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Cross-Protective Immune Responses Induced in Rhesus Macaques by Immunization with Attenuated Macrophage-Tropic Simian Immunodeficiency Virus

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The simian immunodeficiency virus (SIV) macaque model of AIDS has provided a valuable system with which to investigate vaccine approaches for protection against human immunodeficiency virus type 1 (HIV-1) infection. In particular, the ability of macaques persistently infected with attenuated infectious molecular clones of SIV to resist challenge with the pathogenic parental swarm has conclusively demonstrated that protective immunity can be achieved by immunization prior to exposure. The breadth of these protective responses and the immunological correlates of protection, however, have not been identified. In addition, vaccine studies have mainly employed lymphocyte-tropic strains of HIV-1 and SIV. Recent studies have implicated macrophage-tropic strains in the transmission of HIV-1 and have suggested that these virus strains should be examined in vaccine strategies. Macrophage-tropic viruses may confer additional advantages in the induction of protective immunity by replication in antigen-presenting cells. In this study, the immune response of rhesus macaques inoculated with an attenuated macrophage-tropic recombinant of $SIV_{mac}239$ (SIV/17E-Cl) was evaluated with respect to protective immunity by heterologous challenge at various times after infection. Vigorous type-specific neutralizing-antibody responses restricted to SIV/17E-Cl were evident by 2 weeks postinfection. By 7 months, however, cross-reactive neutralizing antibodies emerged which neutralized not only SIV/17E-Cl but also the heterologous primary isolate SIV/DeltaB670. Challenge of SIV/17E-Cl-infected monkeys with SIV/DeltaB670 at various times postinfection demonstrated that protective responses were associated with the appearance of cross-reactive neutralizing antibodies. Furthermore, passive transfer of sera from SIV/17E-Cl-infected animals passively protected two of four naive recipients.

The simian immunodeficiency virus (SIV) macaque model of AIDS provides an excellent system with which to investigate the role of molecularly defined viruses in the pathogenesis of disease and the induction of protective immune responses for vaccine development. In both regards, infectious molecular clones of SIV have provided considerable data on the pathogenesis of diseases caused by lentiviruses (7, 12, 14, 17–19, 34). For example, the molecular clone $SIV_{mac}239$ causes an AIDS-like disease in macaques; a deletion in the *nef* gene results in an infectious virus that is nonpathogenic but replication competent in vivo (14, 15). Studies have shown that monkeys infected with $SIV_{mac}239\Delta nef$ for more than 2 years are protected upon intravenous challenge with a primary culture of $SIV_{mac}251$, the parent isolate from which this clone was derived (7). However, the immune responses responsible for the protection observed in these studies remain unknown.

Vaccines for both human immunodeficiency virus type 1 (HIV-1) and SIV have utilized, for the most part, lymphocyte-tropic strains because they are prevalent during acute infection and late-stage disease (22, 28). $SIV_{mac}239$ is lymphocyte tropic and replicates poorly in macrophages; the virus reproducibly causes AIDS in monkeys but only rarely causes infection of or primary pathological lesions in the central nervous system or lungs of infected animals (15, 34). In contrast, macrophage-tropic viruses derived by in vivo passage of $SIV_{mac}239$ have been shown to be involved in primary central nervous system and lung disease (8, 35). Sequences in the gp120 portion of the *env* gene of SIV have been shown to confer macrophage tropism (1, 2, 26). The role of macrophage-tropic strains in virus transmission and progression to AIDS has not been clearly determined. However, macrophage-tropic viruses may be preferred for the induction of protective immunity because they may infect and elicit systemic and local immune responses in antigen-presenting cells not only in internal lymphoid organs but also in the skin, gut, and other mucosal tissues, thereby providing protection at the mucosal surface. This may be highly relevant for HIV-1 infection and transmission, since recent studies analyzing the viruses present early after infection with HIV-1 provide strong evidence that the transmitted viruses are genotypically homogeneous, compared with the heterogeneity of viruses isolated later in infection (5, 22, 28), and phenotypically macrophage tropic (regardless of route of transmission), compared with the lymphocyte-tropic viruses that predominate later during disease progression (5, 22, 28, 37–40). Thus, an HIV vaccine directed against monocyte-tropic variants at the mucosal surfaces should offer an advantage, given that monocyte-tropic variants are the likely source of transmitted HIV.

In this study, the SIV model was used to examine the infection of rhesus macaques with a molecularly cloned macro-

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phage-tropic virus and to determine the nature and timing of the protective immune response. A macrophage-tropic recombinant virus was constructed by inserting the surface glycoprotein (gp120) and a portion of the transmembrane glycoprotein of a macrophage-tropic strain of SIV (SIV/17E-Br) into the $SIV_{mac}239$ infectious molecular clone (1, 35). This recombinant clone SIV/17E-Cl replicates efficiently in primary rhesus macrophages but poorly in primary rhesus lymphocytes in vitro (1).

Macaques immunized with SIV/17E-Cl rapidly developed antibody responses to this virus, and these responses were monitored for virus neutralization and for antibody avidity and conformation dependence. These antibody responses broadened over time, and the immune responses in the macaques protected the animals against challenge with a heterologous strain of SIV. Further, passive immunization of naive macaques resulted in protection against heterologous virus challenge. This study provides evidence of cross-protective immune responses induced by a macrophage-tropic clone of SIV and demonstrates that protective responses correlate with the broadening of neutralizing antibody.

MATERIALS AND METHODS

Viruses. A macrophage-tropic strain (SIV/17E-Br) was obtained by passage of $SIV_{mac}239$ in rhesus macaques (35). A recombinant molecular clone that contains the surface glycoprotein (gp120) and a portion of the transmembrane glycoprotein (gp41) of SIV/17E-Br in the $SIV_{mac}239$ molecular clone was constructed (1). Stocks of SIV/17E-Cl were prepared by transfection of DNA from the infectious molecular clone into primary macaque peripheral blood lymphocytes (PBL). This stock was used to infect fresh PBL, and a stock containing 10^3 50% tissue culture infective doses ($TCID_{50}$) of virus was obtained. Virus stocks used for neutralization assays were prepared in the human T-cell line CEMx174, and all virus stocks were assayed for infectivity as described below.

