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Evolution of Envelope-Specific Antibody Responses in Monkeys Experimentally Infected or Immunized with Simian Immunodeficiency Virus and Its Association with the Development of Protective Immunity

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Previous studies of attenuated simian immunodeficiency virus (SIV) vaccines in rhesus macaques have demonstrated the development of broad protection against experimental challenge, indicating the potential for the production of highly effective immune responses to SIV antigens. However, the development of this protective immune status was found to be critically dependent on the length of time postvaccination with the attenuated virus strain, suggesting a necessary maturation of immune responses. In this study, the evolution of SIV envelope-specific antibodies in monkeys experimentally infected with various attenuated strains of SIV was characterized by using a comprehensive panel of serological assays to assess the progression of antibodies in longitudinal serum samples that indicate the development of protective immunity. In parallel studies, we also used the same panel of antibody assays to characterize the properties of SIV envelope-specific antibodies elicited by inactivated whole-virus and envelope subunit vaccines previously reported to be ineffective in producing protective immunity. The results of these studies demonstrate that the evolution of protective immunity in monkeys inoculated with attenuated strains of SIV is associated with a complex and lengthy maturation of antibody responses over the first 6 to 8 months postinoculation, as reflected in progressive changes in antibody conformational dependence and avidity properties. The establishment of long-term protective immunity at this time in general parallels the absence of further detectable changes in antibody responses and a maintenance of relatively constant antibody titer, avidity, conformational dependence, and the presence of neutralizing antibody for at least 2 years postinoculation. In contrast to the mature antibody responses elicited by the attenuated SIV vaccines, the whole-virus and envelope subunit vaccines in general elicited only immature antibody responses characterized by poor reactivity with native envelope proteins, low avidity, low conformational dependence, and the absence of neutralization activity against the challenge strain. Thus, these studies establish for the first time an association between the effectiveness of experimental vaccines and the capacity of the vaccine to produce a mature antibody response to SIV envelope proteins and further indicate that a combination of several antibody parameters (including titer, avidity, conformational dependence, and virus neutralization) are superior to any single antibody parameter as prognostic indicators to evaluate candidate AIDS vaccines.

The development of an effective AIDS vaccine is complicated by the ability of human immunodeficiency virus type 1 (HIV-1) to establish a persistent infection via an integrated provirus, to undergo rapid and extensive variations in biological and antigenic properties, to infect both lymphocytes and macrophages, and to be transmitted by multiple routes of exposure (3). Despite these challenges to the host immune system, recent studies with animal lentiviruses have demonstrated effective immunologic control of virus replication and disease in experimentally infected or vaccinated animals (10, 11, 24, 29, 48). These observations indicate the potential for developing an effective vaccine for HIV-1 infection and disease and, most importantly, provide model systems in which to elucidate the nature of immune responses that can control virus replication and prevent the development of disease. Thus, identification of immune parameters associated with protection in these animal model systems can provide immunologic goals for the design of strategies for AIDS vaccines and define prognostic indicators for clinical trials of experimental AIDS vaccines.

Among animal models for HIV-1 infection and disease, the simian immunodeficiency virus (SIV)/rhesus macaque system most closely resembles HIV-1 infections of humans with respect to modes of transmission, the pattern of virus replication, the clinical course of disease progression, and the nature of the virus-specific immune responses (12). In the SIV system, the most broadly protective immune responses have been achieved by inoculation of macaques with naturally or genetically engi-

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neered attenuated strains of virus that establish infection without the development of clinical signs of disease (10, 11, 24, 48). For example, inoculation of macaques with the lymphocyte-tropic SIVmac239 virus strains that possess single or multiple genetic deletions have been shown to elicit broadly protective immunity by 18 months but not by only 5 months postinfection (11, 48). Similar vaccine studies using an attenuated macrophage-tropic (M-tropic) strain of virus, SIV/17E-Cl, demonstrated protective immunity at 8 months postinfection but a lack of protection at 5 months postinfection (10). These vaccine studies, along with trials using other strains of SIV (24), indicate that the evolution of protective immune responses in macaques is a relatively lengthy process and that the exact time course required to achieve protective immunity may be dependent on the nature (biologic and antigenic properties) of the vaccine strain. In support of this maturation hypothesis, Clements et al. (10) demonstrated in initial limited serological assays an evolution of virus-specific antibody that appeared to parallel the development of protective immunity. In addition, Clements et al. (10) and others (23) have also demonstrated that passive transfer of immune serum from macaques inoculated with attenuated SIV can confer protection against virus challenge, indicating an important role for virus-specific antibodies in vaccine protection. Thus, characterization of the evolution of protective immune responses in these experimental vaccine models should provide important information on the nature of protective immunity to SIV and the maturation process required to achieve this immune status.

Therefore, the present study was designed to examine a variety of SIV-specific antibody parameters to characterize the evolution of protective immunity in macaques experimentally vaccinated with various strains of SIV. For comparison, these same antibody parameters were evaluated for other selected SIV vaccines, including inactivated whole-virus vaccine (wv vaccine) and envelope subunit vaccines. The following assays were selected to characterize SIV envelope-specific antibody responses elicited by the various vaccines: (i) antibody titer to native viral glycoproteins and recombinant envelope antigens, (ii) antibody reactivity to conformational and linear envelope determinants (i.e., conformational dependence), (iii) antibody avidity, and (iv) antibody neutralization. The results of these studies reveal a complex maturation of protective immune responses that require about 6 to 8 months following infection to fully develop. In addition, these serological assays identify marked differences in the properties of SIV envelope-specific antibodies associated with protective and nonprotective immune responses.

MATERIALS AND METHODS

Viruses. SIVmac239 (36) was prepared by transfection of the DNA from an infectious molecular clone into rhesus macaque peripheral blood mononuclear cells (PBMC), and a virus stock was expanded in CEMx174 cells.

An M-tropic strain (SIV/17E-Br) was obtained by passage of SIVmac239 in rhesus macaques as previously described (40). A recombinant molecular clone that contains the surface glycoprotein (gp110) and a portion of the transmembrane glycoprotein (gp41) of SIV/17E-Br in the SIVmac239 molecular clone was constructed as previously described (1). Stocks of SIV/17E-Cl were prepared by transfection of DNA from this infectious molecular clone into primary rhesus PBMC. This stock was then used to infect fresh PBMC, and a stock containing ten 50% tissue culture infectious doses ($TCID_{50}$) of virus was obtained. Virus stocks of SIV/17E-Cl for neutralization assays were prepared in the human T-cell/B-cell hybrid line CEMx174 and were assayed for infectivity as described below.

The uncloned SIV/DeltaB670 (4) virus stock was obtained from infection of rhesus PBMC at the Tulane Regional Primate Research Center and represents a heterogeneous primary virus stock. This primary virus stock used as the challenge virus stock will be referred to herein as $SIV/DeltaB670_{Tulane}$. Virus stocks used for neutralization assays were prepared in rhesus PBMC and were assayed for infectivity as described below.

The uncloned SIV/B7, 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.

Viral vaccines. A pool of SIV/DeltaB670 virions was harvested from the infection of H9 cells, concentrated by pressure dialysis, and purified by banding in a 30 to 45% step gradient as previously described (32). This procedure has been shown to promote retention of significant amounts of the virion envelope during the purification process, and this same pool of virus has been used in vaccine studies as previously described (32, 33). For the wv preparations, virions were gradient purified and were then inactivated by incubation at 4°C for 24 h with 0.8% formalin before being combined with adjuvant for immunization (32). The lentil lectin affinity-purified glycoprotein (LL-gp) vaccine, containing SIV gp110 and gp41, was prepared from the same gradient-purified stock of $SIV/DeltaB670_{Tulane}$ and was further fractionated by affinity chromatography using lentil lectin-Sepharose as previously described (33). Recombinant preparations of the $SIV/DeltaB670_{Tulane}$ surface glycoprotein (rgp110) were produced in the baculovirus system and purified by affinity chromatography as previously described (18). The denatured preparation of this baculovirus rgp110 (d-rgp110) was reduced and carboxyamidated as described below for measurement of antibody conformation dependence (26).

Cells. Primary rhesus macaque lymphocytes and macrophages were obtained from heparanized peripheral blood collected from adult macaques as previously described (48). Briefly, the blood was centrifuged at 1,300 × *g* for 15 min, the plasma was removed, and the cells were resuspended in 2× volume with Hanks' buffered saline solution. 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.

To culture rhesus PBMC, cells were resuspended at 10^6 cells per ml in RPMI 1640 supplemented with 10% fetal bovine serum, 50 μg of gentamicin per ml, 2.0 nmol of L-glutamine per ml, 10 mM HEPES 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 then washed and resuspended in the same medium without phytohemagglutinin.

To culture rhesus primary macrophages, cells were resuspended at 2×10^6 cells per ml in RPMI 1640 containing 10% human serum, 50 μg of gentamicin per ml, 2.0 nmol of L-glutamine per ml, 10 mM HEPES buffer, and 20 U of macrophage colony-stimulating factor and 25 U of granulocyte-macrophage colony-stimulating factor (both generous gifts from Genetics Institute, Cambridge, Mass.) per ml and cultured for 5 days. Nonadherent cells were then removed, and the cultures were refed with the same supplemented medium.

