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Immunology

Enhanced immunity to human immunodeficiency virus (HIV) envelope elicited by a combined vaccine regimen consisting of priming with a vaccinia recombinant expressing HIV envelope and boosting with gp160 protein

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ABSTRACT **Transmission studies have suggested that an optimal human immunodeficiency virus type 1 (HIV-1) vaccine should induce both neutralizing antibodies and cytolytic T cells to eliminate free virus and infected cells. A phase I trial in healthy HIV-1-seronegative persons was conducted with a combination HIV-1 vaccine regimen (strain IIIB) consisting of priming with a recombinant vaccinia (vac/env) virus expressing HIV-1 envelope and boosting with a gp160 glycoprotein derived from a recombinant baculovirus (rgp160). T-cell and antibody responses detected after immunization with either vac/env alone or rgp160 alone were generally of low magnitude and transient, and no subject developed neutralizing antibodies. In contrast, recipients of the combination regimen demonstrated *in vitro* T-cell proliferative responses to homologous HIV-1 antigens that were 3- to 10-fold higher than responses with either vaccine alone, and these responses were sustained for >18 months in 75% of recipients. Moreover, both CD8$^+$ and CD4$^+$ cytolytic T cells were detected. Antibody responses (titer, 1:800 to 1:102,400) to homologous HIV envelope developed in all recipients of the combination regimen, and neutralizing antibodies were detected in 7 of 13. Thus, immunization with a live virus vaccine followed by boosting with a soluble protein offers promise for inducing the broad immunity needed in an HIV vaccine.**

Advances in biotechnology have made it possible to utilize recombinant DNA for the design of vaccines for human immunodeficiency virus (HIV), including the production of individual HIV proteins and the construction of recombinant live vectors (1–3). Recombinant live virus vaccines offer some advantages over inactivated virus or recombinant protein vaccines. Replication of the modified live virus or attenuated bacterium in the host can result in amplified and sustained expression of the HIV gene product *in vivo* and in presentation to the immune system of HIV antigens, more closely simulating natural infection (4–7).

Vaccinia virus represents an attractive vector for HIV vaccine development. It can accommodate large pieces of foreign DNA with retention of virion function (8, 9) and has been documented to be safe, immunogenic, and efficacious as a vaccine (10). However, one potential obstacle to the general use of a recombinant HIV-1 vaccinia vaccine is that preexisting immunity to vaccinia can limit its replication and therefore interfere with priming to the nonvaccinia antigens expressed by the recombinant vector (11–13).

Viral proteins can be produced in large quantities through recombinant expression systems (14, 15) and provide an alternative immunogen not dependent on replication in the host. Vaccination with such soluble HIV envelope proteins has been shown to be safe (16). These vaccines have induced humoral antibodies to HIV envelope but to date have elicited T-cell responses to HIV-1 that have been of limited magnitude and duration (16–18). In an attempt to combine the advantages of each of these vaccine preparations, we conducted a study in HIV-seronegative individuals to determine if a combination HIV vaccine approach, consisting of priming with a recombinant vaccinia HIV-1 envelope vaccine and boosting with a recombinant soluble gp160 envelope glycoprotein (rgp160), could elicit the broad immune response likely necessary to provide protection from HIV.

MATERIALS AND METHODS

Study Population. Informed consent was obtained from 13 of the 15 HIV-1-seronegative subjects who had previously participated in a phase I trial evaluating a recombinant vaccinia virus expressing the LAV-BRU strain HIV envelope glycoprotein gp160 (vac/env) (HIVAC-1e, Bristol-Myers Squibb Research Institute, Seattle) (13). Prior to vac/env vaccination, 11 of 13 subjects had received smallpox immunization, and 2 subjects were vaccinia-naive. Twelve to 15 months after their first inoculation with vac/env, these 13 subjects were administered intramuscularly 8 weeks apart two 160-μg doses of an alum-based baculovirus-derived (strain IIIB) rgp160 vaccine (VaxSyn, MicroGeneSys) (Table 1) (16). Four new HIV-1-seronegative volunteers were administered four 160-μg doses of rgp160 alone at study entry and at postvaccination months 1, 6, and 12. All patients were evaluated for local and systemic reactions on days 2, 4, 7, 10, and 14 and weekly for 6 weeks after each vaccination.

