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Hyperimmune antisera against synthetic peptides representing the glycoprotein of human immunodeficiency virus type 2 can mediate neutralization and antibody-dependent cytotoxic activity

(human immunodeficiency virus type 2 envelope/linear immunogenic sites)

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ABSTRACT **Twenty-five 13- to 35-amino-acid-long peptides representing regions of human immunodeficiency virus type 2 (HIV-2), strain SBL6669, envelope proteins were evaluated for their immunogenic activity in guinea pigs. The peptides were selected to provide homologous representation of sites in the HIV-1 envelope proteins that were previously documented to have a particular immunogenic importance. A number of the HIV-2 peptides were found to be capable of inducing strain SBL6669 neutralizing and antibody-dependent cellular cytotoxicity (ADCC) antibodies. Two overlapping peptides covering amino acids 311–337 representing the central and C-terminal part of the variable third (V3) region, terminology according to Modrow *et al.* [Modrow, S., Hahn, B., Shaw, G. M., Gallo, R. C., Wong-Staal, F. & Wolf, H. (1987) *J. Virol.* 61, 570–578], showed the most pronounced capacity to induce neutralizing antibodies. One of the peptides (amino acids 318–337) also induced antibodies mediating ADCC. Two additional regions in the large glycoprotein, gp125, containing linear sites reacting with neutralizing antibodies were identified (amino acids 119–137 and 472–509). The transmembrane protein, gp36, of HIV-2 harbored two regions of importance for induction of neutralizing antibodies (amino acids 595–614 and 714–729). ADCC activity was induced by two additional gp125-specific peptides (amino acids 291–311 and 446–461). Thus, except for the single V3-specific site there was no correlation between linear immunogenic sites stimulating neutralizing antibody and ADCC activity. These findings pave the way for development of synthetic vaccines against HIV-2 and possibly also simian immunodeficiency virus infections. The capacity of such a product to induce protective immunity can be evaluated in macaque monkeys.**

Human immunodeficiency virus (HIV), the causative agent of AIDS, is a retrovirus belonging to the lentivirus subfamily. Antibodies to structural and nonstructural HIV proteins as well as virus-specific cell-mediated immune responses are present in the majority of infected individuals at the early stage of infection (1). In spite of these responses, the infection shows a relentless progression. Considerable individual variation in the time frame of emergence of a state of immunosuppression has, however, been observed. Multiple approaches are being taken to develop a vaccine that can block HIV replication at the time of exposure or at least reduce the impact of primary virus replication so that no immunosuppressive disease will ensue.

The understanding of the role that HIV envelope proteins have in eliciting and mediating protective immunological responses is crucial in projects aiming at vaccine development, since these proteins are the primary targets for antibodies that can neutralize virus infectivity (2, 3). In studies of HIV type 1 (HIV-1), the outer protein gp120 has been shown to exhibit considerable variability (4). Nevertheless, alternate hypervariable and conserved regions exist within this protein (5, 6) and the preserved sequences represent functional modalities. The gp120 and the transmembrane protein gp41 of HIV-1 play, as in the case of other enveloped viruses, a critical role in interaction with the virus receptor (7, 8) in the fusion process with host cell membrane (9) and in syncytium formation (10).

Two related but distinct virus types, HIV-1 and HIV-2, have been identified (11, 12) and both virus serotypes show tropism for CD4-positive lymphocytes and for cells of the monocyte–macrophage lineage (11). Simian immunodeficiency viruses (SIVs), which share sequence homology and genomic organization with the two HIV serotypes, have been isolated from several natural hosts (for review, see ref. 13). SIV is more closely related to HIV-2 than to HIV-1 and both SIV and HIV-2 can infect macaque monkeys (14–16).

In view of the possibility of using the HIV-2 macaque model to evaluate means for immunoprophylactic intervention, we have synthesized 25 peptides originated from different regions of the envelope proteins of the SBL6669 HIV-2 isolate (17). The selection of the peptides was based on identification of regions homologous to important linear immunogenic sites in HIV-1. The peptides have been used for evaluation of their antigenic activity in tests with human and simian sera (18). In this presentation, the immunogenic activities of the peptides is described. They were used for preparation of hyperimmune sera in guinea pigs and for evaluation of the capacity of these sera to react with native protein and to mediate neutralization and antibody-dependent cellular cytotoxicity (ADCC).

