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A Minimally Replicative HIV-2 Live-Virus Vaccine Protects *M. nemestrina* from Disease after HIV-2_{287} Challenge

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M. nemestrina immunized with an apathogenic HIV-2 molecular clone (HIV-2_{KR}) were protected from CD4 decline and disease upon challenge with HIV-2_{287}, after any immunizing virus could be detected. Higher but not lower inocula of HIV-2_{KR} were protective against intravenous inoculation of either 10^5 or 10^1 $TCID_{50}$ of HIV-2_{287}. Protected animals displayed substantial reductions in PBMC proviral burden (1–3 logs), viral titers (1–2 logs), and plasma viral RNA (2–4 logs) compared to unprotected or naive animals as early as 1 week postinfection. Plasma viral RNA became undetectable after 24 weeks in protected animals, but remained high in unprotected animals. No viral RNA was present in the spleen of the protected animal necropsied more than a year after challenge (though viral DNA was still present). No neutralizing responses could be demonstrated, but CTL activity was detected sooner and at higher levels after challenge in protected than in unprotected macaques. In this novel HIV-2 vaccine model, protection was clearly dose-dependent, and clearance of challenge virus RNA from the plasma did not require detectable ongoing replication of the immunizing virus at the time of challenge. © 1998 Academic Press

Key Words: HIV-2; vaccine; animal models; virology—quantitative.

INTRODUCTION

Live-virus vaccination strategies have demonstrated that broad and persistent protection against SIV infection can be achieved (Marthas *et al.*, 1990; Daniel *et al.*, 1992; Lohman *et al.*, 1994; Almond *et al.*, 1995; Clements *et al.*, 1995; Putkonen *et al.*, 1995, 1996; Hirsch *et al.*, 1996; Wyand *et al.*, 1996). However, persistent replication of immunizing virus over a significant period of time has been found to be needed for induction of complete protection, as replication of challenge virus has been detected when animals were challenged earlier after immunization (Wyand *et al.*, 1996), though protection from disease was still generally achieved. Results with potentially safer, but generally less efficacious, strategies, including subunit vaccines, DNA immunization, and inactivated virus vaccines, have also illustrated that failure to protect against infection did not imply failure to protect against occurrence of disease in primate models (Marthas *et al.*, 1992; Hu *et al.*, 1992a,b, 1993; Israel *et al.*, 1994; Lehner *et al.*, 1996; Lu *et al.*, 1996; Mossman *et al.*, 1996). Concerns about the safety of live-virus strategies have been accentuated by the observation that attenuated SIV virus was nonetheless capable of inducing disease when administered to immunologically incompetent (newborn) macaques (Baba *et al.*, 1995). Thus, exploring less replicative immunizing viruses, which may not be capable of producing complete protection against infection, is an appropriate goal, if such an infection still produces an immune state which leads to early control of virus replication and prevents or delays the onset of disease.

Recently, the HIV-2_{KR} molecular clone has been shown to produce only limited infection in *M. nemestrina* (Kraus *et al.*, 1998), characterized by transient detectable viremia (2–24 weeks), and modest and temporary decline in CD4 lymphocyte numbers. HIV-2_{KR} represents a minimally infectious and genetically uniform "immunizing" virus, suitable for exploring protection from virulent isolates in a live-virus vaccination system. To determine the nature and extent of protection induced by HIV-2_{KR} infection, four animals, previously inoculated with higher [10^4

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SFU (syncytia-forming units)] or lower (10^3 SFU) doses of HIV-2_{KR}, were challenged, together with six naive animals, with either high (10^5 $TCID_{50}$) or low (10^1 $TCID_{50}$) doses of HIV-2_{287}, a highly pathogenic HIV-2 isolate (Ho *et al.*, 1996). Following challenge, kinetics of CD4 decline and viral replication were carefully assessed. In order to obtain a preliminary indication of the mechanism(s) involved in protection, serological reactivity, neutralizing activity, and cytotoxic lymphocyte activity were also monitored.

Intravenous inoculation of HIV-2_{287} at either study dose was found to produce prompt and profound decline of CD4 lymphocytes and onset of immunodeficiency disease in all naive animals. Animals initially immunized with a lower dose of HIV-2_{KR} displayed slower decline of CD4 levels, and animals immunized with a higher dose were protected from CD4 decline and effectively cleared virus from the plasma. Viral replication was found to predict eventual outcome, as assessed by quantitative viral culture, viral DNA PCR, and quantitative competitive plasma RNA PCR. In contrast to previously described models of HIV-2 and SIV live-virus immunization (Daniel *et al.*, 1992), *in vitro* immune correlates of protection were not present prior to HIV-2_{287} challenge, as no neutralizing or bulk CTL activity was detectable 14 months after HIV-2_{KR} immunization. However, *gag*-specific CTL activity was observed promptly after challenge in protected animals, suggesting that an anamnestic response may have been primed by exposure to HIV-2_{KR}. This vaccine model represents a novel system of live-virus immunization and protection from pathogenic infection, employing exclusively human immunodeficiency viruses for both immunization and challenge in a readily available primate species (*M. nemestrina*). Findings in this preliminary study suggest that protection may be dose-dependent and that substantially lower levels of replication by a live-virus immunogen may be needed to induce protection from disease than previously demonstrated in SIV live-virus vaccine models.

RESULTS

Protection from CD4 decline

Four animals previously immunized with 10^4 or 10^3 SFU of HIV-2_{KR}, together with six naive animals, were inoculated intravenously with either 10^5 or 10^1 $TCID_{50}$ of HIV-2_{287} (Table 1). By 8 weeks postinoculation (wpi), all six naive animals and animals immunized with the lower dose of HIV-2_{KR} (404 and 407) manifested CD4 decline (Fig. 1). By 16 wpi, their $CD4^+$ lymphocyte numbers declined to below 1% of the preinoculation value. In contrast, only a transient, modest decline in CD4 numbers was noted in animals immunized with 10^4 SFU HIV-2_{KR} (292 and 350), whose $CD4^+$ lymphocyte numbers rose by 26 wpi to levels not significantly different from preinoculation levels (Fig. 1). Decline of CD4 lymphocyte numbers was more prompt in animals challenged with 10^5 $TCID_{50}$ of HIV-2_{287} (compare 292 with 350 and 407 with 404, Fig. 1A, and 096 and 192 with 147 and 172 in Fig. 1B) and slower in animals immunized with the lower dose of HIV-2_{KR} than in naive animals (compare 404 and 407 in Fig. 1A with animals in Fig. 1B). No differences between groups of animals were noted in CD8 lymphocyte numbers or total white blood cell count (data not shown). Naive animals were euthanized 24 weeks after infection, when clinical symptoms of immunodeficiency, principally fever and wasting, were observed. Pneumocystis carinii pneumonia (PCP) was documented in one animal. While animals with a low dose HIV-2_{KR} (404 and 407) experienced eventual CD4 decline, onset of clinical disease was delayed compared to naive animals; however, their clinical condition required euthanasia by 56 wpi. In contrast, animals immunized with the higher dose of HIV-2_{KR} (292 and 350) remained free of symptoms of immunodeficiency. Animal 292 remains alive and asymptomatic, and animal 350 died due to intestinal obstruction at 76 wpi.