Lymphoproliferative Assays. *In vitro* lymphoproliferative responses were assessed as previously described on all individuals 60 days prior to vaccination with vac/env and at 0, 4, 8, 12, and 16 weeks and at 7 and 12 months after vac/env administration (12). After administration of the rgp160 booster, lymphoproliferative assays were again performed on day 0 and weeks 4, 8, 12, 24, and 48–52 after immunization. The HIV antigens utilized included UV-inactivated psoralen-treated HIV-1 (LAV-BRU strain), designated inactivated HIV-1, and baculovirus-derived gp160 protein (IIIB strain)

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Abbreviations: HIV, human immunodeficiency virus; rgp160, recombinant glycoprotein gp160; SI, stimulation index; PBMC, peripheral blood mononuclear cells; LCL, lymphoblastoid cell lines; IL-2, interleukin 2; CTL, cytotoxic T lymphocytes; EIA, enzyme immunoassay; MHC, major histocompatibility complex.

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Table 1. Immunization regimens with vac/env and rgp160

Vaccine dose	Combination vaccine		rgp160 vaccine only	
	Immunogen	Time after priming, mo	Immunogen	Time after priming, mo
Prime	vac/env	0	rgp160	0
Booster	vac/env	2	rgp160	1
Booster	rgp160	10–13	rgp160	6
Booster	rgp160	12–15	rgp160	12

(MicroGeneSys) (13). Non-HIV-1 soluble antigens and mitogens included gradient-purified UV-inactivated vaccinia virus, heat-inactivated herpes simplex virus, tetanus toxoid, candida antigen, and phytohemagglutinin (PHA). [^{3}H]Thymidine incorporation expressed as Δcpm was calculated as the difference between mean cpm of four replicate-stimulated wells and the mean of unstimulated control wells. Preliminary experiments indicated that a positive response required a stimulation index (SI) of >3.0 to the inactivated HIV antigen and an SI of >4.0 to rgp160. The SI was calculated by dividing the mean cpm of the four replicate-stimulated wells by the mean of the unstimulated control wells.

Cytotoxic T-Lymphocyte (CTL) Assays. *CD4$^+$ CTL activity.* Peripheral blood mononuclear cells (PBMC) (10^6 cells per ml) were placed into 96-well plates and stimulated *in vitro* for 7 days with 2.5 μg of gp160 per ml, harvested, and restimulated for an additional week with gp160, autologous irradiated PBMC, and recombinant interleukin 2 (IL-2) (Cetus) at 2 units/ml. On day 14 bulk cultures were cloned by limiting dilution, with autologous irradiated PBMC, gp160 antigen, IL-2, and autologous Epstein–Barr virus-transformed B lymphoblastoid cell lines (LCL) as filler cells. T-cell phenotype was determined by fluorescence-activated cell sorter (FACS) analysis using anti-CD4 and anti-CD8 monoclonal antibodies. Autologous or *HLA-DR*-mismatched allogeneic LCL targets were pulsed with gp160 (LAV), gp120 (SF-2 strain, a gift from K. Steimer, Chiron), or baculovirus control supernatant at 10 μg/ml or were infected with vac/env 5 or New York strain vaccinia (multiplicity of infection = 10:1) for 12–16 hr. Cytolytic activity was evaluated in a 4-hr ^{51}Cr release assay.

CD8$^+$ CTL activity. PBMC (2×10^6) were stimulated for two sequential 1-week periods with 2×10^5 autologous macrophages infected with HIV (BAL strain) in 24-well plates. The effector population was selectively depleted of either CD4$^+$ or CD8$^+$ cells by adherence to AIS MicroCELLector T-25 cell-culture flasks. Target cells included autologous and *HLA*-mismatched LCLs infected with v-*env2* (containing the LAV *env* gene *Kpn* I fragment 5889–8572) or vaccinia (New York strain) for 90 min and labeled overnight with ^{51}Cr for evaluation in a 4-hr lytic assay (19).