MATERIALS AND METHODS

Peptide Synthesis and Immunization Procedure. Twenty-five peptides, representing the envelope proteins of HIV-2, strain SBL6669 [18 from gp125, 1 peptide (A5-22) overlapping the cleavage site between the two proteins, and 6 from the transmembrane protein gp36; Table 1] were synthesized by the method of solid-phase multiple peptide synthesis according to ref. 19. The peptide amino acid sequences were derived from the published sequence of the ISY molecular clone of SBL6669 strain (17, 20). To the peptides that did not have a

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Abbreviations: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; ADCC, antibody-dependent cellular cytotoxicity; V3, variable third.

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Table 1. Amino acid sequences of synthetic peptides representing sites in the envelope glycoproteins of HIV-2

Peptide	Residues	Sequence
A15-77	44–56	ATKNRDTWGTIQC
A15-79	119–137	AVATTSPSGPDMINDTDPC
A4-32	248–269	CTRMMETQPSTWLGFNGTRAEN
A5-2	291–311	NLTILCRRPENKTVVPITLMS
A4-34	296–330	CRRPENKTVVPITLMSGRRFHSQ-KIINKKPRQAWC
A4-37	296–315	CRRPENKTVVPITLMSGRRF
A5-3	307–327	ITLMSGRRFHSQKIINKKPRQ
A4-38	311–330	SGRRFHSQKIINKKPRQAWC
A5-8	318–337	QKIINKKPRQAWCRFKGEWR
A5-9	333–352	KGEWREAMQEVKQTLVKHPR(C)
A7-39	348–367	VKHPRYKGTNDTNKINFTAP
A5-10	363–382	NFTAPEKDSDPEVAYMWTNC
A5-13	411–426	CHIEQIINTWHKVGKN
A5-14	422–438	KVGKNVYLPPREGELS(C)
A5-17	446–461	IANIDVDGDNRTNITF(C)
A5-18	457–471	TNITFSAEVAELYRL(C)
A5-20	472–493	ELGDYKLVEVTPIGFAPTAEKR(C)
A15-80	489–509	TAEKRYSSAPGRHKRGVLVLG(C)
A5-22	505–518	VLVLGFLGFLTTAG(C)
A5-23	573–595	AIEKYLADQARLNSWGCAFRQVC
A5-25	595–614	CHTTVPWVNDTLTPEWNNMT
A15-82	634–649	EQAQIQQEKNMYELQK(C)
A5-28	714–729	HIHKDWEQPDREETEE(C)
A5-29	724–739	EETEEDVGNDVGSRSW(C)
A15-84	830–845	LAVPRRIRQGAEIALL(C)

Peptides A15-77 to A15-80 represent selected regions of gp125, whereas A5-23 to A15-84 are derived from gp36, and A5-22 overlaps the cleavage site between the two. (C), a C-terminal cysteine was added to the peptide to allow coupling to keyhole limpet hemocyanin.

naturally occurring N- or C-terminal cysteine, a cysteine was added at the C terminus. The peptides were cleaved in a multivessel apparatus (19) using the low–high HF procedure (21). Each peptide, both in its crude form and coupled to keyhole limpet hemocyanin by maleimidobenzoyl *N*-hydroxysuccinimide ester coupling (22), was used to immunize pairs of guinea pigs. The animals were given 125 μg of peptide in Freund's complete adjuvant and booster immunizations with the same dose of peptide in Freund's incomplete adjuvant on days 14 and 21 after the first injection. Antisera were collected 10 days after the last injection. Of the four antisera, the one showing the highest antibody titer in tests with homologous peptide was selected for comprehensive analysis of antibody activities. When applicable, a parallel high-titer antiserum was also characterized. In about one-third of the cases, uncoupled peptides gave higher antibody titers than keyhole limpet hemocyanin-coupled peptides.