$SIV_{mac}239$ was prepared by transfection of the DNA from the infectious clone (kindly provided by R. Desrosiers) into PBL, and this stock was expanded in CEMx174 cells. Virus stocks from uncloned viruses (SIV/17E-Br and SIV/DeltaB670) were obtained by infection of CEMx174 cells.

Cells. Primary rhesus macaque lymphocytes and macrophages were obtained from heparinized peripheral blood collected from adult macaques. The blood was centrifuged at 1,300 × *g* for 15 min, plasma was removed, and cells were resuspended in a 2× volume with Hanks buffered saline solution. Peripheral blood mononuclear cells (PBMC) were isolated on either Ficoll-Hypaque or Percoll density gradients. The cells were washed three times with Hanks buffered saline solution and resuspended in medium to culture either lymphocytes or macrophages. When indicated, mononuclear cells were isolated from lymph node biopsied tissue.

To culture PBL, cells were resuspended at 10^6 cells per ml in RPMI 1640 containing 10% fetal bovine serum, gentamicin (50 μg/ml), glutamine (2 nmol/ml), 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) buffer, 100 U of recombinant human interleukin-2 per ml, and 2.0 μg of phytohemagglutinin per ml and cultured for 2 days. The cells were washed and resuspended in the same medium without phytohemagglutinin.

To culture primary macrophages, cells were resuspended at 2×10^6 cells per ml in RPMI 1640 containing 10% human serum, gentamicin (50 μg/ml), glutamine (2 nmol/ml), 10 mM HEPES buffer (RPMI, 10% fetal bovine serum), and 20 U of macrophage colony-stimulating factor per ml and 25 U of granulocyte-macrophage colony-stimulating factor per ml (both gifts from Genetics Institute, Cambridge, Mass.) and cultured for 5 days. Nonadherent cells were removed, and the cultures were refed with the same supplemented medium.

Rhesus macaques. Eight rhesus monkeys born at the Tulane Primate Center were inoculated intravenously via the saphenous vein with 10 $TCID_{50}$ of a cryopreserved preparation of cell-free SIV/17E-Cl propagated in rhesus lymphocytes. All monkeys seroconverted and became persistently PCR positive following infection. Infection was determined by PCR amplification of viral sequences in PBL by using nested primers specific for the viral long terminal repeat (see "PCR" below). Cell-free viremia was assessed by quantitation of viral core antigen in sera by using a commercially available sandwich enzyme-linked immunosorbent assay (ELISA) (Coulter Electronics, Hialeah, Fla.) (20). Of particular interest was either a reciprocal decline in $CD4^+$ (helper) and an increase in $CD8^+$ (suppressor) T lymphocytes or a selective decline in the $CD4^+$ $CD29^+$ helper-inducer T-lymphocyte population. A selective decline in $CD4^+$ $CD29^+$ T lymphocytes has been shown to be a reliable early indicator of disease progression in monkeys infected with SIV/DeltaB670 (27). Clinical status was evaluated by a biweekly physical examination which included complete blood counts, palpation of peripheral lymph nodes and spleen, and monitoring for signs of opportunistic infections.

On day 244 postinfection, two monkeys (L235 and L238) were challenged with 50 animal infectious doses of a cryopreserved preparation of SIV/DeltaB670 grown on rhesus primary phytohemagglutinin blasts. Lymph node biopsies were performed immediately prior to challenge and 14 days postchallenge to evaluate the presence, if any, of sequences specific to the challenge virus at either time point. Lymphocytes derived from these tissues, along with those obtained from the peripheral blood (samples taken up to 6 months postchallenge), were separated by Ficoll-plaque density gradients and subjected to lysis for DNA isolation and PCR.

Three additional animals (M697, M700, and M462) were challenged similarly, except that inoculation with SIV/DeltaB670 was effected on days 178, 192, and 192, respectively, following infection with SIV/17E-Cl.

Infectivity assays. Fivefold dilutions of plasma or supernatant fluids from cultures were inoculated into the wells of a 96-well tissue culture plate containing RPMI 1640 containing 10% human serum, gentamicin (50 μg/ml), glutamine (2 nmol/ml), and 10 mM HEPES buffer, and 10^6 CEMx174 cells were added to each well. The wells were assessed for virus-specific cell cytopathology (CPE) at 3, 5, and 7 days postinfection, and the 7-day results were used to calculate the $TCID_{50}$ by the method of Karber (13).

Neutralization assays. Virus neutralizations were done in 96-well tissue culture plates containing RPMI 1640 plus 10% fetal bovine serum. Fivefold serial dilutions of plasma (heat inactivated at 56°C and clarified by centrifugation) were added to the well with 10 to 100 $TCID_{50}$ of virus and incubated for 1 h at 37°C. CEMx174 cells (10^6 cells) were added to each well, and the development of CPE was recorded at 7 days. This was used to calculate the 50% neutralization endpoint by the method of Karber (13).

Neutralization assays were done in primary macaque macrophages as described above except that primary macaque macrophages were cultured in 96-well plates for 5 days prior to the addition of virus or virus incubated with serial dilutions of plasma. The end point was determined at 14 days by the addition of CEMx174 cells (2×10^6), and CPE was assessed and the 50% neutralization end point was calculated as described above.

Infectious-center assays. Twenty-four-well tissue culture dishes were used to coculture twofold serial dilutions of primary cells (beginning at 10^6 cells) with CEMx174 cells (10^5), and these cells were assessed for virus-induced CPE at 7 and 10 days.