Antibodies to HIV. Serum was obtained from each participant before vaccination and every 2 weeks for the first 12 weeks after vaccination, and at 6, 9, and 12 months after both primary and booster vaccination. Antibodies to HIV-infected cells were assayed by enzyme immunoassay (EIA) (Genetic Systems, Seattle). Antibodies to denatured HIV-1 envelope proteins were determined by diluting sera 1:1 from a 1:100 dilution to a 1:1,622,400 dilution and treating with denatured HIV IIIB lysates (13). The titer was defined as the highest dilution giving a band to gp160, gp120, or gp41 on the immunoblot. All sera were tested concurrently with the same lot of antigen. Neutralizing antibodies to HIV-1 strain IIIB were detected by microneutralization (12). Sera were diluted 1:1 from a 1:5 dilution to a 1:1280 dilution and mixed with 15 $TCID_{50}$ (tissue culture 50% infectious dose) of HIV-1 strain IIIB grown in CEM cells for 1 hr at 37°C; the serum–virus mixture was added to 10^6 PHA-stimulated human PBMCs in 24-well plates. The culture supernatant was assayed for HIV-1 antigen production at 14 days, and a positive serum was defined as inhibition ≥ 1:10 titer. All sera with neutralizing activity were confirmed in a separate syncytia-inhibition assay with CD4-transfected HeLa cells (20). Sera were incubated with 200 syncytia-forming units of the HIV-1/LAV-1 strain and then added to SupT-1 cells in 96-well plates. The fusion-inhibition titer was defined as the serum dilution that reduced the number of syncytia by 50%.

RESULTS

T-Cell Responses to HIV-1 Antigens After Vaccination with the Vaccinia gp160 and Soluble Recombinant Protein Alone. The T-cell responses to HIV-1 and other stimuli after vaccination with vac/env alone have been described (13). Low T-cell proliferative responses to inactivated HIV-1 were detected in 8 of 11 vaccinia-immune individuals during the first 16 weeks after vac/env vaccination [median peak SI of 9.0 (range, 4–24)] (Table 2). These responses diminished rapidly; all 8 no longer exhibited *in vitro* activity to inactivated HIV-1 12 months after immunization. The 2 vaccinia-naive individuals demonstrated strong T-cell responses to inactivated HIV-1 during the first 16 weeks after priming [mean peak SI of 77 (range 20–133)], but again neither responded to inactivated HIV-1 by month 12.

Recipients of gp160 alone demonstrated lower *in vitro* proliferative responses to inactivated HIV-1 than did recipients of vac/env alone (Table 2). After two doses of rgp160, only 1 of 4 subjects responded to inactivated HIV-1. After four doses, only 2 of 4 responded to inactivated HIV, and none of the 4 subjects responded by 6 months after the fourth dose. *In vitro* lymphoproliferative responses to the immu-

Table 2. T-cell proliferative responses to psoralen-UV-inactivated HIV-1

Time of assay	Proliferative responses of vaccine groups								
	Combined vaccine								
	Vaccinia-primed (n = 2)			Vaccinia-naive (n = 11)			rgp160 only (n = 4)		
	Median Δcpm (mean)	Median SI	Responders (%)	Median Δcpm (mean)	Median SI	Responders (%)	Median Δcpm (mean)	Median SI	Responders (%)
After vac-env priming*	2,326 (2231)	9	8/11 (72)	20,568	77	2/2 (100)	NA	NA	NA
Before rgp160 vaccine†	30 (33)	1	0/11 (0)	746	1	0/2 (0)	260 (230)	1	0/4 (0)
Early rgp160 booster‡	12,573 (9434)	43	10/11 (91)	59,194	352	2/2 (100)	422 (716)	3	1/4 (25)
Late rgp160 booster§	7,300 (8484)	37	6/8 (75)	10,011	28	2/2 (100)	210 (760)	1	0/4 (0)

NA, not applicable.
*Data reflects maximal response detected during the 4- to 16-week period after priming with the first and second doses of vac/env.
†Specimen obtained on day of initial rgp160 immunization.
‡Specimen obtained 12 weeks after initial rgp160 immunization.
§Specimen obtained 16 months after last immunization with rgp160 in combined vaccine group and 6 months after last immunization in rgp160-alone group.

nizing rgp160 protein were also low (mean SI of 18.2) and became undetectable 6 months after the fourth immunization.