ELISA. Peptide ELISA was performed as described (23). Immune sera were diluted 1:10 and serial dilutions (1:10) were assayed against the homologous peptide. Whole HIV-2 envelope protein ELISA was performed as follows: antigen was prepared from HIV-2 SBL6669 virus-infected U937 clone 2 cells and culture supernatant was collected when infected cells showed distinct cytopathic changes. Purified virions were pelleted through 30% (wt/vol) sucrose and used (5.0 μg/ml) to coat microtiter plates (Nunc microtiter modules) (24). The plates were coated at room temperature overnight and then blocked for 2 hr with 20% fetal calf serum in phosphate-buffered saline to reduce unspecific binding. Sera diluted 1:100 were incubated for 1 hr at 37°C. Two human HIV-2 antibody-positive antisera were used as positive controls; negative sera controls included both HIV antibody-negative human and guinea pig preimmunization sera. After six washings, horseradish-conjugated rabbit polyclonal antibody to guinea pig IgG (Dakopatts, Glostrup, Denmark) diluted 1:5000 was added to the plates and incubation was continued for 1 hr at 37°C. The substrate 5-orthophenylenediamine solution was then added and the reaction was stopped after 30 min with 4 M H_2SO_4. Samples that showed values above the mean optic density at 490 nm of negative guinea pig sera plus 3 SD were considered positive.

Western Blotting. Western blot analysis was performed as described (12, 24). Disrupted virions were transferred to nitrocellulose strips, and the strips were blocked in 10% nonfat dry milk and individually incubated with guinea pig sera diluted 1:50. After washing, the strips were incubated with peroxidase-conjugated rabbit anti-guinea pig IgG and immunoprecipitation was visualized by using the substrate diaminobenzidine tetrahydrochloride. Human positive HIV-2 antisera, known to react with the majority of virally encoded proteins, were analyzed in parallel with guinea pig sera.

Neutralization Test. Diluted tissue culture supernatant of HIV-2 SBL6669-infected peripheral blood mononuclear cells (50 $TCID_{50}$, 100 μl) was incubated for 1 hr at 37°C with serial dilutions (1:2) of heat-inactivated (56°C for 30 min) guinea pig serum starting at a dilution of 1:20. H9 cells (1×10^5) were added to the virus/serum reaction mixture and incubated for 1 hr at 37°C. Thereafter, the cells were washed and incubated in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum, 1% glutamine, and antibiotics. Medium was changed on days 1 and 4. Seven days after infection, supernatants were collected and analyzed by a HIV-2 antigen-capture ELISA (25). Neutralization was defined as an 80% reduction of the values of optical density at 490 nm in the culture supernatant as compared to the respective preimmune negative guinea pig serum. Tests with all sera were repeated on at least two occasions. The titer was determined as the last dilution step where no virus replication could be detected by ELISA. Serum titers above 20 were considered positive for neutralization.

ADCC Assay. The ADCC assay was performed according to Ljunggren *et al.* (26). The monocytoid cell line U937, clone 2, chronically infected with HIV-2 SBL6669 was used for target cells. Peripheral blood mononuclear cells collected by density centrifugation on Lymphoprep (Nykomed, Oslo) from HIV seronegative donors were used as effector cells. ^{51}Cr-labeled target cells (1×10^4) and effector cells (2×10^5) were mixed with serial dilutions (1:3) of heat-inactivated (30 min at 56°C) guinea pig serum starting at a dilution of 1:30. Supernatants were harvested after 3 hr and released radioactivity was calculated. HIV-2-specific ADCC was determined as the ^{51}Cr release with HIV-2 antibody-positive antiserum minus ^{51}Cr release with a pool of five HIV antibody-negative sera. The spontaneous isotope release never exceeded 10%. HIV antibody-positive antiserum samples with known ADCC titers were included in each test. The reciprocal of the last dilution step with a specific ^{51}Cr release exceeding 2 SD above the release sustained with the HIV antibody-negative sera was taken as the ADCC titer.

