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Immunology

Generation of CD8 suppressor factor and β chemokines, induced by xenogeneic immunization, in the prevention of simian immunodeficiency virus infection in macaques

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ABSTRACT **Previous xenogeneic immunization experiments in rhesus macaques with simian immunodeficiency virus (SIV) grown in human CD4$^+$ T cells consistently elicited protection from challenge with live SIV. However, the mechanism of protection has not been established. We present evidence that xenogeneic immunization induced significant CD8 suppressor factor, RANTES (regulated upon activation, normal T cell expressed and secreted), macrophage inflammatory protein (MIP) 1α, and MIP-1β ($P < 0.001$ - $P < 0.02$). The concentrations of these increased significantly in protected as compared with infected macaques ($P < 0.001$). Xenogeneic stimulation *in vitro* also up-regulated CD8 suppressor factors (SF; $P < 0.001$) and the β chemokines which were neutralized by antibodies to the 3 β chemokines. Recombinant human RANTES, MIP-1α and MIP-1β which bind to simian CCR5, suppressed SIV replication in a dose-dependent manner, with RANTES being more effective than the other two chemokines. The results suggest that immunization with SIV grown in human CD4$^+$ T cells induces CD8-suppressor factor, RANTES, MIP-1α and MIP-1β which may block CCR5 receptors and prevent the virus from binding and fusion to CD4$^+$ cells.**

Complete protection against live pathogenic simian immunodeficiency virus (SIV) infection in macaques has been consistently achieved by immunization with inactivated SIV grown in human T cells (1–4). These encouraging experiments were discontinued, as protection could be induced exclusively by the human CD4$^+$ T cells in which the SIV was grown without any SIV antigen (5). The mechanism of protection was not elucidated, but human cell surface T cell components (HLA class I and II and CD4) were implicated in the protection induced by immunization with SIV grown in human CD4$^+$ T cells (5–13). However, the demonstration that the β-chemokines RANTES (regulated upon activation, normal T cell expressed and secreted), macrophage inflammatory protein (MIP) 1α, and MIP-1β can inhibit replication of macrophage-tropic HIV-1, HIV-2, or SIV (14) by blocking the CCR5 coreceptors (15–19) has raised an alternative mechanism of protection. We have evidence that immunization with recombinant SIV gp120 and p27 in macaques generates CD8 suppressor factors (SF) (14, 20) and the three β-chemokines that were significantly associated with protection (21). We have therefore examined the possibility that *in vivo* xenoimmunization or *in vitro* mixed leukocyte culture reaction might stimulate CD8$^+$ cells to release CD8-SF and the associated β-chemokines. Indeed, herein we show that *in vitro* stimulation of simian T cells with human CD4$^+$ T cells and *in vivo* immunization with SIV grown in human CD4$^+$ T cells (or the latter only) elicit CD8-SF and the integral β-chemokines RANTES, MIP-1α, and MIP-1β. Furthermore, the immunized macaques showed complete protection when challenged with live SIV, as compared with control macaques.

MATERIALS AND METHODS

Immunization of Macaques. Five groups of a total of 18 rhesus macaques were immunized four times by the intramuscular route at about monthly intervals (21, 22). In group A, 7 macaques were immunized with SIVmac 251(32H) derived from the culture supernatant of infected human C8166 cells and purified by ultracentrifugation and gel exclusion chromatography. The inactivated whole virus vaccine was prepared by the addition of 0.8% formaldehyde to SIV and then prepared in immuno-stimulating complexes (ISCOMs) or with muramyl dipeptide (MDP) as described elsewhere (22, 23). In group B, 4 macaques were immunized with inactivated whole measles virus (MV) grown in Vero cells (African green monkey kidney cell line) and administered in ISCOMs or MDP. In group C, 3 macaques were not immunized; in group D, 3 macaques were immunized with recombinant SIVmac 251 gp120 that was expressed in baculovirus and recombinant SIV p27 that was expressed in pGEX-3X as a glutathione *S*-transferase fusion that was cleaved with factor Xa (24). The macaque (928) in group E was immunized with the human CD4$^+$ T cell line (C8166). All but 4 of the 18 macaques were challenged i.v. with 10 mean infective doses of cell-free SIVmac 251 (32H) grown on C8166 cells, and infection was determined by coculture of peripheral blood lymphocytes on C8166 cells and by PCR (22).