PCR. PBMC were isolated from heparanized blood on Ficoll-Hypaque density gradients. When indicated, mononuclear cells were isolated from lymph node biopsies by gentle teasing of biopsied tissue. Cells were washed with RPMI culture medium prior to lysis, and the DNA was purified from detergent-disrupted cells by solvent extraction followed by spooling onto a glass rod. Identification of viral sequences was performed using a nested PCR with conserved sequences in the viral long terminal repeat. Each PCR mixture contained 10 mM Tris-HCl (pH 9.0 at 25°C), 50 mM KCl, 1.75 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 2 mM deoxynucleoside triphosphates, 20 pM 5′ and 3′ oligonucleotide primers, and 2.5 U of *Taq* polymerase (32) (Promega). One microgram of DNA was then amplified by 30 cycles in a DNA Thermocycler (Perkin-Elmer Corp., Norwalk, Conn.). The first cycle consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min plus 10 s for each of 30 cycles. DNA in a second nested round was denatured at 94°C for 1 min, annealed at 45°C for 1 min, and extended at 60°C for 1 min plus 10 s for each of 30 cycles. Fifteen percent of the amplified product was then electrophoresed through a 2% agarose gel and visualized by ethidium bromide staining. The sequences of the long terminal repeat-specific primer pairs used in the first round were (5′) 5′-ata-gtt-gca-gta-cat-gtg-gct-agt-g-3′ and (3′) 5′-tct-ctg-cct-ctt-tct-ctg-taa-tag-ac-3′, and those for the second round were (5′) 5′-ctg-aga-ctg-cag-gga-ctt-tcc-aga-agg-g-3′ and (3′) 5′-agg-cag-aaa-ggg-tcc-tac-aga-cca-ggg-t-3′. To confirm the specificity of the amplified product, fragments were Southern blotted after electrophoresis to a Magna NT Nylon Transfer Membrane (MSI, Westboro, Mass.) and hybridized to a ^{32}P-labeled oligonucleotide complementary to sequences within the primers used in the amplification reaction. The sequence of the oligonucleotide probe was (5′) 5′-agc-agg-tag-agc-ctg-ggt-gtt-c-3′.

Sequencing of SIV gp120. For sequence analysis, amplification of the entire gp120 envelope sequence was performed by nested PCR with the conditions described above and primers (5′) 5′ ttg-agg-gag-cag-gag-aac-tca-tta-3′ and (3′) 5′-cca-ggc-ggc-gac-tag-gag-aga-tgg-gaa-caa-3′ for the first round and (5′) 5′ cct-cca-acg-agc-gct-ctt-cat-3′ and (3′) 5′ cct-gct-gtt-gcg-aga-aaa-ccc-aag-aac-cct-agc-3′ for the second round. Sequences specific for the external domain of gp41 were similarly amplified with primers (5′) 5′-gaa-cat-aca-ttt-att-ggc-atc-cta-g-3′ and (3′) 5′-aag-cag-aaa-ggg-tcc-taa-cag-acc-agg-gt-3′ for the first round and (5′) 5′-cca-ttg-gtc-aaa-cat-ccc-aca-tat-act-gga-3′ and (3′) 5′-cca-ggc-ggc-gac-tag-gag-aga-tgg-gaa-cag-3′ for the second round. Second-round products were cloned into the TA cloning vector (Invitrogen, San Diego, Calif.). Following transformation, colonies containing appropriately sized inserts were selected, and plasmid DNA was purified by alkaline lysis. Inserts containing the envelope region were sequenced by dideoxy sequencing with Sequenase (U.S. Biochemical, Cleveland, Ohio). The resulting sequences were analyzed by using the "dots" alignment program provided by the Mullins laboratory at Stanford University assisted by a SUN sparkstation.

Measurement of SIV envelope glycoprotein-specific antibody conformation dependence. Serum samples from the group of five SIV/17E-Cl-infected monkeys were analyzed for conformation dependence by comparing the serum antibody reactivities with native and denatured viral envelope glycoprotein substrates in a concanavalin A (ConA)-ELISA procedure that maximizes maintenance of protein structure, as described for HIV-1 gp120 immunoassays (31). Preparations of SIV purified by density gradient centrifugation and disrupted with 1% Triton X-100 (TX) were used as a source of native viral envelope glycoproteins. Preliminary experiments utilizing different strains of SIV ($SIV_{mac}251$, $SIV_{mac}239$, SIV/DeltaB670, and SIVB7) as sources of viral glycoprotein antigen in the conformation dependence assay (and in the avidity assay described below) indicated that the level of strain variation in the envelope glycoprotein sequence did not affect the reactivity patterns observed with the polyclonal macaque immune serum. Therefore, in the experiments whose results are presented here, the SIVB7 strain was used as the standard reference glycoprotein antigen. SIVB7, kindly provided by Edmundo Kraiselburd (University of Puerto Rico Primate Center), is a noninfectious virus produced by a CEMx174 cell line infected with a reverse transcriptase-defective variant of SIVmmH3.

To produce denatured envelope glycoproteins, the purified SIVB7 virus was treated with dithiothreitol to reduce disulfide bonds and then with iodoacetic acid to achieve an irreversible carboxymethylation of the reduced sulfhydryl groups (6). These reaction conditions were chosen because they should quantitatively disrupt envelope glycoprotein disulfide bonds and affect tertiary protein structure without extensive alterations in protein secondary structural properties, as would be expected from treatments with chaotropic salts or ionic detergents.

Immunolon II Microtiter plates (Dynatech Laboratories) were incubated with 2.5 μg of ConA (Vector Laboratories) per well in 50 μl of phosphate-buffered saline (PBS) (pH 7.4) for 1 h at room temperature and then washed two times with PBS containing 0.1% TX (PBS-TX). The ConA-coated plates were then used to adsorb the envelope glycoproteins from preparations of native or denatured SIVB7 (3 μg/50 μl in each well). It is estimated that 3 μg of SIVB7 contains about 90 ng of envelope glycoprotein. The ConA-adsorbed viral glycoproteins were washed four times with PBS-TX and then blocked with 100 μl of 5% nonfat dry milk in PBS (BLOTTO) per well for 1 h at room temperature. After the blocking solution was removed, 50 μl of appropriately diluted test monkey serum was added to each well and incubated for 1 h at room temperature. All test sera were diluted in BLOTTO to produce an A_{450} of about 1.0 in the standard ConA-ELISA procedure. After the serum incubation, the wells were washed with PBS-TX, and 50 μl of a 1:1,000 dilution of peroxidase-conjugated anti-human immunoglobulin G in BLOTTO per well was added for a 1-h incubation at room temperature. The wells were once again washed with PBS-TX, and 200 μl of TMBlue (TSI) substrate was added and left for approximately 15 min before color development was terminated by the addition of 50 μl of 1 N sulfuric acid per well. Antibody reactivity to the ConA-anchored native or denatured envelope glycoprotein substrates was then determined by measuring the A_{450}. A conformation index was then calculated from the ratio of antibody reactivities to native and denatured envelope glycoprotein substrates. Thus, the conformation index is a direct measure of the conformation dependence of a particular antibody sample, i.e., the larger the conformation index, the greater the requirement for native envelope glycoprotein structure.