RESULTS

A Large Proportion of Guinea Pig Anti-HIV-2 env Peptide Antisera Have a Capacity to React with Whole Viral Antigens. All guinea pig immune antisera, except the one obtained from the animal immunized with peptide A5-22, consistently reacted with their homologous peptide with titers ranging from 10^3 to 10^7 (Table 2). Nineteen of the 25 antisera reacted with the intact gp125 or gp36 protein in whole HIV-2 ELISA as shown in the table. None of the guinea pig sera obtained prior to immunization reacted in ELISA against peptides or whole HIV-2 antigen. A strict correlation was observed between reactivity in whole antigen ELISA and in Western blot assay (Table 2, Fig. 1), with 17 antisera reacting to either gp125 (13 antisera) or to gp36 (4 antisera). Three of the antisera (A4-37,

Table 2. Antibody activity of guinea pig hyperimmune antisera against peptides representing HIV-2 glycoprotein sites in homologous peptide and whole antigen ELISA, neutralization (NA), and ADCC assays

Anti-peptide antiserum	Antibody activity				
	In homologous peptide ELISA	In whole antigen ELISA	In WB assay	In NA test*	In ADCC test†
A15-77	10^5	1.57	+++	—	—
A15-79	10^4	0.39	++	80	—
A4-32	10^4	0.30	++	—	—
A5-2	10^7	0.36	+	—	270
A4-37	10^6	0.32	−	—	—
A4-34	10^6	‡	−	—	—
A5-3	10^5	0.34	++	—	—
A4-38	10^6	0.55	+++	>320	—
A5-8	10^7	0.32	+++	>320	90
A5-9	10^7	‡	+++	—	—
A7-39	10^7	‡	−	—	—
A5-10	10^5	0.30	++	—	—
A5-13	10^5	‡	−	—	—
A5-14	10^7	0.69	++	—	—
A5-17	10^7	0.38	−	—	90
A5-18	10^4	0.72	+++	—	—
A5-20	10^7	1.141	++	40	—
A15-80	10^4	1.064	++	80	—
A5-22	10^1	‡	−	—	—
A5-23	10^5	0.36	+	—	—
A5-25	10^5	0.47	+	40	—
A15-82	10^4	0.40	−	—	—
A5-28	10^3	0.89	+	80	—
A5-29	10^4	1.80	+	—	—
A15-84	10^4	‡	−	—	—

+++, Strong; ++, moderate; +, weak; and −, no reaction in WB assay.
*Reciprocal titers above 20 are indicated.
†Reciprocal titers above 30 are indicated.
‡Extinction value lower than cutoff limit.

A5-17, and A15-82) gave a low extinction value in whole antigen ELISA but did not show any reaction in Western blots. The reverse situation was encountered with A5-9, which reacted strongly with gp125 in Western blot but was completely negative in whole antigen ELISA. This antiserum is likely to recognize an epitope normally hidden in the gp125 but that is exposed in the denatured condition used in Western blotting.

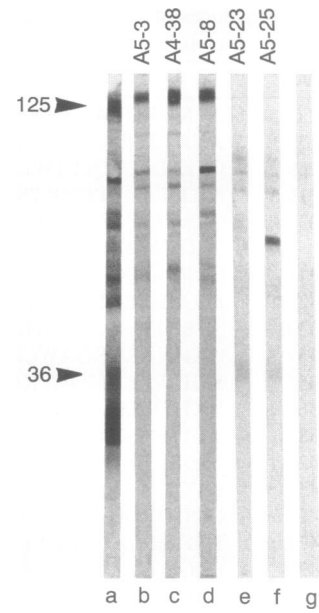

FIG. 1. Western blot reactivity of antisera from guinea pigs hyperimmunized with synthetic peptides representing sites in the envelope protein of SBL6669 HIV-2 isolate. Lanes: a, human HIV-2-positive antiserum reacting with several viral encoded proteins, including the glycoproteins gp125 and gp36; b–d, immune guinea pig antisera reacting with the outer envelope protein gp125; e and f, immune guinea pig antisera reacting with the transmembrane protein gp36; g, preimmunization guinea pig control serum.

Anti-HIV-2 env Peptide Antisera Mediate Neutralization and ADCC Activity. Seven of the 25 anti-peptide guinea pig antisera neutralized homologous HIV-2 virus in titers ranging between 40 and 320 (Table 2, Fig. 2). All the antisera that showed neutralizing activity also reacted in both whole antigen ELISA and Western blot. The two antisera (A4-38 and A5-8) with the highest neutralization activity were directed against overlapping peptides (amino acids 311–330 and 318–337) representing a region located centrally and C-terminally within and just outside the variable third (V3) region. The four regions recognized by additional sera showing neutralization encompassed amino acids 119–137 (A15-79), 472–509 (represented by two overlapping peptides, A5-20 and