Immunization and challenge of rhesus macaques. (i) SIV/17E-Cl. Rhesus macaques born at the Tulane Regional Primate Center receiving the M-tropic attenuated virus vaccine SIV/17E-Cl were inoculated, challenged, and monitored for signs of infection and disease as previously described (10). Briefly, monkeys were inoculated intravenously via the saphenous vein with 10 $TCID_{50}$ of a cryopreserved preparation of cell-free SIV/17E-Cl. Following infection with the attenuated virus vaccine SIV/17E/Cl, each macaque was challenged with 50 infectious doses (ID_{50}) of a cryopreserved preparation of $SIV/DeltaB670_{Tulane}$ grown on rhesus primary PBMC at 5 months (monkeys M697, M700, and M462 were challenged on days 178, 192, and 192 postinfection, respectively), at 8 months (monkeys L235 and L238 were challenged on day 244 postinfection), or subsequently at approximately 2 years (monkeys L235 and L238 were rechallenged at 28 months; monkeys L462 and M118 were challenged at 28 months; monkey M688 was challenged at 17 months; and monkey M689 was challenged at 27 months postinfection).

(ii) SIVmac316/Δnef and SIVmac239/Δ3. Rhesus macaques receiving genetically engineered attenuated strains of either SIVmac316/Δnef (*nef* deletion) or SIVmac239/Δ3 (triple deletion of *nef*, *vpr*, and *vpu*) were inoculated, challenged, and monitored for signs of infection and disease as previously described (48). Briefly, the macaques were inoculated intravenously with either the *nef*-deleted or triple-deletion virus vaccine (equal to 5 ng of p27 antigen) and were subsequently challenged with 10 ID_{50} of the uncloned stock of SIVmac251 at 8, 20, or 72 weeks postinfection.

(iii) wv and envelope subunit vaccines. Rhesus macaques born at the Tulane Regional Primate Center were immunized with wv or viral subunit preparations, challenged, and monitored for signs of infection and disease as follows: monkeys H633, G633, H497, and H609 received 560 μg of inactivated whole virus (inactivated total viral protein) coupled with MDP and were intravenously challenged with 10 ID_{50} of $SIV/DeltaB670_{Tulane}$ as previously described (32); monkeys G069, G441, G067, and G607 were intramuscularly immunized with 80 μg of the LL-gp subunit preparation containing SAF-1 and were intravenously challenged with 10 ID_{50} of $SIV/DeltaB670_{Tulane}$ as previously described (33); monkeys I379, J778, K662, K831, and K701 received four intramuscular immunizations of 100 μg each of native rgp110; monkeys J492, K614, K912, K029, and K721 received four intramuscular immunizations of 100 μg each of denatured, carboxyamidated rgp110 and were intravenously challenged with 10 ID_{50} of $SIV/DeltaB670_{Tulane}$ as previously described (34).

Infectivity assays. Fivefold serial dilutions of plasma or supernatant fluids from cultures were added to the wells of a 96-well tissue culture plate containing RPMI 1640 supplemented with 10% human serum, 50 μg of gentamicin per ml, 2 nmol of L-glutamine per ml, and 10 mM HEPES buffer. CEMx174 cells (10^6) were added to each well, and the wells were assessed for virus-specific cytopathology at 3, 5, and 7 days postinfection. The 7-day results were used to calculate the $TCID_{50}$ by the method of Karber (21).

Neutralization assays. Virus neutralizations were performed in 96-well tissue culture plates containing RPMI 1640 containing 10% fetal bovine serum as previously described (10). Briefly, fivefold serial dilutions of plasma (heat inactivated at 56°C and clarified by centrifugation) were added to the well with 10 to 50 $TCID_{50}$ of virus and incubated for 1 h at 37°C. CEMx174 cells (10^6) were then added to each well, and the development of cytopathology was recorded at 14 days. Results were used to calculate the 50% neutralization endpoint by the method of Karber (21).

Measurement of SIV envelope glycoprotein-specific antibody endpoint titers by ConA ELISA. Serum samples from SIV-infected or immunized macaques were analyzed for their reactivity to native viral envelope glycoprotein substrate in a concanavalin A (ConA) enzyme-linked immunosorbent assay (ELISA) that maximizes the maintenance of protein structure, as previously described for HIV-1 gp120 immunoassays (37, 38). Preparations of SIV purified by density gradient centrifugation and disrupted with 1% Triton X-100 (TX) were used as sources of viral envelope glycoproteins, herein referred to as native viral glycoproteins. Preliminary experiments using different strains of SIV (SIVmac251, SIVmac239, SIV/DeltaB670, and SIV/B7) as sources of viral glycoprotein antigen indicated that the level of strain variation in the envelope glycoprotein sequence did not affect the reactivity patterns observed with polyclonal macaque immune sera. Therefore, in the experiments presented here, the SIV/B7 strain was used as the standard reference glycoprotein (10). For endpoint titers to rgp140 (Table 2), the antigen is baculovirus rgp140, which represents all of the surface glycoprotein gp110 and the ectodomain of the transmembrane glycoprotein gp41. Due to purification steps necessary to isolate this protein, the rgp140 is substantially denatured and most likely represents a relatively linear form of the uncleaved glycoprotein (18).

Immulon II microtiter plates (Dynatech Laboratories) were incubated with 5.0 μg of ConA (Vector Laboratories) per well in 100 μl of phosphate-buffered saline (PBS-A; pH 7.4) for 1 h at room temperature and then washed two times with PBS-A. The ConA plates were used to adsorb the envelope glycoproteins from TX-disrupted SIV/B7 (1 to 3 μg of total viral protein, as determined by Bio-Rad *DC* protein assay, per 50 μl in each well) overnight at 4°C. It is estimated that 3 μg of SIV/B7 contains about 90 ng of envelope glycoprotein. The ConA-adsorbed viral glycoprotein wells were then washed four times with PBS-A and blocked with 100 μl of 5% nonfat dry milk in PBS-A (BLOTTO) per well for 1 h at room temperature. After the blocking solution was removed, 50 μl of serial twofold-diluted monkey serum was added to each well and incubated for 1 h at room temperature. The sample wells were again washed four times with PBS-A, and 50 μl of peroxidase-conjugated anti-monkey immunoglobulin G (Sigma), diluted 1:20,000 in BLOTTO, per well was added for a 1-h incubation at room temperature. The wells were once again washed four times with PBS-A, and 200 μl of TM-Blue (Intergen) substrate was added and left for approximately 20 min at room temperature before color development was determined by the addition of 50 μl of 1 N sulfuric acid per well. Antibody reactivity to the ConA-anchored glycoprotein substrates was then determined by measuring the optical density (OD) at 450 nm, using an automated plate reader (Titertek). Endpoint titers were determined by the last twofold dilution whose OD was twice that of normal monkey serum at the lowest dilution (1:100) or an OD of 0.100, whichever value was greater.

Measurement of SIV envelope glycoprotein-specific antibody conformational dependence. Serum samples from SIV-infected or immunized macaques were analyzed for conformational dependence by comparing the serum antibody reactivities to native and denatured viral envelope glycoprotein substrates in a ConA ELISA. Preparations of gradient purified, TX-disrupted SIV/B7 were again used as a source of native viral envelope glycoproteins. To produce denatured envelope glycoproteins, 1 mg of the purified SIV/B7 was treated with 1 mM dithiothreitol in a volume of 1 ml for 15 min at room temperature to reduce disulfide bonds. Following the dithiothreitol treatment, 2 mM iodoacetamide (prepared in 1 N sodium hydroxide) was added to the virus to achieve an irreversible carboxyamidation of the reduced sulfhydryl groups. The denatured virus was then purified and concentrated by spinning through a Centricon filter (Amicon, Inc., Beverly, Mass.). The final protein concentration was then determined by using the Bio-Rad *DC* protein assay. 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 (26).

For these conformational assays, native or denatured viral envelope glycoproteins prepared from TX-disrupted SIV/B7 or denatured SIV/B7, respectively, were captured onto 96-well microtiter plates by using ConA as described above for the standard ConA ELISA. Test sera, diluted in BLOTTO to produce an OD at 450 nm of about 1.0 to 1.5 in the endpoint titer ConA ELISA procedure, were reacted in triplicate wells with either the ConA-anchored native or denatured glycoprotein substrate, thoroughly washed with PBS-A, and then incubated with 50 μl of a 1:20,000 dilution of peroxidase-conjugated anti-monkey immunoglobulin G (Sigma) in BLOTTO per well for 1 h at room temperature. The wells were once again washed four times with PBS-A, incubated with 200 μl of TM-Blue (Intergen) substrate for approximately 20 min at room temperature, and developed as described above for the standard ConA ELISA. A conformation ratio was then calculated from the ratio of antibody reactivities to native versus denatured envelope glycoprotein substrates. Thus, the conformation ratio is a direct measure of the conformational dependence of a particular antibody sample; i.e., the larger the conformation ratio, the greater the requirement for native envelope glycoprotein structure.