Lymphoproliferative Responses After Priming with vac/env and Boosting with rgp160. Administering rgp160 boosters to vac/env recipients markedly increased T-cell responses to HIV-1 antigens (Table 2). Four weeks after the first booster, 12 of 13 subjects including 10 of the 11 subjects who had received vaccinia immunization prior to their vac/env vaccination showed robust T-cell responses to HIV-1. The median proliferative response to HIV-1 12 weeks after immunization with vac/env and boosting with gp160 was 12,573 Δcpm versus peak responses after vac/env immunization alone and rgp160 alone of 2326 Δcpm and 716 Δcpm, respectively (Table 2) [$P < 0.01$ for the combination versus single vaccine recipients (Mann–Whitney test)]. These responses to HIV-1 persisted, with 90% of subjects still responding to inactivated HIV-1 at 6 months, 80% at 12 months, and 75% at 18 months after rgp160 boosting. All 11 previously vaccinia-primed subjects also demonstrated marked enhancement of lymphoproliferative responses to gp160 after boosting with rgp160. The median SI to gp160 increased from 1.3 prior to boosting to 78 4 weeks after the first booster and 388 after the second booster ($P < 0.01$).

The 2 vaccinia-naive individuals also had increased responses to HIV after rgp160 immunization. The mean SI to inactivated HIV-1 increased to 352 (Table 2) and remained strongly reactive 18 months after boosting. Responses to gp160 were even stronger, with a mean SI of 866 4 weeks after the second booster and 122 18 months after boosting.

Responses to non-HIV antigens including PHA, candida, tetanus toxoid, and herpes simplex virus did not significantly change in any subject. No changes in circulating $CD4^+$ cell number, immunoglobulin concentrations, or hematologic parameters occurred after administration of either the individual or combination vaccine regimens.

Antibody Response After Vaccination with vac/env or gp160 Alone. Vac/env vaccination alone elicited antibodies to HIV-1 envelope protein in only 3 (2 vaccinia-naive and 1 vaccinia-primed) subjects. Antibodies were demonstrable only by Western blot analysis, and no neutralizing activity or binding to infected cells (EIA) were detected. Peak antibody titers in the vaccinia-naive individuals were 1:200 and 1:800 and were no longer measurable 7 and 12 months later. In the one responding vaccinia-primed subject, antibody to HIV-1 was detected only at a 1:100 dilution and was no longer detectable 16 weeks after vac/env immunization.

Among the 4 subjects who received rgp160 alone, no neutralizing antibodies were detected at any time after vaccination (Table 3). EIA antibody developed in 1 subject after the third dose of vaccine but lasted for <4 weeks. Western blot antibodies developed in all 4 recipients, with median peak titers after 4 doses of rgp160 of 1:3200. However, only 1 of these 4 subjects still possessed detectable Western blot antibodies 6 months after the fourth dose, and no individuals were reactive at 12 months.

Enhanced Antibody Response in Recipients of vac/env Followed by rgp160. Recipients of the combination vaccine regimen demonstrated higher antibody titers to HIV-1 envelope than recipients of either vac/env or rgp160 alone, and neutralizing antibodies developed in 7 of 13 subjects. Prior to boosting, only 1 of 13 vac/env subjects had detectable antibodies to HIV-1 envelope (Table 3). The initial booster of rgp160 induced prompt, high-titer antibodies in both the vaccinia-naive and vaccinia-primed individuals (Fig. 1). Nine of 11 vaccinia-primed individuals demonstrated antibodies to gp160 2 weeks after the initial booster with rgp160, consistent with an anamnestic response (Fig. 1). After rgp160 boosting, Western-blot antibodies to HIV envelope protein were detected in all 13 subjects, and EIA antibodies were detected in 10 subjects (Table 3). While antibody titers decreased over time, 10 of 12 subjects still had antibodies to denatured gp160 18 months after the booster.