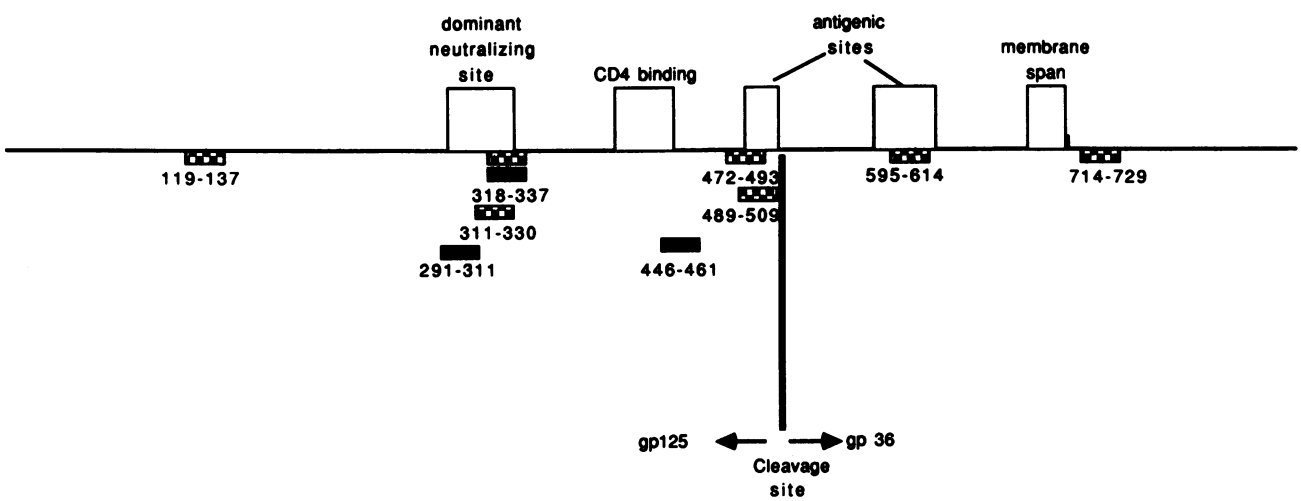

FIG. 2. Linear immunogenic sites in the HIV-2 envelope glycoproteins, which are targets for neutralization antibody and ADCC activities. In the upper part of the figure areas homologous to biologically important HIV-1 regions are indicated. In the lower part of the figure target regions for neutralizing antibodies are represented by checkered bars and target regions for ADCC activity are represented by solid bars.

A15-80; amino acids 472–493 and 489–509, respectively), 595–614 (A5-25), and 714–729 (A5-28). Moderate titers of neutralizing antibodies occurred in 3 of the antisera—A15-79, A15-80, and A5-28.

Distinct ADCC activity was found in 3 (A5-2, A5-8, A5-17) of the 25 sera (Table 2) and all of these sera were positive in whole antigen ELISA and/or Western blot. ADCC titers ranged between 90 and 270. A single antiserum, A5-8, collected from the animals immunized with the peptide encompassing amino acids 318–337 showed significant ADCC activity in combination with high neutralizing antibody titers.

DISCUSSION

This study demonstrates that hyperimmune antisera against peptides representing certain regions of the envelope proteins of HIV-2 strain SBL6669 can mediate neutralization of homologous virus and ADCC with cells infected with the same virus. A number of target regions for antibodies showing such activities were found. Since the HIV-2 peptides used were selected based on results from similar studies on HIV-1 (1), it is of interest to compare the data on biologically important linear immunodominant sites in the two types of HIV.

Neutralizing antibodies were induced by immunization with peptides representing five different sites in the HIV-2 envelope proteins—three sites in gp125 and two sites in gp36. The most active immunogenic site was located in the central and C-terminal part of the V3 region and possibly also involved a stretch of amino acids outside the C-terminal cysteine of V3. The role of V3 as the principle neutralizing site in HIV-1 has been highlighted by several studies evaluating the immunogenicity of peptides (27, 28). Postinfection antibodies reacting with the HIV-1 V3 loop can mediate neutralization (29, 30) and also inhibit membrane fusion of the virus (27). Furthermore, monoclonal antibodies against the HIV-1 V3 region can block virus infectivity (31). The V3 region has been described to harbor both strain- and type-specific sites and it appears in fact even to include some site(s) shared between HIV-1 and HIV-2 (32). Sequencing of the V3 region of a large number of HIV-1 strains has shown that these different specificities may be reflected in various amino acid substitutions in different positions within this region (33). No published data on the immunogenicity of the V3 region of HIV-2 or the closely related SIV are available, but it has been predicted that the structural characteristics of the V3 region of HIV-1 and HIV-2 should show considerable divergence.

