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Protection of Macaques against Infection with Simian Type D Retrovirus (SRV-1) by Immunization with Recombinant Vaccinia Virus Expressing the Envelope Glycoproteins of Either SRV-1 or Mason-Pfizer Monkey Virus (SRV-3)

BRIAN A. BRODY,[1] ERIC HUNTER,[1]* JOHN D. KLUGE,[2] RICK LASAROW,[3] MURRAY GARDNER,[3] AND PRESTON A. MARX[2]†

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294,[1] and California Primate Research Center[2] and Department of Pathology, University of California,[3] Davis, California 95616

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Rhesus macaques were immunized with live vaccinia virus recombinants expressing the envelope glycoproteins (gp70 and gp22) of simian type D retrovirus (SRV), serotype 1 or 3. All of the animals immunized with either the SRV-1 *env* or the SRV-3 *env* vaccinia virus recombinant developed neutralizing antibodies against the homologous SRV. In addition, both groups developed cross-reactive antibodies and were protected against an intravenous live-virus challenge with SRV-1. The four control animals immunized with a vaccinia virus recombinant expressing the G protein of respiratory syncytial virus were not protected against the same SRV-1 challenge. Although SRV-1 and SRV-3 immune sera showed cross-neutralization, they failed to neutralize a separate, more distantly related serotype, SRV-2, in an in vitro assay. These findings are consistent with the known degree of serologic and genetic relatedness of these three SRV strains.

A naturally occurring immunodeficiency disease of Asian macaques is caused by a family of infectiously transmitted type D retroviruses (for a summary, see reference 11). These simian type D retroviruses (SRVs) are classified into five distinct neutralization serotypes, 1 through 5 (7, 24, 25, 31), but they seem to use a common cell surface receptor (29). SRV infection is prevalent in wild-caught and colony-bred macaques and is one of the leading causes of death among species of Asian macaques in several United States primate research centers (10). The prototype type D retrovirus was isolated in 1970 from a spontaneous breast tumor in a rhesus monkey (*Macaca mulatta*) and was called Mason-Pfizer monkey virus (M-PMV) (16). Experimental transmission of M-PMV into young rhesus monkeys at that time showed that it was nononcogenic but instead was immunosuppressive (8). These findings were later confirmed by the reisolation of M-PMV from the frozen primary tumor and experimental induction of fatal immunodeficiency disease in young rhesus monkeys (5). In cross-neutralization studies with rhesus monkey antisera, M-PMV was more closely related to SRV-1 than to SRV-2 (24). M-PMV was thus designated SRV-3 and will be referred to as such in this article. SRV-1, SRV-2, and SRV-3 have each been molecularly cloned and totally sequenced (27, 30, 32), and fatal immunodeficiency has been induced with the cloned SRV-1 (13). In keeping with its partial serologic relatedness to SRV-1, the *env* amino acid sequence of SRV-3 was found to be more closely related to that of SRV-1 than that of SRV-2 (32). In the surface (SU) domain of the *env* gene, SRV-1 and SRV-3 have 83% identity, whereas SRV-2 has 58% identity with either SRV-1 or SRV-3.