Neutralizing antibodies developed after the first booster with rgp160 in 2 subjects and after the second booster of rgp160 in 5 subjects (Table 3). Two of 2 vaccinia-naive and 5 of 11 vaccinia-primed individuals developed neutralizing antibodies. All 7 also demonstrated inhibition of HIV-1 strain LAV BRU in the CD4-HELA cell syncytia-inhibition assay. Neutralizing antibodies persisted for a shorter duration (mean, 18 weeks) than Western-blot antibodies. Only 1 vaccinia-primed subject had neutralizing antibodies at 12

Table 3. Antibody responses to HIV-1

	Response of vaccine groups in antibody assays					
	Combined vaccine					
	Vaccinia-primed (n = 11)		Vaccinia-naive (n = 2)		rgp160 only	
Time of assay	Responders/n	Median titer	Responders/n	Median titer	Responders/n	Median titer
	Western blot (Ab to denatured virus)					
Before rgp160 vaccine*	0/11	<1:100	1/2	1:400	0/4	<1:100
4 wk after two doses rgp160	11/11	1:3200	2/2	1:102,400	1/4	<1:100
4 wk after four doses rgp160	NA	—	NA	—	4/4	1:3200
16 mo after last rgp160†	8/10	1:400	2/2	1:25,600	1/4	1:400
	EIA (Ab to infected cells)					
Before rgp160 vaccine*	0/11	<1.0	0/2	<1.0	0/4	<1.0
4 wk after two doses rgp160	8/11	1.6‡	2/2	4.8‡	0/4	<1.0
4 wk after four doses rgp160	NA	—	NA	—	1/4	<1.0
16 mo after last rgp160†	0/10	<1.0	1/2	1.1‡	0/4	<1:10
	Neutralization (Ab to infected virus)					
Before rgp160 vaccine*	0/11	0	0/2	0	0/4	<1:10
4 wk after two doses rgp160	5/11	1:20	2/2	1:40	0/4	<1:10
4 wk after four doses rgp160	NA	—	NA	—	0/4	<1:10
16 mo after last rgp160†	0/11	<1:10	1/2	1:10	0/4	<1:10

*Specimen obtained on day of initial rgp160 immunization.
†Specimen obtained 16 months after last immunization with gp160 in combined vaccine group and 6 months after last immunization in rgp160-alone group.
‡Titer represented as "R" value (ratio of the absorbance value of the specimen/mean absorbance of the negative control.

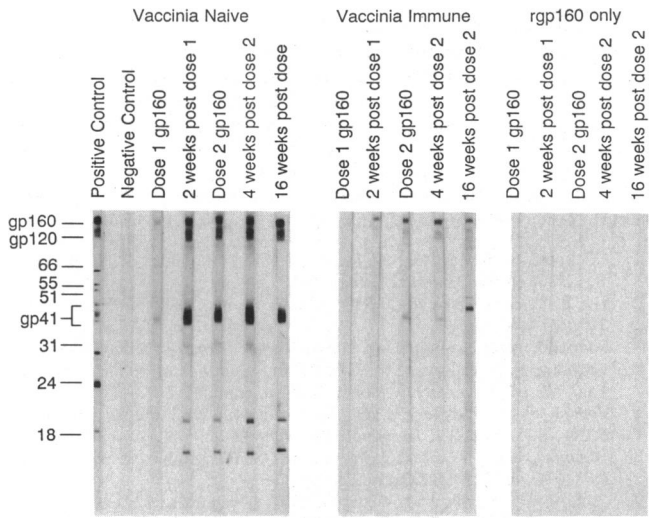

FIG. 1. Temporal profiles of antibody responses to HIV-1 envelope proteins after priming with vac/env and boosting with rgp160. All sera (except the specimens obtained 16 weeks after dose 2 of rgp160) were tested concurrently with the same lot of antigen. The vaccinia-naive patient had a low titer of antibody to gp160 (1:400) 1 year after vac/env and just prior to the first dose of rgp160. The titer 2 weeks after dose 1 was 1:25,600 and was 1:102,400 at the time of dose 2 and at 4 and at 16 weeks after dose 2. Reactivity at 19 and 16 kDa was detected after vac/env immunization in this and other vac/env recipients. The vaccinia-immune recipient lacked antibodies to gp160, gp120, and gp41 at all time points after vac/env immunization and at the time of dose 1. The titer of gp160 antibody 2 weeks after dose 1 was 1:1600 and increased to 1:3200 at weeks 4 and 16 after dose 2. Only minimal responses to gp160 (1:100) were seen after 2 doses of rgp160 in the subject who received only rgp160.

months, while 1 vaccinia-naive individual had neutralizing antibodies at both 12 and 18 months after rgp160 boosting.

