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Immunology

# Protection of macaques with a simian immunodeficiency virus envelope peptide vaccine based on conserved human immunodeficiency virus type 1 sequences

(envelope epitopes/AIDS/immunity)

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ABSTRACT **This report describes the vaccination of rhesus macaques with peptides selected from regions of the simian immunodeficiency virus (SIV) envelope that are hydrophilic, immunoreactive, and highly homologous with corresponding conserved envelope sequences of the human immunodeficiency virus (HIV). The peptides, produced as β-galactosidase fusion proteins, induced virus-neutralizing and peptide-specific antibodies. After challenge with virulent virus, controls became virus positive and developed gradually rising antibody titers to SIV over 63 weeks. Immunized macaques developed a postchallenge anamnestic response to SIVenv antigens within 3–6 weeks followed by a gradual, fluctuating decline in SIV antibody titers and partial or total suppression of detectable SIV. Virus suppression correlated with prechallenge neutralizing antibody titers. Although the average CD4$^+$ cell count in the blood of immunized macaques remained constant, the control macaques exhibited a progressive decrease developing about week 55 after challenge. The conserved nature of the HIV and SIV peptides and the similar humoral immunoreactivity in the respective hosts suggest that homologous HIV peptides may be important components of a successful immunization strategy.**

Extensive efforts are being devoted to the development of an effective vaccine to human immunodeficiency virus (HIV), the retrovirus associated with acquired immunodeficiency syndrome (AIDS) (1, 2). Initial attempts to immunize chimpanzees with subunit and vaccinia recombinant vaccines (3, 4), or to protect them by passive transfer of antibodies from HIV patients (5), failed to prevent persistent infection following challenge by HIV. An alternative to the chimpanzee model has been developed using the simian immunodeficiency viruses (SIV), which causes a fatal disease similar to AIDS in humans. The SIVs are the closest known relatives of HIV on the basis of sequence homology and biological and functional similarities (6–14).

Studies with SIV (15–17) demonstrated that whole virus vaccines are able to inhibit infection or delay onset of the disease. Although these results are encouraging, HIV vaccine strategies based on inactivated whole virus may have limitations because of the difficulty of determining the absence of residual infectious virus in large-scale production lots of product. An alternate approach is the use of recombinant products. Recently protection of two chimpanzees from HIV infection was demonstrated after vaccination with the recombinant envelope glycoprotein gp120 (18). Another alternative is the use of small peptides. We report here our studies using envelope (env) peptides that are highly conserved in the SIV and HIV isolates sequenced to date. These epitopes were presented as bacterial recombinant fusion products of a β-galactosidase (βgal) immunocarrier and were tested for their ability to protect macaques against SIV infection and disease. The selection of the βgal immunocarrier was based on its demonstrated efficiency of presentation of alphavirus envelope peptides in rodents (19, 20).

The conserved HIV peptides (21) served as a point of reference for selection of SIVmac-env sequences (10) to be used in immunization of macaques. We have previously shown that the antibody reactions (percent reactivity and mean titers) of SIV/Mne-infected macaque sera with the selected SIVenv βgal antigens were very similar to those of HIV-infected human sera with the corresponding four HIVenv–βgal antigens (21, 22). This correlation is further evidence for the close similarity between the SIV macaque model and HIV infection in humans. It also suggests that the SIV model system is suitable for assessing a vaccination strategy based on SIV/HIV envelope peptides.

## MATERIALS AND METHODS

**Selection and Preparation of SIVenv–βgal Antigens.** Four SIVenv epitopes (two from gp120, SIV88-NVTESFDAWEN and SIV500-RYTTGGTSRNKR, and two from gp32, SIV582-EKYLEDQAQLNAWGCAFRQVC and SIV647-EEAQIQQEKNMYELQKLNSWD) that appeared to be sufficiently homologous in sequence and location to the highly conserved HIV-env epitopes as well as being hydrophilic in nature were selected (22). Oligonucleotides coding for these peptides were prepared and inserted at the 5′ end of the gene under the *Escherichia coli trp* expression element (22). The four recombinant SIVenv-βgal polypeptides were expressed in bacteria and were purified (95%) by high-pressure liquid chromatography (HPLC) as described (22).

**Virus and Animal Titration.** Isolation of the SIV/Mne clone E11S has been described (14). E11S cells contain two indistinguishable SIV/Mne proviruses integrated at unique sites in the cellular DNA. A stock of 1–3 × $10^5$ tissue culture infectious dose (TCID) per ml of this virus (titrated in AA2 cells) was prepared from these cells and used for the current study. A $10^3$ dilution of this stock was used to challenge macaques in the present study.

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Abbreviations: HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; βgal, β-galactosidase; TCID, tissue culture infectious dose; PBL, peripheral blood lymphocyte; p.c., postchallenge; RIP, radioimmunoprecipitation.