Production and Assay of CD8-SF Activity from Enriched CD8$^+$ T Cells. Peripheral blood mononuclear cells (PBMCs) from the immunized or control macaques were separated *in vitro* and CD8$^+$ cells were enriched by depletion of CD4$^+$ cells, B cells, and monocytes by panning, using OKT4 and antibodies to human Ig. The enriched CD8$^+$ cells were then cultured with phytohemagglutinin (PHA; 10 μg/ml) for 3 days. Preparation of CD8-SF was then carried out as described (20, 21). PHA-stimulated CD8$^+$ cells were cultured at a concentration of 3×10^6 cells per ml in RPMI 1640 medium containing 10% fetal calf serum and 10% human interleukin (IL) 2 preparation

Abbreviations: ISCOMs, immunostimulating complexes; MCP, macrophage chemotactic protein; MIP, macrophage inflammatory protein; MV, measles virus; RANTES, regulated upon activation, normal T cell expressed and secreted; SF, suppressor factors; SIV, simian immunodeficiency virus; PBMC, perpheral blood mononuclear cell; PHA, phytohemagglutinin; IL, interleukin; RT, reverse transcriptase; SI, stimulation indices; MDP, muramyl dipeptide.

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(TLF, Biotest Diagnostics, Danville, NJ). After a 2-day incubation (at 37°C, in 5% CO_2), the culture supernatant was collected, and the cells were reconstituted with fresh medium; this was repeated up to three times. At each passage the cellular density was adjusted to 3×10^6 cells per ml. The collected supernatants were filtered through a 0.45-μm filter and stored at −70°C for the CD8-SF activity assay. For the *in vitro* experiments, 5×10^5 PBMCs per ml, obtained from naive macaques, were cocultured with an equal number of irradiated (25,000 rads; 1 rad = 0.01 Gy) C8166 cells, L cells (transfected with HLA-I and untransfected cells), autologous PBMCs, or in RPMI 1640 medium alone, supplemented with heat-inactivated 10% autologous serum, 2 mM of glutamine, and penicillin (100 units/ml) and streptomycin (100 μg/ml). After 7 days of culture, the viable cells were separated by density-gradient centrifugation with Lympho-Prep (Nycomed, Oslo), $CD8^+$ cells were enriched, and CD8-SF was prepared as described above.

Enriched $CD4^+$ cells (about 95%) were prepared from simian PBMCs by negative selection with mAb to $CD8^+$, as described above. The CD4 cells were stimulated for 3 days with PHA (10 μg/ml) in RPMI containing 10% fetal calf serum. The cells were washed, pelleted (10×10^6 cells), and then incubated with 1 ml of SIV mac 251 J5 molecular clone propagated in simian PBMCs [provided by M. Cranage, Centre for Applied Microbiology and Research (Porton Down, Salisbury, U.K.]; the reverse transcriptase (RT) activity per 10^6 $CD4^+$ cells was 35,000 cpm. To assay the activity of CD8-SF, 100 μl of $CD8^+$ cell culture supernatant diluted at 1:2 and 1:5 was added at the start of the culture to 2×10^5 SIV-infected $CD4^+$ cells in 96-well plates. After incubation for 2 days, 100 μl of the culture fluid was removed and replaced with 100 μl per well of diluted (1:2 or 1:5) $CD8^+$ cell supernatant or control medium. This was repeated every 2 days for up to 14 days, and the RT activity was determined by using the RT detection and quantification method (Quan-T-RT assay kit, Amersham).

In some experiments, the infected $CD4^+$ cells were cocultured with enriched $CD8^+$ cells derived from immunized or unimmunized macaques by using a semipermeable transmembrane device (Costar). In this system, 5×10^5 of $CD8^+$ cells (in 200 μl) were added to the upper chamber of a 6.5-mm-diameter (0.4-μm pore) polycarbonate transwell culture insert. SIV-infected $CD4^+$ cells (5×10^5 cells in 200 μl) were added into 24-well cluster plates (bottom chamber). The cultures were refed at 2- or 3-day intervals with 20% IL-2 medium, and the RT activity in the bottom chamber was assayed on day 7.

Inhibition of SIVmac 251 Replication by Recombinant β Chemokines. To assay the effects of chemokines on SIVmac 251 replication in $CD4^+$ T cells, increasing concentrations (0.25–250 ng/ml) of recombinant RANTES, MIP-1α, MIP-1β, and macrophage chemotactic protein (MCP) 1 were added to CD 4^+ cells (1×10^6 cells per 100 μl) 30 min before infection. After infection, $CD4^+$ cells (2×10^5 cells per well) were plated on to 96-well plates and cultured in 20% IL-2 medium containing the same doses of chemokines. The cells were refed with fresh culture medium and the supernatant was assayed for the RT activity.