Measurement of envelope glycoprotein-specific antibody avidity. The avidity index values of serum antibodies to the viral envelope glycoproteins (10, 11) were determined by measuring the resistance of antibody-envelope glycoprotein complexes in the ConA-ELISA to 8 M urea. For these avidity assays, the ConA-anchored native viral envelope glycoprotein substrate was prepared from TX-disrupted SIVB7 in microtiter plates as described above for the conformation dependence assays. Test sera, diluted to give an A_{450} of about 1.0, were reacted with the ConA-glycoprotein substrate as described above, and then the wells were washed with PBS-TX. Triplicate wells containing the glycoprotein immune complexes were then treated in parallel for 5 min with either PBS or a solution of 8 M urea in PBS. Following this treatment, the sample wells were washed thoroughly with PBS-TX, incubated with TMBlue, and monitored at 450 nm, as described above. The avidity index was then calculated from the ratio ($A/B \times 100\%$) of the absorbance value obtained with the urea treatment (A) to that observed with the PBS treatment (B). Antibodies with avidity index values of $<30\%$ are designated low-avidity antibodies, those with values of 30 to 50% are designated intermediate-avidity antibodies, and those with values of $>50\%$ are designated high-avidity antibodies (11).

RESULTS

Recombinant macrophage-tropic virus SIV/17E-Cl. The recombinant molecular clone SIV/17E-Cl contains the gp120 sequence and a portion of the gp41 sequence (to amino acid 730) from the macrophage-tropic/neurotropic virus strain SIV/17E-Br in the background of the infectious molecular clone $SIV_{mac}239$ (1, 14, 35). A comparison of the cell tropisms of the two parental viruses and the recombinant molecular clone (Table 1) showed that the *env* sequences from the macrophage-tropic strain SIV/17E-Br conferred a tropism for macrophages upon $SIV_{mac}239$. Although the recombinant clone replicates to a level comparable to that of the parental virus (SIV/17E-Br) in primary rhesus macrophages in vitro, it replicates relatively poorly in primary PBL. Thus, this molecular clone appears to be attenuated in its ability to replicate in primary lymphocytes.

TABLE 1. Cell tropism of SIV strains

Virus	$TCID_{50}$[a]		
	CEMx174	Rhesus PBL	Rhesus macrophages
$SIV_{mac}239$	10^5	10^4	10^1
SIV/17E-Br	10^5	10^4	10^4
SIV/17E-Cl	10^3	10^2	10^4

[a] $TCID_{50}$ produced in the indicated cell type and titrated in CEMx174 cells.

Inoculation of rhesus macaques with SIV/17E-Cl. Eight rhesus macaques were inoculated intravenously with 10 $TCID_{50}$ of SIV/17E-Cl that had been grown in primary rhesus lymphocytes. All monkeys seroconverted and became persistently PCR positive following inoculation. Five of these monkeys were serially monitored for infectious virus by culture of both PBL and macrophages (Table 2). At early times (7 to 14 days) postinoculation, the virus could be cultured from all animals from both populations; thereafter, the virus could not be cultured from lymphocytes and could only periodically be cultured from macrophages. However, macrophage cultures were persistently positive by PCR analysis for SIV/17E-Cl envelope sequences for up to 55 weeks after inoculation.

The development of an AIDS-like disease was monitored in all eight SIV/17E-Cl-infected monkeys by detection of viral p26 in serum and by flow cytometric measurement of changes in T-lymphocyte populations in the peripheral blood; $CD4^+$ (helper) and $CD8^+$ (suppressor) T lymphocytes, as well as the $CD4^+$ $CD29^+$ (helper-inducer) T-lymphocyte population, were monitored in all animals. A selective decline in $CD4^+$ $CD29^+$ T lymphocytes has been shown to be a reliable early indicator of disease progression in monkeys infected with the pathogenic isolate SIV/DeltaB670 (27). Unlike the case for infection of macaques with the lymphocyte-tropic strain $SIV_{mac}239$ (14, 34), no significant changes were observed in any T-lymphocyte populations, and none of the SIV/17E-Cl-infected animals had detectable SIV p26 antigenemia at any time postinfection (data not shown). This low level of virus

TABLE 2. Isolation of virus from inoculated monkeys

Animal	Cells[a]	Isolation of virus on day:					
		7	21	28	195	218	258
L235	Macrophages	+	−	−	−	−	−
	PBL	+	−	−	−	−	−
L238	Macrophages	+	−	−	−	−	−
	PBL	+	−	−	−	−	−
L471	Macrophages	+	−	+	−	+	−
	PBL	+	−	−	−	−	−
L652	Macrophages	+	−	+	−	+	−
	PBL	+	−	−	−	−	−
M118	Macrophages	+	−	+	−	+	−
	PBL	+	−	−	−	−	−

[a] Cells were cultured from the peripheral blood as described in Materials and Methods. For macrophages, virus was assayed both by titration of supernatants and by cocultivation with CEMx174 cells at 14 days after isolation. For lymphocytes, virus was assayed both by titration of supernatants and by development of virus-induced CPE at 7 and 14 days after isolation.