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**Animals and Immunization.** Appropriate doses of the recombinant fusion proteins were determined by initially immunizing rhesus macaques with 4, 40, or 400 μg of HIV-647–βgal. Accordingly, three rhesus monkeys weighing 4–5 kg (3X7, 4GP, and 4GI) were injected i.m. with a mixture of 4 μg of each of the four HPLC-purified SIVenv–βgal fusion proteins with complete Freund's adjuvant on day zero, with incomplete Freund's adjuvant on day 15, and without adjuvant on day 35. Animals received a final immunization on day 387 with 40 μg of each fusion protein without adjuvant. The same protocol was followed for three control macaques (4GC, 4HS, and 3XP), except that the antigen was the βgal carrier molecule only. The monkeys were monitored weekly for evidence of loss of appetite, reduced physical activity, diarrhea, and other physical signs associated with SIV infection. At the time of each monthly bleeding, the monkeys were weighed and examined for lymphadenopathy. Clinical laboratory procedures included standard blood chemistry analysis, complete blood counts, and flow cytometry including T4- and T8-positive lymphocytes.

In conducting the research described in this report, we adhered to the *Guide for the Care and Use of Laboratory Animals* (23). The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

**Virus Isolation.** Macaque peripheral blood lymphocytes (PBLs) were isolated as described (14) and PBLs ($3–10 \times 10^6$) were added to $5 \times 10^6$ cells of the AA-2 CL1 cell line (M.G.L. and R.E.B., unpublished data). Cultures were maintained for 7–9 weeks and assayed for the presence of reverse transcriptase activity at periodic intervals as described (12).

**Anti-Peptide Antibody ELISA.** Tests were performed in triplicate on 2-fold serial dilutions of monkey sera (starting 1:8) on microtiter plates coated with the different synthetic SIV peptides or their bovine serum albumin conjuate (Peninsula Laboratories). End point titers were determined as 3-fold above backgrounds and at OD ≥ 0.05.

**Anti-SIV Antibody ELISA.** In view of the proven crossreactivity of sera from SIV-infected animals with the HIV-2 antigen, the macaque sera were run in duplicate in a HIV-2 ELISA kit (Genetic Systems, Seattle). Values of milli-OD per min (background ≤ 0.3) were used as ELISA units.

**Immunoblots.** Immunoblot analyses of anti-SIV antibodies were performed with various sera (diluted 1:100) on Immobilon membranes (Millipore, Bedford, MA) containing purified disrupted viral proteins (16).

**Radioimmunoprecipitation (RIP) Assay.** AA-2 cells ($2 \times 10^7$; cell line obtained through the AIDS Research and Reference Reagent Program, AIDS Program, National Institute of Allergy and Infectious Diseases, National Institutes of Health from Michael Hershfield) chronically infected with SIV/Mne CL E11S were incubated with 100 μCi (1 Ci = 37 GBq) of [$^{35}$S]methionine and [$^{35}$S]cysteine. Cell lysate ($2 \times 10^7$ cpm) was incubated with 10 μl of monkey sera and protein A-Sepharose. Washed pellets were resuspended and run on 10–20% SDS/PAGE. Normal monkey serum and mock-infected cell lysates were used as negative controls, and serum from a SIV/Mne-infected macaque was used as a positive control.

**Virus-Neutralizing Antibody Assay.** Approximately 5000 cells of the AA-2 CL 1 cell line (M.G.L. and R.E.B., unpublished data) were plated on 96-well flat-bottom Costar microtiter plates that had been coated with 10 μg of poly(L-lysine). One to $9 \times 10^2$ TCID of SIV/Mne CL E11S was incubated for 2 hr at 37°C with various dilutions of heat-inactivated (56°C, 30 min) plasma or sera and then added to the AA-2 CL1 cells for 1 hr at 37°C. The virus was removed, cells were washed, and the plates were incubated. The number of giant cells in each well was determined after 4–6 days of cell growth. Control wells contained virus and preinoculation sera and typically yielded 20–50 giant cells per well. A serum dilution was considered to have virus neutralization activity if it inhibited the formation of giant cells by ≥50%. The lowest serum dilution used was 1:12. Where a range is given, both wells gave a roughly 50% reduction or else separate experiments yielded these values.

**Polymerase Chain Reaction (PCR) Analysis.** About 1.0 μg of DNA isolated from macaque PBLs was used as template for PCR amplification using primers (CATGTAGCTAGTGGATTCCATAGAAGCAGAAGT and ACTGCTCCTTCCCCTTTCCA) homologous to regions of the SIVmne CL E11S polymerase gene. After completion of 30 cycles, products were separated in 1% agarose gels, and DNA was transferred to nylon membranes by the procedure of Southern (24) and hybridized to 2–10 ng of $^{32}$P-labeled ($5 \times 10^6$ cpm) oligonucleotide probe (AATTTTAAAAGAAGGGGAGGAATAGGGG). Blots were exposed to x-ray film (Kodak XAR-5) for 90 hr. A negative control was DNA extracted from uninfected AA2 cells and a positive control was from SIV/Mne CL E11S AA-2-infected cells.

