Requirements for simian immunodeficiency virus antigen-specific *in vitro* proliferation of T cells from infected rhesus macaques and sooty mangabeys

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The measurement of cell-mediated immunity against the etiologic agent of human AIDS (HIV) in the non-human primate model of AIDS (simian immunodeficiency virus, SIV) has been difficult. In general, culture of peripheral blood mononuclear cells from HIV-1- and SIV-infected humans and monkeys, respectively, with purified inactivated HIV and SIV virus preparations has given inconsistent or negative proliferative responses. However, we describe herein an assay which consists of coculturing monocytes that have been pulsed with inactivated SIV_{smm} with nylon-wool-purified autologous T cells, leading to antigen-specific T-cell proliferation. The proliferative response, which predominantly occurs in CD4+ T cells, is major histocompatibility complex (MHC) class II-restricted and requires antigen processing. This assay will greatly facilitate the identification of the immunodominant epitopes recognized by T cells in sooty mangabeys, which are naturally infected but remain clinically asymptomatic, and in rhesus macaques, in which experimental infection leads to clinical symptomatology similar to human AIDS, eventually resulting in death.

AIDS 1990, 4:399–407

Keywords: AIDS, animal models of human AIDS, cell-mediated immunity in AIDS, SIV_{smm}, non-human primate models of AIDS.

Introduction

As a step toward delineation of T-cell-mediated immunity against the etiologic agent of human AIDS, our laboratory has been conducting studies on non-human primates naturally or experimentally infected with the simian immunodeficiency virus (SIV) [1]. A large majority (> 75%) of the sooty mangabeys (*Cercocebus atys*) at the Yerkes Regional Primate Research Center are naturally infected with SIV (the virus isolated being termed SIV_{smm}) without any apparent pathology or clinical sequelae. Experimental inoculation of 10^4 tissue culture infective dose (TCID) of SIV_{smm} into healthy rhesus macaques (*Macaca mulatta*), however, results in the development of clinical diseases and death, very similar to human AIDS [2]. Thus these two non-human primate species provide a unique opportunity to differentiate protective from pathological immune responses to SIV_{smm}.

Whereas cell-mediated immunity is thought to play a major role in the host defense against viral infection, the study of cell-mediated immunity against HIV and SIV is complicated by the ability of the virus to replicate and cause lysis of CD4+ T cells and monocytes, the two cell types which are integrally involved in the induction and effector function of cell-mediated immunity [3–5]. To address this issue, our laboratory has developed a T-cell proliferation assay in which enriched populations of monocytes are first pulsed with ultraviolet radiation-inactivated SIV_{smm}, washed free of excess virus, and then cocultured with autologous T cells. This procedure overcomes the problems associated with the inhibitory ef-

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Sponsorship: This work was supported by grants NIH-RO1-AI-27057 and NIH-DRR-00165.

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Date of receipt: 15 August 1989; revised: 15 January 1990.

fect of intact virus and/or other contaminants which may be involved in non-specific inhibition in the pathways involved in antigen processing, presentation, and antigen-specific T-cell proliferation. Such an assay provides a useful tool for (1) the fine dissection of the cell subpopulations involved in the immune response during SIV infection; (2) the sequential monitoring of SIV_{smm}-specific T-cell immunity; (3) the processing requirements and the role of antigen-processing cells (APCs) during the course of infection, and (4) the fine comparative analysis of the T-cell SIV-specific response of the two species. In addition, this assay has facilitated the isolation of CD4+ and CD8+ SIV_{smm}-specific cloned T-cell lines from both experimentally infected rhesus macaques and asymptomatic sooty mangabeys, which will provide valuable reagents for the delineation of the immunodominant epitopes of the SIV_{smm} that induce T-cell immunity in these two species.

Materials and methods

Animals

Adult rhesus macaques (*Macaca mulatta*) and sooty mangabeys (*Cercocebus atys*) housed in individual cages or in open pens at the Yerkes Regional Primate Research Center were the source of peripheral blood samples utilized during the course of this study.

All animals used in this study were maintained in accordance with the instructions of the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, and the Department of Health and Human Services guidelines, 'Guide for the Care and Use of Laboratory Animals'. All animals used in this study were free of retrovirus D infection and were seronegative for simian T-cell leukemia virus type I (STLV-I) at the time of these studies,

Media

Media used throughout these studies consisted of RPMI-1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mmol/l L-glutamine and 10% heat-inactivated (56°C, 30 min) fetal calf serum. For cloning purposes, the medium was supplemented with 1000 U/ml of recombinant interleukin 2 (r-IL2) (Hoffman–La Roche, Nutley, New Jersey, USA).

Antigens, mitogens and reagents

SIV_{smm} was originally isolated from asymptomatic sooty mangabeys [1] by coculture with human phytohemagglutinin P (PHA-P) blasts. A large pool of the SIV_{smm} was either grown in the HTC cell line by our laboratory or purchased commercially by contract (Advanced Biotechnologies, Silver Spring, Maryland, USA). The pooled 2000× concentrated SIV_{smm} preparation contained 5×10^{10} virus particles/ml and was aliquoted in NUNC vials in 1 ml phosphate-buffered saline (PBS), pH 7.4, and stored at −196°C until use. The virus preparation was inactivated by two sequential exposures to a germicidal ultraviolet lamp (Sylvania Electric Products, Danvers, Massachusetts, USA) for 2 min at a distance of 3 cm from the lamp in a laminar flow hood, followed by the addition of 1% final concentration of psoralen in dimethylsulfoxide (DMSO; Sigma Chemical Company, St Louis, Missouri, USA) according to the procedure described by Hanson *et al.* [6]. Instead of psoralen, β-propionolactone at a final concentration of 0.015% can be utilized for inactivation of SIV as described previously [7] without affecting the antigenicity of the SIV preparation as measured by the *in vitro* proliferation assay. The effectiveness of the inactivation process was assessed by adding inactivated virus to cells in tissue culture and measuring reverse transcriptase (RT) activity. Protein content was determined using the Biorad (Richmond, California, USA) protein estimation kit. Two different strains of cytomegalovirus (CMV) were used. One consisted of the CMV strain and the other a rhesus CMV isolate (obtained courtesy of Dr T. McGraw, University of California, Davis, California, USA). Both the viruses were grown in large batches in the human WI-38 cell line, pooled, and shown to contain approximately 2×10^8 $TCID_{50}$ CMV/ml. The virus was aliquoted and stored at −196°C until use. CMV was also inactivated prior to use by a procedure similar to that described for SIV_{smm} above.