Measurement of SIV envelope glycoprotein-specific antibody avidity. The avidity index values of serum antibodies to the native viral envelope were determined by measuring the resistance of antibody-envelope glycoprotein complexes in the ConA ELISA to 8 M urea (17, 35, 43). For these avidity assays, the ConA-anchored native viral envelope glycoprotein substrate was prepared from gradient-purified, TX-disrupted SIV/B7 as described above for the standard ConA ELISA. All test sera were diluted in BLOTTO to produce an OD at 450 nm of about 1.0 to 1.5 in the endpoint titer ConA ELISA procedure and were plated in two sets of triplicate wells. After the serum incubation, triplicate wells were treated in parallel for 5 min with either PBS-A or a solution of 8 M urea in PBS-A. Following this treatment, the wells were thoroughly washed with PBS-A, incubated with TM-Blue, and developed as described above. The avidity index was then calculated from the ratio of the absorbance value obtained with urea treatment to that observed with PBS-A treatment multiplied by 100. Antibodies with avidity index values of <30% are designated low avidity, those with index values between 30 and 50% are designated intermediate avidity, and those with values >50% are designated high avidity (17).

While measurements of antibody conformational dependence and antibody avidity were performed at the dilution producing an OD at 450 nm of 1.0 to 1.5 in the endpoint titer ConA ELISA procedure, experiments using several different dilutions within this linear range were performed to ensure that the variation in actual values was within 10%.

RESULTS

Evolution of protective immune responses in monkeys inoculated with the attenuated SIV/17E-Cl virus vaccine. To characterize the time course of the evolution of the antibody response to viral envelope proteins in macaques that were inoculated with the highly attenuated, M-tropic recombinant virus SIV/17E-Cl, longitudinal serum samples taken at monthly intervals postinoculation were analyzed for antibody titer, conformational dependence, and avidity, using the ConA ELISA procedure. We have previously reported that these macaques were protected from infection and disease when challenged with the heterologous, primary isolate $SIV/DeltaB670_{Tulane}$ following infection with the attenuated SIV/17E-Cl strain for ≥8 months. In contrast, no protection was observed at 5 months postinoculation (10). Furthermore, passive serum transfer experiments from rhesus macaques inoculated with SIV/17E-Cl for ≥8 months resulted in protection of two of four naive recipients, suggesting an important role for SIV-specific antibody in this attenuated virus vaccine protection (10). In the present study, we analyzed in more detail the envelope-specific antibody responses to the native envelope glycoproteins in the ConA ELISA at 5 and 8 months postinoculation with the attenuated SIV/17E-Cl virus vaccine and also examined the maintenance of these antibody responses at 18 to 28 months postinoculation.

Representative results, shown in Fig. 1, demonstrated that the development of envelope-specific serum antibodies to native envelope glycoproteins were initially detected by about 2 to 4 weeks postinfection and reached maximum titers at around 6 months. In addition to the amount of envelope-specific antibody (endpoint titer), the qualitative properties of the antibody response gradually evolved during this time, as evidenced by the continued changes in the values measured for antibody conformational dependence, avidity, and virus neutralization. While immune sera taken at all time points preferentially recognized native envelope antigens better than the denatured antigen substrate (as indicated by conformation ratios of >1), the conformational dependence of the envelope-specific antibodies displayed a continuous decrease during the first 6 months postinfection, decreasing from a high of 4 to 6 at 1 month postinfection to a relatively steady value of about 2 after 6 to 8 months of infection. These changes in conformational dependence suggest a steady increase in the concentration of antibodies directed to more linear determinants of the

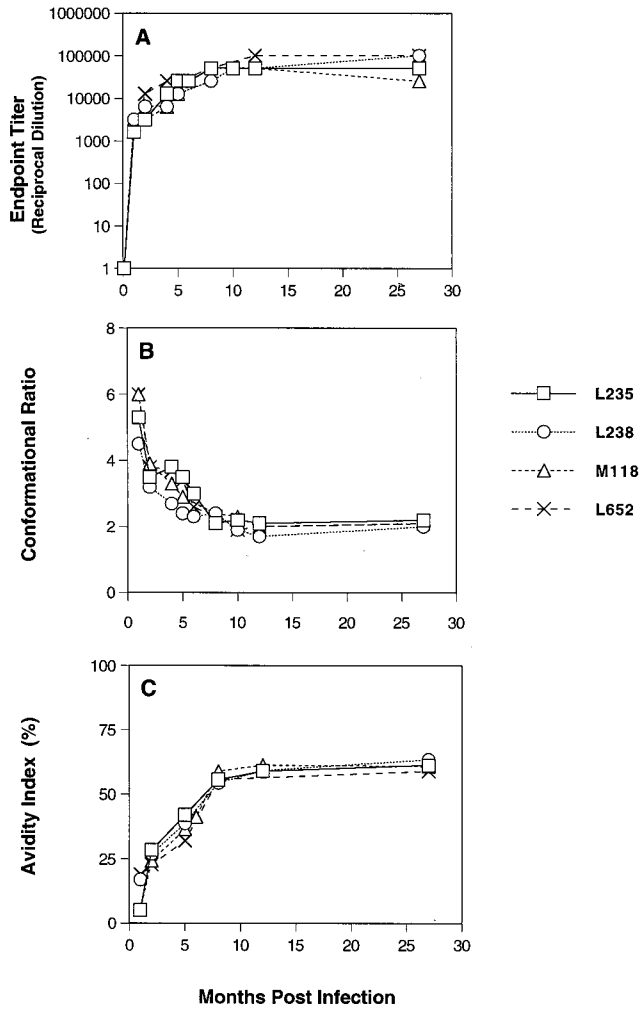

FIG. 1. Evolution of protective envelope-specific antibody responses in monkeys inoculated with the attenuated SIV/17E-Cl virus vaccine. Longitudinal samples were obtained from four rhesus macaques at the indicated times after inoculation with SIV/17E-Cl. (A) The reciprocal endpoint titer was determined by measuring activity to the native viral envelope glycoproteins in the ConA ELISA. (B) The conformational dependence of serum antibodies to SIV envelope glycoproteins was determined by measuring the serum reactivity to native viral glycoproteins compared to that of denatured viral glycoproteins in the ConA ELISA. (C) The avidity of serum antibodies for SIV glycoproteins was determined by measuring the resistance of serum antibody-envelope glycoprotein immune complexes to disruption by treatment with 8 M urea in the ConA ELISA. All three techniques are described in Materials and Methods.

SIV envelope proteins during the first several months postinfection. The third antibody assay was measurement of envelope-specific antibody avidity index, as measured by the relative stability of the antigen-antibody complexes to an 8 M urea wash. A similar evolution of antibody avidity was also observed during the first 6 to 8 months postinfection, as shown by the steady increase from low-avidity antibody (less than 30%) observed during the first 6 months postinfection to the high-avidity antibodies (greater than 50%) maintained after about 7 months postinfection.

Finally, we analyzed the serum samples for the ability to neutralize both the immunizing M-tropic SIV/17E-Cl virus and the heterogeneous primary isolate SIV/DeltaB670$_{Tulane}$, used as the challenge virus. As summarized in Table 1, neutralizing antibodies to both viruses emerged early, by about 2 to 4 weeks postinfection. Interestingly, while the presence of neutralizing antibodies to both viruses was maintained for 2 years postinoculation, neutralization titers appeared to peak around 4 months postinoculation and then gradually decreased over the 2-year observation period.

Taken together, the results of these serological assays reveal a continuous and complex maturation of SIV envelope-specific antibody responses over the first 6 to 8 months postinfection that is reflected by changes in antibody avidity, conformational dependence, and neutralization properties. Once these fully mature humoral immune responses are established, they appear to be maintained at a relatively steady state for at least 2 years postinfection.

Analysis of envelope-specific antibodies associated with protective and nonprotective immune responses in macaques inoculated with SIV/17E-Cl. To address the issue of whether in vitro antibody assays might serve to predict protection of immunized rhesus macaques from virus challenge, we further analyzed day-of-challenge serum samples from SIV/17E-Cl-inoculated macaques. Analyses of serum samples from macaques challenged at 5 months and those challenged either at 8 months or at 18 to 28 months revealed marked differences in envelope-specific antibody reactivity profiles (Table 2). Envelope-specific serum reactivity to the native envelope glycoproteins demonstrated antibody endpoint titers that were only slightly (on average two- to threefold) higher in monkeys that were challenged at 8 months or at 18 to 28 months (average titers of 1:51,200 and 1:66,560, respectively) compared to the monkeys that were challenged at 5 months (average titer of

TABLE 1. Virus neutralization titers of SIV/17E-Cl-inoculated monkeys

Animal	Mo postinfection	Virus neutralization[a]	
		SIV/17E/Cl	SIV/DeltaB670$_{Tulane}$
L235	1	3.0	5.8
	2	4.0	6.0
	3	ND	7.5
	4	4.8	6.8
	5	3.8	ND
	8	3.5	4.7
L238	1	3.0	4.9
	2	4.0	6.3
	3	ND	5.8
	4	4.8	6.7
	5	4.1	ND
	8	4.3	3.5
L652	1	3.8	7.0
	2	4.3	4.6
	3	ND	7.0
	4	4.5	5.4
	5	4.4	ND
	8	4.4	3.0
M118	1	4.0	3.3
	2	4.3	ND
	3	ND	6.3
	4	3.5	6.0
	5	4.6	>4.2
	8	4.0	2.8

[a] Virus neutralizations were performed on rhesus macaque primary cells and CEMx174 cells, and the ability to neutralize the SIV/17E-Cl immunization virus or the SIV/DeltaB670$_{Tulane}$ challenge virus is represented as the log of the 50% endpoint titer. ND, not determined.