Chemokine Assay. RANTES, MIP-1α, MIP-1β, MCP-1, or IL-8 was assayed in the $CD8^+$ cell culture supernatants, generated by *in vivo* immunization, by xenogeneic HLA class I-transfected L cells, or by autologous or PHA stimulation, by using the specific ELISA kits (R & D Systems). Optimum conditions were established with the $CD8^+$ cell culture supernatant diluted 1:8 and all the results were corrected for the dilution factor and presented in pg/ml.

Lymphoproliferative Assay. The lymphocyte proliferative response to xenogeneic or autologous stimulation was carried out by one-way stimulation. Aliquots of 2×10^5 PBMCs were plated into 96-well plates and stimulated with an equal number of irradiated C8166 or autologous PBMCs or 5×10^4 transfected L cells for 7 days. In addition, 2×10^5 PBMCs from the same macaques were stimulated with PHA (10 μg/ml) for 3 days. Twenty-four hours before the cultures were terminated, [^{3}H]thymidine (1 μCi per well; 1 Ci = 37 GBq) was added, the cells were harvested onto filter papers discs, and the [^{3}H]thymidine uptake was determined by scintillation counting of β emissions. Stimulation indices (SI) were calculated by the ratio of counts in stimulated and unstimulated cells.

Neutralization of the CD8-SF Activity with Anti-Chemokine Antiserum. CD8-SF was induced by xenogeneic stimulation with irradiated C8166 cells of $CD8^+$ cells derived from naive macaques or by PHA stimulation of primed $CD8^+$ cells derived from macaques immunized with SIV gp120/p27 by the targeted iliac lymph node route (21). The $CD8^+$ cell supernatant was pretreated with goat polyclonal antibodies (purchased from R & D Systems) to RANTES (200 μg/ml) and MIP-1α or MIP-1β (100 μg/ml) for 30 min at room temperature and was then added to SIV-infected simian $CD4^+$ cells. The CD8-SF-containing supernatant, treated or untreated with the antibodies, was replaced every 2 days, and after 7 days the RT activity was assayed. Normal goat serum IgG (200 μg/ml) was used as a control antibody. The results were expressed as percent neutralization = 1 − (cpm of control − cpm of treated supernatant)/(cpm of control − cpm of untreated supernatant) × 100.

RESULTS

Production of Suppressor Factor and β Chemokines by $CD8^+$ T Cells Derived from Macaques Immunized with SIV Grown in Human Cells. The possibility was examined that xenogeneic immunization elicits CD8-SF and β-chemokines (14, 20, 21). Significantly greater CD8-SF was produced by PBMCs of macaques immunized with SIV grown in human cells (Table 1, group A; 77.1 ± 8.5%) than those (group D) immunized i.m. with recombinant SIVgp120 and p27 in alum (44.7 ± 1.9%) that was free of human cellular antigens ($P < 0.01$). To exclude the possibility that ISCOMs or MDP may have been responsible for eliciting the CD8-SF, MV grown in Vero cells and prepared in ISCOMs or in MDP was used to immunize macaques by the i.m. route. PBMCs from these (group B) and the three unimmunized macaques (group C) generated very little CD8-SF (20.3 ± 2.9% and 26.1 ± 3.7%, respectively), which was significantly less than that elicited by i.m. immunization with SIV grown in human cells ($P < 0.001$). Hence, a very significant increase in CD8-SF was found in the protected macaques immunized *in vivo* with SIV grown in the human $CD4^+$ T cell line (group A), when compared with the infected macaques (groups B and C) immunized with MV or with unimmunized controls ($P < 0.001$).

The three β-chemokines generated by the $CD8^+$-enriched T cells were then assayed in four groups of macaques. The results showed the highest levels of RANTES, MIP-1α, and MIP-1β, but not the control IL-8, in macaques immunized with SIV grown in human $CD4^+$ T cells (Table 1, group A). Significant increases in RANTES ($P < 0.05$ or $P < 0.02$), MIP-1α ($P < 0.001$), and MIP-1β ($P < 0.05$ or $P < 0.02$) were found in macaques immunized with the SIV grown in human T cells, as compared with each of the three other groups, with the exception of MIP-1α in the unimmunized and MIP-1β in the gp120- and p27-immunized macaques (Table 1). Furthermore, a significant increase in RANTES ($t = 2.916$, $P < 0.02$), MIP-1α ($t = 3.655$, $P < 0.01$), and MIP-1β ($t = 2.7065$, $P < 0.02$) was found in the seven protected macaques immunized with the vaccine prepared in human $CD4^+$ T cell (group A) as compared with the seven infected controls. CD8-SF and β-chemokines were also assayed in all macaques after challenge with live SIV but the results showed no significant differences as compared with the prechallenge data (not

