symbols). There were 7.5 times as many gag-specific CTL in the line grown in the presence of serum.

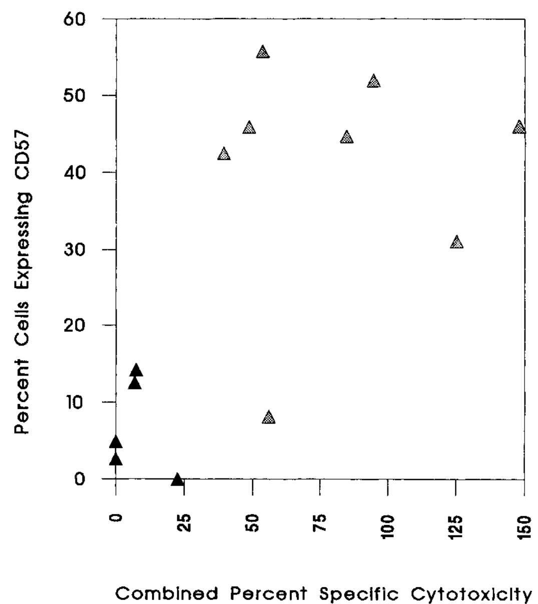


Figure 5. Correlation between surface expression of CD57 on $CD8^+$ cells and the total HIV-specific lysis, which represents the sum of env-, RT-, and gag-specific lysis ($r = 0.65$). Cells grown in unsupplemented AIM V (▲) have low levels of CD57 expression and specific cytotoxicity, whereas cells grown in serum or plasma supplemented AIM V (▲) more frequently express CD57 and exhibit HIV-specific cytotoxicity. No significant correlation was seen for CD28, CD38, or HLA-DR expression and specific cytotoxicity.

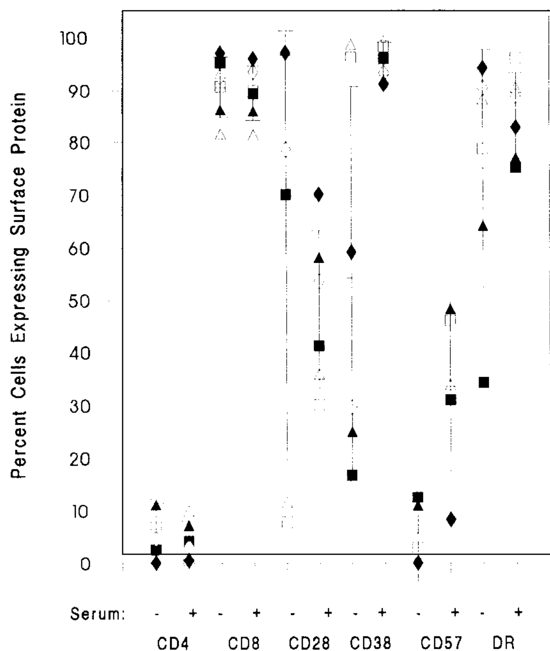


Figure 6. Flow cytometry analysis of cell-surface markers after 16 to 22 days in culture reveals increases in the number of cells expressing CD57, CD38, and HLA-DR after growth in the presence of serum. The overwhelming majority of cells are CD8⁺, independent of the presence of serum. Differences in CD28 expression were not consistent in the six cell lines tested [143 (Δ), 203 (□), 219 (◇), 221 (◆), 307 (▲), 322 (■)]. Data for cell line 169 were not included because there were too many nonviable cells.

of 54.2 ± 33.2% in serum-free media to 95.8 ± 2.4% in the presence of serum ($P < 0.04$). DR expression increased somewhat from a mean of 74.9 ± 22.6% in serum-free media to 84.9 ± 8.2% in 5% human AB serum, but this difference was not statistically significant.

For subject 203, the expression of surface protein markers on CD8⁺ T cell lines was compared to that on freshly isolated CD8⁺ cells. Expression of CD28 (29.7% on freshly isolated T cells) decreased to 7.7% in serum-free media, but was stable in 5% human AB serum (30.1%). There was also a marked decrease in CD57 expression in serum-free media (2.6%) compared to 5% human AB serum (46.0%) or freshly isolated CD8⁺ PBMC (42.3%). In both cell lines, the percent of cells staining with CD38 and HLA-DR increased from that of the circulating CD8⁺ T cells. Of freshly isolated CD8⁺ T cells, 77.6% expressed CD38 and 38.9% expressed DR. In the serum-free T cell line, 96.1% of the cells were CD38⁺ and 78.6% HLA-DR⁺. Virtually all CD8⁺ cells in the 5% human AB serum-exposed line expressed both CD38 (98.1%) and HLA-DR (95.5%).

Plasma versus Serum in the Generation of HIV-Specific Cytotoxic T Cells

The three sources of sera (FCS, human AB, and autologous human) were roughly comparable in their ability to support the growth of antigen-specific CTL. However, when plasma and sera derived from the same source were directly compared in cell lines from subjects 169 and 307, there was substantially more antiviral CTL activity by the sera-exposed T cells (Table I). Both subjects' cell lines were grown in autologous plasma and sera. For subject 169, there was substantial cytolysis of env-expressing targets only in the cell line grown in serum. This result was found for both autologous and allogeneic sources of serum. The 307 cell line grown in serum had about twice the HIV-specific cytotoxicity as the line grown in plasma: 39% vs. 14% against env-expressing targets (after subtracting the lacZ background), 25% vs. 13% against RT; and 21% vs. 12% against gag. However, the plasma-supplemented line maintained substantially higher levels of CD4⁺ T cells than the other conditions tested. On day 21, the plasma supplemented line had 16.3% CD4⁺ and 75.4% CD8⁺ cells, whereas the 5% autologous serum-supplemented line had 5.6% CD4⁺ and 87.8% CD8⁺ cells. The differences in the percentage of CD8⁺ cells in these lines will translate into slight differences in effective E:T ratios, and may therefore contribute somewhat to the lower lysis seen. This increase in the persistence of CD4⁺ cells with plasma was also seen in a second subject (143), whose plasma-supplemented line contained 42% CD4⁺ cells on day 21, whereas the 5% human AB serum-supplemented line contained 9% CD4⁺ and 82% CD8⁺ cells.