The type D retroviruses spread among macaques by close physical contact, most likely by the percutaneous inoculation of cell-free virus in saliva via biting and scratching (20). Natural resistance to SRV-induced disease correlates with the development of neutralizing antibodies to the surface and transmembrane envelope glycoproteins, gp70 and gp20, respectively (18). Immunization of rhesus macaques at the California Primate Research Center with a Formalin-inactivated SRV-1 vaccine elicited neutralizing antibodies and protected the animals from experimental infection by SRV-1 (26). Subsequent immunization with denatured, nonglycosylated SRV-1 envelope antigens expressed in yeast cells did not elicit neutralizing antibody (17) or protect against experimental infection with this virus (9). At the Washington Primate Research Center, pigtailed macaques (*M. nemestrina*) immunized with a recombinant vaccinia virus expressing the envelope glycoproteins of SRV-2 were protected against challenge infection with this virus (15). Protection correlated with the induction of antibodies that neutralized SRV-2 infectivity and mediated lysis of SRV-2-infected cells by antibody-dependent cell-mediated cytotoxicity. The vaccinated monkeys also showed positive SRV-2-specific lymphoproliferative responses. Whether these monkeys are also protected against challenge from the serologically distinct SRV-1 remains to be determined. Because of the close serologic relatedness of SRV-1 to SRV-3, we thought it likely that recombinant vaccines containing *env* from these two SRV strains would show cross-protection in vivo. We therefore immunized rhesus monkeys with vaccinia virus recombinants expressing the envelope glycoproteins of SRV-1 or SRV-3 and challenged both groups with SRV-1.

Eleven healthy young adult (3 to 7 years old) rhesus monkeys from the immunodeficiency disease-free colony at the California Primate Research Center were confirmed to be free of SRV, simian immunodeficiency virus, and simian T-lymphotropic virus strain 1 by enzyme-linked immunosorbent assay (ELISA) and Western immunoblot (24). The

* Corresponding author.
† Present address: Primate Research Institute, University of New Mexico, Alamogordo, NM 88330.

presence of viremia was excluded by cocultivating peripheral blood mononuclear cells (PBMC) with Raji cells. Four animals served as the control group, four received the SRV-3 recombinant, and three were immunized with the SRV-1 recombinant. Each animal was housed individually in standard open cages according to National Institutes of Health guidelines for adult rhesus monkeys.

In order to create the vaccinia virus recombinants, a fragment containing the entire coding domain of the *env* gene of SRV-3 (M-PMV) was subcloned into M13mp19 from the infectious molecular clone pSHRM15 (28). A unique *Sma*I restriction site was introduced 46 nucleotides upstream of the *env* initiation codon by oligonucleotide-directed mutagenesis (33). Similarly, a unique *Sal*I site was introduced immediately following the termination codon of the *env* gene. The 1.8-kb *Sma*I-*Sal*I fragment was removed, blunt-ended with Klenow enzyme, and cloned into the *Sma*I site of the vector pSC11 (6) downstream of the $P_{7.5}$ promoter. The recombinant construct was called pSC-M-PMV. The SRV-1 *env* gene was excised from plasmid pNL-3 (kindly provided by Paul Luciw) by *Eco*RI digestion, leaving 90 nucleotides upstream and 4 nucleotides downstream of the *env* gene, and similarly cloned into pSC11. This plasmid construct was termed pSC-SRV. Recombinant vaccinia viruses capable of producing blue plaques in the presence of 500 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma, St. Louis, Mo.) per ml were generated by published protocols (22, 23). One of these was picked for each recombinant and purified by two more successive rounds of infection and selection. A final purified plaque was used to produce a small stock of each recombinant virus. The SRV-3 recombinant was designated Vac.SRV-3.*env*, and the SRV-1 recombinant was similarly termed Vac.SRV-1.*env*. Virus stock titers were determined on CV-1 cells as described before (6).

Recombinant vaccinia viruses were assayed for their ability to express and process the $Pr86^{env}$ precursor protein of the SRVs to the mature surface (gp70) and cell-associated transmembrane (gp22) glycoproteins in a metabolic labeling experiment. At 5 h postinfection (p.i.), CV-1 cells infected with either Vac.SRV-3.*env* or Vac.SRV-1.*env* were labeled in a 15-min pulse with [^{3}H]leucine and then chased in unlabeled medium for 4 h. This chase period has been shown previously to be sufficient for conversion of a majority of Pr86 to gp70 and gp22 glycoproteins in CV-1 cells infected with simian virus 40 recombinant virions expressing the M-PMV envelope glycoprotein gene (2). The results of this protein expression experiment are shown in Fig. 1, where it can be seen that the precursor protein is processed normally to gp70 and gp22 when expressed from the vaccinia virus vector. Recognition by the goat antiserum of the Vac.SRV-1.*env* proteins is somewhat reduced in comparison to those of Vac.SRV-3.*env* owing to the use of SRV-3 as the antigen. However, recognition of gp22 is similar in both, presumably because of the high degree of amino acid sequence homology between the transmembrane (TM) glycoproteins of SRV-1 and SRV-3. In the absence of the SRV proteinase and virus assembly, the gp22 protein is not further processed to the normally virus-associated gp20 (4); nevertheless, these surface-expressed glycoproteins can induce cell-cell fusion in Raji cells infected with either vaccinia virus recombinant (3).