The mitogens concanavalin A and PHA-P were purchased from Sigma Chemical Company (St Louis, Missouri, USA) and Difco Laboratories (Detroit, Michigan, USA), respectively. Pokeweed mitogen was purchased from Gibco Laboratories (Grand Island, New York, USA). Paraformaldehyde and the inhibitors leupeptin and chloroquine were purchased from Sigma. Tetanus toxoid was obtained from Massachusetts Biological Laboratories (Boston, Massachusetts, USA) and contained 0.33 mg protein/ml or 420 LFU/ml.

Isolation of peripheral blood mononuclear cells and preparation of APC-, CD4+, and CD8+-enriched T-cell subpopulations

T cells were purified from Percoll gradient-purified PBMCs by passage over a nylon wool column as previously described [8]. The APCs were obtained by plating an aliquot of the PBMCs at a concentration of 5×10^6/ml in 7 × 7 cm polystyrene Petri dishes (Corning Glass, New York, USA). The plates were incubated for 2 h at 37°C in a 7% CO_2 humidified atmosphere, and the non-adherent cells were removed by careful aspiration followed by the addition and removal of two cycles of 5 ml medium. The adherent cells remaining in the plate were removed by the addition of 1 mmol/l ethylenediaminetetraacetic acid (EDTA) in PBS and placement of the Petri plate on ice for 20 min followed by use of a sterile cell scraper. The adherent cells (APCs) were washed twice with medium prior to use.

Enriched populations of CD4+ and CD8+ T cells were obtained by treatment of nylon-wool-purified T cells with monoclonal anti-CD4 (Leu3a) and anti-CD8 (Leu2a) antibodies (Becton-Dickinson, Mountain View, California,

USA) followed by rosette depletion with ox red blood cells conjugated with affinity-purified goat anti-mouse immunoglobulin as described by Mayer *et al.* [9] and by Young and Lehner [10]. Flow microfluorometric analysis of an aliquot of these enriched populations of CD4+ and CD8+ T cells using fluorescein-conjugated Leu3a and Leu2a reagents showed that these cells were < 95% pure subpopulations. Typically, 20 ml heparinized blood yielded 5–8 × 10^7 mononuclear cells from which nylon-wool-purified T cells, CD4+- and CD8+-enriched T cells and APCs were prepared for use in the proliferation assay.

Proliferation assay

For the standard assay, APCs prepared as described above were adjusted to 2 × 10^6 viable cells/ml and 0.05 ml dispensed into each well of a 96-well flat-bottomed microtiter plate. To each well was added 0.1 ml medium containing 10 µg ultraviolet and psoralen-inactivated SIV_{smm} or CMV (except for dose–response studies). Controls received medium alone. The microtiter plates were then incubated for 3 h at 37°C in a 7% CO_2 humidified atmosphere. Subsequently, the APCs were washed with medium four times using a 12-channel multipipetter. The nylon-wool-purified T cells or enriched populations of CD4+ and CD8+ cells were adjusted to 4 × 10^6 viable cells/ml and 0.1 ml dispensed into appropriate wells of the microtiter plates. Cultures were performed in triplicate. The microtiter plates were then incubated for 6 days at 37°C in a 7% CO_2 humidified atmosphere, and 16 h prior to harvest each well was pulsed with 1 µCi methyl-^{3}H-thymidine (specific activity 2 Ci/mmol, New England Nuclear, Boston, Massachusetts, USA) in 0.02 ml medium. The details of the mitogen-induced proliferation assay and the allogeneic mixed lymphocyte culture assays have been published elsewhere.

Cloning of SIV-specific CD4+ and CD8+ T cell lines

Nylon-wool-purified T cells from appropriate animals were cocultured with autologous APCs (4 × 10^6/ml T cells and 1 × 10^6/ml APCs) pulsed with inactivated SIV_{smm} (100 µg/ml). The cultures were incubated for 7 days at 37°C in a 7% CO_2 humidified atmosphere. After incubation, the cells were washed, layered over a Ficoll-Hypaque gradient, and centrifuged at 450 **g** for 30 min. The cells at the interface were then subjected to limiting dilution whereby the cell suspension was diluted to contain approximately 0.3 cell/0.1 ml; the suspension was then dispensed into individual wells of a 96-well round-bottomed plate which contained irradiated (25 Gy) 5 × 10^4 autologous PBMCs in a volume of 0.1 ml. The medium consisted of RPMI-1640 supplemented with penicillin, streptomycin, L-glutamine 20% heat-inactivated fetal calf serum, and 1000 units r-IL2/ml. Positively growing cultures were expanded *in vitro* in similar media. Bulk cultures were then obtained and the majority of the cells cryopreserved. An aliquot was then placed in 30 ml flasks with autologous APCs pulsed with SIV_{smm}. A total of 2 × 10^6 cloned T cells were cultured with irradiated (25 Gy) 5 × 10^5 APCs pulsed with 50 µg SIV_{smm}. After 7 days in culture, the cells were washed, layered over Ficoll-Hypaque gradient, centrifuged at 450 **g**, and the cells at the interface collected, washed in medium, and tested for antigen specificity. The 48 h *in vitro* proliferation assay was utilized to test for specificity. The cloned T-cell lines were maintained in medium containing 1000 units r-IL2/ml and restimulated every 14 days with autologous APCs pulsed with inactivated SIV_{smm}. Aliquots of each cloned T-cell line were also subjected to phenotypic analysis using a battery of monoclonal antisera and flow microfluorometry.

