Simian Immunodeficiency Virus-Specific Cytotoxic T-Lymphocyte Induction through DNA Vaccination of Rhesus Monkeys

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In view of the growing evidence that virus-specific cytotoxic T lymphocytes (CTL) play an important role in containing the early spread of human immunodeficiency virus type 1 (HIV-1) in infected individuals, novel vaccine strategies capable of eliciting HIV-1-specific CTL are being pursued in attempts to create an effective AIDS vaccine. We have used the simian immunodeficiency virus of macaques (SIV_{mac})/rhesus monkey model to explore the induction of AIDS virus-specific CTL responses by DNA vaccination. We found that the inoculation of rhesus monkeys with plasmid DNA encoding SIV_{mac} Env and Gag elicited a persisting SIV_{mac}-specific memory CTL response. These CTL were CD8⁺ and major histocompatibility complex class I restricted. These studies provide evidence for the potential utility of DNA inoculation as an approach to an HIV-1 vaccine.

In view of the growing evidence that virus-specific cytotoxic T lymphocytes (CTL) play an important role in containing the early spread of human immunodeficiency virus (HIV-1) in infected individuals (10, 16, 25), an HIV-1 vaccine should be capable of eliciting such an effector cell response. However, many of the HIV-1 vaccine strategies that have received the greatest attention to date, including the use of recombinant HIV-1 envelope glycoprotein immunogens, do not elicit virus-specific CTL (11). To induce HIV-1-specific CTL, viral antigen must be processed as peptide fragments to facilitate their association with major histocompatibility complex (MHC) class I molecules for presentation to CD8⁺ T cells (18, 26). Since MHC class I processing of viral antigen occurs most efficiently in the course of intracellular synthesis of viral proteins, the approaches to HIV-1-specific CTL induction that have been pursued most actively are those using live vectors. However, these strategies have, in general, given disappointing results in primate protection studies. Immunization with regimens that include recombinant vaccinia virus or *Mycobacterium bovis* BCG constructs has not protected macaques against challenge with most simian immunodeficiency virus (SIV) isolates (1, 4, 7–9, 24). Novel vaccine strategies capable of eliciting HIV-1-specific CTL should, therefore, be pursued.

Recent studies have raised the possibility that DNA-based vaccination may prove useful for generating virus-specific CTL responses. DNA vectors expressing proteins of influenza virus (15, 19, 23), HIV (6, 12), rabies virus (22), lymphocytic choriomeningitis virus (13, 27, 28), and malaria (17) have been shown to induce CTL responses in mice. These responses have been raised by injections of DNA in saline (13, 15, 17, 19, 23, 28) and by gene gun-mediated delivery of DNA (6, 12, 28). In the present study, we have evaluated the efficacy of DNA immu-

nization for the induction of SIV_{mac}-specific CTL in rhesus monkeys.

The immunogens in the studies presented here include five SIV_{mac} plasmids (11a). One of these, designated pJW4303/SIV239.sgp110, encoded the receptor binding subunit of the envelope glycoprotein of SIV_{mac}239. Three encoded all of the extracellular domains of the envelope glycoproteins of SIV_{mac}239, SIV_{mac}251, and SIV_{mac}316. These are designated pJW4303/SIV239.sgp130, pJW4303/SIV251.sgp130, and pJW4303/SIV316.sgp130. The final DNA, pCMV/SIV239.dpol, expressed non-infectious SIV_{mac}239 particles. SIV239.dpol encoded all the SIV_{mac} genes except for *pol* and *nef*. The transcriptional control elements for the pJW4303 plasmids (1a) included ~2,000 bp from the cytomegalovirus immediate-early promoter (including intron A) and polyadenylation sequences from the bovine growth hormone gene. Envelope sequences in the pJW4303 vector were cloned in frame with leader sequences modeled on those of the tissue plasminogen activator, a construction that rendered Env expression independent of Rev (2). The transcriptional control elements for the pCMV vector include ~750 bp from the cytomegalovirus immediate-early promoter and a downstream intron and polyadenylation signal from the rat preproinsulin II gene (see pBC12/CMV in reference 3). Plasmid DNAs were grown in *Escherichia coli* DH5 α or HB101 and purified by two equilibrium centrifugations on CsCl gradients.

Nucleotide inoculations were administered in clusters, at weeks 1 and 3, weeks 11 and 13, and again at weeks 21 and 23. At the first two inoculations, four rhesus monkeys (894, L37, L44, and 8RK) were given each of the three SIV_{mac}239 DNA constructs by three routes: intravenous (500 μ g), intramuscular (500 μ g), and gene gun (four inoculations of 7.2 μ g DNA per inoculation). Three monkeys (L116, 8AV, and 8R5) received the three SIV_{mac}239 DNAs by gene gun only (four inoculations for each construct). At the fourth through sixth inoculations, both groups received the same inoculations as the first two plus two gene gun inoculations of the SIV_{mac}251 DNA and two

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TABLE 1. SIV_{mac} Env-specific lysis in nucleotide vaccine-elicited effector T lymphocytes 24 weeks after initial immunization

Immunization and effector cells ^a	% Release ^b		
	vac-env	vac-cont	Specific
Control			
8R7	20	21	0
8B7	11	14	0
Intramuscular, intravenous, and gene gun			
894	37	19	18
L37	39	23	16
L44	33	16	17
8RK	62	26	36
Gene gun			
L116	48	29	19
8AV	25	15	10
8R5	21	9	12

^a Effector cells were PBL from the indicated monkeys stimulated in vitro with paraformaldehyde-fixed, vaccinia virus-SIV_{mac} Env-infected B-LCL as described in the legend to Fig. 1. The construction of these recombinant vaccinia viruses was described previously (14).