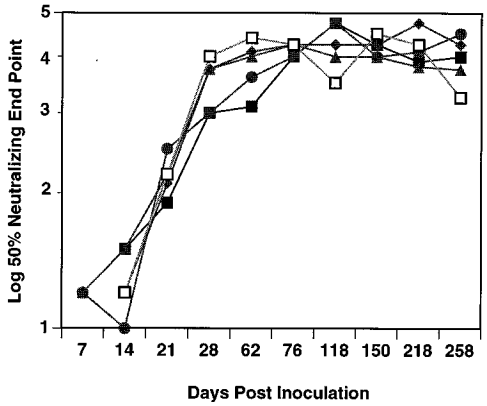

FIG. 1. Neutralizing-antibody titers (log_{10}) that increased over time in the sera from monkeys inoculated with SIV/17E-Cl. Neutralization assays were done against SIV/17E-Cl virus. The results are the averages of at least two independent assays, as described in Methods and Materials, and are for monkeys L235 (■), L238 (●), M118 (□), L652 (◆), and L471 (▲).

replication in animals infected with SIV/17E-Cl probably reflects the cell tropism of the virus and the pattern of replication of SIV in macrophages in vivo. Five monkeys have been monitored for 21 months, and four of them (two challenged and two unchallenged) have become virus negative by culture and intermittently negative by PCR analysis of PBMC.

Development of neutralizing-antibody responses. In contrast to the low level of replication of the recombinant virus, there was rapid development of a humoral immune response in all of the animals. By 14 days postinoculation, all five monkeys monitored serially had neutralizing antibodies directed against the infecting strain, SIV/17E-Cl (Fig. 1). These titers rose rapidly, peaked at 5 months postinfection, and remained constant throughout the following year. Neutralization assays were routinely done with a T-cell line (CEMx174); however, when the assays were done with primary rhesus macrophages, an equivalent level of neutralizing antibody was measured. The production of high levels of neutralizing antibodies in monkeys infected with SIV/17E-Cl is in direct contrast to the absence or low levels of neutralizing antibodies made in response to infection with the parental strain $SIV_{mac}239$. Thus, envelope sequences in SIV/17E-Cl that confer macrophage tropism also appear to be responsible for eliciting a strong neutralizing-antibody response in vivo.

To determine the specificity of the neutralizing antibodies, neutralizations were done at monthly intervals with the following virus strains: (i) $SIV_{mac}239$, (ii) the uncloned parental strain SIV/17E-Br, (iii) an additional recombinant clone (SIV/17E-Fr) that contains the entire *env* gene from SIV/17E-Br, and (iv) a heterologous primary isolate of sooty mangabey monkey origin, SIV/DeltaB670 (Table 3). Within the first 150 days postinoculation, sera obtained from the SIV/17E-Cl-infected monkeys, with the exception of that from monkey L652, neutralized only SIV/17E-Cl. In contrast, samples obtained later neutralized not only SIV/17E-Cl but also SIV/17E-Br, the recombinant virus SIV/17E-Fr, and the heterologous isolate SIV/DeltaB670 (Table 3). The neutralizing titer against the heterologous primary isolate, SIV/DeltaB670, was never as high as those observed with the homologous clone, SIV/17E-Cl (Table 4). To examine whether the homologous neutralizing-antibody response to SIV/DeltaB670 in macaques was lower than that observed for SIV/17E-Cl, the neutralizing titers to SIV/DeltaB670 in the sera from monkey C344, a long-term survivor of SIV/DeltaB670 infection (one of only two survivors of over 400 animals inoculated with this virus), were determined. Indeed, the titers were 2 log units less than those for SIV/17E-Cl, indicating that there appears to be a weaker neutralizing-antibody response to SIV/DeltaB670 in infected animals (Table 3). Nevertheless, the pattern of neutralizing activity was highly consistent in multiple samples taken throughout the infection in all of the animals tested and with respect to repeated determinations performed on the same sample. The rapid appearance of broadly reactive neutralizing antibodies in monkey L652 remains unexplained (Table 4). For the majority of these animals, a broadening of neutralizing antibody from type-specific to group-specific activity was observed 6 to 8 months after infection (Table 4).

TABLE 3. Determination of neutralizing-antibody specificity

Monkey	Neutralization titer[a]			
	SIV/17E-Cl	SIV/17E-Br	SIV/17-Fr	SIV/DeltaB670
L235	4.3	0.7	0.7	1.0
L238	4.8	0	2.3	1.0
L471	4.5	0	2.3	1.0
L652	3.4	1.8	3.0	1.75
M118	4.5	0.9	2.6	1.2
C344[b]	4.2	NT[c]	NT	2.1

[a] 50% neutralization end point (log_{10}) for sera taken 219 days postinoculation.
[b] Long-term survivor of SIV/DeltaB670 infection.
[c] NT, not tested.

The ability of antiserum raised against SIV/17E-Cl late in the infection to neutralize SIV/DeltaB670 was surprising given the genetic diversity of the two viruses. Unlike SIV/17E-Cl, which consists of a single genotype of $SIV_{mac}251$ lineage, SIV/DeltaB670 is a primary isolate consisting of a swarm of genetic variants cultured from the lymph node of a rhesus monkey infected with SIV from a sooty mangabey monkey. Sequence comparison of the V1 hypervariable regions of *env* found within the SIV/DeltaB670 genetic quasispecies identified a divergence of less than 10%, whereas a similar comparison of these sequences with SIV/17E-Cl showed an average divergence of over 25%. To place this difference in perspective, a comparison of SIV/17E-Cl V1 sequences with those obtained from the Los Alamos database for $SIV_{mac}251$, SIV_{mne}, SIV/STM, and HIV-2ROD showed divergences of 9.5, 16, 30, and 40%, respectively. Thus, persistent infection with the attenuated molecular clone SIV/17E-Cl induces over time neutralizing antibodies against a genetically diverse strain of SIV.