Out of the two overlapping HIV-2 V3-specific peptides inducing neutralizing antibodies, the most C-terminal one in addition stimulated production of ADCC antibodies. This may reflect the occurrence of overlapping neutralization and ADCC-mediating sites in this region. The V3 HIV-2 ADCC site appears to have a different location than the HIV-1 V3-specific ADCC site identified with a murine monoclonal antibody (34). It should be noted that the two additional peptides stimulating production of ADCC antibodies [amino acids 291–311 (the N-terminal end of the V3 region) and 446–461] lacked the capacity to mediate induction of neutralizing antibodies. Thus, overall there is a poor correlation between linear sites responsible for neutralization and ADCC, which may not be surprising since an independent occurrence of antibodies carrying these activities was found in human sera (26).

It should be emphasized that both HIV-2-infected humans and monkeys mobilize high titers of antibodies reacting with V3-associated peptides inducing neutralizing and in one case ADCC antibodies (18). HIV-2 gp125 envelope peptides from two regions besides the V3 site were found to be capable of inducing neutralizing antibodies. These were amino acid regions 119–137 and 472–509, the latter of which was covered by two overlapping peptides. Amino acids 119–137 are within a variable region corresponding to the V1 region of HIV-1 (5). Previous studies by us (18) have shown, in analogy to what has been previously demonstrated for HIV-1 (35), the existence of a highly antigenic region at the C-terminal conserved end of the gp125 HIV-2 glycoprotein. Although Palker *et al.* (35) did not identify neutralizing and fusion inhibition activity of antisera against peptides representing this region, for HIV-2, we found that two overlapping peptides covering amino acids 472–509 could mediate neutralizing responses.

Two peptides representing regions of gp36 were shown to elicit neutralizing antibodies. Both regions are relatively conserved among the different HIV-2 isolates (20). The first one (amino acids 595–614) is located C-terminally to a highly conserved immunogenic area that is recognized by the majority of sera from HIV infected individuals (36, 37) and has offered the possibility of discriminating between infection with the two different serotypes of the virus (37, 38). Whereas the antigenicity of this site, represented by peptide A5-23 (amino acids 573–595), is exceptionally high, the neighboring peptide A5-25 (amino acids 595–614) capable of inducing neutralizing antibodies has low antigenicity (18). The second target region for neutralization on the transmembrane protein gp36 (amino acids 714–729) has been predicted to be located inside the lipid layer. This region is highly conserved among the different HIV-1 isolates, but it is more variable among HIV-2 serotypes. It appears analogous to a neutralization target area found in the HIV-1 gp41 protein (39). Several groups (40, 41) have shown that HIV-1 antisera to the corresponding peptide can neutralize the virus and block fusion activity. Peptides in this region of HIV-1 and HIV-2 have low to moderate antigenicity (18).

The data obtained in this study are highly encouraging with regard to future possibilities of developing a synthetic HIV-2 vaccine. The capacity of sera neutralizing homologous HIV-2 virus to block infectivity of heterologous HIV-2 virus strains and also SIV remains to be evaluated. The different peptides capable of inducing neutralizing antibodies in guinea pigs may be used in different combinations to immunize other species of animals for evaluation of production of neutralizing antibodies and also protection against infection or disease. This kind of study with HIV-2 as well as homologous SIV peptides and virus challenge need to be made with both homologous and related heterologous virus strains. A prime target is cynomolgus macaques monkeys, which can be infected with both SIV and HIV-2 (15, 16) and in which SIV infection permissive (42) and HIV-2 complete blocking of replication (43) has been demonstrated. Further recent findings favoring the possibilities of inducing antibody-mediated protection against lentivirus infection in cynomolgus macaque monkeys are results of passive immune prophylaxis experiments showing blocking of both HIV-2 and SIV challenge virus infection (44).

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