TABLE 1

Animals Used in HIV-2_{287} Challenge Experiment

Animal	HIV-2_{KR} Inoculum (SFU)	HIV-2_{287} Inoculum ($TCID_{50}$)	CD4 decline after HIV-2_{287} Inoculation	Immunodeficiency disease
J92096	None	10^5	Rapid	Rapid
T92192	None	10^5	Rapid	Rapid
F91368	None	10^5	Rapid	Rapid
M91392	None	10^5	Rapid	Rapid
F92147	None	10^1	Rapid	Rapid
J92172	None	10^1	Rapid	Rapid
J90292	10^4	10^5	None	None
F90350	10^4	10^1	None	None
F90407	10^3	10^5	Rapid	Delayed
F90404	10^3	10^1	Rapid	Delayed

Viral replication kinetics

To determine if limitation of virus replication correlated with protection from CD4 lymphocyte decline, quantitative cultures of PBMC were performed, proviral DNA was determined by quantitative PCR, and plasma viral RNA was measured using an EIA QC–PCR specific for HIV-2_{287}. Virus was isolated from all animals after HIV-2_{287} inoculation. As few as 100 PBMC were required for reisolation at the peak of viremia (Fig. 2). Viral DNA burden in PBMC was of comparable magnitude (Fig. 3). At 2 wpi, proviral burden was dramatically lower in the protected animal given high-dose HIV-2_{287} (292—$10^{1.3}$) than in the naive ($10^{4.0}$ and $10^{3.5}$ for 096 and 192, respectively) or the unprotected (407—$10^{3.5}$) animals given the same inoculum. In contrast, little difference was seen at 2 wpi in animals which received the low dose of HIV-2_{287}.

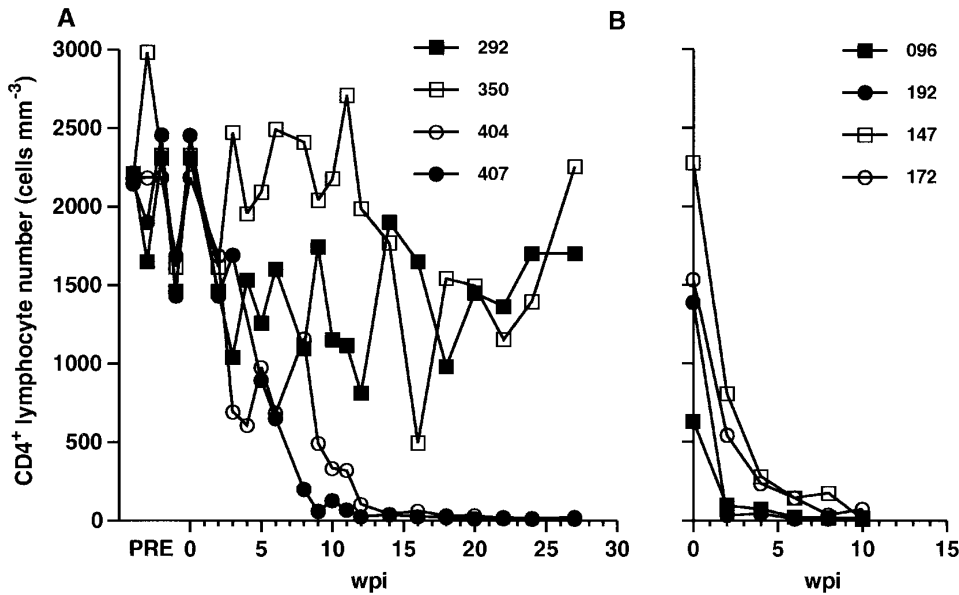

FIG. 1. Effect of HIV-2_{287} challenge on $CD4^+$ lymphocyte numbers. Absolute $CD4^+$ lymphocyte counts (cells mm^{-3}) of KR-experienced animals (A) and naive animals (B) challenged with HIV-2_{287} are shown. Weeks postimmunization, wpi. Animal 292 (■—high KR, high 287), 350 (□—high KR, low 287), 404 (○—low KR, low 287), and 407 (●—low KR, high 287). Animal 096 (■—naive, high 287), 192 (●—naive, high 287), 147 (□—naive, low 287), and 172 (○—naive, low 287). (A) PRE indicates CD4 lymphocyte number determinations made over the 4 months prior to challenge (time not to scale).

By 4 wpi, however, animal 350 displayed a lower proviral burden ($10^{1.8}$) than did the naive ($10^{2.2}$ and $10^{2.9}$ for 147 and 172, respectively) or the unprotected (404—$10^{5.2}$) animals given low-dose HIV-2_{287}. Over time, proviral burden in all groups of animals appeared to approach a common mean ($\sim 10^3$).

Similar findings were noted early in the course of plasma viremia. Among the group receiving 10^5 $TCID_{50}$ HIV-2_{287}, plasma RNA copy numbers were lower in the protected (292—$10^{5.4}$ copies ml^{-1}) than in the naive ($10^{8.2}$ and $10^{8.3}$ copies ml^{-1} for 096 and 192, respectively) or unprotected (407—$10^{8.2}$ copies ml^{-1}) animals at 1 wpi (Fig. 4). Peak viremia was delayed in the group receiving 10^1 $TCID_{50}$ HIV-2_{287}, but a reduction was still seen at 2 wpi in the protected animal (350—$10^{6.2}$ copies ml^{-1}), compared to the naive ($10^{7.4}$ and $10^{7.1}$ copies ml^{-1} for 147 and 172, respectively) and unprotected (404—$10^{6.6}$ copies ml^{-1}) animals. In contrast to the gradual increase in proviral burden in all animals, plasma viremia declined and diverged progressively in protected and unprotected animals. Among the high-dose HIV-2_{287} group, plasma viremia was 2.3–4.4 logs lower in the protected animal (292—$10^{3.4}$ copies ml^{-1}) than in naive ($10^{7.8}$ and $10^{5.7}$ copies ml^{-1} for 096 and 192) or unprotected (407—$10^{6.9}$ copies ml^{-1}) animals by 16 wpi. Differences in the low-dose HIV-2_{287} group were also evident. Viremia in the protected animal (350—$10^{2.4}$ copies ml^{-1}) was 1–3.4 logs lower than in the naive (147—$10^{5.8}$ and 172—$10^{3.4}$) or unprotected (404—$10^{3.5}$) animals at 1 wpi, and it was 1.2 logs lower (350—$10^{4.2}$) than in the unprotected animal (404—$10^{5.4}$ copies ml^{-1}) at 16 wpi. Substantial decline in plasma RNA in naive animals (147—$10^{4.5}$ and 172—$10^{3.2}$ copies ml^{-1}) at 16 wpi probably reflected destruction of the producing cell population.