Avidity and conformation dependence of antibody responses to SIV/17E-Cl. In light of the kinetics of the appearance of neutralizing-antibody responses in the macaques infected with SIV/17E-Cl, we sought to examine the evolution of SIV enve-

TABLE 4. SIV/DeltaB670-specific neutralization titers

Day postinfection	SIV/DeltaB670-specific neutralization titer[a] with monkey:				
	L235	L238	L471	L652	M118
90	NT[b]	0	NT	1.25	NT
118	NT	0	0	1.25	0
150	0	0	0	1.0	0
195	0.7	0	0.7	1.75	0
219	1.0	1.0	1.0	1.75	1.2
244	1.0	1.0	NT	NT	NT

[a] 50% neutralization end point (log_{10}).
[b] NT, not tested.

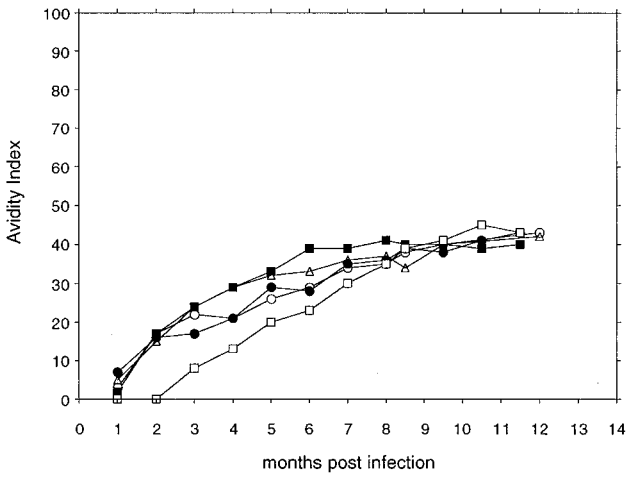

FIG. 2. Avidity of serum antibodies for SIV envelope glycoproteins. The antibody avidity index was determined by measuring in a ConA-ELISA the resistance of test serum antibody-envelope glycoprotein immune complexes to disruption by treatment with 8 M urea, as described in Materials and Methods. Longitudinal serum samples were obtained from the following five macaques at various times after infection with SIV/17E-Cl: L235 (■), L238 (●), M118 (□), L652 (◆), and L471 (▲).

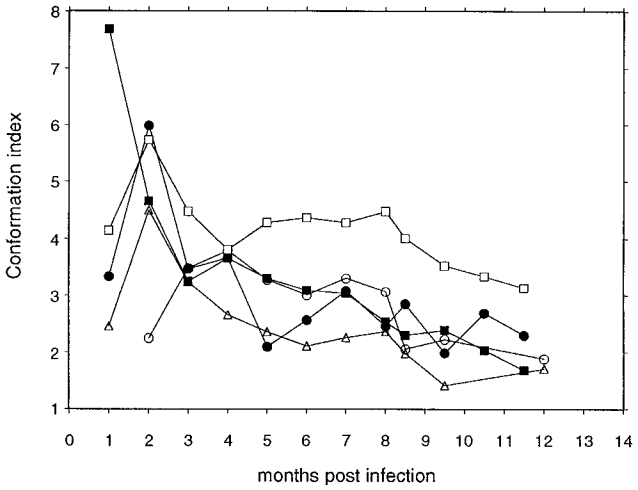

FIG. 3. Conformation dependence of serum antibodies to SIV envelope glycoproteins. The conformation dependence of envelope glycoprotein-specific serum antibodies elicited by infection with SIV/17E-Cl was determined by measuring in a ConA-ELISA the antibody reactivities against native SIV envelope glycoproteins and against denatured envelope glycoproteins prepared by reduction and carboxymethylation of protein sulfhydryl groups, as described in Materials and Methods. Longitudinal serum samples were obtained from the following five macaques at various times after infection with SIV/17E-Cl: L235 (■), L238 (●), M118 (□), L652 (◆), and L471 (▲). The conformation index is defined as the ratio of serum reactivity with native envelope glycoprotein to that with denatured envelope glycoprotein.

lope glycoprotein-specific antibody responses with respect to their avidity and conformation dependence, properties which have been frequently used as immune correlates of protection. For these purposes, native viral glycoproteins from purified SIV preparations were anchored onto ConA in microtiter plates for the respective immunoassays. Serum antibody avidities were determined by measuring the stabilities of antibody-antigen complexes to a urea wash, as described previously (11). In this functional assay, avidity index values of below 30% are considered to indicate low-avidity antibodies, values of between 30% and 50% are considered to indicate intermediate-avidity antibodies, and values of above 50% are considered to indicate high-avidity antibodies.

The data in Fig. 2 demonstrate that the envelope glycoprotein-specific antibody responses in the five SIV/17E-Cl-infected monkeys increase in avidity over the first 7 months postinfection and apparently level off at an intermediate avidity of about 40% thereafter. The relatively slow evolution of antibody avidity in the glycoprotein-specific antibody responses indicates an ongoing maturation of humoral immune responses to this chronic infection during at least the first 7 months postinfection. At this time, the antibody avidity appears to reach a maximum level that is maintained even after a subsequent virus challenge at 8 months postinfection. The slow increase in the avidity of antibodies to the SIV envelope glycoprotein is in distinct contrast to the relatively rapid increase in avidities observed during other viral infections (33), perhaps indicating an important escape mechanism by which SIV eludes immune responses soon after infection.

Previous studies have indicated a correlation between antibody populations specific for conformation-dependent epitopes and the presence of broadly neutralizing antibody reactivity to HIV-1 (9, 25, 36) and SIV (21). Therefore, the second antibody parameter measured was the conformation dependence of the envelope glycoprotein-specific antibody responses in the longitudinal panel of serum samples from the SIV/17E-Cl-infected macaques. For this assay, the reactivity of the serum antibodies at a standard dilution was measured in parallel against ConA-anchored native viral glycoprotein and against denatured viral glycoprotein produced by reductive carboxymethylation of protein cysteine sulfhydryl groups. Thus, this assay compares the reactivity of serum antibodies with a native viral glycoprotein complex with that with envelope glycoproteins in which all disulfide bonds have been irreversibly reduced to alter protein tertiary structure, without deliberate denaturation of the envelope protein secondary structure.