Recombinant vaccinia viruses were grown and purified for animal vaccination by sucrose gradient centrifugation as described previously (14, 21). Vac.RSV.G (originally designated vAG301), a vaccinia virus recombinant in the same wild-type vaccinia virus background as the SRV recombinants that expresses the respiratory syncytial virus (RSV) G protein (1), was prepared in order to act as a vaccinating agent for the control group of animals.

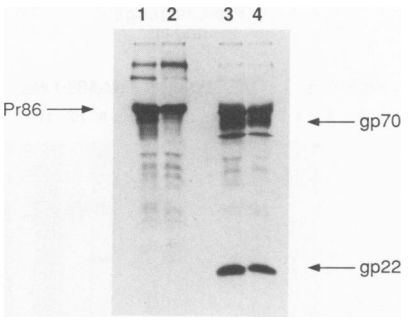

FIG. 1. Confluent monolayers of CV-1 cells were infected with either recombinant vaccinia virus Vac.SRV-1.*env* or Vac.SRV-3.*env* at a multiplicity of infection of 2. Labeled proteins, from cell lysates, representing a 15-min pulse and a 4-h chase were reacted with goat antiserum prepared against disrupted SRV-3 virions. Immunoprecipitation was initiated by the addition of fixed *Staphylococcus aureus* (strain Cowan) cells. Precipitates were analyzed by 10% polyacrylamide–sodium dodecyl sulfate gel electrophoresis. Lanes 1 and 2 show the Env precursor protein Pr86 for Vac.SRV-3.*env* and Vac.SRV-1.*env*, respectively. Lanes 3 and 4 show the processing of Pr86 to the mature proteins gp70 and gp22 for Vac.SRV-3.*env* and Vac.SRV-1.*env*, respectively.

Three rhesus macaques received the vaccinia virus-SRV-1 *env* recombinant (Vac.SRV-1.*env*), four received the vaccinia virus-SRV-3 *env* recombinant (Vac.SRV-3.*env*), and four controls received the respiratory syncytial virus recombinant (Vac.RSV.G). All animals were immunized three times, at weeks −14, −8, and −2 from date of challenge, with 10^8 PFU of the respective vaccinia virus recombinants by skin scarification. On day 0, all monkeys were inoculated intravenously with 1.5 ml containing 2×10^5 syncytium-inducing units of SRV-1.

SRV-1-specific antibody titers of 1:100 to 1:200 were detected in both groups of SRV-immunized animals at 3 weeks after the first boost (−5 weeks) (Table 1). On the day of live-virus inoculation (day 0), which was 2 weeks after the second boost, the ELISA titers were 1:200 to 1:400 for all seven SRV vaccinates except one of the SRV-3 vaccinates, for which the ELISA titer was <1:100. Western blot analysis done on the day of challenge showed that all SRV-vacci-