Table 1. Production of CD8-SF and β-chemokines from $CD8^+$-enriched T cells in five groups of macaques and the association with protection from SIV infection

Group	CD8-SF, %		Chemokine, pg/ml				Immunity
	1:2	1:5	RANTES	MIP-1α	MIP-1β	IL-8	
A							
IXV SIV-ISCOM	95	55	1,080	2,560	680	928	Protected
1YB SIV-ISCOM	92	17	1,976	3,576	1,472	3,296	Protected
4053 SIV-ISCOM	40	ND	640	1,864	320	5,112	Protected
1XD SIV-MDP	59	18	3,808	4,456	3,024	3,336	Protected
3B SIV-MDP	94	ND	648	2,168	328	1,864	Protected
4083 SIV-MDP	64	48	2,496	2,520	1,936	4,368	Protected
4094 SIV-MDP	96	ND	1,768	2,128	1,424	2,896	Protected
Mean ± SEM	77.1 ± 8.5	34.5 ± 9.9	1,774 ± 429	2,753 ± 351	1,312 ± 360	3,400 ± 399	
	$t = 6.127$*		$t = 2.916$	$t = 3.655$	$t = 2.706$	NS	
	$P < 0.001$		$P < 0.02$*	$P < 0.01$*	$P < 0.02$*		
B							
8791 MV-ISCOM	18	ND	720	248	240	4,416	Infected
8789 MV-ISCOM	26	ND	640	288	240	5,016	Infected
4060 MV-MDP	13	ND	560	392	264	4,312	Infected
1IH MV-MDP	24	ND	560	376	256	3,232	Infected
Means ± SEM	20.3 ± 2.9		620 ± 38	326 ± 35	250 ± 6	4,244 ± 371	
	$t = 6.298$*		$t = 2.678$	$t = 6.869$	$t = 2.879$	NS	
	$P < 0.001$		$P = 0.037$	$P < 0.001$	$P = 0.028$		
C							
9008 nil	19.5	0	416	792	416	2,528	Infected
9012 nil	26.3	0	400	1,968	360	5,288	Infected
9027 nil	32.5	0	280	2,528	400	2,192	Infected
Mean ± SEM	26.1 ± 3.7	0	365 ± 42	1,762 ± 511	392 ± 16	3,326 ± 980	
	$t = 5.473$*		$t = 3.265$	$t = 1.595$	$t = 2.497$	NS	
	$P < 0.001$		$P = 0.017$	$P = 0.186$	$P = 0.047$		
D							
25 gp120/p27/Alum	42.7	2.9	192	272	408	4,608	Not challenged
87 gp120/p27/Alum	48.5	16.3	1056	536	792	10,184	Not challenged
205 gp120/p27/Alum	42.9	0	512	464	600	3,624	Not challenged
Mean ± SEM	44.7 ± 1.9	6.4 ± 5	587 ± 252	424 ± 79	660 ± 110	6,138 ± 2,042	
	$t = 3.706$†		$t = 2.385$	$t = 6.464$	$t = 1.849$	NS	
	$P < 0.01$		$P = 0.044$	$P < 0.001$	$P = 0.107$		
E							
928 C8166 cells	77.1	62.1	780	1,348	684	4,348	Not challenged

ND, not done; NS, not significant.
*CD8-SF from seven protected were compared with seven infected macaques (groups B and C).
†CD8-SF compared with those from group A.

presented). Thus, SIV infection of the seven control macaques did not stimulate increases in CD8-SF or alter the level of β-chemokines generated by mitogenic stimulation of their $CD8^+$ cells.

A finding that has defied interpretation in the past was that immunization with SIV grown on human cells elicited protection against SIV grown on human cells but not when challenged with SIV grown on macaque cells (5, 23, 25, 26). The specificity of protection of SIV grown on human cells might be accounted for by the recent finding of SIV-specific coreceptors on macaque but not on human cells (15, 27). Thus, β-chemokines induced by SIV grown on human cells will be targeted to CCR5 but not to coreceptors with different ligand specificities. Indeed, CD8 cells derived from macaques immunized with human CD4 cells (C8166) or with SIV grown on human CD4 cells inhibited SIV grown on human cells (83.7 and 82.9%; Table 2) assayed by coculture of $CD8^+$ cells with infected $CD4^+$ cells. However, the same cells showed markedly decreased inhibition of SIV grown on macaque cells (15.9% and 44.7%, respectively), comparable with that found in unimmunized macaques (20.5 and 23%, respectively). The results of these experiments suggest that β-chemokines generated by $CD8^+$ cells from macaques immunized with SIV grown on human $CD4^+$ cells will inhibit SIV replication if SIV was propagated on human rather than macaque cells, because the latter may use alternative coreceptors to which the specific ligands have not been generated.