TABLE 2. Characterization of antibody responses to viral envelope glycoproteins in monkeys inoculated with an attenuated M-tropic strain of SIV (17E-Cl)

Animal	Time (mo) of challenge	Status of protection	Endpoint titer[a]	Conformation ratio[b]	Avidity index[c] (%)	Virus neutralization[d]	
						SIV/17E-Cl	SIV/DeltaB670$_{Tulane}$
M462	5	No	6,400	3.8	30	3.2	5.0
M697	5	No	25,600	2.6	39	3.9	4.9
M700	5	No	25,600	2.8	45	4.2	3.5
Avg[e]	5	No	19,200	3.1	38	3.8	4.8
L235	8	Yes	51,200	2.1	56	4.3	4.7
L238	8	Yes	51,200	2.2	54	4.8	3.5
Avg	8	Yes	51,200	2.2	55	4.6	4.1
L235	28	Yes	51,200	2.1	61	2.8	1.9
L238	28	Yes	102,400	1.9	63	3.0	3.3
L652	28	Yes	102,400	2.1	59	3.3	2.8
M118	28	Yes	25,600	2.4	61	2.8	1.4
M688	18	Yes	51,200	1.7	61	3.7	0.5
Avg	>18	Yes	66,560	2.0	61	3.1	2.0
M689	28	No	6,400	3.8	45	3.3	0.9

[a] Antibody endpoint titers were measured to the native envelope glycoproteins in the ConA ELISA.
[b] Determined by comparing reactivities of native and denatured glycoproteins in the ConA ELISA.
[c] Determined by measuring the relative stability of the native viral envelope antigen-antibody complexes to an 8 M urea wash in ConA ELISA. Values were calculated by using the equation (urea-washed wells/PBS-washed wells) × 100, where values $x \geq 50\%$, $50\% > x > 30\%$, and $x \leq 30\%$ have been defined high, intermediate, and low avidity, respectively; $x < 5$ designates urea values too low to measure.
[d] Virus neutralizations were performed on rhesus macaque primary cells and CEMx174 cells, and the ability to neutralize the SIV/17E-Cl immunization virus or the SIV/DeltaB670$_{Tulane}$ challenge virus is represented as the log of the 50% endpoint titer.
[e] Average values calculated for groups of monkeys.

1:19,200). Despite only minimal differences in envelope-specific antibody titer, changes in the conformational dependence of the serum antibodies were evident as the monkeys developed protective immune responses. All of the immune sera preferentially recognized native envelope antigens better than the denatured antigen substrate (as determined by conformational ratios of >1). However, the early immune serum samples taken at 5 months postinoculation displayed the highest conformation ratios (averaging 3.1), whereas immune serum from monkeys challenged at 8 months or later displayed substantially lower conformational ratios (averaging about 2). These data indicate that while envelope-specific antibody responses are always predominantly directed to conformational determinants, the evolution of protective immune responses may be associated with an increase in antibodies specific for linear determinants on the SIV envelope glycoproteins, suggesting a broadening of the humoral immune response to include recognition of additional determinants. Differences in antibody avidity were also observed as the monkeys developed protective immune responses. Monkeys challenged at 5 months displayed antibody avidities ranging from low to intermediate, with an average avidity index of 38%. In contrast, both monkeys challenged at 8 months and five of the six monkeys challenged or rechallenged at 18 to 28 months displayed high-avidity antibodies, with average index values of 55 and 61%, respectively.

Interestingly, one of the monkeys (M689) failed to be protected from virus challenge at 28 months postinoculation. A closer analysis of the day of challenge serum from this monkey revealed antibody values similar to those observed in the monkeys challenged at 5 months (lower titer and avidity and higher conformational ratios). This was surprising since the virus-specific antibody response reflected a relatively immature envelope-specific antibody response, despite persistent SIV/17E-Cl infection for more than 2 years. This lack of antibody maturity was further mirrored by a lack of protection from heterologous challenge.

Finally, we analyzed day-of-challenge serum samples for the ability to neutralize both the immunizing SIV/17E-Cl virus vaccine and the challenge virus SIV/DeltaB670$_{Tulane}$. As summarized in Table 2, day-of-challenge sera from all monkeys at all three time points were able to effectively neutralize both the immunizing M-tropic virus SIV/17E-Cl and the heterogeneous challenge virus SIV/DeltaB670$_{Tulane}$. These results suggest that the presence of neutralizing antibody, whether to the immunizing virus strain or to the heterologous challenge strain, is a necessary but not sufficient component of protection.

In general, these data indicate that protective immune responses are associated with the generation of high levels of envelope-specific antibodies that display high conformational dependence, high avidity, and neutralization of both the vaccine and challenge viruses in vitro. Additionally, these observations suggest that successful immunization may be associated with the generation of a mature antibody response and that the best vaccines are those that can reach this maturity in the shortest amount of time.

Analyses of protective immune responses in monkeys immunized with genetically engineered deletion mutants of SIV. Wyand et al. (48) recently described a detailed serological analysis of immune serum taken from a panel of 19 rhesus macaques inoculated for various lengths of time with mutants of SIVmac239 containing either single or multiple deletions before virus challenge with a biological clone of SIVmac251. The panel of monkeys studied was similarly divided between those that were protected (10 of 19) or unprotected (9 of 19) from the experimental virus challenge, as summarized in Table 3. The results of their studies indicated no definitive correla-

TABLE 3. Characterization of envelope-specific antibody responses in animals immunized with genetically engineered deletion mutants of SIVmac239 (Δnef and Δ3)

Vaccine and animal	Conformation ratio[a]	Avidity index[b] (%)	Virus neutralization (%)		Status of protection
			$SIVmac251_{Lab}$[c]	$SIVmac251_{BC}$[d]	
2-mo challenge					
Avg[e]	3.9	11	1:274	57.7	2/7 protected
SIVmac316/Δnef					
R03	>6	<5	1:20	40	No
R49	5.3	<5	1:60	19	No
8Q0	3.6	21	1:960	86	No
SIVmac239/Δ3					
R54	3.7	16	1:480	81	No
*R08	—	—	<1:20	1	No
R24	3.5	15	1:80	85	Yes
92A	5.1	14	1:320	93	Yes
5-mo challenge					
Avg	2.8	41	1:1,787	73.3	3/6 protected
SIVmac316/Δnef					
R30	3.0	31	1:320	37	No
92E	2.5	31	1:640	68	No
H093	3.2	61	1:5,120	95	Yes
SIVmac239/Δ3					
91Q	3.3	43	1:3,840	59	No
99R	2.2	42	1:480	88	Yes
R15	2.5	38	1:320	93	Yes
18-mo challenge					
Avg	3.1	55	1:4,067	90.2	5/6 protected
SIVmac316/Δnef					
R19	2.3	74	1:2,560	96	Yes
H17	5.2	33	1:120	86	No
SIVmac239/Δ3					
280-91	2.2	62	1:960	96	Yes
296-91	5.3	53	1:80	87	Yes
H061	3.6	62	1:320	85	Yes
R21	2.3	44	1:160	91	Yes

[a] Determined by comparing the reactivities of native and denatured glycoproteins in the ConA ELISA; $x > 6$ designates denatured antigen values too low to measure; — designates both native and denatured values too low to measure.

[b] Determined by measuring the relative stability of the native viral envelope antigen-antibody complexes to an 8 M urea wash in ConA ELISA, and values were calculated by using the equation (urea-washed wells/PBS-washed wells) × 100, where values $x \geq 50\%$, $50\% > x > 30\%$, and $x \leq 30\%$ have been defined high, intermediate, and low avidity, respectively; $x < 5$ designates urea values too low to measure; — designates both PBS and urea values too low to measure.

[c] Virus neutralizations were performed against the tissue culture lab-adapted $SIVmac251_{Lab}$ virus by Wyand et al. as previously described (48) and are expressed as the reciprocal dilution that yielded 50% virus neutralization.

[d] Virus neutralizations were performed against the $SIVmac251_{BC}$ challenge virus by David Montefiori as previously described (48) and are expressed as percentage of virus neutralization.

[e] Average values calculated for groups of monkeys (*R08 was not included since all values too low to measure).

tions between the level of protection and various immune parameters, including antibody titer and neutralization activity against the lab-adapted strain of SIVmac251 ($SIVmac251_{Lab}$). However, there was an apparent association detected between protection and the presence of neutralizing antibodies to the biological clone of SIVmac251 ($SIVmac251_{BC}$) used as the challenge virus; i.e., all 10 protected monkeys displayed an average in vitro neutralizing activity of ≥90% against the challenge virus stock. However, the association between protection and neutralization activity was not absolute, as a number of the serum samples taken from the unprotected monkeys also contained neutralization levels above 50%. In light of the serological data obtained with monkeys immunized with the SIV/17E-Cl attenuated vaccine strain, it was of interest to evaluate some additional antibody parameters as potential components of protection in this panel of 19 immune sera.