TABLE 1. Antibody titers in SRV-immunized macaques

Immunizing virus and macaque no.	ELISA titer vs SRV-1		
	−5 wk	Day 0	+9 wk
Vac.SRV-1.*env*			
22207	1:100	1:200	1:200
23040	1:100	1:200	1:400
21671	1:100	1:200	<1:100
Vac.SRV-3.*env*			
23454	1:200	1:400	1:800
21013	1:200	1:400	1:800
20229	1:100	<1:100	1:100
20278	1:200	1:400	1:200
Vac.RSV.G (controls)			
20577	<1:100	<1:100	1:200
21896	<1:100	<1:100	1:1600
18546	<1:100	<1:100	<1:100
21828	<1:100	<1:100	1:400

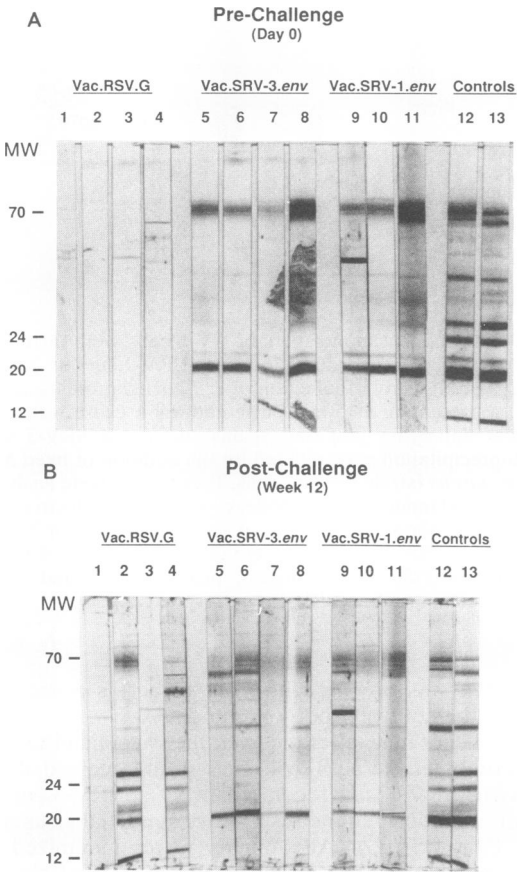

FIG. 2. Humoral immune responses as determined by immunoblot on the day of live virus challenge (A) and at week 12 postchallenge (B). Lanes 1 to 4, sera from control monkeys (20577, 21896, 18546, and 21828) vaccinated with recombinant vaccinia virus expressing the RSV G protein; lanes 5 to 8, sera from monkeys (23454, 21013, 20229, and 20278) vaccinated with Vac.SRV-3.*env*; lanes 9 to 11, sera from monkeys (22207, 23040, and 21671) vaccinated with Vac.SRV-1.*env*; lanes 13 and 14, SRV-1 and M-PMV (SRV-3) reference sera, respectively, from naturally infected monkeys. The SRV-1 antigen (2 μg per lane) was obtained from Advanced Biotechnologies, Bethesda, Md. Sizes are indicated in kilodaltons.

nated monkeys had strong antibody reactions against the two SRV-1 envelope glycoproteins, gp70 and gp20 (Fig. 2A). In all SRV vaccinates, low titers of neutralizing antibody (1:2 to 1:8) were first detected 1 week after the second boost (−1 week) (Table 2). On the day of live-virus inoculation (day 0), neutralizing antibody titers of 1:8 to 1:16 were present in all SRV vaccinates, including the animal with the low ELISA titer. The titers of neutralizing antibody were generally severalfold higher against the homologous virus than against the heterologous serotype. None of the four controls had any serologic antibody response to SRV-1 or SRV-3 prior to challenge. All SRV vaccinates and controls had developed neutralizing antibody titers of 1:80 to 1:640 to vaccinia virus by the day of challenge (data not shown). Prior to challenge, T-cell proliferation to heat-inactivated SRV-1 was minimal (stimulation index [S.I.] ≤ 2.0) in all vaccinates and controls (data not shown).