The Effect of *in Vitro* Xenogeneic Stimulation on the Production of CD8-SF and β-Chemokines. Xenogeneic stimulation of simian cells was then investigated *in vitro*. Human $CD4^+$ T cells (C8166) were irradiated and used to stimulate simian PBMCs in one-way mixed lymphocyte reaction (Fig. 1). Significant variations in SI were found among the means of the

Table 2. Effect of CD8-SF derived from macaques immunized with SIV grown on human $CD4^+$ cell line (C8166) on *in vitro* replication of SIV grown on human or macaque cells

In vivo immunization of macaques	% *in vitro* inhibition of SIV replication by coculture of simian $CD8^+$ with $CD4^+$ cells infected with SIV grown on	
	Human $CD4^+$ cells*	Macaque $CD4^+$ cells†
Human $CD4^+$ cells*	83.7 ± 4.2	15.9 ± 6.2
SIV grown on human $CD4^+$ cells*	82.9 ± 0.5	44.7 ± 12.2
Unimmunized	20.5 ± 17.5	23 ± 14

*C8166 cell line.
†From PBMCs.

four groups of macaques (ANOVA: $F = 72.902$, $P < 0.001$). As expected the human T cell line induced a significant increase in T cell proliferation of the simian cells with a mean SI of 24.8 ± 4.9, as compared with unstimulated cells ($t = 8.634$, $P < 0.001$). Mitogenic stimulation with PHA yielded the highest SI (67.5 ± 10.9) but autologous $CD4^+$ T cell stimulation failed to induce a significant increase in SI (Fig. 1). $CD8^+$ T cells from these cultures were then used to generate CD8-SF (14, 20, 21). CD8-SF showed significant variation among the four group means at dilutions of 1:2 (ANOVA: $F = 45.719$, $P < 0.001$) and 1:5 (ANOVA: $F = 5.912$, $P < 0.02$). $CD8^+$ T cells from all five macaques stimulated *in vitro* with irradiated xenogeneic C8166 cells generated CD8-SF, yielding 70.8 ± 2.2% inhibition of SIV replication (Fig. 1), which was significantly greater at both 1:2 and 1:5 dilutions ($t = 12.353$, $P < 0.001$; and $t = 3.797$, $P < 0.01$, respectively) than unstimulated cells or as compared with autologous stimulation of $CD8^+$ T cells (29.9 ± 2.3%; $t = 13.202$, $P < 0.001$; and $t = 3.270$, $P < 0.02$, respectively). These results suggest that xenogeneic stimulation *in vitro* generate CD8-SF and that this is not related to the T cell proliferative response, because PHA elicited the highest SI and failed to yield significant CD8-SF.

The CD8 culture supernatants prepared for the CD8-SF were then used to assay the three β-chemokines (Fig. 1). Significant variation in the concentrations of RANTES, MIP-1α, and MIP-1β were found among the four group means of macaques (ANOVA: $F = 4.161$, 3.885, and 4.309, respectively; $P < 0.05$). The concentration of RANTES but not MIP-1α or MIP-1β was significantly increased in xenogeneic stimulation (702 ± 177 pg/ml; $t = 3.328$, $p = 0.029$), as compared with unstimulated cells (109.2 ± 14.9 pg/ml) or with autologous stimulation (127 ± 33 pg/ml; $t = 3.185$, $P < 0.02$) of $CD8^+$ T cells (Fig. 1). The MCP-1 control showed no significant differences between these groups, and PHA failed to stimulate significant increases in any of the chemokines.

To confirm that HLA antigen is involved in induction of CD8-SF, L cells transfected with HLA class I (B7) (28) were used to stimulate simian lymphocytes (Fig. 1). HLA class I induced significant simian T cell proliferation (SI = 10.6 ± 1.6), as compared with stimulation by L cells alone ($P < 0.001$). HLA class I-transfected L cells also generated very significant CD8-SF (73.2 ± 2.4% and 50.8 ± 6.1% at 1:2 and 1:5 dilutions), compared with L cells alone (20.6 ± 6.9 and 0.7 ± 0.7, respectively $P < 0.001$). The four β-chemokines in the CD8-SF were then assayed and as with xenogeneic stimulation, HLA class I-transfected L cells showed that RANTES was significantly increased in concentration (1,117 ± 320 pg/ml), as compared with L cells alone (221 ± 44 pg/ml; $t = 2.768$, $P < 0.05$).