The results of the envelope-specific antibody avidity and conformational dependence assays are summarized in Table 3. In general, the data indicated a time-dependent maturation of antibody responses in monkeys inoculated with either the single- or triple-deletion mutant of SIV. Antibody avidity values showed a steady increase at the 2-, 5-, and 18-month time points, while the conformational dependence decreased over the same time interval. For example, antibody avidity values averaged about 11% for the 2-month serum samples, 41% for the 5-month serum samples, and 55% for the 18-month serum samples. Thus, the antibodies produced in the inoculated monkeys steadily progressed from low to intermediate to high avid-

ity at 2, 5, and 18 months postinfection, respectively. Average conformational dependence values initially decreased from 3.9 at 2 months to 2.8 at 5 months and were maintained thereafter, averaging 3.1 at 18 months. These serological analyses demonstrate an evolution and long-term maintenance of antibody responses elicited by the SIV deletion mutants that is similar to the progression of antibody responses observed in monkeys immunized with the SIV/17E-Cl attenuated virus vaccine.

The data summarized in Table 3 demonstrate a strong association between antibody avidity measurements and the level of protection observed in the experimentally immunized and challenged monkeys. Overall, 8 of the 10 monkeys that were protected from virus challenge displayed antibody avidity values greater than 35%, corresponding to the presence of intermediate- to high-avidity antibody. In contrast, eight of the nine monkeys that were not protected from virus challenge displayed low to intermediate antibody avidities that were less than 35%. The time-dependent evolution of antibody avidity also appears to parallel the maturation of protective immune responses. For example, five of six monkeys were protected from challenge at 18 months postinfection, and serum antibodies from these five protected monkeys displayed intermediate to high avidity values (>35%); in contrast, serum antibodies from the single unprotected monkey displayed the lowest antibody avidity of 33%, a value considered just into the intermediate avidity range. Of the six monkeys challenged at 5 months postinfection, three were protected and three were not protected by the attenuated virus vaccine. In this group, the three protected monkeys displayed intermediate to high avidity values (38, 42, and 61%), while two of the three unprotected monkeys displayed low antibody avidity of 30%. All seven monkeys challenged at 2 months postinoculation displayed only low-avidity antibody, and only two of these animals were protected from virus challenge.

These data demonstrate that the presence of antibody avidity values of >35% is associated with protection in 16 of 19 monkeys contained in this study. Measurements of antibody avidity, however, do not appear to be an absolute predictor of protection, as 2 of 19 monkeys were protected with only low-avidity antibody, and 1 monkey with intermediate-avidity antibody was not protected. The accuracy of antibody avidity as a prognostic indicator of a protective immune response is similar to the degree of association reported by Wyand et al. (48) for in vitro serum neutralization of the SIVmac251 challenge stock. As summarized in Table 3, all 10 of the protected monkeys displayed serum neutralization activities against the challenge SIVmac251$_{BC}$ virus stock of above 80%, suggesting that protective immune responses were associated with the production of antibodies that could effectively neutralize the challenge virus stock. However, this virus neutralization also cannot be viewed as an absolute component of protection, as 4 of the 10 unprotected monkeys in the study also displayed serum neutralization activities in the range of 50 to 90%. In addition, neutralization of the lab-adapted strain of SIVmac251 was not associated with protection in these same monkeys, indicating again that the neutralization results are not an absolute component of protection and are largely dependent on the virus strain used.

Finally, the data in Table 3 demonstrate that, in general, there is a strong association between the presence of high-avidity antibody and virus neutralization of the SIVmac251$_{BC}$ challenge strain used in the neutralization assay. All of the monkeys that developed intermediate- to high-avidity antibody displayed high levels of neutralization activity against the challenge SIVmac251$_{BC}$ virus in the serum neutralization assay. Once again, this association was not absolute, as sera from three monkeys (8Q0, R24, and 92A) challenged at 2 months postinoculation displayed virus neutralization activities greater than 85% but antibody avidity values less than 20%. The observation of high levels of serum neutralization activity by apparently low-avidity antibodies raises a number of interesting questions about the mechanism of virus inactivation by these serum samples taken at 2 months postinoculation.

Characterization of antibody responses elicited by inactivated whole-virus and envelope subunit vaccines to SIV. With the development of serological parameters characteristic of protective immune responses elicited by live attenuated SIV vaccines, it was of interest to assay these same serological properties for envelope-specific antibodies produced by other experimental SIV vaccines that we have previously evaluated and found to be ineffective in eliciting virus-specific immune responses capable of preventing virus infection and disease. These experimental SIV vaccines include inactivated whole virus (wv) (32), lectin-purified envelope glycoproteins (LL-gp) (33), and baculovirus-produced recombinant SIV gp110, both as a native (rgp110) antigen and denatured (d-rgp110) by reduction and carboxyamidation (34). Each of these SIV immunogens was adjuvanted with MDP and used in an immunization regimen that consisted of at least three doses of vaccine prior to experimental virus challenge. The vaccinated monkeys were subsequently challenged with 10 ID_{50} of the primary isolate SIV/DeltaB670$_{Tulane}$ after the final immunization and then monitored for evidence of infection and disease. While initial evaluations of the inactivated wv and LL-gp vaccines indicated a high level of protection from infection (32), subsequent studies suggested that this protection was not due to virus-specific immune responses but perhaps due to antibodies produced to cellular proteins contained in the virus preparation (22, 42). In contrast, the baculovirus-derived rgp110 vaccines failed to provide any protection against virus infection and the development of disease (34). Thus, these vaccine recipients provide a useful panel in which to determine the properties of envelope-specific antibodies associated with nonprotective immune responses to SIV for a comparison to the antibody properties observed above in macaques immunized with attenuated SIV strains.

To characterize the antibody responses elicited by the wv and envelope subunit vaccines, serum samples collected on the day of virus challenge were assayed for antibody titer, conformational dependence, and avidity, using the ConA ELISA procedure. The reactivity of each serum sample was measured against the native SIV envelope glycoproteins (viral gp) and against rgp140 to further compare the specificity of the serum antibodies for native and denatured envelope antigen determinants, respectively. Finally, each serum sample was also tested in neutralization assays to determine its capacity to inactivate the challenge virus SIV/DeltaB670$_{Tulane}$. The results of these serological assays are summarized in Table 4, with a representative serum sample from a monkey immunized with the SIV/17E-Cl included as a reference for a protective immune response to SIV challenge infection.

The results of these studies revealed distinguishing differences in the properties of the antibodies elicited by the various vaccines. The inactivated wv vaccine produced envelope-specific antibodies that were more reactive with native viral glycoproteins compared to the denatured rgp140 antigens (average titers of 1:7,200, compared to 1:650), that were predominantly directed to conformationally dependent determinants (average ratio of 1.6), and that reacted with a low intermediate avidity to both the native viral glycoprotein and rgp140 antigens (average avidity values of 32 and 36%, respectively). The LL-gp vaccine elicited antibodies that, while displaying quantitative properties similar to those of the wv vac-

TABLE 4. Envelope-specific antibody responses to SIV envelope-based vaccines

Vaccine and animal	Endpoint titer[a]		Conformation ratio[b]	Avidity index[c] (%)		Virus neutralization[d]
	Viral gp	rgp140		Viral gp	rgp140	
Attenuated virus[e] (17E-Cl)	51,200	51,200	2.2	55	62	4.0
wv						
Avg[e]	7,200	650	1.6	32	36	0.6
H633	12,800	800	1.7	30	29	1.8
G633	3,200	600	1.4	31	30	0.0
H497	6,400	800	1.3	35	39	0.5
H609	6,400	400	1.8	33	46	0.0
LL-gp						
Avg	2,400	5,600	1.3	19	61	1.6
G069	3,200	6,400	1.1	18	69	0.5
G441	800	3,200	1.6	21	62	2.1
G067	3,200	6,400	0.9	20	51	2.8
G607	3,200	6,400	1.0	16	61	0.9
Native rgp110						
Avg	2,400	40,960	1.2	<5	81	0.8
I379	800	25,600	1.2	<5	79	0.0
J778	3,200	51,200	0.8	<5	86	0.0
K662	1,600	25,600	1.1	<5	69	0.0
K831	3,200	51,200	2.2	<5	79	3.2
K701	3,200	51,200	0.8	<5	93	ND
Denatured rgp110						
Avg	360	40,960	0.2	<5	77	2.0
J492	200	51,200	0.3	<5	83	2.1
K614	800	51,200	0.3	<5	81	1.8
K912	400	25,600	0.4	<5	71	2.1
K029	200	51,200	0.0	<5	69	2.1
K721	200	25,600	0.1	<5	80	2.1

[a] Antibody endpoint titers were measured to the native envelope glycoproteins in the ConA ELISA. To control for the presence of antibodies to cellular proteins in the wv and LL-gp immune sera, a mock virus preparation (depleted of viral proteins) was captured as the antigen in the ConA ELISA. Results of these mock virus endpoint titer ELISAs revealed that only about 25% of the serum reactivity was directed to cellular proteins, indicating that the predominant antibody reactivity was in fact to SIV proteins in the ConA ELISA.

[b] Determined by comparing reactivities of native and denatured glycoproteins in the ConA ELISA.