Note the discrepancy of late viremia in protected and unprotected HIV-2_{KR} experienced animals (Fig. 4B, 52 wpi), where plasma viral copies declined to the level of undetectability ($10^{2.3}$ copies ml^{-1}) in protected animals, yet remained high ($10^{7.9}$, $10^{6.4}$ copies ml^{-1}) in unprotected animals 404 and 407. Plasma viral RNA could not be detected in animals 292 and 350 after 24 wpi (not shown). Plasma viremia remained undetectable 22 months after HIV-2_{287} inoculation in animal 292, and proviral DNA also declined to below 10^2 copies/10^6 PBMC (not shown). In addition, viral RNA was undetectable in the spleen of animal 350 at the time of necropsy (though viral DNA was still present), but could be easily isolated from splenic tissue of animal 407 or earlier plasma from macaque 350 (Fig. 5).

Serological response to HIV-2_{287} challenge

To investigate the mechanism of control of viral replication in protected animals, serological reactivity of KR-immunized animals was characterized by Western blotting. Prior to challenge with HIV-2_{287}, minimal serological reactivity could be demonstrated. After challenge, envelope reactivity actually declined in animal 407 (Fig. 6A, lanes C and D, note loss of gp130 bands), and p26 antibody was not detectable 52 wpi (lane D), whereas animals 292 and 350 exhibited anamnestic responses to envelope proteins (Fig. 6B, lanes H and L, respectively). All animals displayed detectable serological reactivity to p26 and an initial increase in p26 antibody after HIV-2_{287}

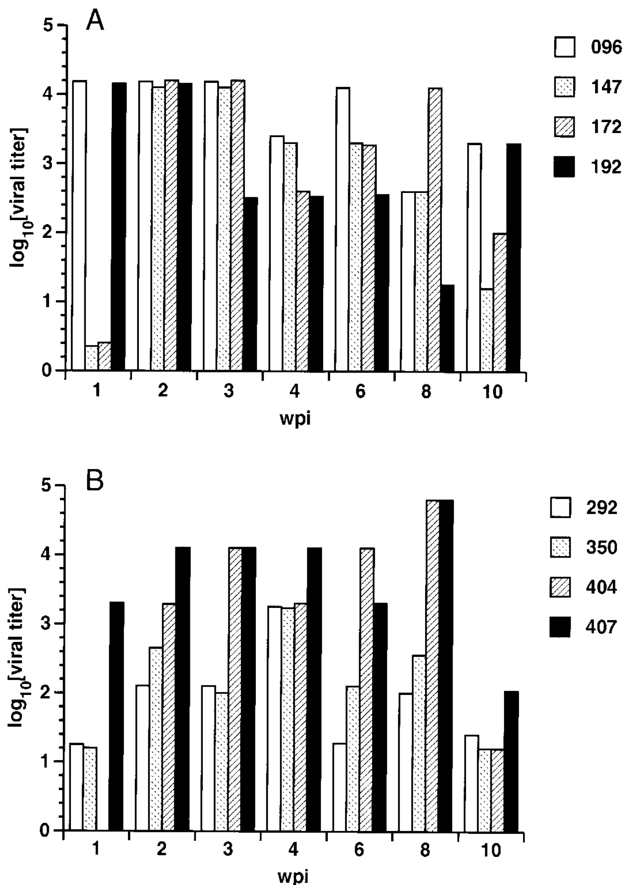

FIG. 2. Quantitative culture of PBMC. PBMC from challenged animals were serially diluted 10-fold starting with 1×10^6 cells and cocultured with 1×10^6 fresh PHA/IL-2 stimulated allogenic *M. nemestrina* PBMC. Titers are expressed as the log of the maximal dilution yielding a positive culture by p26 EIA of supernatant within 8 weeks. (A) Naive animals 096, 147, 172, and 192. (B) KR-immunized animals 292, 350, 404, and 407. Solid black and white bars denote animals receiving the high inoculum of HIV-2_{287}, and stippled and gray bars denote animals receiving the low inoculum. No virus could be detected from animal 404 at 1 wpi.

inoculation (Fig. 6) consistent with an accelerated response. The inability to detect envelope antibody in animal 407 after HIV-2_{287} challenge (Fig. 6A, compare lanes B and C) may be explained by the extremely high early viremia (Fig. 4B, 1 wpi, solid bars, $>10^8$ copies ml^{-1}). Neutralizing activity, as assessed by syncytial inhibition on HT4-6C, could not be detected in the sera of HIV-2_{KR}-infected macaques either before or after challenge with HIV-2_{287}, while control sera from HIV-2 infected individuals produced 50% inhibition at titers from 1:80 to 1:160 (not shown). Using a more sensitive constant-serum, varying-virus p26-based neutralization assay to detect HIV-2 replication in human PBMC, pooled serum from SIV_{mac239}- and SIV_{mac251}-infected macaques exhibited significant neutralizing activity, producing a 2 log_5 reduction in the titer of HIV-2_{287} (Table 2, left) and a 1.25 log_{10} reduction in the titer of HIV-2_{KR} (Table 2, right); however, no significant neutralizing activity of KR-infected macaque sera against either virus could be demonstrated either before HIV-2_{287} challenge (0 wpi) or up to 58 weeks after HIV-2_{287} challenge.