Antisera

Monoclonal anti-MHC class I monomorphic antiserum (clone W6/32) was purchased from Serotec Ltd (Indianapolis, Indiana, USA) and monoclonal anti-MHC class II monomorphic antiserum was produced in our laboratory (clone L-243), obtained from the American Type Culture Collection (ATCC; Rockville, Maryland, USA). Other monoclonal antisera were purchased from Becton-Dickinson, (Mountain View, California, USA). Normal mouse serum was obtained from Balb/c mice from our animal colony at Emory University. The mouse serum was absorbed with human PBMCs and kaolin and passed through a 0.22 µm millipore filter prior to use.

Results

Specificity of the *in vitro* proliferative response of PBMCs from rhesus macaques and sooty mangabeys

A series of preliminary experiments was performed to establish optimum culture conditions, dose of virus, and cell concentrations. Data from these initial studies showed that the maximum proliferative response of nylon-wool-purified T cells cocultured with autologous APCs occurred between days 5 and 6 of culture. The optimal ratio of T cells and APCs was determined to be 4 × 10^5 and 1 × 10^5 cells, respectively, in a total volume of 0.2 ml. The optimum concentration of ultraviolet light- and psoralen-treated virus preparations used for pulsing of autologous APCs that gave maximum proliferative responses ranged between 5 and 10 µg/culture. Such values were used throughout these studies unless otherwise noted.

The specificity of these assays was established utilizing nylon-wool-purified T cells and autologous APCs from PBMCs of three uninfected rhesus macaques, three rhesus macaques that were experimentally infected with SIV_{smm}, three SIV_{smm} virus-negative and seronegative sooty mangabeys, and three SIV_{smm} virus-positive and seropositive sooty mangabeys. All animals were asymptomatic at the time of this study. As seen in Fig. 1, while T cells from all the animals responded to CMV-pulsed autologous APCs and to allogeneic cells in MLR, only T cells from the SIV_{smm}-infected rhesus macaques and sooty mangabeys showed proliferation with autologous APCs pulsed with SIV_{smm}. These data demonstrate that only T cells from SIV_{smm}-infected animals proliferate in response to autologous APCs pulsed with SIV_{smm}. The inability of T cells from uninfected or SIV_{smm}-seronega-

tive animals to respond to autologous APCs pulsed with SIV_{smm} was not secondary to defective T cells, since these T cells do respond to autologous APCs pulsed with CMV, autologous cells and polyclonal mitogens. Of interest is our consistent observation that the magnitude of the proliferative response by T cells from SIV-positive sooty mangabeys to autologous APCs pulsed with SIV_{smm}, CMV or allogeneic cells was always lower than similar responses by T cells from the rhesus macaques experimentally infected with SIV_{smm}. The response of T cells from both species to polyclonal mitogens, however, was similar (data not shown). In addition, the T-cell response from these two species to r-IL2 *in vitro* was also very similar (data not shown). These data suggest that the lower response of T cells to autologous APCs pulsed with SIV_{smm}, CMV or allogeneic cells is not secondary to an intrinsic defect of T cells from sooty mangabeys to proliferate *in vitro*. Furthermore, the difference was not secondary to kinetics of the proliferative response (day of harvest), or to the dose of viral antigens used for pulsing, or to cell concentrations of T cells and APCs. The reasons for this difference are not clear at present. However, it could reflect the differences in the secretion of other lymphokines and co-stimulator molecules in such an assay system.

Both CD4 + and CD8 + T cells proliferate in response to autologous APCs pulsed with SIV_{smm} and CMV

Nylon-wool-purified T cells and enriched populations of CD4+ and CD8+ cells from three uninfected rhesus macaques, three SIV_{smm}-infected rhesus macaques, three virus-negative and seronegative sooty mangabeys, and three SIV_{smm} virus-positive and seropositive sooty mangabeys were assayed for their proliferative response to autologous APCs pulsed with varying doses (0.1, 1.0, 5, 10, and 25 μg/10^5 APCs) of ultraviolet and psoralen-inactivated SIV_{smm} or CMV. Maximum proliferative response was obtained with the use of 10 μg of the virus preparation used for pulsing the autologous APCs; hence, for brevity, only the data obtained with this concentration are shown in Fig. 2. As can be seen, whereas unseparated T cells and enriched populations of CD4+ and CD8+ T cells from both SIV_{smm}-positive and -negative animals responded to autologous APCs pulsed with CMV, the proliferative response to autologous APCs pulsed with SIV_{smm} was seen only with unseparated and enriched populations of CD4+ and CD8+ T cells from SIV_{smm}-infected