[c] Determined by measuring the relative stability of the native viral envelope antigen-antibody complexes to an 8 M urea wash in ConA ELISA, and values were calculated by using the equation (urea-washed wells/PBS-washed wells) $\times$ 100, where values $x \geq 50\%$, $50\% > x > 30\%$ and $x \leq 30\%$ have been defined high, intermediate, and low avidity, respectively; $x < 5$ designates urea values too low to measure.

[d] Virus neutralizations were performed on rhesus macaque primary cells and CEMx174 cells, and the ability to neutralize the SIV/DeltaB670$_{\text{Tulane}}$ challenge virus is represented as the log of the 50% endpoint titer. ND, not determined.

[e] Average values calculated for groups of monkeys.

cine recipients, differed in certain qualitative antibody properties. For example, the LL-gp vaccine serum antibodies displayed only slightly higher reactivity to the rgp140 antigen (average titers of 1:5,600) compared to the native viral glycoprotein (average titers of 1:2,400). However, there were more marked differences in the antibody avidity values to rgp140 (average avidity values of 61%) compared to native viral glycoprotein (average avidity values of only 19%), and the LL-gp vaccine serum antibodies were somewhat less conformationally dependent than the antibodies elicited by the wv vaccine (average ratio of 1.3 compared to 1.6). These differences in antibody specificities of the wv and LL-gp vaccine antibodies presumably reflect structural differences in the respective envelope antigens resulting from the detergent solubilization and lectin affinity chromatography procedures used to isolate the envelope proteins from purified virus.

Even more striking differences were observed in the serological properties displayed by the serum antibodies produced by the two baculovirus recombinant envelope subunit vaccines, native and denatured rgp110. In contrast to the wv and LL-gp vaccine serum antibodies, the antibodies elicited by both recombinant vaccines produced a substantially higher titer and avidity to rgp140 (average titers of 1:41,000 for both, and average avidity values ranging from 77 to 81%) compared to the native viral envelope protein (average titers ranging from 1:2,400 for native rgp110 and 1:360 for denatured rgp110, with low avidity values of <5% for both). The two recombinant glycoprotein vaccines could also be distinguished by the conformational dependence of the serum antibodies. The native rgp110 vaccine sera preferentially recognized conformational determinants on the envelope glycoprotein (average conformational ratio of 1.2), while the denatured rgp110 vaccine sera reacted preferentially with linear envelope determinants (average conformational ratio of 0.2).

Differences in ability of the SIV wv and viral subunit vaccines to elicit neutralizing antibodies, as measured in vitro,

were also observed. In general, the presence of neutralizing antibody in vitro failed to correlate with protection in vivo in any of monkeys immunized with the wv or subunit vaccines. While several of these monkeys displayed detectable neutralizing antibody titers to the challenge virus SIV/DeltaB670$_{Tulane}$, the presence of this neutralizing antibody was not associated with the evolution of a mature antibody response (as characterized by the low titers, low conformational dependence, and low avidities to native viral gp compared to the SIV/17E-Cl prototype monkey) or with protection. In addition, the highest neutralizing titers were observed in the monkeys immunized with the denatured rgp110 vaccine, an unexpected observation since this immune serum preferentially reacted with the denatured rgp140 form of the viral envelope. These neutralization results again raise questions about the mechanism of virus inactivation by these serum samples and suggest that the inability of these vaccinates to elicit high avidity and conformationally dependent antibodies is indicative of an immature and nonprotective immune response, as ultimately demonstrated by the lack of protection from virus challenge.

DISCUSSION

In this study, we used a panel of serological assays to characterize the nature of antibody responses produced in response to experimental infection with attenuated strains of SIV. The results of these studies reveal a number of generalizations about the properties of antibody responses to the SIV envelope glycoproteins in monkeys infected with a variety of attenuated strains of virus. (i) The production of SIV envelope-specific antibodies involves a complex maturation process that is reflected in the evolution of antibody avidity and conformational dependence over the first 6 to 8 months postinfection. (ii) This maturation process evidently requires only low levels of viral antigen presentation, as the attenuated SIV strains examined here at best establish transient plasma viremia. (iii) Once established, the mature antibody responses are maintained for at least 2 years with relatively constant titer, avidity, and conformational dependence, suggesting ongoing immune stimulation. (iv) The time required for maturation of SIV envelope-specific antibodies in general parallels the time required for the development of protective immunity to experimental challenge with SIV. (v) No single serological measurement can provide an absolute predictor of protection; however, protection can be associated most reliably with a combination of antibody properties that include high titer to native envelope glycoproteins, high avidity, and in vitro neutralization of the challenge virus.

The elucidation of a clearly defined, complex, and lengthy maturation of SIV-specific antibody responses provides an interesting context in which to evaluate the potential efficacy of candidate vaccines in that the immunization protocol must result in sufficient stimulation to achieve immune maturation. In this study, infections of monkeys with various attenuated strains of SIV were all successful in producing broadly protective immunity, if sufficient time was allowed for adequate maturation of the immune responses. In contrast, all of the inactivated vaccines (wv or envelope subunit) examined here produced only immature antibody responses, characterized by low titer, lower conformational dependence, low avidity, and failure to provide protection from experimental challenge. Additionally, the antibody responses associated with nonprotective immunity in macaques inoculated with attenuated SIV for less than 6 months displayed immature properties similar to those found in the inactivated wv and envelope subunit vaccine recipients. These observations emphasize the importance of designing immunization protocols that provide for sustained presentation of native viral envelope antigens to allow sufficient time for complete maturation of immune responses. In this regard, attenuated virus strains and DNA vaccines appear to provide optimum potential for achieving the necessary maturation to protective immunity. However, it should be noted that certain genetic alterations to SIV can in fact produce highly attenuated virus strains that are incapable of eliciting protective immunity even after 8 months, presumably because of inadequate levels of virus replication and antigen presentation that produce immature immune responses (31a). Similarly, current DNA vaccines may be unable to sustain envelope antigen presentation for sufficient time to complete maturation to protective immunity (25).

The relatively gradual maturation of antibody responses to SIV infection in the presence of constant antigen presentation is a distinct contrast to the relatively rapid maturation of antibodies observed in most acute viral infections (39, 49), thus raising a number of questions about the capacity of the immune system to initiate effective immune responses to SIV. The observed maturation period does not appear to be restricted to infections with attenuated strains of SIV, as similar evolution of antibody titer, conformational dependence, and avidity have been observed in monkeys infected long-term with pathogenic SIV/DeltaB670$_{Tulane}$ and SIVmac251 (unpublished data). In addition, analyses of antibody responses to equine infectious anemia virus (EIAV) in experimentally infected ponies reveals an evolution of viral envelope specific antibodies over the first 6 to 8 months postinfection that closely resembles the maturation process described here for SIV (15). Based on these observations in two lentivirus systems, we propose that a gradual maturation of antibody responses may be a general property of lentivirus infections and that the early, immature antibody responses may in fact serve to amplify rather than restrict early stages of lentivirus infection. In this regard, it is interesting that immature antibody responses are associated with vaccine enhancement of EIAV replication and disease (30) and that enhancing antibodies to SIV are found predominantly at early times postinfection before immune maturation has occurred (28). Thus, it will be important to carefully evaluate other lentivirus infections, including HIV-1 infection, to test this model more thoroughly.

The exact mechanism(s) responsible for the delayed maturation of antibodies to SIV envelope proteins remains to be determined. The observed maturation of antibody responses described here can theoretically be attributed to an evolution in antibody that results from (i) changes in the viral envelope sequences during virus replication, (ii) quantitative changes in the antibodies directed to immunorecessive and immunodominant envelope determinants, and/or (iii) qualitative changes in the antibodies directed to a constant set of envelope determinants. Previous studies described by Clements et al. demonstrate a maturation of SIV envelope-specific antibody in the absence of viral envelope sequence variation (10), suggesting that antigenic variation is not required for the observed maturation of immune responses. The current observations of an evolution of antibody conformational dependence (increasing antibodies to linear envelope determinants) concomitant with increasing antibody avidity seem to be most consistent with the latter two models for antibody evolution. While EIAV infections, in general, reveal a similar temporal pattern of virus-specific antibody evolution, the specific changes in certain antibody parameters differ from those observed here in SIV infections. For example, envelope-specific antibodies produced during EIAV infection steadily increase in conformational dependence, as antibody avidity also increases over the first 6 to 8 months postinfection (15). In contrast, SIV envelope-specific antibodies decrease in conformational dependence while in-

creasing in antibody avidity. Thus, further experiments are necessary to elucidate the exact mechanisms associated with the maturation of antibody responses to lentivirus envelope proteins.

In addition to providing a conceptual model for designing effective AIDS vaccine strategies, the studies described here address the practical problem of identifying reliable in vitro correlates of protective immunity. To date, no single humoral or cellular in vitro immunological assay has been proven as a definitive immune correlate of protection. The recent study by Wyand et al. demonstrates that in vitro neutralization of the challenge virus stock is necessary, but not sufficient, as a prognostic indicator of a protective immune status in monkeys immunized with attenuated strains of SIV; i.e., all protected monkeys displayed high levels of neutralizing antibodies against the challenge virus, but about one-half of the serum samples from unprotected monkeys also displayed substantial in vitro neutralization activity against the challenge virus (48). The serum neutralization data (Tables 1 and 2) obtained in this study of monkeys inoculated with SIV/17E-Cl also indicate that serum neutralization of the challenge virus (SIV/DeltaB670$_{Tulane}$) is a necessary but insufficient indicator of protection. For example, the serum neutralization data in Table 1 indicated the presence of neutralizing antibody to both the vaccine and challenge strains of SIV by 1 month postinoculation, while protection from virus challenge was observed only at $\geq$8 months postinfection. These data indicate that while the lack of in vitro serum neutralization of the virus challenge stock is strongly associated with the absence of protective immunity, the presence of antibodies that neutralize the challenge virus in vitro can reliably only indicate the potential for protection against virus challenge.