Following the challenge with 2×10^5 syncytium-inducing units of SRV-1, given intravenously 2 weeks after the last boost (week 0), all four controls quickly became viremic, as detected by isolation of virus from cocultured PBMC at 1 and 2 weeks p.i. (Table 3). Two of the controls (20577 and 18546) remained viremic at 4, 6, and 9 weeks p.i., while the other two controls (21896 and 21828) became nonviremic. The number of infected PBMC, as determined by the endpoint dilution assay (24) at 2 weeks p.i., was greater (1:750) in the persistently infected controls than in those that became nonviremic (1:1,565 and 1:25,000). The two controls that remained viremic made only a weak antibody response to the infection, as measured by ELISA, Western blot, and neutralization assays, whereas the two controls that cleared the infection made a stronger antibody response to viral *env* and core antigens (Tables 1 and 2, Fig. 2B). By contrast, all of the SRV-1- and SRV-3-vaccinated animals remained nonviremic for up to 9 weeks p.i. Following challenge with live SRV-1, about half of the SRV vaccinates showed a one- to threefold rise in antibody titer, as measured by ELISA or neutralization, versus the homologous virus (Tables 1 and 2). Increases in neutralizing antibody titer against the heterologous virus after challenge were less marked. After challenge, no increased staining density was observed in the Western blots from the protected animals (Fig. 2) and no significant reactivity to core proteins could be detected. None of the SRV-1 or SRV-3 immune sera were able to neutralize SRV-2 in vitro (data not shown). At 2 months following challenge, the SRV-1-specific T-cell proliferative responses had increased severalfold (S.I. = 3.0 to 6.0) in three of four SRV-1 and two of four SRV-3 *env* vaccinates (data not shown). At 3 months p.i., the two persistently infected control animals were ill with immunodeficiency disease, whereas all other animals remained healthy.

These results demonstrate that immunization of rhesus macaques with live recombinant vaccinia virus expressing the envelope glycoproteins of either SRV-1 or SRV-3 protects against challenge infection with SRV-1. Cross-protection in vivo is correlated with the vaccine induction of cross-reacting SRV-1 and SRV-3 binding and neutralizing antibody in vitro. The rise in SRV-1 neutralizing antibody titer occurring soon after challenge in most of the protected vaccinates (Table 2) suggests that a transient infection occurred but was promptly cleared by the immunized animals. The lack of established infection in the protected animals is also indicated by the absence of antibody to the viral core protein (p27) on Western blot (Fig. 2B) and the failure to isolate virus from PBMC (Table 3). These findings extend those made previously with the vaccinia virus-SRV-2 envelope vaccine (15) and indicate that the envelope glycoproteins of SRV-1 and SRV-3 are also sufficient for eliciting protective immunity against SRV-1.

The degree of humoral immunity induced by these vaccines was similar to that found in animals convalescing from SRV-1 and SRV-3 infection (18) and was sufficient to confer protection against a high challenge dose of SRV-1. The results confirm the serologic relatedness of SRV-1 and SRV-3 previously shown by cross-neutralization in vitro (24) by showing cross-protection of SRV-3 *env*-vaccinated monkeys against SRV-1 in vivo. The lack of close relatedness of these serotypes with SRV-2 is demonstrated by the absence of cross-neutralization of SRV-2 by the SRV-1 and SRV-3 immune sera. Although SRV-1 and SRV-3 *env* vaccines should cross-protect against each other, neither of these vaccines would be expected to protect against infection with the serologically distinct SRV-2 strain.