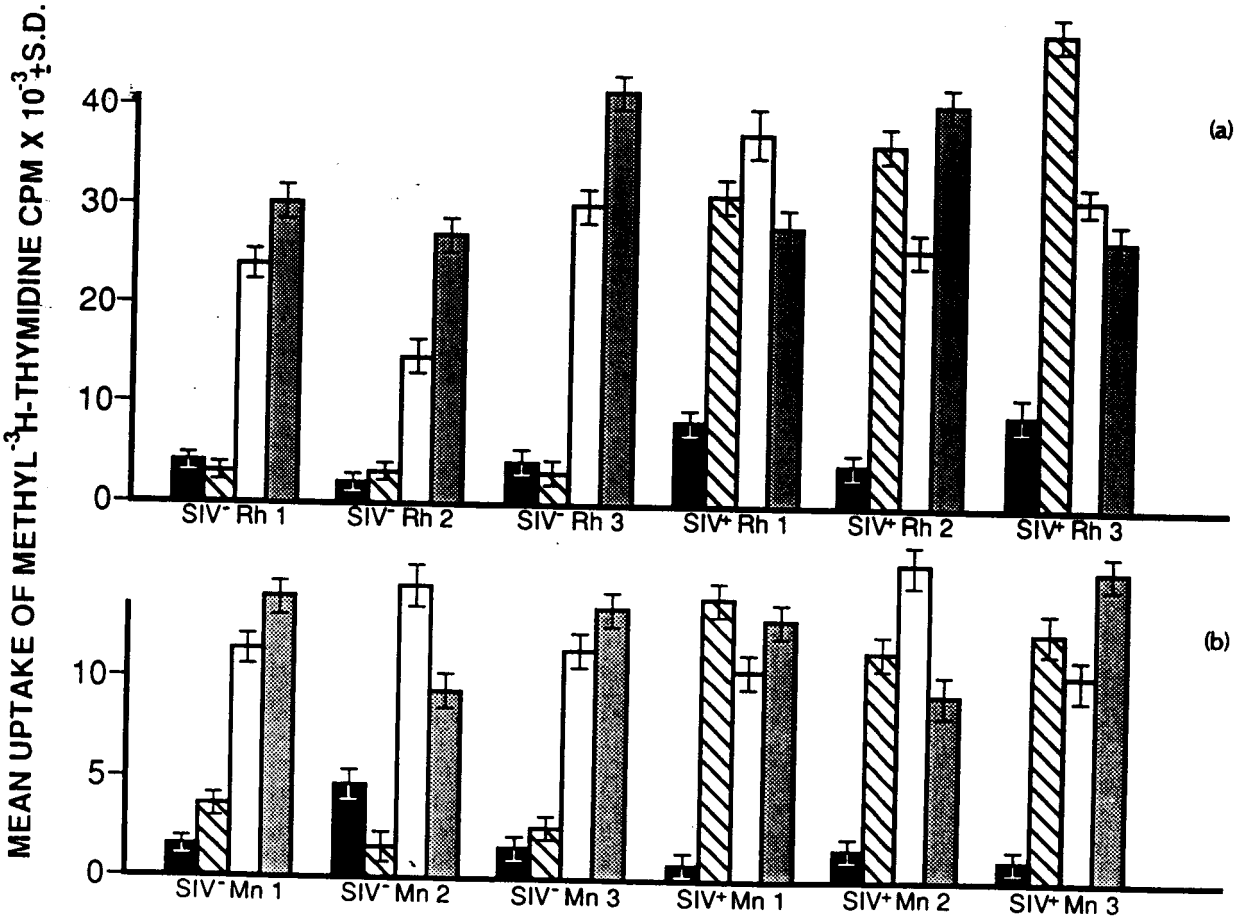

Fig. 1. Specificity of the *in vitro* response of nylon-wool-purified T cells cocultured with autologous antigen-presenting cells (APCs; 4 × 10^5 T cells and 1 × 10^5 APCs per culture) pulsed with control medium (■), 10 μg simian immunodeficiency virus (SIV_{smm}) per culture (▨), or 10 μg rhesus cytomegalovirus (CMV-Rh) per culture (□). In addition, T cells were cocultured with allogeneic peripheral blood mononuclear cells (□). Cultures were incubated in microtiter plates for 6 days and proliferation measured as described in Materials and methods. (a) Response of three uninfected rhesus macaques (SIV− Rh1, 2, and 3) and three rhesus macaques experimentally infected with SIV_{smm} (SIV+ Rh1, 2, and 3) for periods of 15, 18, and 20 months. (b) Response of three SIV virus-negative and seronegative (SIV− Mn1, 2, and 3) and three SIV virus-positive and seropositive sooty mangabeys (SIV+ Mn1, 2, and 3).

Table 1. Effect of antibodies against monomorphic major histocompatibility complex (MHC) class I and class II antigens on the *in vitro* proliferative responses of nylon-wool-purified T cells to autologous, antigen-presenting cells (APCs) pulsed with simian immunodeficency virus (SIV_{smm}) or cytomegalovirus (CMV)*.

Source of T cells	Addition of	Uptake of ^{3}H-TdR (mean c.p.m. ± s.e.m.) × 10^{-3}			
		APCs with SIV_{smm}	% Inhibition†	APCs with CMV	% Inhibition†
SIV-infected rhesus 1					
	Medium	15.4 ± 2.1	–	21.4 ± 1.6	–
	Anti-class I	11.3 ± 0.8	26.3	15.3 ± 0.7	28.7
	Anti-class II	3.4 ± 1.2	78.1	7.2 ± 1.0	65.2
	NMS	16.4 ± 1.8	−6.9	18.5 ± 2.1	13.7
SIV-infected rhesus 2					
	Medium	24.7 ± 1.7	–	32.7 ± 2.7	–
	Anti-class I	16.3 ± 2.0	34.0	21.4 ± 3.0	34.6
	Anti-class II	6.3 ± 0.5	74.7	14.6 ± 0.9	55.3
	NMS	20.3 ± 1.9	17.8	26.9 ± 3.1	17.4
SIV-positive mangabey 1					
	Medium	9.7 ± 0.5	–	13.2 ± 0.6	–
	Anti-class I	8.9 ± 0.9	7.6	9.6 ± 0.5	17.2
	Anti-class II	3.3 ± 0.7	66.3	4.3 ± 0.3	67.8
	NMS	7.9 ± 0.6	18.8	12.3 ± 0.7	6.9
SIV-positive mangabey 2					
	Medium	7.4 ± 0.4	–	11.4 ± 0.9	–
	Anti-class I	6.0 ± 0.4	18.4	7.8 ± 0.6	31.4
	Anti-class II	3.1 ± 0.2	57.5	4.2 ± 0.3	63.6
	NMS	7.0 ± 0.7	5.7	9.3 ± 0.5	19.4

*Nylon-wool-purified T cells (4 × 10^5/culture) were cocultured with autologous APCs (1 × 10^5/culture) that were pulsed with 10 µg of either SIV/SMM or CMV; 10 µl of undiluted monomorphic MHC class I, 10 µl of undiluted MHC class II antiserum, 10 µl of normal mouse serum (NMS), or 10 µl of medium was then added to each appropriate culture and the cultures harvested on day 6. T cells and APCs were obtained from two rhesus macaques that were experimentally infected with SIV/SMM for periods of 17 and 21 months and two adult sooty mangabeys that were SIV/SMM-seropositive, as determined by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. †% Inhibition was calculated as follows: 1 − (mean c.p.m. of cultures receiving antiserum)/(mean c.p.m. of cultures receiving medium) × 100. ^{3}H-TdR, methyl-^{3}H-thymidine.