In our previous study of monkeys inoculated with the attenuated M-tropic SIV/17E-Cl, we reported an apparent relationship between protection from heterologous virus challenge and the development of broadly neutralizing antibodies that were able to inactivate both the SIV/17E-Cl vaccine strain and a laboratory stock of the SIV/DeltaB670 (hereby designated SIV/DeltaB670$_{Baltimore}$) in standard in vitro assays (10). In contrast to this previous study, the present analyses of serum neutralization in monkeys inoculated with SIV/17E-Cl (Tables 1 and 2) did not indicate an association between the development of neutralizing antibodies to the challenge virus SIV/DeltaB670$_{Tulane}$ and the development of protective immunity. The reason for this apparent discrepancy appears to be attributable to important differences in the neutralization sensitivity of the virus stocks used in the previous and present studies. The actual standard challenge virus stock of SIV/DeltaB670$_{Tulane}$, which was propagated only in rhesus PBMC, is evidently readily inactivated by serum antibodies in the standard neutralization assay, even when the serum is taken from monkeys immunized with attenuated SIV/17E-Cl for only 1 month (Table 1). In contrast, the SIV/DeltaB670$_{Baltimore}$ virus used in the previous studies was passaged one to two times in CEMx174 cells, apparently yielding a virus stock that is much less complex and much more resistant to in vitro neutralization compared to the parental SIV/DeltaB670$_{Tulane}$. Thus, in our previous studies using the SIV/DeltaB670$_{Baltimore}$, we observed neutralizing antibodies only in serum taken at $\geq$8 months postinoculation with the attenuated SIV, whereas no serum neutralization was detectable with this virus stock at earlier times postinoculation. While the basis for the differences in neutralization sensitivity between the SIV/DeltaB670$_{Tulane}$ and SIV/DeltaB670$_{Baltimore}$ stocks is currently under investigation, these observations emphasize that minor changes in neutralization assay conditions can markedly affect the observed levels of serum neutralization, a problem which complicates comparisons of results obtained in different labs. Despite the apparently contradictory results between the present and previous studies, the earlier neutralization data obtained with SIV/DeltaB670$_{Baltimore}$ does indicate changes in the neutralization specificity of serum antibodies during the first 6 to 8 months postinoculation with the attenuated SIV vaccine. This evolution of more broadly neutralizing antibodies is consistent with the current model of a maturation of antibody responses during this time period.

Among the serological parameters evaluated in this study, in vitro measurements of antibody avidity provided a useful discrimination between mature and immature antibody responses and between protective and nonprotective responses to SIV. Virus-specific antibody avidity assays have previously been used to distinguish between mature and immature antibody responses to rubella virus (16, 17, 31) and hepatitis virus (45, 47) and to characterize antibody responses to infections with other viruses, including Epstein-Barr virus (2, 14), varicella-zoster virus (19, 20), human herpesvirus 6 (46), respiratory syncytial virus (27), cytomegalovirus (5, 6), and HIV-1 (7–9, 13, 41, 44). This serological assay differentiates polyclonal serum populations based on the relative stability of individual antigen-antibody interactions to a particular chemical treatment, in this case 8 M urea. Thus, observed avidity values cannot be associated reliably with any single property of the antigen or antibody but must be viewed as an average of numerous variables, including antibody affinity, conformational dependence, and the molecular nature of antigen-antibody interactions (ionic, hydrophobic, etc.). Changes in antibody avidity can therefore reflect both changes in antibody populations to a single antigenic determinant and changes in the specificity of antibodies from different determinants of an antigen. Further experiments are necessary to elucidate the physical and functional properties of low- and high-avidity antibodies to SIV envelope proteins as fractionated by treatment of the envelope antigen-serum antibody complex with urea.

The serological analyses presented in this report indicated that the presence of intermediate- to high-avidity antibodies to the SIV envelope proteins was characteristic of protective immune responses, while low-avidity antibodies were typically associated with nonprotective immune responses. In this regard, the antibody avidity assay appears to provide a useful complement to serum neutralization as an in vitro indicator of protective immunity. While neither antibody avidity nor serum neutralization provided an absolute in vitro correlate of in vivo protection, it is important to note that the combination of antibody avidity and serum neutralization of the challenge virus was 100% accurate in predicting in vivo protection in the immunized monkeys included in these studies. Based on these observations, we propose that a panel of serological parameters including antibody titer, conformational dependence, antibody avidity, and neutralization of the challenge virus be used in combination as prognostic indicators of in vivo protection in experimental trials of candidate AIDS vaccines.

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REFERENCES

1. **Anderson, M. G., D. Hauer, D. P. Sharma, S. V. Joag, O. Narayan, M. C. Zink, and J. E. Clements.** 1993. Analysis of envelope changes acquired by SIVmac239 during neuroadaptation in rhesus macaques. Virology **195:**616–626.