Neutralization of the CD8-SF Activity with Anti-Chemokine Antisera. Specific involvement of the three β-chemokines within CD8-SF that were generated by xenogeneic stimulation was tested by inhibition with antibodies to the three β-chemokines. The three combined goat antibodies to RANTES, MIP-1α, and MIP-1β showed up to 99% (±19) inhibition of the CD8-SF activity measured by SIV RT production (Fig. 2). Antibodies to each chemokine elicited 6.3–18.3% neutralization and that by the control goat IgG was 6.3% (±3.4), as was found previously with HIV, using human cell lines (14). Inhibition of the chemokines by antibodies was similar when PHA was used to stimulate *in vivo*-primed simian cells, instead of the xenogeneic human C8166 cells (Fig. 2). These results suggest that the combined antibodies to RANTES, MIP-1α, and MIP-1β neutralize the inhibitory effect of these chemokines on SIV replication in simian $CD4^+$ T cells. Furthermore, this neutralization effect is comparable whether the CD8-SF is

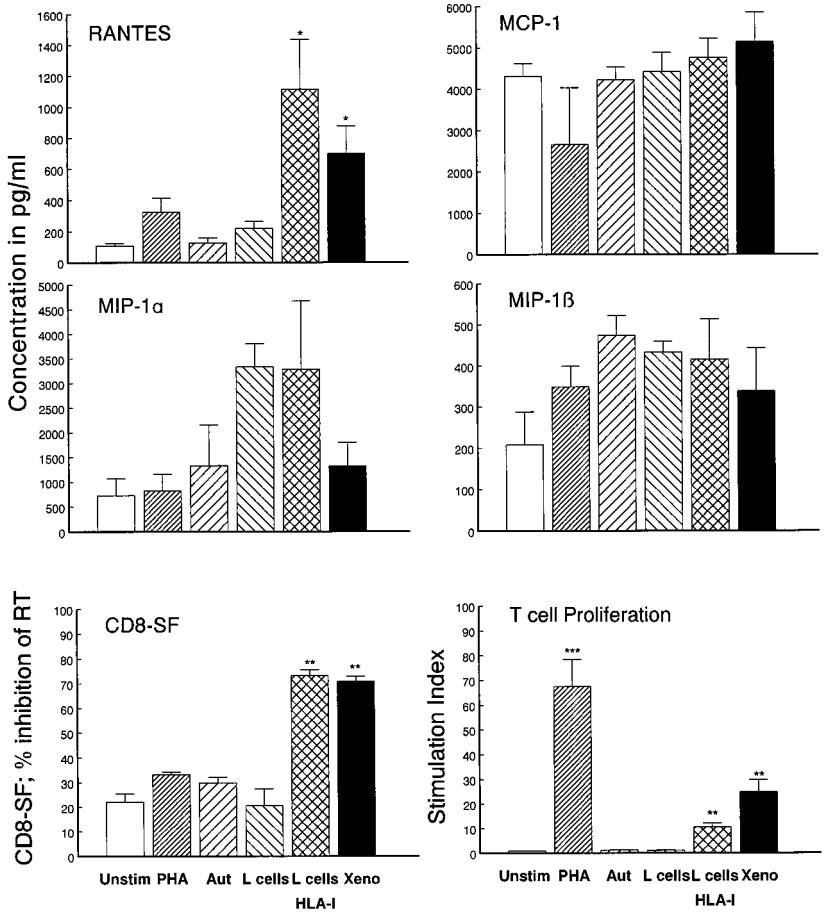

FIG. 1. Lymphocyte proliferation and production of CD8-SF and β-chemokines generated by stimulation with xenogeneic (C8166) or HLA class I-transfected L cells of PBMCs from four naive rhesus macaques. In parallel experiments, PBMCs from macaques were stimulated with irradiated xenogeneic HLA class I-transfected L cells or autologous mononuclear cells or with PHA at 10 μg/ml. Untreated PBMCs from these macaques and L cells were used as controls.