Cytotoxic Immune Responses After Vaccination. Consistent with the strong $CD4^+$ proliferative T-cell responses, gp160-specific $CD4^+$ T-cell clones with cytolytic activity were readily detected up to 10 months after gp160 boosting in both a vaccinia-naive and a vaccinia-immune recipient of the combination vaccine regimen. A representative clone from a vaccinia-primed individual is shown in Fig. 2. This clone both proliferated in response to HIV-1 envelope proteins and lysed autologous targets, but not HLA class II-mismatched allogeneic targets, that had been pulsed with either homologous IIIB or heterologous SF-2 HIV viral envelope antigens or infected with vac/env (Fig. 2).

During the conduct of this trial, techniques to express HIV-1 envelope genes in autologous stimulators and targets with vectors other than vaccinia recombinants were not available for analysis of $CD8^+$ CTL responses. Recently, we developed a method to detect $CD8^+$ CTLs using HIV-1 infected autologous macrophages as stimulator cells. A vaccinia-naive patient who had recently received vac/env and rgp160 immunization had with this method detectable $CD8^+$ cytolytic responses to HIV-1 envelope at 3, 4, 6, and 9 months after rgp160 boosting (months 7, 8, and 10 after the initial vac/env priming) (Fig. 3). This cytolytic activity was specific for HIV envelope and was MHC class I-restricted. The envelope-specific cytolytic activity was mediated by $CD8^+$ T cells, as 80% of the lytic activity remained after depletion of $CD4^+$ cells ($\leq$2% of the residual T-cell population expressed CD4) and only minimal lytic activity was seen after removal of $CD8^+$ cells. The absence of similar HIV envelope-specific $CD8^+$ CTL in unimmunized seronegative controls indicated the lytic activity resulted from the immunization.

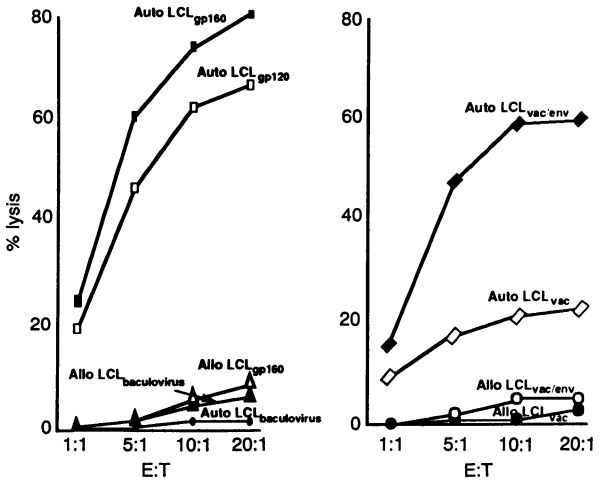

FIG. 2. Cytolytic (CTL) reactivity of an HIV-1-specific $CD4^+$ clone derived from an individual primed with vac/env and boosted with rgp160. E:T is the effector-to-target cell ratio. (*A*) Cytolytic reactivity was assessed against autologous or *HLA-DR*-mismatched allogeneic LCL pulsed with gp160, gp120, or baculovirus control supernatant at 10 μg/ml for 12–16 hr at 37°C. (*B*) Cytolytic reactivity was examined against targets infected with vac/env 5 or New York strain vaccinia virus.

DISCUSSION

Both cell-free and cell-associated HIV can be found in human semen and blood and have been shown to establish infection in experimental animals (21–23). Thus, optimal protection from HIV infection will likely require the presence of adequate neutralizing antibodies to bind cell-free virus and adequate cytolytic effector cells to eliminate virally infected cells during the early stages of infection. This requirement for a broad effector response may be difficult to achieve with one type of recombinant subunit vaccine, since different presentations of immunogens vary in efficiency for inducing individual components of the immune response. In general, recombinant proteins can effectively prime $CD4^+$ cells and induce antibody responses but are generally ineffective at inducing $CD8^+$ CTL because of a failure to associate intracellularly with class I MHC antigens (24). Consistent with this paradigm, $CD4^+$ clones that proliferate in response to HIV or lyse HIV-1-infected cells have been detected after immunization of humans with gp160 protein, but it has not been possible to identify $CD8^+$ CTL from these vaccinees (25). By contrast, recombinant vaccinia viruses infect target cells, resulting in intracellular expression of viral genes, and effectively induce class I-restricted $CD8^+$ CTL responses to the