The data in Fig. 3 indicate that the serum antibodies present at all time points tested resulted in conformation index values ranging from about 1.5 to 8, indicating that the serum antibodies were on average two- to eightfold more reactive with the native viral glycoprotein substrate than with the denatured viral glycoprotein antigen. It is interesting that the highest levels of antibody conformation dependence were observed at 2 months postinfection (average index = 5) and that the conformation index in general decreased at later time points to an average of 2.5 at the time of virus challenge. The conformation index remained unchanged for the several months postchallenge. The predominance of conformation-dependent antibody produced in SIV/17E-Cl-infected monkeys is reminiscent of the case for the serum antibodies produced in HIV-1-infected individuals (25, 36) and in monkeys experimentally infected with various strains of SIV (21). To our knowledge, however, this is the first time that this parameter has been reported for serum samples taken over the course of an experimental infection in monkeys, thereby revealing the early dominance of conformation-dependent antibodies and an unexpected decline in the relative abundance of conformation-dependent antibodies with the progression of the persistent infection.

Lack of heterogeneity of *env* sequences in macaques. To examine whether the broadening of the neutralizing-antibody response and increased avidity of the antibody for the viral glycoprotein were due to the development of heterogeneity in *env* protein composition, the genetic diversity of gp120 sequences in virus present in two of the animals that were chal-

TABLE 5. Challenge of SIV/17E-Cl-infected macaques with a heterologous strain of SIV

Monkey	Day of challenge	Neutralization titer[a] for:		Protection
		SIV/17E-CL	SIV/DeltaB670	
M697	178	3.9	0.7	−
M462	192	3.9	0	−
M700	192	3.0	0	−
L235	243	4.3	1.0	+
L238	243	4.8	1.0	+

[a] 50% neutralization end point ($\log_{10}$).

TABLE 6. Glycoprotein-specific antibody titer at challenge in monkeys infused with SIV/17E-Cl antiserum

Monkey	gp140 titer[a]	PCR postchallenge
N261	1,600	−
N549	800	+
N644	1,600	−
M954	1,600	+
Donor serum	6,400	

[a] Reciprocal dilution.

lenged (L235 and L238) was examined at 9 days after inoculation in the peripheral blood mononuclear cells and at 244 days after inoculation in mononuclear cells from both lymph nodes and the peripheral blood. The entire gp120 sequence (nucleotides 6342 to 8222 [30]) was directly amplified from mononuclear cell DNA. Sequences specific for the external domain of gp41 (nucleotides 7682 to 10195 [30]) were similarly amplified, cloned, and sequenced. Very few nucleotide changes were detected in all clones examined. A comparison of early and late samples failed to reveal any significant difference in either the number of mutations or location of these changes, a finding that suggests that the observed changes were likely due to errors introduced by *Taq* polymerase. This low level of virus heterogeneity is in direct contrast to infection with the lymphocyte-tropic molecular clone $SIV_{mac}239$, which rapidly generates genetic diversity within this same time frame (3, 4). This finding is probably due to the low level of replication observed in the SIV/17E-Cl-infected animals relative to that observed for $SIV_{mac}239$. These data suggest that the development of cross-reactive neutralizing antibodies was likely not due to the generation and selection of antigenic variants or to the presence of a widely divergent virus swarm in these monkeys but rather may reflect the recognition of less-immunogenic viral epitopes by the immune system as these responses mature.

Challenge of SIV/17E-Cl-infected macaques with a heterologous strain of SIV. To determine whether the broadened neutralization response that appeared after 7 months postinfection in the SIV/17E-Cl-infected macaques could confer broad-spectrum protective immunity against a heterologous virus challenge, infected macaques were inoculated intravenously with 50 animal infectious doses of rhesus monkey-grown SIV/DeltaB670 either prior to 7 months (three macaques) or after 8 months (two macaques) postinfection (Table 5). Lymph node biopsies were performed immediately prior to and 14 days after challenge and, together with PBMC-derived cells, were evaluated for SIV/DeltaB670-specific sequences by PCR. SIV-specific sequences, when present, were further analyzed by sequence analysis of cloned PCR products containing the V1 hypervariable domain of gp120 (27). No evidence of infection with SIV/DeltaB670 could be identified in either these samples or PBMC samples obtained during the next 6 months from either of the monkeys (L235 and L238) which had broadly reactive neutralizing responses. In contrast, all three macaques (M697, M462, and M700) challenged earlier in infection had SIV/DeltaB670-specific sequences in both PBMC and lymph node-derived mononuclear cells; only one of these animals (monkey M697) had detectable neutralizing antibody to SIV/DeltaB670 at challenge. Multiple SIV/DeltaB670 variants found within the challenge inoculum were identified in monkey M700; this animal died of an AIDS-like disease 95 days postchallenge. The major variant found within the challenge inoculum was identified in monkey M462, whereas only a minor variant (representing less than 10% of the variants) could be identified in monkey M697. A similar analysis of naive monkeys inoculated with SIV/DeltaB670 has shown that the major variant present in the initial inoculum consistently emerges as the dominant form following in vivo infection (unpublished results). Taken together, these data suggest that the clonal emergence of a minor form of the inoculum observed in monkey M697 may represent a variant that has escaped the low level of neutralizing antibody detected in the serum of this animal at challenge. Both M462 and M697 have lived beyond 280 days postchallenge.

Passive protection of naive rhesus macaques. To further define the role of humoral immunity in the protective responses induced by SIV/17E-Cl infection, sera from the two monkeys that were protected from SIV/DeltaB670 challenge were collected, heat inactivated, and evaluated for the ability to passively protect naive recipient monkeys (Table 6). Four monkeys were infused with 10 ml of SIV/17E-Cl antiserum per kg of body weight, with two control monkeys receiving similar amounts of normal monkey serum, and challenged intravenously with 50 animal infectious doses of SIV/DeltaB670 7 h later (29). Infection was assessed by PCR of PBMC DNA at weekly intervals postinoculation. As expected, SIV *env*-specific antibody titers in the monkeys that received immune serum at challenge were four to eight times lower in the recipients than in the donor serum. Both control monkeys were virus PCR positive at 7 days postinoculation. SIV/DeltaB670 infection was evident at 7 days postinoculation in one monkey (N549) that received immune serum; a second animal (M954) was PCR positive at 14 days. The remaining two monkeys remained PCR negative for up to 6 months postinoculation and demonstrated no other signs of virus infection (e.g., seroconversion or clinical disease).