Table 2. Effect of incubation temperature for the pulsing of antigen-presenting cells (APCs) and the effect of paraformaldehyde fixation (PF) of APCs prior and post pulsing with simian immunodeficiency virus (SIV_{smm}) or cytomegalovirus (CMV).

Experiment	T cells cocultured with autologous APCs pulsed with	Temperature of incubation	Uptake of ^{3}H-TdR (mean c.p.m. ± s.e.m.) × 10^{-3}	
			Rhesus	Mangabey
1*	Medium	4°C	0.4 ± 0.06	0.2 ± 0.05
	SIV_{smm}	4°C	2.3 ± 0.07	0.4 ± 0.07
	CMV	4°C	1.8 ± 0.4	0.8 ± 0.1
	Medium	37°C	0.2 ± 0.05	0.6 ± 0.05
	SIV_{smm}	37°C	28.1 ± 1.3	8.0 ± 0.5
	CMV	37°C	34.7 ± 4.1	13.0 ± 1.0
2†	Medium + 1% PF prior		1.8 ± 0.4	0.4 ± 0.1
	SIV_{smm} + 1% PF prior		2.3 ± 0.3	0.6 ± 0.07
	CMV + 1% PF prior		1.1 ± 0.2	0.7 ± 0.09
	Medium + 1% PF prior		1.0 ± 0.06	0.3 ± 0.08
	SIV_{smm} + 1% PF prior		19.3 ± 0.9	0.6 ± 0.09
	CMV + 1% PF prior		10.5 ± 0.8	0.9 ± 0.1
	SIV_{smm}		28.9 ± 1.5	8.9 ± 0.5
	CMV		34.7 ± 5.2	14.7 ± 0.9

*APCs were pulsed with medium (control), 10 µg SIV_{smm} or 10 µg CMV at 4°C or 37°for 3 h, washed, then cocultured with autologous T cells (1 × 10^5 APCs with 4 × 10^5 T cells) from a rhesus macaque experimentally infected with SIV/SMM and a virus-positive, seropositive sooty mangabey naturally infected with SIV/SMM. †APCs from an experimentally infected rhesus macaque and a virus-positive, seropositive sooty mangabey were treated with 1% fresh paraformaldehyde for 5 min prior to pulsing or after 3 h of pulsing at 37°C with medium (control), 10 µg SIV/SMM or 10 µg CMV. Subsequently, the APCs were washed and cocultured with autologous T cells (1 × 10^5 APCs with 4 × 10^5 T cells). ^{3}H-TdR, methyl-^{3}H-thymidine.

Table 3. Effect of antigen-processing inhibitors on the ability of antigen-presenting cells (APCs) to present simian immunodeficiency virus (SIV_{smm}) or cytomegalovirus (CMV) to autologous T cells*.

APCs and T cells from	Inhibitor (concentration)	Uptake of ^{3}H-TdR (mean counts/min ± s.e.m.) ACPs pulsed with			
		SIV_{smm}	%Inhibition	CMV	%Inhibition
SIV-negative rhesus 1	Medium	210 ± 10	–	21742 ± 2346	–
	Leupeptin (1 mmol/l)	900 ± 91	–	8621 ± 1597	60.1
	Chloroquine (0.1 mmol/l)	1147 ± 162	–	2261 ± 138	89.6
SIV-positive rhesus 1	Medium	12107 ± 1357	–	18954 ± 926	–
	Leupeptin (1 mmol/l)	3617 ± 61	70.2	4737 ± 532	75.1
	Chloroquine (0.1 mmol/l)	3556 ± 45	70.7	2145 ± 586	88.7
SIV-positive rhesus 2	Medium	7458 ± 914	–	27349 ± 1562	–
	Leupeptin (1 mmol/l)	3446 ± 149	53.8	7948 ± 689	71.0
	Chloroquine (0.1 mmol/l)	2729 ± 304	63.5	5564 ± 733	79.7
SIV-positive mangabey 1	Medium	5327 ± 426	–	9013 ± 699	–
	Leupeptin (1 mmol/l)	1438 ± 159	67.0	5362 ± 701	40.5
	Chloroquine (0.1 mmol/l)	726 ± 88	86.4	3367 ± 765	62.7
SIV-positive mangabey 2	Medium	4951 ± 231	–	8136 ± 432	–
	Leupeptin (1 mmol/l)	1926 ± 435	61.1	4747 ± 361	41.7
	Chloroquine (0.1 mmol/l)	823 ± 54	83.4	3012 ± 732	63.0

*APCs from one uninfected rhesus macaque and two rhesus macaques experimentally infected with SIV_{smm} and two SIV virus-positive seropositive sooty mangabeys were pulsed with either 10 µg inactivated SIV_{smm} or 10 µg CMV per 10^5 cells in the presence of medium (control) leupeptin or chloroquine for 3 h at 37°C in a 7% CO_2 humidified atmoshpere. The APCs were then washed and cocultured with autologous T cells (1×10^5 APCs and 4×10^5 T cells) for a period of 6 days and the proliferative response measured as described in the Materials and methods section. ^{3}H-TdR, methyl-^{3}H-thymidine.

animals. Although both CD4+ and CD8+ T cells proliferate when cocultured with autologous APCs pulsed with either SIV_{smm} or CMV, it is clear that the predominant response was seen with CD4+ T cells in both species. Again, as described above, the relative magnitude of the proliferative response was greater by T cells and CD4+ T cells from rhesus macaques than by T cells and CD4+ T cells from sooty mangabeys. Of interest was the observation that depletion of CD8+ T cells from PBMCs of all three rhesus macaques resulted in an enhanced proliferative response by enriched CD4+ T cells cocultured with autologous APCs pulsed with either SIV_{smm} or CMV. A similar enhancement of the proliferative response was not seen with CD8+ T-cell-depleted, CD4+ T-cell-enriched populations of cells from sooty mangabeys.