Our results confirm that, in contrast to infections with lentiviruses such as human and simian immunodeficiency

TABLE 2. Neutralizing antibody titers in SRV-immunized macaques[a]

Immunizing virus and macaque no.	Antibody titer							
	−8 wk		−1 wk		Day 0		+9 wk	
	SRV-1	SRV-3	SRV-1	SRV-3	SRV-1	SRV-3	SRV-1	SRV-3
Vac.SRV-1.*env*								
22207	<1:2	<1:2	ND[b]	ND	1:4	1:4	1:32	1:4
23040	ND	<1:2	1:8	<1:2	1:16	1:4	1:16	1:4
21671	<1:2	<1:2	1:8	<1:2	1:16	1:4	1:32	1:4
Vac.SRV-3.*env*								
23454	<1:2	<1:2	<1:2	1:4	1:4	1:16	1:32	1:8
21013	<1:2	<1:2	<1:2	1:4	1:2	1:8	1:8	1:16
20229	ND	<1:2	ND	ND	1:4	1:8	1:8	1:16
20278	<1:2	ND	1:8	1:2	1:4	1:8	1:8	1:4
Vac.RSV.G (controls)								
20577	ND	<1:2	<1:2	<1:2	<1:2	<1:2	1:4	<1:2
21896	<1:2	<1:2	ND	<1:2	<1:2	<1:2	1:64	1:4
18546	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2
21828	<1:2	<1:2	<1:2	<1:2	<1:2	<1:2	1:32	1:2

[a] Monkey sera were heated at 50°C for 30 min and used to block infection of Raji cells as described before (25).
[b] ND, not determined.

viruses, neutralizing antibody titers generally correlate well with naturally occurring and vaccine-induced protection in type D retrovirus infections of macaques (19). This finding is similar to that previously shown in certain type C retrovirus infections of lower animals (for review, see reference 12). The role of cellular immunity in the type D retroviral vaccine protection has not been determined. The detection of antibodies mediating antibody-dependent cell-mediated cytotoxicity by the vaccinia virus-SRV-2 *env* vaccine indicates that potential means of vaccine protection other than neutralization may also have been elicited (15). Interestingly, the presence of cytotoxic T-cell killing in the type D retrovirus-infected or -vaccinated monkeys has not yet been demonstrated. Insofar as the type D retroviruses are spread among macaques primarily in saliva (20), neutralizing antibodies alone may be capable of conferring protection against infection. The increase in SRV-1-specific T-cell proliferation observed 2 months after challenge in most of the vaccine-protected animals was probably the response to transient infection and is consistent with the anamnestic humoral antibody response also seen following challenge. With most other successful vaccines, including the SRV-1 inactivated whole virus (26) and vaccinia virus-SRV-2 *env* recombinant (15), immune protection has similarly occurred after transient infection from the challenge virus. Indeed, strong, lasting immunity in these SRV-vaccinated monkeys will probably result from this initial infection that is successfully cleared by the immunized animals. However, in the present study, the duration of immunity remains to be determined.

TABLE 3. Viremia in SRV-1-challenged macaques[a]

Immunizing virus and macaque no.	Viremia at time postchallenge:				
	Wk 1	Wk 2	Wk 4	Wk 6	Wk 9
Vac.SRV-1.*env*					
22207	−	−	−	−	−
23040	−	−	−	−	−
21671	−	−	−	−	−
Vac.SRV-3.*env*					
23454	−	−	−	−	−
21013	−	−	−	−	−
20229	−	−	−	−	−
20278	−	−	−	−	−
Vac.RSV.G (controls)					
20577	+	+	+	+	+
21896	+	+	−	−	−
18546	+	+	+	+	+
21828	+	+	−	−	−

[a] Infectious SRV-1 was isolated by cocultivation of PBMC with Raji cells and confirmed by immunofluorescence as described before (25).

The control of SRV infection in macaques in captivity, particularly in breeding colonies, is an urgent need from both the practical and scientific viewpoints. This goal can be accomplished by a test and removal program, which, however, requires serial assays for type D retrovirus antibodies and infectious virus (19). It should also now be possible to protect macaques from natural infection with SRV-1, SRV-2, or SRV-3, the most common serotypes in U.S. primate facilities (10), by using a polyvalent vaccinia virus vaccine expressing SRV-2 and either SRV-1 or SRV-3 envelope glycoproteins.

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