DISCUSSION

The infection of rhesus macaques with an attenuated macrophage-tropic clone of SIV induced immune responses that protected against challenge with a highly virulent primary isolate that differs by 16% overall in gp120 nucleotide sequence and by 25% in the sequence of the V1 hypervariable region of gp120. The induction of this protective response occurred 6 to 8 months postinfection in concert with a plateau in the avidity of gp120-specific antibody responses and a broadening of neutralizing-antibody responses. These responses were mainly directed toward conformation-dependent epitopes, because binding of antibody could be abrogated by denaturation of viral glycoproteins.

The ability of sera from vaccinated macaques to passively protect two of four naive recipients lends further support to the role of antibody in protection and demonstrates that sterilizing immunity can be induced by an attenuated SIV vaccine. How-

ever, these findings do not address whether these humoral immune responses are the sole mediators of protection. Indeed, the failure to protect all of the monkeys in the passive protection experiment argues that additional responses (e.g., cellular immune responses) may also be employed by an attenuated virus vaccine to act in concert with antibody to achieve optimal protection.

High-avidity and conformation-dependent antibodies have been implicated as important correlates for protective antibody responses to HIV-1 infection (9, 25), suggesting that these properties could be useful in defining the evolution of protective antibody responses in the macaques immunized with the live SIV/17E-Cl. Both the avidity and conformation dependence data reveal a maturation of SIV envelope-specific responses that requires at least 6 months postinfection. The requirement for this lengthy maturation process may explain the reported failure of attenuated SIV vaccines at 4 months postinoculation compared with the broad protection observed at 2 years postinoculation with the same attenuated SIV strain (14). In this regard, it has also been reported that broadly protective immune responses to equine infectious anemia virus evolve after 6 to 8 months postinfection in naturally and experimentally infected horses (23). Taken together, these observations suggest that protective immunity to lentiviruses requires a complex evolution of immune responses produced during a relatively lengthy maturation process in infected animals. The stringent requirements for the production of protective immune responses to lentivirus infections may explain the failure of less-rigorous immunizations with inactivated whole virus and subunit vaccines against SIV (24).

Since previous studies have correlated broadly neutralizing antibody responses to SIV and HIV-1 with conformation-dependent antibodies, we sought to examine the conformation dependence of envelope-specific antibody responses in relation to the appearance of type- and group-specific neutralizing antibodies and the establishment of protective immunity in macaques immunized with the SIV/17E-Cl attenuated macrophage-tropic strain. The results of these studies revealed a predominance of conformation-dependent serum antibodies at all time points tested postinoculation but also revealed an unexpected decline in the level of conformation-dependent antibodies as the infection progressed. This latter observation suggests that antibodies to linear determinants of the viral envelope glycoprotein become increasingly prevalent over the first 6 months postinfection, when their level appears to reach a stable plateau. One cannot from these data make a definitive correlation between the evolution of protective antibodies to SIV and the generation of linear or conformation-dependent antibody populations. Thus, it will be important to determine further the relationship between type- and group-specific neutralizing activities and linear and conformation-dependent antibodies to the SIV envelope. Taken together, the antibody avidity and conformation dependence assays indicate a progressive maturation of antibody responses to the attenuated macrophage-tropic SIV vaccine strain for about 7 months postinfection, suggesting that this may be the minimum time required for the evolution of a protective immune response to SIV.

The utilization of viral proteins conferring macrophage tropism may offer a selective advantage over the lymphocyte-tropic analogs that have dominated vaccine development thus far. Macrophage-tropic strains of HIV-1 appear to be transmitted most efficiently and to establish initial infections in humans (37–40) and are therefore the initial targets for vaccine-induced protection. The selective presentation of viral proteins by macrophages may also be preferred for the induction of protective immunity at the mucosal surfaces involved in sexual transmission of HIV-1. The studies described in this report suggest that macrophage-tropic strains may also be better inducers of protective responses. These responses may be attributable to the different routes of antigen processing and presentation that occur in macrophages. Viral antigens in infected macrophages would be expected to present antigen in the context of both major histocompatibility complex classes I and II. The vigorous early induction of neutralizing-antibody responses observed in SIV/17E-Cl-infected monkeys is in striking contrast to the absence of these responses in monkeys infected with $SIV_{mac}239$, which differs by only seven amino acid residues in gp120 (1). This may be due to the initial widespread infection of antigen-presenting cells in the peripheral blood because of the macrophage tropism of the virus. In addition, the macrophage-tropic virus may cause early infection of a broader range of cells in vivo, particularly antigen-presenting cells in the skin, gut, and other lymphoid organs. This could increase the immune response detected in the serum but may also elicit important protective local immune responses in the animal. The low level of replication of the macrophage-tropic virus in the CD4 lymphocytes may also contribute to the vigorous immune response. These cells are required for their helper function in the induction of immune responses, and infection by SIV or HIV has been shown to impair these helper functions. The investigation of the pattern of early infection and replication of macrophage-tropic viruses in vivo will be an important aspect in understanding the development of the protective immune response observed in this study.

This study examined the immune response and the clinical outcome of a strongly macrophage-tropic recombinant of SIV, and results similar to those of Lohman et al. (16) were obtained. It provides evidence that this virus elicits a vigorous immune response and that with time this immune response broadens to protect the animal against challenge with a heterologous, virulent strain of virus. This macrophage-tropic molecular clone differs from its lymphocyte-tropic parent only in the *env* gene; thus, these changes have altered the virus cell tropism and host immune response. These results provide a model system in which to examine the development of macrophage-tropic-based attenuated and subunit vaccine strategies.

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