Effect of monoclonal antibodies against cell surface antigens on the *in vitro* proliferative response

Nylon-wool-purified T cells from two SIV_{smm}-infected but asymptomatic rhesus macaques and two SIV_{smm}-infected sooty mangabeys were cocultured with autologous APCs pulsed with either SIV_{smm} or CMV. To triplicate cultures of each combination of T cells and antigen-pulsed APCs was added 1, 5, 10 or 20 µl undiluted anti-MHC class I, MHC class II, normal mouse serum (all at 1.0 µg/ml antibody) or medium (control). For the purposes of brevity, only the data with the use of 10 µl of each serum are reported. As seen from Table 1, the addition of 10 µl anti-MHC class II antiserum significantly ($P < 0.01$) inhibited the proliferative response of T cells from both species to autologous APCs pulsed with either SIV_{smm} or CMV. In addition, in most cases significant inhibition was also seen with the addition of 10 µl of anti-MHC class I antiserum but not to the same level as seen with anti-MHC class II antiserum. This pattern of inhibition was not secondary to the dose of anti-MHC class I or class II antisera used.

The proliferative response of T cells to autologous APCs pulsed with SIV_{smm} requires antigen processing

To delineate the processing requirements of the SIV_{smm} by APCs, experiments were performed in which APCs were pulsed at 4° or 37°C. As seen in Table 2, optimum T-cell proliferation from both species was seen with APCs pulsed at 37°C. In addition, treatment of APCs with paraformaldehyde prior to pulsing with SIV_{smm} failed to induce optimal proliferation of autologous T cells. However, significant proliferation was observed with APCs from rhesus macaques that had been treated with paraformaldehyde after pulsing with SIV_{smm} or CMV (Table 2). A similar assay performed with T cells and APCs from SIV-seropositive sooty mangabeys essentially gave similar results except that paraformaldehyde fixing of autologous APCs either prior to or after SIV_{smm} pulse failed to induce significant proliferation of T cells (Table 2). Although the proliferative response of T cells from sooty mangabeys is comparatively weak and thus subject to interpretation, these data suggest that T cells from sooty mangabeys may either additionally require costimulator accessory molecules or predominantly recognize different forms of processed SIV_{smm} and CMV antigens. Experiments performed with antigen-processing inhibitors such as leupeptin and chloroquine showed that treatment of APCs during pulsing with SIV_{smm} or CMV with these inhibitors significantly inhibited the proliferative response of autologous T cells from both rhesus macaques and sooty mangabeys (Table 3). The inhibition of this proliferative response was not secondary to the presence of residual amounts of these inhibitors