^b The ⁵¹Cr release assay was performed as described in the legend to Fig. 1 at an effector/target ratio of 20:1. Target cell B-LCL were infected with a vaccinia virus-Env (vac-env) or a control vaccinia virus-equine herpesvirus gH gene (vac-cont) construct.

gene gun inoculations of the SIV_{mac}316 DNA. For gene gun inoculations, 7.2 µg of DNA was precipitated onto 1.44 mg of 1- to 3-µm gold beads in the presence of calcium chloride and spermidine (5).

A number of different techniques for expanding CD8⁺ memory CTL from peripheral blood lymphocytes (PBL) of the vaccinated rhesus monkeys were considered. While peptide restimulation of PBL results in the greatest expansion of effector cells with the lowest background killing, this type of assay can be done only on PBL of monkeys that have been selected for specific MHC class I haplotypes (24). Lectin stimulation of PBL is the least sensitive approach to expanding CTL in vitro; however, low backgrounds are generally seen with this technique (14). The use of recombinant vaccinia virus-infected, fixed, autologous B-LCL is of intermediate efficiency in expanding CTL in vitro and is associated with consistent but acceptable background levels of killing in assays (20). The latter approach was selected for the present studies.

SIV_{mac}-specific CTL activity in PBL of these vaccinated monkeys was assessed, as previously described (20), at 1, 4, and 7 weeks following each cluster of vaccinations. Autologous herpesvirus papio-immortalized B lymphoblastoid cell lines (B-LCL) infected with recombinant vaccinia viruses carrying either SIV_{mac} env or SIV_{mac} gag were paraformaldehyde fixed. These viral antigen-expressing B-LCL were cultured with PBL at stimulator/responder ratios of 1:2. On day 3, 40 U of recombinant interleukin-2 per ml was added to the cultures, and PBL were cultured for another 3 days. Effector cells were assessed in a 5-h ⁵¹Cr release assay using autologous B-LCL infected with recombinant vaccinia virus-SIV_{mac} constructs or a control vaccinia virus-equine herpesvirus glycoprotein gH construct. PBL of naive rhesus monkeys stimulated and assayed in this manner do not lyse SIV_{mac}-expressing target cells. Past experience has indicated that a measured specific release mediated by PBL of virus-infected or vaccinated monkeys of greater than 10% is reproducible.

By 14 weeks following the initial vaccinations, SIV_{mac} Env-

FIG. 1. PBL of DNA-immunized rhesus monkeys lysed SIV_{mac} Gag- and Env-expressing autologous B-LCL. Monkeys were immunized by intramuscular and intravenous routes and by a gene gun (A) or by a gene gun only (B). Target B-LCL were infected with recombinant vaccinia viruses carrying the SIV_{mac} gag, env, or control (equine herpesvirus 1 gH) genes. Effector cells were Ficoll dextran-isolated PBL obtained prior to and following immunization of the monkeys. PBL were cultured for 3 days at 5×10^6 /ml with 1×10^7 stimulator cells per ml. Recombinant human interleukin-2 (40 U/ml) was added to the cultures on day 3, and cells were cultured for another 3 days. Stimulator cells were autologous B-LCL infected with recombinant vaccinia virus-SIV_{mac} Gag or vaccinia virus-SIV_{mac} Env and fixed with 1.5% paraformaldehyde-phosphate-buffered saline for 30 min. ⁵¹Cr-labeled target cells were incubated for 5 h with effector cells at effector/target ratios of 20:1, 10:1, and 5:1 (solid, stippled, and shaded bars, respectively). Spontaneous release varied from 10 to 20%. Specific release was calculated as (experimental release - spontaneous release)/(100% release - spontaneous release) $\times$ 100. The values shown for percent specific lysis represent the lysis of target cells infected with a recombinant vaccinia virus expressing a SIV_{mac} gene minus lysis of target cells infected with a recombinant vaccinia virus expressing the control gene. ND, not determined.