$CD8^+$ cytotoxic lymphocyte activity correlates with protection

To determine if cytotoxic lymphocyte activity was involved in protection from CD4 decline, PBMC from naive and HIV-2_{KR}-immunized animals were exposed to two *in vitro* cycles of stimulation, and bulk-stimulated populations analyzed for CTL activity. No HIV-2 *gag*-specific CTL activity was detected 14 months after immunization with either low or high doses of HIV-2_{KR}, just before HIV-2_{287} challenge (Table 3). However, in the months following HIV-2_{287} challenge, specific lysis of *gag*-expressing BLCL targets was detected in both animals previously exposed to high-dose HIV-2_{KR} (292 and 350). In contrast, specific lysis was either not detected or

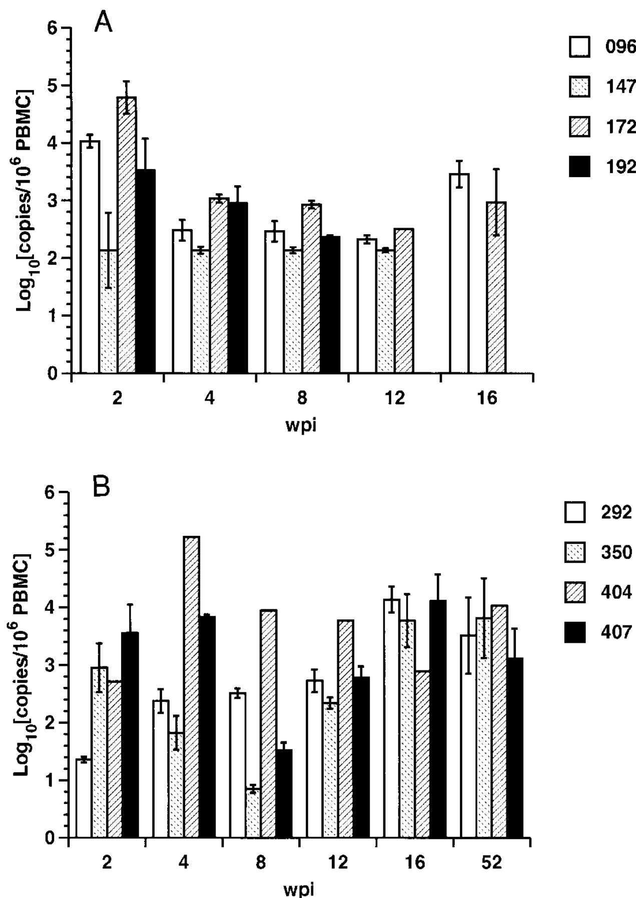

FIG. 3. $CD4^+$ lymphocyte proviral burden in protected and unprotected animals. Shown are log_{10} average proviral copies/10^6 PBMC. Each value represents two or more determinations on two or more samples. Error bars represent standard deviations. (A) Naive animals. (B) HIV-2_{KR}-immunized animals. Solid black and white bars denote animals receiving the high inoculum of HIV-2_{287}, and stippled and gray bars denote animals receiving the low inoculum.

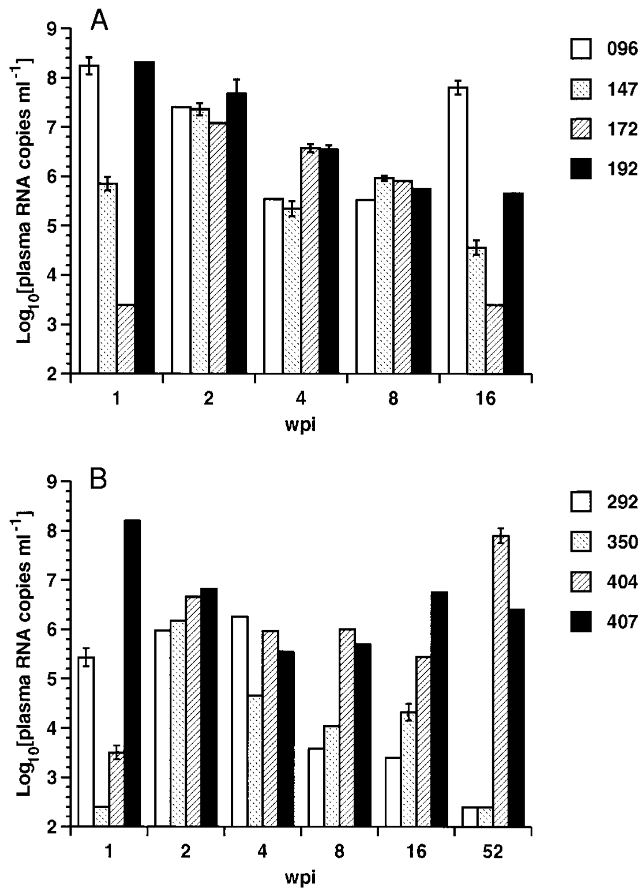

FIG. 4. Plasma RNA levels in protected and unprotected animals. Values shown represent $\log_{10}$ of calculated RNA copies/ml plasma using duplicate to quadruplicate samples. Columns with error bars represent standard deviations of values obtained by three independent RNA extraction techniques. (A) Naive animals 096, 147, 172, and 192. (B) KR-experienced animals 292, 350, 404, and 407. Solid black or white bars denote animals which received 10^5 $TCID_{50}$ HIV-2_{287}, and stippled or gray bars denote animals receiving 10^1 $TCID_{50}$ HIV-2_{287}.

detected only at low levels (<3% specific lysis), in negative control animals (165, 296), naive animals challenged with HIV-2_{287} (096, 192, 368), and in animals previously exposed to a low dose of HIV-2_{KR} (404, 407). In animal 292, still surviving, CTL activity was present at all time points measured, and, in animal 350, significant CTL activity (>4% specific lysis) was present on two of three occasions. Both animals protected from lethal HIV-2 disease had *gag*-specific CTL activity detected up to 6 months after HIV-2_{287} challenge. To determine the phenotype of effector cells responsible for the observed CTL activity, specific lysis of HIV-2 *gag*-expressing BLCL targets by positively selected CD8-enriched mononuclear cells was compared to that exhibited by unselected and CD8-depleted PBMC effectors. The major effector population responsible for *in vitro* activity consisted of CD8, presumably MHC class I-restricted, cytotoxic lymphocytes (Fig. 7).

DISCUSSION

Inoculation with SIV viral strains of reduced pathogenicity has previously been shown to prevent infection or disease progression after challenge with pathogenic SIV (Marthas *et al.*, 1990; Daniel *et al.*, 1992; Lohman *et al.*, 1994; Almond *et al.*, 1995; Clements *et al.*, 1995; Putkonen *et al.*, 1995, 1996; Hirsch *et al.*, 1996; Wyand *et al.*, 1996). While protection from infection is elicited by attenuated homologous clones (Daniel *et al.*, 1992; Almond *et al.*, 1995) 8 months or more after immunization, protection has been noted to be incomplete when heterologous virus is used for challenge (Putkonen *et al.*, 1990; Clements *et al.*, 1995; Abimiku *et al.*, 1995; Letvin *et al.*, 1995; Petry *et al.*, 1995; Vogt *et al.*, 1995; Wyand *et al.*, 1996) or when challenge is performed too early after inoculation. Moreover, the degree of protection may be related to the level of replication of the immunizing virus (Daniel *et al.*, 1992).