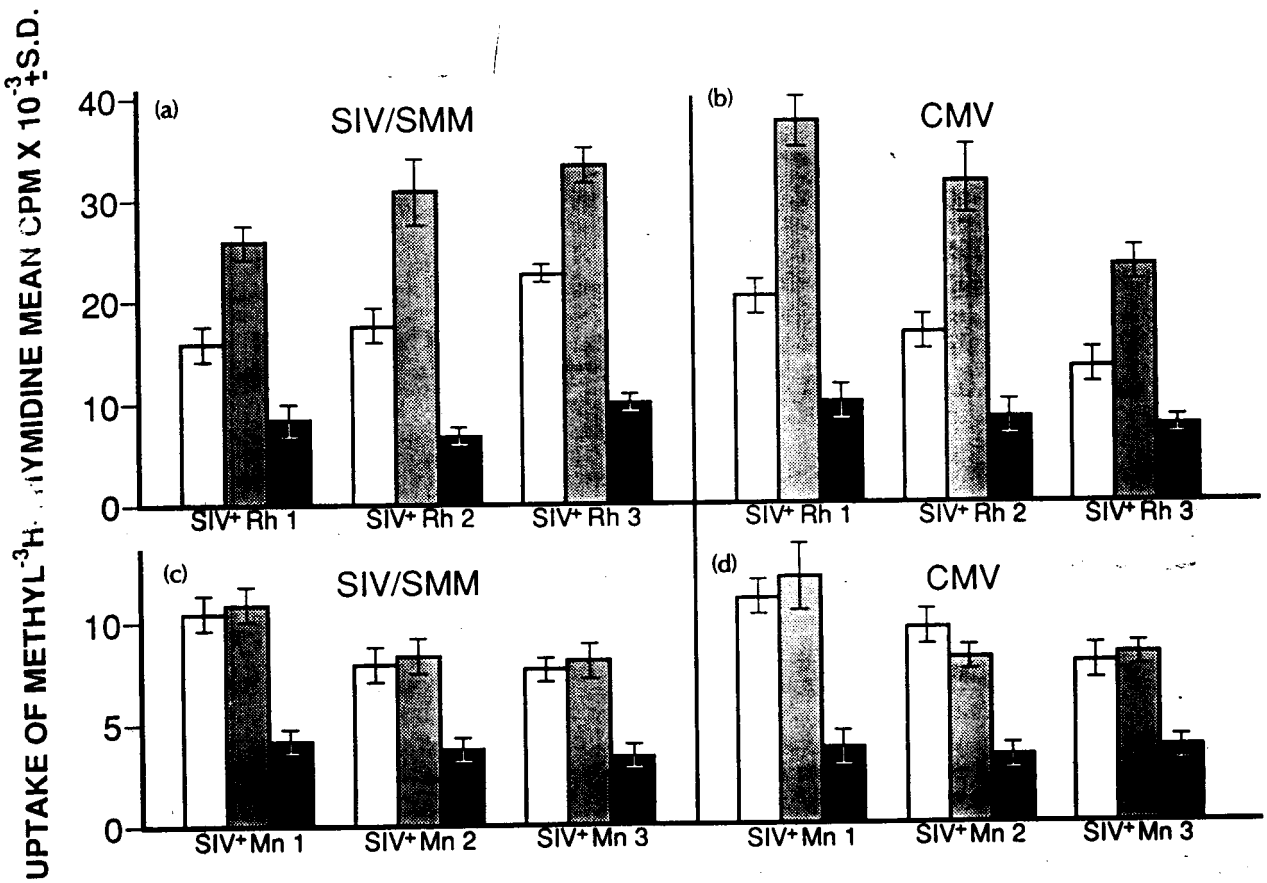

Fig. 2. *In vitro* proliferative response of unseparated nylon-wool-purified T cells (□), enriched CD4+ T cells (□), and enriched CD8+ T cells (■), each cocultured with autologous antigen-presenting cells (APCs) pulsed with 10 μg of ultraviolet- and psoralen-inactivated simian immunodeficiency virus (SIV_{smm}; a and c) or rhesus cytomegalovirus (CMV-Rh; b and d). Each culture consisted of 4×10^5 T cells and 1×10^5 APCs. (a and b) Response of cells from three rhesus macaques (SIV^+RH1, 2, and 3) experimentally infected with SIV_{smm} for periods of 16, 19, and 24 months. Media controls gave mean c.p.m. ± s.d. values of 2146 ± 219, 1930 ± 137, and 848 ± 62 for unfractionated CD4+ and CD8+ cells from Rh1; 2356 ± 337, 1525 ± 62, and 948 ± 91 for similar cells from Rh2; and 3456 ± 437, ± 144, and 1136 ± 54 for similar cells from Rh3, respectively. (a) Response to SIV_{smm}. (b) Response to CMV-Rh. (c, d) Response of cells from three SIV virus-positive, seropositive naturally infected sooty mangabeys (SIV^+, Mn1, 2, and 3). Note: media controls gave mean c.p.m. ± s.d. values of 1210 ± 42, 897 ± 56, and 323 ± 54 for unfractionated CD4+- and CD8+-enriched cells from Mn1; 1013 ± 42, 537 ± 97, and 232 ± 16 for similar cells from Mn2; and 1532 ± 231, 602 ± 48, and 211 ± 41 for similar cells from Mn3, respectively. (c) Response to SIV_{smm}. (d) Response to CMV-Rh.

in the APC cultures. The final wash fluid of the culture after treatment with leupeptin and chloroquine failed to demonstrate inhibition of fresh T cell and APC cultures assayed separately for SIV- or CMV-specific proliferation.

Derivation of SIV_{smm}-specific CD4+ and CD8+ T-cell lines

Nylon-wool-purified T cells from SIV_{smm}-seropositive sooty mangabeys and experimentally infected rhesus macaques were cocultured with autologous APCs pulsed with an optimal dose of SIV_{smm} for a period of 7 days. The cell cultures were then cloned using a limiting dilution assay whereby the cells were diluted to 0.3 cell/well. Positive clones were expanded using media containing r-IL-2 and then restimulated with autologous SIV_{smm}-pulsed APCs. Several T-cell lines were thus prepared and examined for antigen specificity and cell surface phenotype. As seen in Table 4, two CD4+ and one CD8+ SIV_{smm}-specific T-cell lines have been established from a rhesus macaque. In addition, one CD4+ and two CD8+ T cell lines specific for SIV_{smm} have been established from naturally infected sooty mangabeys. These cell lines are specific for SIV_{smm} since proliferative responses were seen only when they were cocultured with autologous APCs pulsed with SIV_{smm} but not with CMV or tetanus toxoid. These T-cell lines will be valuable as reagents to examine differences, if any, that may exist between the epitopes of SIV_{smm} that are recognized by experimentally infected rhesus macaques and naturally infected sooty mangabeys.

Discussion

The data presented in this communication demonstrate the presence of *in vitro* T-cell proliferative responses to ultraviolet light and psoralen-inactivated SIV_{smm} presented by autologous APCs. The proliferative responses are antigen-specific in that T cells only from SIV_{smm}-infected rhesus macaques and SIV_{smm}-seropositive sooty mangabeys proliferated *in vitro*. This failure to proliferate by cells from uninfected monkeys was not sec-

Table 4. Specificity of CD4+ and CD8+ cloned T-cell lines from simian immunodeficiency virus (SIV_{smm})-infected rhesus macaques and naturally infected sooty mangabeys*.

T cell line	Species†	Phenotype	Uptake of ^{3}H-TdR (mean c.p.m. ± s.e.m.)			
			Medium	SIV	CMV	TT
EU87-SIV-003	Rhesus	CD4+	323 ± 44	46411 ± 2337	478 ± 24	513 ± 119
EU87-SIV-007	Rhesus	CD4+	237 ± 26	28924 ± 1319	354 ± 44	391 ± 72
EU87-SIV-0012	Mangabey	CD4+	412 ± 35	33414 ± 1566	397 ± 52	736 ± 66
EU87-SIV-004	Rhesus	CD8+	1014 ± 59	14362 ± 736	1225 ± 103	2022 ± 217
EU87-SIV-009	Mangabey	CD8+	298 ± 32	21298 ± 904	416 ± 59	518 ± 60
EU87-SIV-010	Mangabey	CD8+	256 ± 27	9414 ± 331	381 ± 26	564 ± 74

*The T-cell lines were cultured with autologuos antigen-presenting cells (APCs; 2×10^5 T cells and 5×10^4 APCs) pulsed with either medium (control), SIV_{smm}(10 µg/culture), cytomegalovirus (CMV; 10 µg/culture) or tetanus toxoid (TT; 5 µg/culture) for a period of 48 h. The cultures were harvested after an 8 h pulse with 1 µCi methyl-^{3}H-thymidine (^{3}H-TdR) and the mean counts/min of triplicate cultures calculated. †Rhesus, cell lines derived from rhesus macaques; mangabey, cell lines derived from sooty mangabeys.