FIG. 2. SIV_{mac} -specific effector T cells elicited in monkeys by DNA vaccination are $CD8^+$. $CD8^+$ and $CD8^-$ PBL were isolated by incubating the cultured cells with anti- $CD8$ monoclonal antibody-bound immunomagnetic beads (Dyna, Oslo, Norway). After a 2-day incubation, the magnetic beads were removed from the purified $CD8^+$ or $CD8^-$ cells with a magnet. The live cells were then isolated by Ficoll diatrizoate density gradient centrifugation and assessed for effector function. The $CD8^+$ cell-depleted populations contained $<2\%$ $CD8^+$ cells as assessed by flow cytometry. Effector/target ratios: 20:1 (solid bars), 10:1 (stippled bars), 5:1 (hatched bars), and 3:1 (open bars).

specific lytic activity was detected in PBL of all the vaccinated monkeys (Table 1; Fig. 1). SIV_{mac} Gag-specific lytic activity was demonstrated in PBL of two of the seven vaccinated monkeys. The lower incidence of Gag- than Env-specific CTL responses may simply reflect the fact that all five vaccine DNAs expressed Env but only one expressed Gag. The groups of vaccinated monkeys that received nucleotide inoculations only by gene gun appeared to develop slightly lower levels of SIV_{mac} -specific lytic activity than those receiving inoculations intramuscularly, intravenously, and with a gene gun. While not shown in Fig. 1, SIV_{mac} -specific lytic activity in PBL persisted without diminution in all of the monkeys between the second and third clusters of vaccinations. PBL obtained from the animals during this interval were used to characterize the elicited effector T-cell response (Table 1 and Fig. 2).

The phenotype and MHC restriction of the nucleotide vaccine-elicited memory T cells were defined. $CD8^+$ and $CD8^-$ effector cells isolated by positive selection using immunomagnetic beads were assessed for SIV_{mac} Env- and Gag-specific target cell lysis (Fig. 2). The SIV_{mac} -specific effector cells were present only in the $CD8^+$ lymphocyte populations. To assess the MHC class I restriction of the SIV_{mac} -specific CTL-target cell interactions, the MHC class I alleles expressed by cells of the vaccinated monkeys were characterized. A lysate was prepared from radiolabeled B-LCL derived from each of these monkeys. MHC class I molecules were precipitated from each lysate by using a monoclonal antibody specific for a shared-framework determinant of MHC class I, and the expressed molecules were separated on the basis of charge by one-dimensional isoelectric focusing. Thus, each major band in the lane of a gel represented a different MHC class I allele. Little sharing of MHC class I alleles between these monkeys was found (Fig. 3). Effector cells from four of the vaccinated mon-

FIG. 3. One-dimensional isoelectric focusing analysis of MHC class I molecules expressed by cells of rhesus monkeys. Monkey identification numbers are listed above the gel. The position to which β_2 microglobulin (β_2m) migrates is marked. BB7.7 radioimmunoprecipitates were prepared from rhesus monkey B-LCL, and one-dimensional isoelectric focusing analysis was performed as previously described (21). Briefly, monoclonal antibody BB7.7 (American Type Culture Collection, Rockville, Md.) was used to immunoprecipitate MHC class I molecules from 0.1% Nonidet P-40 lysates of rhesus monkey B-LCL metabolically labeled for 4 h with 100 μ Ci of ^{35}S -Label (ICN Biomedicals; Costa Mesa, Calif.). Radioimmunoprecipitates were subjected to neuraminidase type VIII digestion (Sigma, St. Louis, Mo.) prior to electrophoresis using a Hoefer Scientific Instruments (San Francisco, Calif.) slab gel apparatus.

keys were assessed for SIV_{mac} -specific cell lysis of targets whose expressed MHC class I molecules were characterized (Table 2). Lysis of SIV_{mac} Env- or Gag-expressing autologous but not allogeneic targets was seen. These observations indicate that the nucleotide vaccine-elicited SIV_{mac} -specific effector cells were $CD8^+$, MHC class I-restricted CTL.

TABLE 2. Nucleotide vaccine-elicited effector T lymphocytes lyse autologous but not allogeneic, MHC class I-mismatched target cells

Effector cells ^a and target antigen	% Specific lysis for the following target cells ^b :				
	Autologous ^c	012	458	009	018
L37					
Gag	22	0	0	0	0
Env	20	0	3	0	1
L44					
Gag	16	0	0	0	0
Env	21	0	1	2	0
L116, Gag	19	1	1	2	0
8RK, Env	29	4	2	0	1

^a Effector cells were PBL stimulated in vitro with paraformaldehyde-fixed, vaccinia virus- SIV_{mac} Env- or Gag-infected B-LCL as described in the legend to Fig. 1. The construction of these recombinant vaccinia viruses was described previously (14).

^b The ^{51}Cr release assay was performed as described in the legend to Fig. 1 at an effector/target ratio of 20:1. Target cell B-LCL were infected with vaccinia virus-Env, vaccinia virus-Gag, or a control vaccinia virus-equine herpesvirus gH gene construct.

^c Target cells were either autologous or allogeneic B-LCL selected according to their MHC class I phenotype as defined in the study shown in Fig. 3.

These studies indicate that nucleotide vaccination can elicit persisting immunodeficiency virus-specific CTL in nonhuman primates. Although these monkeys were not protected from a live SIV_{mac} challenge with cell-free SIV_{mac} (11a), these initial studies must be seen as encouraging. There is every reason to expect that more potent SIV_{mac}-specific CTL might be elicited in macaques through the use of nucleotide vectors that express viral proteins at higher levels and/or combining this vaccine approach with other immunization modalities.

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