DISCUSSION

AIM V medium by itself fails to support the efficient outgrowth of specific antiviral CTL from mitogen-stimulated PBMC from HIV-seropositive patients. T cells grown from the PBMC of one donor in another serum-free medium, X-VIVO 20, also failed to develop HIV-specific cytotoxicity. However, the addition of as little as 1% autologous plasma or serum supports the ex vivo development of antiviral CTL. We and others have previously shown that anti-HIV cytotoxicity is predominantly MHC-restricted and TcR-mediated.[13,19,23] Serum-free media did support the development of nonspecific cytotoxicity (data not shown) and vigorous cell growth.

The early preclinical and clinical studies of LAK cell generation were performed using serum-supplemented media.[15,20] But AIM V medium has also been used to generate LAK cells successfully and to generate specific CTL against EBV-transformed B-LCL and melanoma and renal cell tumors.[4,5,7,11,17,24] This apparent contradiction with our data can be partially explained by the fact that LAK activity represents a mix of cytolytic activity by several distinct cell types, triggered predominantly in an MHC-unrestricted manner by non-TcR cell-surface receptors. LAK cell lines are short-term cultures (3 to 7 days) in which pharmacological levels of IL-2 are used

to activate both natural killer (NK) ($CD16^+$, $CD3^-$) cell activity as well as LAK-like activity in a subset of $CD3^+$ and $CD8^+$ T cells. These cells can lyse a wide range of targets in vitro such as autologous fresh tumor cells, NK-resistant and -sensitive cultured tumor cells, and allogeneic blasts. They are also effective at antibody-directed cellular cytotoxicity.

In some circumstances, however, it has been possible in serum-free media to generate $CD8^+$ T cell lines and clones which lyse immortalized cell lines and tumors via TcR engagement.[4,17,24] We do not know whether serum is required for antigen-specific CTL generation from the circulating lymphocytes of HIV-seronegative individuals. In preliminary experiments, serum was required to generate a secondary CTL response to a viral peptide antigen from normal donor PBMC, but was not required to generate an in vitro CTL response to autologous B-LCL. These results suggest that the choice of APCs or cytokines in the medium may play a role in whether or not serum is required for ex vivo expansion.

HIV-infected subjects differ from uninfected subjects in that they have an unusually high frequency of circulating $CD8^+$ T cells that express cell surface HLA-DR and/or CD38 as well as diminished numbers of functioning helper $CD4^+$ cells.[8,22] Circulating $CD8^+$ cells also express significantly lower levels of CD28 compared to those from HIV-seronegative individuals.[21] Increased expression of CD38 and DR, together with loss of CD28 cell-surface staining, occur when $CD8^+$ cells become activated CTL. Giorgi has noted a worse prognosis in HIV-infected subjects who have a high frequency of circulating $CD38^+$ $CD8^+$ T cells.[8] These differences in the starting cell populations may mean that the conditions required for ex vivo expansion of T cells from HIV-infected subjects may differ from those required for growing cell lines derived from healthy, uninfected donors.

The reduced numbers of T cells expressing CD38 and CD57 in cell lines grown in the absence of serum or plasma could be due to the death of specific cell populations or to downregulation of expression of these markers. In any event, T cells bearing these activation markers appear to be the effective antiviral CTL. It is also not clear from this study whether serum might be required to generate functionally active CTL from naive precursor $CD8^+$ cells and/or from memory CTL.

Diminished lysis of some targets by effector cells generated in AIM V media has been previously reported. Finkelstein and Miller demonstrated that murine LAK cells grown in AIM V media are not as efficient at killing TNP-modified syngeneic ConA blasts as cells generated in media supplemented with 10% head inactivated FCS.[5] However, a 1:1 mix of the serum containing media with AIM V media could completely restore lysis. In a bispecific antibody (BsAb)-directed cellular immunotherapy model, Lamers et al. reported that mixed media, containing 78% RPMI, 20% AIM V, and 2% autologous or pooled human serum, was much more efficient at inducing antibody-directed cytolysis than AIM V alone.[12] These experiments would have detected a combination of both antigen-specific and nonspecific lysis. Therefore, our study is the first clear-cut evidence that a serum factor(s) may be required for antigen-specific CTL generation at least under certain conditions.

We also noted decreased specific killing by cell lines from two subjects generated in plasma compared with those grown in the presence of serum obtained from the same source. This result needs to be verified. We did not find any inhibition of CTL generation by the heparin used to obtain plasma (data not shown). This finding suggests that a factor released by activated platelets during clot formation may stimulate generation of antigen-specific CTL ex vivo. Our study also suggests that plasma may enhance the outgrowth of $CD4^+$ cells in culture, at least in some patients. The possibility of a potential role for activated platelets in directing T cell activation toward a CTL response is intriguing but will require further supportive evidence.

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