In this study, animals previously inoculated with 10^4 SFU, but not 10^3 SFU, of the avirulent HIV-2_{KR} molecular clone were protected from CD4 decline and immunodeficiency disease after challenge with HIV-2_{287} (Ho *et al.*, 1996). Dose dependence of protection, with regard to the inoculum of immunizing virus, was suggested by the difference in CD4 decline in animals immunized with the higher and lower doses, as well as the slower CD4 decline and longer survival of "unprotected" HIV-2_{KR}-experienced animals compared to naive controls. Viremia after infection with HIV-2_{KR} was of comparable magnitude and duration in all immunized animals, so that dose-dependent protection could not be foreseen. Protection was not simply a function of the greater amount of viral protein administered to animals 292 and 350, however, as two macaques administered 200× more viral antigen in the form of a noninfectious *nef*-deletion mutant, were not protected from CD4 decline after HIV-2_{287} challenge (not shown).

In both SIV and SHIV vaccination systems, the level of viral replication early after infection has been demon-

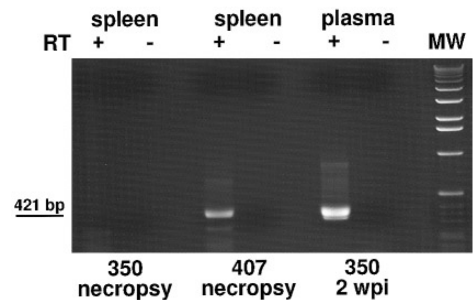

FIG. 5. Inability to detect viral transcripts in the spleen of protected animal 350. Ethidium bromide-stained agarose gel of RT–PCR products from splenic tissue from protected animal 350 and unprotected animal 407, as well as plasma RNA from animal 350 obtained 2 wpi. PCR on each sample was performed in either the presence (+) or the absence (−) of reverse transcription.

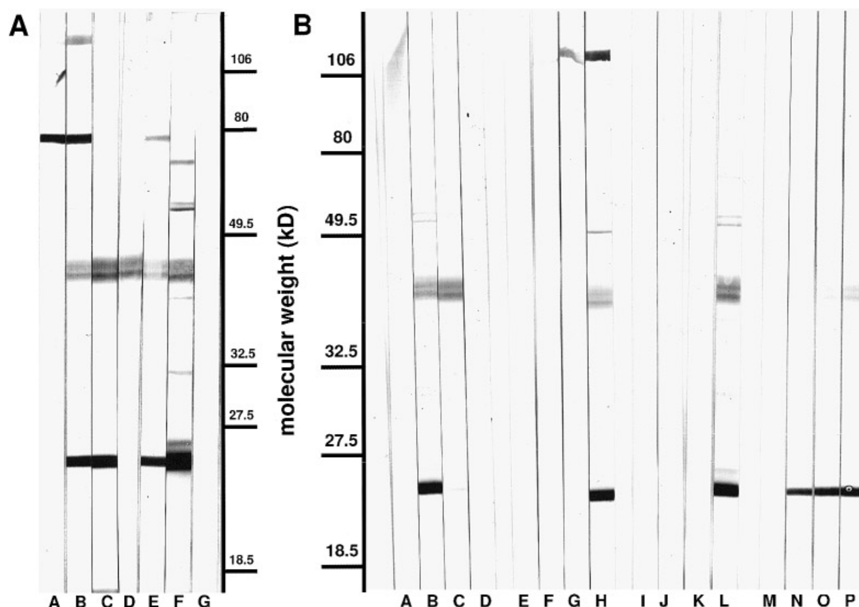

FIG. 6. Serological reactivity of HIV-2_{KR} immunized animals. (A) Animal 407: Lanes A, B, and E—2, 24, and 52 wpi with HIV-2_{KR}; lanes C and D—16 and 52 wpi with HIV-2_{287}; lane F—HIV-2-seropositive human control; lane G—negative control. (B) Lane A—*M. nemestrina*-negative control; lane B—*M. nemestrina*-positive control; lane C—human HIV-2-positive control; lane D—normal human serum. Lanes E–H, animal 292; lanes I–L, animal 350; lanes M–P, animal 404. Lanes E, I, and M: prior to HIV-2_{KR} infection; lanes F, J, and N: 24 wpi HIV-2_{KR}; lanes G, K, and O: 52 wpi HIV-2_{KR}; lanes H, L, and P—16 wpi HIV-2_{287}.

strated to predict progression to disease (Letvin *et al.,* 1995; Hirsch *et al.,* 1996), and in human HIV-1 infection, viral burden has recently been shown to be a strong predictor of outcome (Mellors *et al.,* 1996). In this study, administration of a low (10^1 $TCID_{50}$) rather than high (10^5 $TCID_{50}$) inoculum of HIV-2_{287} resulted in a 1- to 2-week delay and a slight decline in the magnitude of maximal viremia noted, and animals challenged with the low dose of HIV-2_{287} also displayed slower rates of CD4 decline. Protected animals exhibited lower levels of both plasma RNA (2–4 logs) and proviral burden (1–3 logs) than unprotected KR-experienced animals or naive animals inoculated in the same fashion. Though the number of immunized macaques included in this initial trial was limited, none of the naive animals survived longer than 24 wpi, and all the animals immunized with the higher dose of HIV-2_{KR} were protected. The large magnitude of the differences in viral replication and CD4 lymphocyte depletion between protected and unprotected animals strongly suggests that control of viremia is causally associated with the observed difference in outcomes. The prolonged control of viral replication in protected animals in this trial, together with the observed absence of detectable viral RNA in the spleen of the protected animal which was euthanized, emphasizes that the eventual outcome of protection from disease, but not from infection, may be both a reasonable and achievable goal of vaccine protection and underscores the importance of viral quantification to the understanding of vaccine effects.

TABLE 2

Neutralization Activity of Sera from HIV-2_{KR}-Immunized HIV-2_{287}-Challenged Animals on PBMC

	HIV-2_{287}		HIV-2_{KR}			
Animal serum	0 wpi[a]	58 wpi	0 wpi[a]	18 wpi	27 wpi	58 wpi
J90292	3.50[b]	3.50	5.50	5.50	4.50	4.50
F90350	4.50	3.50	5.50	4.50	4.50	4.50
F90404	3.50	4.50	5.50	4.50	4.50	5.50
F90407	3.50	3.50	5.50	4.50	4.50	4.50
Negative control	3.70 ± 0.40			4.75 ± 0.43		
Positive control	1.70 ± 0.00			3.50 ± 0.00		

Note. Negative control, HIV-2_{287} or HIV-2_{KR} virus without serum. Positive control, pooled sera from SIV infected macaques.