ondary to defective T cells since aliquots of the same T cells proliferated when cocultured with autologous APCs pulsed with CMV or when cultured with non-specific mitogens and allogeneic cells. These results provide compelling evidence for physiologically relevant cell-mediated immunity during SIV infection. The finding that inhibitors of antigen processing such as leupeptin and chloroquine were effective in inhibiting the appropriate presentation of SIV_{smm} to T cells from SIV_{smm}-infected monkeys (see Table 3) suggests that the T-cell responses being measured were predominantly against processed forms of SIV_{smm}. The observation that autologous APCs from sooty mangabeys treated with 1% paraformaldehyde prior to pulsing with SIV or CMV do not present the appropriate antigens is not unexpected. However, the ability of autologous APCs that had been treated with 1% paraformaldehyde after pulsing with SIV or CMV to present antigens to T cells from rhesus macaques raises some interesting questions. These data may be interpreted to indicate that T cells from sooty mangabeys naturally infected with SIV_{smm} may recognize forms of processed SIV_{smm} antigens different from those recognized by T cells from rhesus macaques experimentally infected with SIV_{smm}. On the other hand, perhaps APCs from sooty mangabeys process SIV_{smm} peptides in a manner distinct from that of rhesus macaques. Finally, it is also quite possible that different pathways of T-cell activation occur in the two species. Further studies are needed to address this issue.

The consistent differences in the magnitude of the proliferative responses of T cells and isolated enriched populations of CD4+ T cells cocultured with either SIV_{smm}- or CMV-pulsed autologous T cells from these two species warrants discussion. It was reasoned that such lower responses of T cells from sooty mangabeys may be secondary to a higher relative frequency of recirculating CD8+ T cells in the peripheral blood of this species [11], which, in contrast to CD4+ T cells, are known to proliferate to a lesser extent. It is quite possible that chronic SIV_{smm} infection of sooty mangabeys leads to a higher frequency of virus-infected CD4+ T cells as compared with experimental infection of rhesus macaques. Such increased virus replication may result in lysis of the infected cells and thus lead to a decrease of CD4+ T cells from PBMCs of spoty mangabeys in our assay system. On the other hand, it is possible that such differences are either secondary to autologous cytolytic T lymphocyte activity in cultures of CD4+ T cells from sooty mangabeys, as described with human T cells by Siliciano *et al.* [12], or due to specific requirements of other cofactors not efficiently being synthesized or secreted by CD4+ T cells and/or APCs from sooty mangabeys. Studies are currently under way to address this issue.

A small antigen-specific proliferative response was observed with CD8+ T cells. This response was apparently MHC class I-restricted. It is not clear whether this represents minor access of endocytosed antigen to the MHC class I processing pathway compared with a processing-independent response. The isolation of cloned T-cell lines specific for SIV_{smm} from experimentally infected rhesus macaques and naturally infected sooty mangabeys, as shown in Table 4, will provide valuable reagents to ascertain possible differences in the SIV_{smm} epitopes that are recognized by T cells from those two monkey species. It remains to be seen whether these cloned T-cell lines have cytotoxic potential against SIV_{smm}-infected target cells.

In summary, we describe a T-cell proliferation assay which allows for the evaluation of both the monocyte and T-cell contributions to cell-mediated immunity. Such an assay should prove useful in sequentially monitoring the function of these two cell types during the course of SIV infection. In addition, such as assay might help provide clues in determining why sooty mangabeys are chronically infected with SIV, yet remain clinically asymptomatic.

Acknowledgements

The authors are deeply grateful to the staff of the Yerkes Regional Primate Research Center who were extremely helpful in providing us with the appropriate specimens from the monkeys. The authors are grateful to Dr Kirk Ziegler for his critical review of the manuscript and to Ms Cecile Long for her editorial assistance.

References

1. FULTZ PN, MCCLURE HM, ANDERSON DC, *ET AL.*: Isolation of a T-lymphotropic retrovirus from naturally infected sooty mangabey monkeys (*Cercocebus atys*). *Proc Natl Acad Sci USA* 1986, 83:5286–5290.
2. MCCLURE HM, ANDERSON DC, FULTZ PN, *ET AL.*: Spectrum of disease in macaque monkeys chronically infected with SIV_{smm}. *Vet Immunol Immunopathol* 1989, 21:13–24.
3. KANNAGI M, YETZ JM, LETVIN NL: *In vitro* growth characteristics of simian T-lymphotropic virus type III. *Proc Natl Acad Sci USA* 1985, 82:7053–7057.
4. KLATZMANN D, CHAMPAGNE R, CHAMARET S, *ET AL.*: T Lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. *Nature* 1984, 312:767–768.
5. SATTENTAU QJ, CLAPHAM PR, WEISS RA, *ET AL.*: The human and simian immunodeficiency viruses HIV-1, HIV-2 and SIV interact with similar epitopes on their cellular receptor, the CD4 molecule. *AIDS* 1988, 2:101–105.
6. HANSON CV, RIGGS JL, LENETTE EH: Photochemical inactivation of DNA and RNA viruses by psoralen derivatives. *J Gen Virol* 1978, 40:345–358.
7. NISHANIAN PG, OJO-AMAIZE E, WITTENBOGAART CH, *ET AL.*: A simple and rapid method for preparation of HIV expressing targets for functional assays. *J Immunol Methods* 1988, 107:261–271.
8. EISEN SA, WEDNER HJ, PARKER CW: Isolation of pure human peripheral blood T lymphocytes using nylon wool columns. *Immunol Commun* 1972, 1:571–578.
9. MAYER L, FU SM, KUNKEL HG: Rapid generation of T cell hybridomas. *J Immunol Methods* 1985, 81:271–277.
10. YOUNG C, LEHNER TA: A comparative investigation of three methods of separation of CD4 and CD8 cells from human peripheral blood cells. *J Immunol Methods* 1988, 107:31–40.
11. AHMED-ANSARI A, BRODIE AR, FULTZ P, *ET AL.*: Flow microfluorometric analysis of peripheral blood mononuclear cells from primates—correlation of phenotype with immune function. *Am J Primatol* 1989, 17:107–131.
12. SELICIANO RF, LAWTON T, KNALL C: Analysis of host virus interactions in AIDS with anti gp-120 T cell clones: effect of HIV sequence variation and a mechanism for CD4+ cell depletion. *Cell* 1988, 54:561–575.