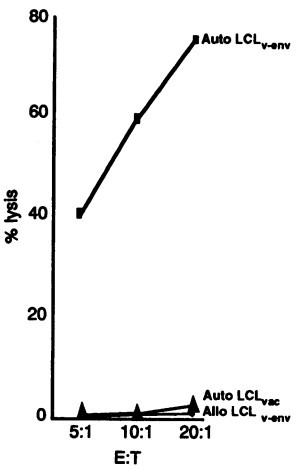

FIG. 3. HIV-1 envelope-specific $CD8^+$ CTL in a seronegative vaccinee 8 months after the rgp160 booster. Effector cells were derived from bulk PBMCs stimulated with autologous HIV-infected macrophages. Target cells were LCLs infected with vac/env (autologous and allogeneic) and vaccinia (autologous).

product of the inserted gene (26, 27). Thus, $CD8^+$ responses to HIV and simian immunodeficiency virus envelope proteins have been detected after immunization of primates with vac/env vaccines (15, 27).

The immune responses detected to the HIV vaccines in our trial appear to follow these models. Vac/env immunization alone induced poor antibody responses even among previously vaccinia-naive recipients, presumably because of limited presentation of antigen to B cells. rgp160 alone elicited better antibody responses than vac/env, although the most consistent responses were only to denatured HIV-1 envelope, and no neutralizing activity was detected. *In vitro* T-cell responses to inactivated HIV-1 were more pronounced and sustained among recipients of vac/env alone than the alum-based rgp160 recombinant vaccine. It should be noted that the schedule for immunization with vac/env priming and gp160 boosting differed from the immunization intervals with rgp160 alone. However, in subsequent studies with subjects receiving four injections of a 4 times higher dose of rgp160 (640 μg) than that used in this study, neutralizing antibodies were still not detectable despite a higher titer antibody response to denatured HIV envelope. These results suggest that the epitopes recognized by neutralizing antibodies are not efficiently presented by rgp160 protein.

Our results with rgp160 alone and vac/env alone are similar to those of others. Dolin and associates (16, 17, 28) and Kovacs *et al.* (29) reported that multiple doses of rgp160 do elicit antibodies to HIV-1 envelope by Western blot and occasionally EIA antibodies (16–29), but fusion inhibition and neutralizing antibodies are infrequent. Graham *et al.* (28) utilizing this same vac/env vaccine in a larger group of vaccinia-naive recipients demonstrated a similar lack of a sustained antibody response to gp160 HIV-1 envelope.

Combining the two vaccines in one immunization regimen resulted in quantitatively higher and more sustained antibody and T-cell responses than that induced by either vaccine alone. Moreover, this regimen was associated with the development of two important functional immune responses—cytolytic T cells of both the $CD4^+$ and $CD8^+$ phenotype and neutralizing antibodies. The proliferative T-cell responses induced by the combined vaccine approach appeared durable, with reactivity still detectable 18 months after boosting. In addition, the CD4 proliferative and cytotoxic responses recognized distantly related HIV strains. Several relevant issues require additional investigation: the durability of the $CD8^+$ CTL responses; whether more frequent boosting, use of more potent adjuvants, or boosting with envelopes derived from other strains of HIV-1 will increase the frequency and duration of neutralizing antibodies to HIV-1 envelope; and the effect of combination vaccines on mucosal immunity.

In summary, our data suggest that the use of combination vaccine regimens, consisting of priming with a live recombinant vaccinia virus expressing a HIV subunit protein (or possibly alternative live vectors such as avipox, adenovirus, or bacillus Calmette–Guérin) followed by boosting with soluble recombinant protein warrants further study for the development of an immunization strategy for HIV. Since the initial report of our study, Graham and colleagues (30) have also used rgp160 to boost vaccinia-naive recipients after priming with vac/env and observed similar augmented antibody responses as reported here. In addition, using an analogous recombinant-vaccinia and soluble-protein immunization regimen for simian immunodeficiency virus (SIV) in vaccinia-naive macaques, one of us has recently demonstrated protection of macaques from subsequent SIV challenge (31). Thus, this approach may have the potential to elicit the broadly based immune responses that almost certainly will be necessary to protect against HIV-1 infection.

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