[a] 0 wpi, time of HIV-2_{287} inoculation.

[b] All numbers shown represent the geometric mean titer in log dilutions ($\log_5$ for HIV-2_{287}, $\log_{10}$ for HIV-2_{KR}).

The immunological mechanism controlling viral replication in protected animals remains unclear. While high levels of neutralizing antibody have been noted to be present in some long-term HIV-1 survivors (Sattentau 1996), and in macaques protected with live-attenuated SIV (Putkonen *et al.,* 1990; Daniel *et al.,* 1992; Cao *et al.,* 1995; Clements *et al.,* 1995; Wyand *et al.,* 1996), no evidence was found to indicate that neutralizing activity was responsible for the observed protection in this study, although anamnestic serological responses were seen in all animals except for animal 407. It is tempting to

TABLE 3

CTL Activity of *M. nemestrina* after HIV-2_{287} Challenge

	Exposure		Percent specific lysis at E:T of 25:1 after HIV-2_{287} exposure (months)							
			0		1		2		6	
Animal	HIV-2_{KR}	HIV-2_{287}	vac[a]	gag[b]	vac	gag	vac	gag	vac	gag
J90292	High	High	2	4	1	23	2	13	0	22
F90350	High	Low	0	3	1	11	0	4	0	7
F90404	Low	Low	0	2	0	0	0	0	3	4
F90407	Low	High	0	0	4	4	0	0	NT	NT
J92096	None	High	0	0	0	0	NT	NT	NT	NT
F91368	None	High	NT[c]	NT	1	1	0	0	NT[d]	NT[d]
T92192	None	High	0	0	0	0	2	0	NT[d]	NT[d]
F92296	None	None	NT	NT	0	2	1	0	0	0
F92165	None	None	0	0	1	0	3	1	0	2

[a] vac, autologus BLCL infected with wild-type vaccinia virus.
[b] gag, autologus BLCL infected with rVV expressing HIV-2 *gag*.
[c] NT, not tested.
[d] Animal died of AIDS.

speculate that the high early viremia (Fig. 4B) in this unprotected, immunized animal obscured or prevented an antibody response.

Animals protected from CD4 decline generated HIV-2-specific $CD8^+$ cytotoxic lymphocyte activity more frequently and at greater levels than animals which progressed to CD4 decline and disease (Table 3), though no bulk CTL activity was detectable prior to challenge. This might represent an anamnestic reponse, whereby exposure to the challenge virus resulted in rapid boosting of preexisting CTL activity from below the limit of detection to levels which controlled early virus replication. In contrast, the slower primary reponse in naive animals could have allowed initial virus replication to overwhelm host defenses. High levels of cytotoxic lymphocyte activity have been found in long-term survivors of HIV-1 (Rinaldo *et al.*, 1995) and are associated with long-term suppression of SIV and HIV-2 replication in other macaque models (Reimann *et al.*, 1994; Heeney *et al.*, 1994; Gallimore *et al.*, 1995; Mellors *et al.*, 1996; Johnson, 1996;). The substantial CTL activity present in protected animals in this trial strongly suggests a relationship with protection from CD4 decline and the persistent control of viremia observed in this model. However, CTL activity alone has not been shown to predictably lead to protection (Letvin *et al.*, 1995; Hulskotte *et al.*, 1995; Yasutomi *et al.*, 1995) or reduction in viral load in other studies. The recent development of suitable vaccinia expression systems for HIV-2_{287} *gag* should allow confirmation that the observed activity is directed against the challenge strain in future experiments. Alternatively, HIV-2-specific CD4 T-cell helper activity elicited by HIV-2_{KR} immunization might have accelerated primary immune responses to HIV-2_{287}. Other immune factors, such as CD8-mediated suppression via chemokine inhibition of replication, might also have facilitated protection. While some researchers have found elaboration of chemokines by PHA-stimulated $CD8^+$ lymphocytes to correlate with protection (Lehner *et al.*, 1996), no differences in the plasma concentration of RANTES were noted between protected and unprotected animals in this study (D. Looney and A. L. DeVico, unpublished).

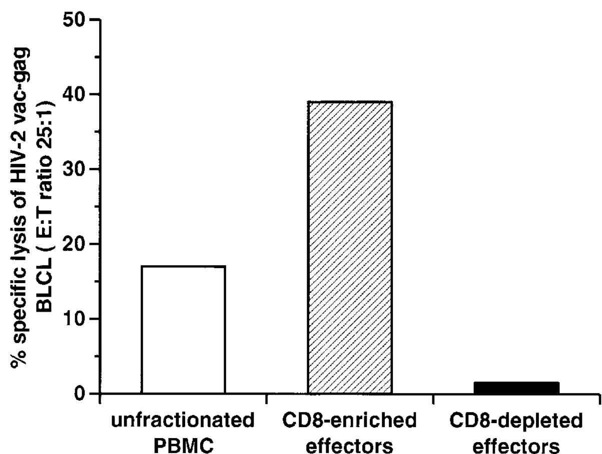

FIG. 7. Phenotype of CTL effectors in animal 292. Specific lysis exhibited by unfractioned PBMC (open bar) or fractions enriched (cross-hatched) or depleted (solid bar) of $CD8^+$ lymphocytes against autologous BLCL infected with recombinant vaccinia expression HIV-2_{KR} *gag*. Cytolytic assays were performed using PBMC from animal 292 obtained 4 wpi at an effector:target ratio of 25:1.

A larger trial is now under way to confirm and expand upon these initial results to explore whether live-virus vaccines which produce only transient infection, poten-

tially more acceptable for eventual human trial, might nonetheless confer significant protection from development of immunodeficiency disease.

MATERIALS AND METHODS

Cells

HeLa-CD4 (HT4-6C), Molt4/Clone 8 (Molt4/8), and peripheral blood mononuclear cells (PBMC) were grown in RPMI containing 10% heat-inactivated fetal calf serum (FCS) with 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. PBMC were stimulated with 5 μg/ml PHA-P for 24–48 h (Sigma, St. Louis, MO) and cultured in media containing 100 U/ml human IL-2 (Schiapparelli Biosystems, Columbia, MD).