2. **Andersson, A., V. Vetter, L. Kreutzer, and G. Bauer.** 1994. Avidities of IgG directed against viral capsid antigen or early antigen: useful markers for significant Epstein-Barr virus serology. J. Med. Virol. **43:**238–244.
3. **Barker, E., S. W. Barnett, L. Stamatatos, and J. A. Levy.** 1995. The human immunodeficiency viruses, p. 1–96. *In* J. A. Levy (ed.), The Retroviridae. Plenum Press, New York, N.Y.
4. **Baskin, G. B., L. N. Martin, S. R. S. Rangan, B. J. Gormus, M. Murphey-Corb, R. H. Wolf, and K. F. Soike.** 1986. Transmissible lymphoma and simian acquired immunodeficiency syndrome in rhesus macaques. JNCI **77:**127–139.
5. **Blackburn, N. K., T. G. Besselaar, B. D. Schoub, and K. F. O'Connell.** 1991. Differentiation of primary cytomegalovirus infection from reactivation using the urea denaturation test for measuring antibody avidity. J. Med. Virol. **33:**6–9.
6. **Boppana, S. B., and W. J. Britt.** 1995. Antiviral antibody responses and intrauterine transmission after primary maternal cytomegalovirus infection. J. Infect. Dis. **171:**1115–1121.
7. **Brostrom, C., A. Sonnerberg, and M. Sallberg.** 1995. Human immunodeficiency virus type-1 infected patients with no disease progression display high avidity antibody to autologous V3 sequences. J. Infect. Dis. **171:**509–511.
8. **Chargelegue, D., C. M. O'Toole, and B. T. Colvin.** 1993. A longitudinal study of the IgG antibody response to HIV-1 p17 gag protein in HIV-1^+ patients with haemophilia: titre and avidity. Clin. Exp. Immunol. **93:**331–336.
9. **Chargelegue, D., C. M. Stanley, C. M. O'Toole, B. T. Colvin, and M. W. Steward.** 1995. The absence or loss of antibodies of high affinity to human immunodeficiency virus (HIV) is associated with disease progression in HIV-1 infected patients. J. Infect. Dis. **172:**897.
10. **Clements, J. E., R. C. Montelaro, M. C. Zink, A. M. Amadee, S. Miller, A. M. Trichel, B. Jagerski, D. Hauer, L. N. Martin, R. P. Bohm, and M. Murphey-Corb.** 1995. Cross-protective immune responses induced in rhesus macaques by immunization with an attenuated macrophage-tropic simian immunodeficiency virus. J. Virol. **69:**2737–2744.
11. **Daniel, M. D., F. Kirchhoff, S. C. Czajak, P. K. Sehgal, and R. C. Desrosiers.** 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the *nef* gene. Science **258:**1938–1941.
12. **Desrosiers, R. C.** 1990. The simian immunodeficiency viruses. Annu. Rev. Immunol. **8:**557–578.
13. **Devash, Y., T. Calvelli, D. Wood, K. Reagan, and A. Rubinstein.** 1990. Vertical transmission of HIV-1 is correlated with the absence of high affinity/avidity maternal antibodies to the gp120 principal neutralizing domain. Proc. Natl. Acad. Sci. USA **87:**3445–3449.
14. **Gray, J. J.** 1995. Avidity of EBV VCA-specific IgG antibodies: distinction between recent primary infection, past infection and reactivation. J. Virol. Methods **52:**95–104.
15. **Hammond, S. A., S. J. Cook, D. L. Lichtenstein, C. J. Issel, and R. C. Montelaro.** 1997. Maturation of the cellular and humoral immune responses to persistent infection in horses by equine infectious anemia virus is a complex and lengthy process. J. Virol. **71:**3840–3852.
16. **Hedman, K., J. Hietala, A. Tiilikainen, A.-L. Hartikainen-Sorri, K. Raiha, J. Suni, P. Vaananen, and M. Pietilainen.** 1989. Maturation of immunoglobulin G avidity after rubella vaccination studied by an enzyme linked immunosorbent assay (avidity-ELISA) and by haemolysis typing. J. Med. Virol. **27:**293–298.
17. **Hedman, K., and I. Seppala.** 1988. Recent rubella virus infection indicated by a low avidity of specific IgG. J. Clin. Immunol. **8:**214–221.
18. **Javaherian, K., A. J. Langlois, S. Schmidt, N. Kaufmann, N. Cates, J. P. M. Langedijk, R. H. Meloen, R. C. Desrosiers, D. P. W. Burns, D. P. Bolognesi, and S. D. Putney.** 1994. The principal neutralizing determinant of simian immunodeficiency virus differs from that of human immunodeficiency virus. Proc. Natl. Acad. Sci. USA **256:**320.
19. **Junker, A. K., and P. Tilley.** 1994. Varicella-zoster virus antibody avidity and IgG-subclass patterns in children with recurrent chickenpox. J. Med. Virol. **43:**119–124.
20. **Kangro, H. O., S. Manzoor, and D. R. Harper.** 1991. Antibody avidity following varicella-zoster virus infections. J. Med. Virol. **33:**100–105.
21. **Karber, G.** 1931. Beitrag zue kollektiven Behandlung pharmakologischer Reinhenversuche. Arch. Exp. Pathol. Pharmakol. **162:**480–487.
22. **Langlois, A. J., K. J. Weinhold, T. J. Matthews, M. L. Greenberg, and D. P. Bolognesi.** 1992. The ability of certain SIV vaccines to provoke reactions against normal cells. Science **255:**292–293.
23. **Lewis, M. G., W. R. Elkins, F. E. McCutchan, R. E. Benveniste, C. Y. Lai, D. C. Montefiori, D. S. Burke, G. A. Eddy, and A. Shafferman.** 1993. Passively transferred antibodies directed against conserved regions of SIV envelope protect macaques from SIV infection. Vaccine **11:**1347–1355.
24. **Lohman, B. L., M. B. McChesney, C. J. Miller, E. McGowan, S. M. Joye, K. A. Van Rompay, E. Reay, L. Antipa, N. C. Pedersen, and M. L. Marthas.** 1994. A partially attenuated simian immunodeficiency virus induces host immunity that correlates with resistance to pathogenic virus challenge. J. Virol. **68:**7021–7029.
25. **Lu, S., J. Arthos, D. C. Montefiori, Y. Tasutomi, K. Manson, F. Mustafa, E. Johnson, J. C. Santoro, J. Wissink, J. I. Mullins, J. R. Haynes, N. L. Letvin, M. S. Wyand, and H. L. Robinson.** 1996. Simian immunodeficiency virus DNA vaccine trial in macaques. J. Virol. **70:**3978–3991.
26. **Means, G. E., and R. E. Feeney.** 1971. Chemical modifications of proteins, p. 216–222. Holden-Day, Inc., San Francisco, Calif.
27. **Meurman, O., M. Waris, and K. Hedman.** 1992. Immunoglobulin G antibody avidity in patients with respiratory syncytial virus infection. J. Clin. Microbiol. **30:**1479–1484.
28. **Montefiori, D. C., K. A. Reimann, N. L. Letvin, J. Zhou, and S.-L. Hu.** 1995. Studies of complement-activating antibodies in the SIV/macaque model of acute primary infection and vaccine protection. AIDS Res. Hum. Retroviruses **11:**963–970.
29. **Montelaro, R. C., and D. P. Bolognesi.** 1995. Vaccines against retroviruses, p. 605–656. *In* J. A. Levy (ed.), The Retroviridae. Plenum Press, New York, N.Y.
30. **Montelaro, R. C., C. Grund, M. Raabe, B. Woodson, R. F. Cook, S. Cook, and C. J. Issel.** 1996. Characterization of protective and enhancing immune responses to equine infectious anemia virus resulting from experimental vaccines. AIDS Res. Hum. Retroviruses **12:**413–415.
31. **Morgan-Capner, P., and H. I. J. Thomas.** 1988. Serological distinction between primary rubella infection and reinfection. Lancet **i:**1397.

31a. **Murphy-Corb, M., and R. Desrosiers.** Unpublished data.

32. **Murphey-Corb, M., L. N. Martin, B. Davison-Fairburn, R. C. Montelaro, M. Miller, M. West, S. Ohkawa, G. B. Baskin, J.-Y. Zhang, S. D. Putney, A. C. Allison, and D. A. Eppstein.** 1989. A formalin-inactivated whole SIV vaccine confers protection in macaques. Science **246:**1293–1297.
33. **Murphey-Corb, M., R. C. Montelaro, M. A. Miller, M. West, L. N. Martin, B. Davison-Fairburn, S. Ohkawa, G. B. Baskin, J.-Y. Zhang, G. B. Miller, S. D. Putney, A. C. Allison, and D. A. Eppstein.** 1991. Efficacy of SIV/DeltaB670 glycoprotein-enriched and glycoprotein-depleted subunit vaccines in protecting against infection and disease in rhesus monkeys. AIDS **5:**655–662.
34. **Ohkawa, S., K. Xu, L. A. Wilson, R. C. Montelaro, L. N. Martin, and M. Murphey-Corb.** 1995. Analysis of envelope glycoprotein-specific antibodies from SIV-infected and gp110-immunized monkeys in ACC and ADCC assays. AIDS Res. Hum. Retroviruses **11:**395–403.
35. **Polanec, J., I. Seppala, S. Rousseau, and K. Hedman.** 1994. Evaluation of protein-denaturing immunoassays for avidity of immunoglobulin G to rubella virus. J. Clin. Lab. Anal. **8:**16–21.
36. **Regier, D. A., and R. C. Desrosiers.** 1990. The complete nucleotide sequence of a pathogenic molecular clone of simian immunodeficiency virus. AIDS Res. Hum. Retroviruses **6:**1221–1231.
37. **Robinson, J. E., D. Holton, J. Liu, H. McMurdo, A. Murciano, and R. Gohd.** 1990. A novel enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies to HIV-1 envelope glycoproteins based on immobilization of viral glycoproteins in microtiter wells coated with concanavalin A. J. Immunol. Methods **132:**63–71.
38. **Robinson, J. E., D. Holton, S. Pacheo-Morell, J. Liu, and H. McMurdo.** 1990. Identification of conserved and variant epitopes of human immunodeficiency virus type 1 (HIV-1) gp120 by human monoclonal antibodies produced by EBV-transformed cell lines. AIDS Res. Hum. Retroviruses **6:**567–579.
39. **Salmi, A. A.** 1991. Antibody affinity and protection in virus infections. Curr. Opin. Immunol. **3:**503–506.
40. **Sharma, D. P., M. C. Zink, M. Anderson, R. Adams, J. E. Clements, S. V. Joag, and P. Narayan.** 1992. Derivation of neurotropic simian immunodeficiency virus from exclusively lymphocytetropic parental virus: pathogenesis of infection in macaques. J. Virol. **66:**3550–3556.
41. **Simon, F., C. Rahimy, A. Krivine, M. Levine, J. M. Pepin, D. Lapierre, E. Denamur, L. Vernoux, A. DeCrepy, P. Blot, E. Vilmer, and F. Brun-Vezinet.** 1993. Antibody avidity measurement and immune complex dissociation for serological diagnosis of vertically acquired HIV-1 infection. J. Acquired Immune Defic. Syndr. **6:**201–207.
42. **Stott, E. J.** 1991. Anti-cell antibody in macaques. Nature **353:**393.
43. **Thomas, H. I. J., and P. Morgan-Capner.** 1991. Rubella-specific IgG1 avidity: a comparison of methods. J. Virol. Methods **31:**219–228.
44. **Thomas, H. I. J., S. Wilson, C. M. O'Toole, C. M. Lister, A. M. Saees, R. P. F. Watkins, and P. Morgan-Capner.** 1996. Differential maturation of avidity of IgG antibodies to gp41, p24 and p17 following infection with HIV-1. Clin. Exp. Immunol. **103:**185–191.
45. **Ward, K. N., W. Dhaliwal, K. L. Ashworth, E. J. Clutterbuck, and C. G. Teo.** 1994. Measurement of antibody avidity for hepatitis C virus distinguishes primary antibody responses from passively acquired antibody. J. Med. Virol. **43:**367–372.
46. **Ward, K. N., J. J. Gray, M. E. Joslin, and M. J. Sheldon.** 1993. Avidity of IgG antibodies to human herpesvirus-6 distinguishes primary from recurrent infection in organ transplant recipients and excludes cross-reactivity with other herpesviruses. J. Med. Virol. **39:**44–49.
47. **Wregitt, T. G., J. J. Gray, S. Aloyisus, M. Contreras, and J. A. Barbara.** 1990. Antibody avidity test for recent infection with hepatitis C virus. Lancet **335:**789.
48. **Wyand, M. S., K. H. Manson, M. Garcia-Moll, D. Montefiori, and R. C. Desrosiers.** 1996. Vaccine protection by a triple deletion mutant of simian immunodeficiency virus. J. Virol. **70:**3724–3733.
49. **Zinkernagel, R. M.** 1996. Immunology taught by viruses. Science **271:**173–178.