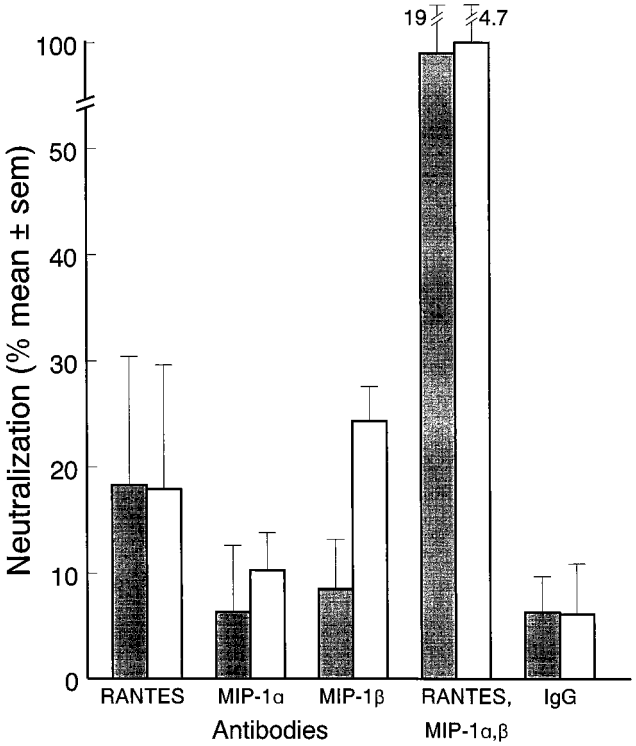

FIG. 2. Neutralization of the CD8-SF activity with anti-chemokine antiserum. CD8-SF induced by xenogeneic or PHA stimulation was neutralized with single or mixed antibodies to RANTES, MIP-1α, and MIP-1β, and the effect on SIV replication was determined by an assay of RT activity. The results are expressed as percent neutralization.

generated by xenogeneic or mitogenic stimulation of $CD8^+$ cells.

Inhibition of SIV Replication by Recombinant β-Chemokines. Dose-dependent inhibition by recombinant β-chemokines of SIV replication was assayed by RT activity (Fig. 3). RANTES was most effective, because >90% inhibition was achieved with 25 ng/ml, whereas MIP-1β at 250 ng/ml and higher concentrations of MIP-1α were required to reach the same levels of inhibition. However, a combination of the three β-chemokines (each at 2.5 ng/ml) elicited >90% inhibition. The control MCP-1 failed to yield significant inhibition of RT activity (Fig. 3).

DISCUSSION

Xenoimmunization of rhesus macaques with SIV grown in human $CD4^+$ T cells elicited significant increase in CD8-SF ($P < 0.001$) and the β-chemokines RANTES, MIP-1α, and MIP-1β ($P < 0.02$, $P < 0.01$, and $P < 0.02$. respectively). These may prevent macrophage-tropic HIV-1 (14) and both the latter and T cell-tropic SIV (29) from binding and infecting $CCR5^+$ $CD4^+$ cells. Indeed, CD8-SF and the three β-chemokines were significantly increased in seven macaques protected from SIV when immunized with SIV grown in human $CD4^+$ T cells, as compared with the seven infected control macaques. These results suggest that the mechanisms of protection elicited by xenogeneic immunization and by targeted iliac lymph node (TILN) immunization with recombinant SIV gp120 and p27 (21) share significant production of CD8-SF and β-chemokines, both of which inhibit SIV replication. It is noteworthy that i.m. immunization with SIV gp120 and p27 elicits lower levels of CD8-SF and β-chemokines than TILN immunization. Indeed, in another experiment i.m. immunization of six macaques with the same vaccine failed to protect them from rectal mucosal challenge with SIVmac 251 J5 molecular clone (T.L., L.A.B., M. Cranage, L.T., C.D., G. Hall, and M. Dennis, unpublished results).

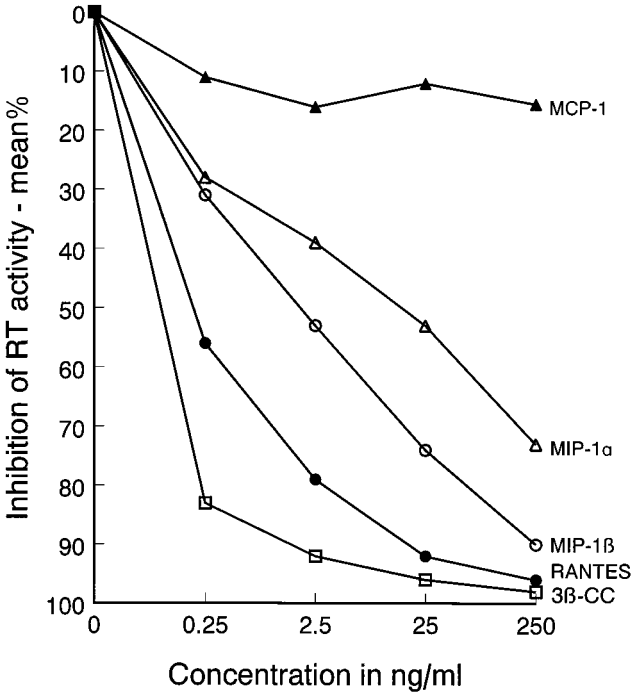

FIG. 3. Dose-dependent inhibition of SIVmac 251 replication in simian $CD4^+$ cells by human recombinant β-chemokines. PHA-stimulated $CD4^+$ cells were incubated for 30 min with the β chemokines RANTES, MIP-1α, and MIP-1β or control MCP-1 (each at 0.25–250 ng/ml) before SIV infection. SIV was added in the presence or absence of β-chemokines and incubated for 2 h. The level of SIV replication was tested on day 7 after infection by the RT assay in the culture supernatant. The result of suppression of SIV replication was expressed as the mean percent inhibition of RT activity.