Viruses

The immunizing viral stock was prepared from Molt4/8 cells infected with HIV-2_{KR} (Kraus *et al.,* 1998) and titered on HT4-6C (Chesebro *et al.,* 1991; Kimpton and Emmerman, 1992). HIV-2_{287} was derived by serial passage of HIV-2_{EHO} (Dr. Luc Montagnier, Pasteur Institute) in *M. nemestrina.* Animal F87265 (265) was inoculated intravenously with 10^6 $TCID_{50}$ cell-free HIV-2_{EHO} as well as 10^7 autologous PBMC infected *in vitro.* Ten milliliters of whole blood obtained from macaque 265 at 44 wpi was transfused into animal F89071 (071), and then 10 ml of blood from animal 071 at 20 wpi was inoculated into F89287 (287). The HIV-2_{287} challenge stock was derived from coculture of lymph node mononuclear cells of animal 287 with fresh stimulated allogenic macaque PBMC. Virus stocks were prepared as clarified supernatants (3000 *g* for 20 min at 4°C) and aliquoted and stored at −80°C until use. HIV-2_{287} virus for neutralization assays was obtained from coculture of plasma from infected animal 192 at 1 wpi with human PBMC.

Animal immunization

Animals J90292 and F90350 (292 and 350) were inoculated with 10^4 SFU and macaques F90404 and F90407 (404 and 407) were immunized with 10^3 SFU of HIV-2_{KR} (Kraus *et al.,* 1998). Challenge was performed 14 months after immunization with HIV-2_{KR}. Animals 292 and 407 were inoculated intravenously with 10^5 $TCID_{50}$ HIV-2_{287} and animals 350 and 404 with 10^1 $TCID_{50}$ of HIV-2_{287}. Naive animals J92096, T92192, F91368, and M91392 (096, 192, 368, and 392) were inoculated with 10^5 $TCID_{50}$ and F92147 and J92172 (147 and 172) were inoculated with 10^1 $TCID_{50}$ of HIV-2_{287} (Table 1). Negative control animals included J92165 and F92296 (165 and 296).

Analysis of lymphocyte subsets and flow cytometry

Lymphocytes purified from buffy coats of freshly collected EDTA anticoagulated whole blood were stained to enumerate T-cell subsets using FITC-conjugated anti-CD4 (Leu 3a) and phycoerythrin-conjugated anti-CD8 (Leu 2a) monoclonal antibodies according to the manufacturer's directions (Becton–Dickinson, Mountain View, CA) for flow cytometric analysis (Coulter).

DNA and RNA extraction

PBMC were isolated by 93% Ficoll–Paque (Pharmacia) gradient centrifugation of blood diluted 1:1 with Hanks' balanced salt solution (HBSS). DNA from PBMC (3–10 × 10^6) was extracted using QIAamp columns (QIAGEN) according to the manufacturer's instructions. For viral RNA, acid–citrate–dextrose anticoagulated plasma (400 μl to 4 ml) was ultracentrifuged (100,000 *g,* 1 h, 4°C), pellets were lysed in 200 μl RNAzol (Tel-Test Inc., Friendswood, TX), and RNA was isolated according to the manufacturer's directions. For quantitation, RNA was resuspended in 10–40 μl diethylpyrocarbonate (DEPC)-treated H_2O.

Quantitative culture

Lymphocytes were serially diluted 1:10 in duplicate, starting with 10^6 cells, into 24-well tissue culture plates containing 10^6 allogenic PHA/IL-2 stimulated *M. nemestrina* PBMC. Wells were sampled weekly and fed with fresh cells as needed, and p26 antigen was determined by Coulter SIV Antigen Capture EIA. Cultures were considered positive if the supernatant p26 exceeded background by threefold. Titers were calculated as the geometric mean of the maximal dilution of cells which gave positive cultures.

Quantitative PCR

Quantitative competitive PCR (QC–PCR) was performed on aliquots of genomic DNA or 10-μl volumes of RNA, diluted so that equivalence occurred between 10^3 and 10^5 copies of QC template. QC template was produced by cloning a 51-bp synthetic sequence into a *Spe*I–*Sma*I deletion in the wild-type PCR product in pBS-KS^-. RNA was transcribed using SP6 RNA polymerase (Riboprobe kit, Promega). Template RNA (10^1–10^6 copies) was added to 100 pmol of downstream primer 126 (5′-CAA-AGC-CAA-TTG-GTG-TTA-TC-3′) in a final volume of 14.2 μl, denatured at 68°C for 8 min, chilled on ice, and added to reverse transcriptase mix (25 mM Tris–HCl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 100 μg/ml BSA, 1 mM dNTP, 10 mM DTT) and 200 U of M-MLV reverse transcriptase (GIBCO-BRL, Life Technologies) in a final volume of 20 μl. Reverse transcription (37°C, 45 min) was followed by 2 min at 90°C and 2 min at 4°C. For QC–PCR of viral DNA, QC template DNA was added directly to 100- to 500-ng samples of genomic DNA in 20 μl H_2O. For PCR, 20-μl samples were added to 100 μl PCR reaction mix containing 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 200 μM each dNTP, 7 mM digoxigenin-11–dUTP, 1 μM each of primer 124 (5′-AGT-CTC-

ATA-GCC-AAC-ATT-GA-3′) and primer 126, and 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim). Samples were denatured at 94°C for 45 s, followed by 35 cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 60 s, and final extension for 7 min at 72°C.

EIA was performed in duplicate on each sample. Reaction products (20 μl) were mixed with 50 μl of 2× hybridization solution (12× SSC, 10 mM Tris–HCl, pH 7.5, 10 mM EDTA) and 30 μl of 50 nM 3′-biotinylated capture probe (wild-type probe env144: 5′-AGT-GCA-GAA-GTG-TCA-GAA-CTG-TAT-AAA-TTG-GA-3′ or QC probe env145: 5′-TGG-GCT-ACG-TCT-TGC-TGG-CGT-TCG-CGA-CGC-GA-3′). Samples were denatured (95°C, 5 min), cooled on ice (1 min), and hybridized (20°C, 45 min). Streptavidin-coated plates (Pierce, Rockford, IL) were washed three times with buffer A (100 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20), incubated with blocking solution (0.05% casein and 100 μg/ml salmon sperm DNA in buffer A, 45 min, 20°C), and washed 3×. Aliquots of the hybridization reaction (50 μl) were incubated in duplicate wells (20°C, 1 h), wells washed 3×, 50 μl anti-digoxigenin alkaline phosphatase conjugate (1:750 dilution, buffer A) added, and plates incubated (37°C,45 min). Substrate (100 μl, 3.8 mM *p*-nitrophenyl phosphate, 0.5 mM $MgCl_2$, 87 μl diethanolamine, pH 9.5) was added to each well, and absorbance read at 405 nM (605 nM background) at 30-min intervals. Controls included *M. nemestrina* genomic DNA, standard curves of WT and QC plasmid, and titrated WT plasmid with constant QC plasmid copies.

Qualitative RT–PCR from spleen viral RNA

RNA was extracted from the spleen of macaques 350 and 407. After mincing, tissue was dissolved in RNAzol, and RNA obtained as described above. Samples were reverse-transcribed (Access RT–PCR, Promega) and amplified (35 cycles, 94°C 58°C) with 5′ primer 290 (5′-CAA-TAA-AAC-CAT-GTG-TTA-AAT-TAA-CC-3′) and 3′ primer 244 (5′-GCA-CAA-TAC-CTA-AAT-CTT-AAA-CTA-TCC-3′), according to the manufacturer's directions.

Western blot analysis

Sera were heat-inactivated at 56°C for 30 min before incubation with Western blot strips. Strips were prepared using 200× concentrated HIV-2_{KR} virus (100,000 *g*, 1 h) grown in Molt4/8 cells. Pelleted virus was lysed in sample buffer (65 mM Tris–HCl, 2% SDS, 10% glycerol, 0.01% bromphenol blue, 4% 2-β-mercaptoethanol, pH 8.0), run on 12% polyacrylamide gels, and transferred to PVDF membranes (Immobilon) at 200 mA overnight in a Hoefer semidry blotting apparatus. Strips were blocked (5% powdered milk, 0.1% Tween 20, and 5% horse serum, 100 mM Tris, 1 h), incubated with sera (1:50 in PBS) overnight at 4°C, and washed 3× (PBS with 0.05% Tween 20), and protein bands developed using protein G horseradish peroxidase conjugate (1:5000) and diaminobenzidine substrate solution (Sigma).

Virus neutralization

Virus neutralization was performed as previously described by syncytial focus assay using HT4-6C cells (Ho *et al.*, 1996) and by reduction of titer on PBMC. Neutralization assays on PBMC were performed by diluting virus serially (1:5 or 1:10 in duplicate) for each test serum in U-bottomed microtiter wells and adding a fixed 1:40 final dilution of each serum to each well. Neutralization activity against both HIV-2_{KR} and HIV-2_{287} was determined in separate experiments. Sera of challenged animals 292, 350, 404, and 407, at 0, 18, 27, and 58 wpi for HIV-2_{KR} and 0 and 58 wpi for HIV-2_{287}, were used. Virus/sera mixtures were incubated for 45 min at 4°C and 45 min at 37°C, and then 5×10^5 PHA/IL-2 treated PBMC were added to each well. Cells were infected for 3 h at 37°C, washed 3× with PBS, and incubated in RPMI medium containing 100 U/ml IL-2. Endpoints were determined by p26 antigen capture assay of culture well supernatants on day 8 for HIV-2_{KR} and on day 10 for HIV-2_{287}. Titration of virus alone, and in the presence of neutralizing positive control sera (pooled sera from SIV_{mac239}- and SIV_{mac251}-infected macaques, courtesy P. Johnson) were included. Results are reported as the virus titer in the presence or the absence of sera, expressed as the geometric mean log dilution which was positive.

Recombinant vaccinia virus

A *Kpn*I–*Eco*RI fragment of pEPG containing a portion of the leader sequence and the complete *gag* coding region (HIV-$2_{SBL-ISY}$ nucleotides 421–2653) was blunt-ended and cloned into the *Sma*I site of the vaccinia insertion vector pVV1 (Weir *et al.*, 1982) just downstream of the vaccinia growth factor promoter (VGFP) and inserted between the viral thymidine kinase sequences, to create pVV-ISY *gag*. The pVV-ISY *gag* vector was transfected into human TK^- 143B cells previously infected with VV-WR wild-type vaccinia virus. Recombinant vaccinia virus expressing HIV-2 *gag* (VV*gag*) was obtained as described (Panicali and Paoletti, 1982; Mackett *et al.*, 1984) by three cycles of plaque purification of BUdR-resistant TK^- viral clones screened for *gag* sequences using a ^{32}P-labeled pEPG probe (Steffy *et al.*, 1992). Viral stocks were grown on HeLa cells and stored at −80°C until use.

Target cell lines

Autologous B-lymphoblastoid cells lines (BLCL) were established by infecting 10^5 PBMC in 100 μl of R15 (RPMI medium with 15% FCS) with 100 μl supernatant from S394-1X1055 cells producing *Herpesvirus papio* (gift of Dr. Sharp, Southwest Foundation for Biomedical Re-

search) in 96-well U-bottomed microtiter plates (Costar, Cambridge, MA). BLCL were expanded, but not cloned.

Generation of CTL lines

CTL effectors were generated *in vitro* by two, weekly, sequential stimulation cycles by modification of previously described methods (Kent *et al.,* 1995). Stimulator cells were prepared using autologous BLCL, infected with VV *gag* (m.o.i. 10, 16 h), exposed to UV light (10 min at 4 cm distance) and γ-irradiation (8000 rad), and added to lymphocytes (10 responder:1 stimulator). Autologous PBMC filler cells were added (1 responder:1 filler) to cultures during the second stimulation. Each well, containing 10^6 responder cells/ml in R15 medium, was pulsed 48 h following stimulation with 10 U/ml of rIL-2 (Hoffman–LaRoche, Nutley, NJ).

Cytolytic activity assay

Cytolytic activity was measured in a standard 4-h ^{51}Cr release assay as previously described (Kent *et al.,* 1995, 1996). Autologous BLCL target cells (1.0×10^6) infected with VV*gag* were labeled (0.1 mCi $Na^{51}CrO_4$, NEN Products, Boston, MA) in 1 ml of medium and plated at 5×10^3 targets/well. Effector cells were added to targets at varying E:T ratios (total volume 200 μl) and incubated for 4 h at 37°C. The percent specific release was calculated by standard formula. Standard deviation of triplicate wells was always less than 10% and spontaneous release was always less than 25% of the maximal release.

Phenotype of CTL effectors

To determine the phenotype of cells mediating cytolytic responses, CD8 lymphocytes were depleted by adsorption to antibody coated flasks (A.I.S., Menlo Park, CA) according to the manufacturer's directions (Kent *et al.,* 1995, 1996). Nonadherent (CD8 depleted) cells were aspirated and adherent (CD8 enriched) cells gently removed. Staining with phycoerythrin-conjugated Leu 2a (Becton–Dickinson, Mountain View, CA) confirmed that the CD8-enriched population contained >94% and the CD8-depleted population <6% $CD8^+$ T cells.

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