The *in vivo* results were corroborated with *in vitro* experiments, showing that stimulation of simian PBMCs with irradiated human $CD4^+$ T cells or HLA-I-transfected L cells generated significant increase in CD8-SF ($P < 0.001$) and RANTES ($P < 0.05$) but not the control chemokine MCP-1. The specificity of xenogeneic stimulation of CD8-SF and the constituent β-chemokines was demonstrated by complete neutralization of the CD8-SF inhibition of SIV replication by antibodies to the β-chemokines. It appears that neutralization of the inhibitory effect of β-chemokines with antibodies to the latter, first shown with HIV and human cell lines (14), can be achieved by generating CD8-SF either by PHA stimulation of primed $CD8^+$ T cells or the xenogeneic mixed leukocyte reaction of naive $CD8^+$ T cells.

The potency of the three β-chemokines to inhibit SIV replication in simian $CD4^+$ T cells was then examined. Dose-dependent inhibition of SIV replication was carried out with the three β-chemokines. RANTES was the most effective chemokine requiring a 10-fold lower concentration than MIP-1β to inhibit SIV replication. A combination of RANTES with the two other chemokines decreases the concentration of chemokines from 25 to 2.5 ng/ml to yield >90% inhibition of SIV replication. The combined concentration of the three β-chemokines derived from $CD8^+$ cells of the seven SIV immunized and protected macaques varied from 2.8 to 11.3 ng/ml, suggesting that SIV inhibitory concentrations have been achieved. In contrast the MV-immunized controls (in the same preparation of ISCOMs or MDP) yielded only 1.1–1.2 ng/ml and all macaques were infected.

Recent studies suggest that in addition to CCR5 other SIV-specific coreceptors may be found exclusively on macaque cells (15, 27). This finding may account for the observation that

immunization with SIV grown on human cells elicits protection against SIV grown on human but not if grown on macaque cells (5, 22, 23, 25, 26). Indeed, any β-chemokine to CCR5 induced by SIV grown on human cells will be targeted to CCR5 but not to coreceptors specific for macaque cells that bind different ligands. This has been demonstrated with CD8-SF, derived from macaques immunized with human CD4 cells or SIV grown on human CD4 cells, by inhibiting SIV grown on human but not SIV grown on macaque cells. Inhibition of SIV replication by CD8-SF and the three β-chemokines *in vitro* is consistent with the *in vivo* reports that xenogeneic protection in 26 of the 28 macaques is dependent on the challenge SIV being grown on xenogeneic cells (22, 23, 25, 26). Furthermore, it should be emphasized that in the *in vivo* experiments (25, 26), the same macaques that were protected when challenged with SIV grown in human cells (16 of 17) were subsequently infected (14 of 14) when rechallenged with SIV grown on macaque cells. Hence, the propagation of SIV on human C8166 cells may have encouraged CCR5 coreceptor usage. However, when SIV is grown on simian cells, the coreceptor usage may have been altered to allow propagation of SIV variants that use other simian coreceptors, thereby escaping the CCR5 inhibitory responses induced by prior xenoimmunization. The ability to escape immune inhibition by coreceptor switch is consistent with the switch in HIV from CCR5 to CCR2b, CCR3, and CXCR4 reported in HIV-1-infected humans (30).

Xenoimmunization with SIV grown in human $CD4^+$ T cells elicits CD8-SF with RANTES, MIP-1α, and MIP-1β that can block the CCR5 receptors and prevent SIV replication. Recent evidence suggests that β-chemokines binding to CCR5 receptors may also induce internalization of the receptors, thereby down-regulating cell surface expression of the SIV binding coreceptors (31) and preventing SIV infection. This mechanism might be responsible for the potent sterilizing immunity against SIV infection elicited by xenogeneic immunization in macaques (1–5). It is of considerable interest to determine whether the proposed protective mechanism might also be involved in resistance to HIV infection in alloimmunized humans, as may occur in pregnancies and blood transfusions.

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