## RESULTS

Three control macaques (animals 4GC, 4HS, and 3XP) were immunized with βgal only, and three macaques (3X7, 4GP, and 4GI) were immunized with a mixture of all four SIVenv–βgal fusion polypeptides (SIV88, SIV500, SIV582, and SIV647) (Table 1). Sera from all monkeys were evaluated in various tests: (*i*) ELISA against synthetic peptides (or peptides conjugated to bovine serum albumin), immunodots against βgal only, immunodots against SIVenv–βgal antigens [in the presence of excess βgal (21, 22)], immunoblots with

Table 1. Serological data on immunized macaques prior to SIV challenge

| | Prechallenge titer of immunized monkeys on various days of immunization | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 3X7 | | | 4GP | | | 4GI | | |
| Immunoassay | Day 43 | Day 387 | Day 394/409 | Day 43 | Day 387 | Day 394/409 | Day 43 | Day 387 | Day 394/409 |
| ELISA-88* | 64 | <8 | <8 | 128 | 8 | 8 | 128 | <8 | <8 |
| ELISA-500* | <8 | <8 | <8 | 8 | <8 | <8 | 64 | <8 | <8 |
| ELISA-582* | >4096 | 64 | 512 | >4096 | 64 | 512 | >4096 | 64 | 1024 |
| ELISA-647* | 512 | 16 | 256 | 32 | <8 | 64 | 32 | <8 | 8 |
| WB gp32† | + | − | + | ± | − | ± | ++ | − | ± |
| RIP gp32 + gp120/160‡ | + | ± | + | + | ± | + | ++ | ± | + |
| Neutr. antibody§ | 48 | 12–24 | 96 | 48 | 24 | 48 | <12 | <12 | 12–24 |

All control animals immunized with the βgal carrier molecule only did not react in any of these assays.
*Performed with different synthetic SIV peptides. Reciprocals of end point titers are listed.
†Only antibodies to gp32 were detected in immunoblots at the indicated intensities.
‡In RIP assays only envelope polypeptides were detected at the indicated intensities.
§Neutralizing antibody. Reciprocals of neutralization titers are listed.

Table 2. Virus isolation and neutralizing antibody data after challenge

| | Virus isolation results and neutralizing antibody titers at various intervals after challenge | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Week 0 | | Week 3–4 | | Week 6 | | Week 8 | | Week 12 | | Week 17 | | Week 29 | | Week 37 | | Week 49 | | Week 56 | |
| Macaque | SIV | NAB | SIV | NAB | SIV | NAB | SIV | NAB | SIV | NAB | SIV | NAB | SIV | NAB | SIV | NAB | SIV | NAB | SIV | NAB |
| Immunized | | | | | | | | | | | | | | | | | | | | |
| 3X7 | − | 96 | − | 96 | − | 96 | − | NT | − | NT | − | 48 | − | 48 | − | 48 | − | NT | − | NT |
| 4GP | − | 48 | − | 48–96 | + | 24 | − | NT | − | NT | − | 48 | − | 48 | − | 48 | − | NT | − | NT |
| 4GI | − | 12–24 | + | 12–24 | + | 24 | + | NT | + | NT | + | 192 | − | 192 | − | 96–192 | − | NT | − | NT |
| Control | | | | | | | | | | | | | | | | | | | | |
| 3XP | − | <12 | + | 12–24 | + | <12 | + | NT | + | 48 | + | 96 | + | 192 | + | 192 | + | NT | + | NT |
| 4HS | − | <12 | + | <12 | + | <12 | − | NT | + | 96 | + | 192 | + | 192 | + | 192 | + | NT | + | NT |
| 4GC | − | ≤12 | + | ≤12 | − | 12 | + | NT | + | 96 | + | 192 | + | 96 | − | 96 | − | NT | + | NT |

SIV, virus isolation negative (−) or positive (+). NAB, reciprocal of neutralizing antibody titer, which yielded 50% reduction in giant cell formation.

whole disrupted SIV/Mne virions (11); (*ii*) RIP assays (with $^{35}$S-labeled SIV/Mne polypeptides); and (*iii*) *in vitro* neutralizing antibody assays. Prior to immunization, all animals were negative in all of the above tests. Within 2 weeks after the third injection (day 43 or 50) all animals developed high antibody titers (>1:100,000 by immunodots) to the βgal immunocarrier. Until challenged, all control monkeys were negative in all of the SIV-specific tests. Rhesus monkeys immunized with the SIVenv–βgal mixture developed specific antibodies against the SIV peptides by days 43–50 (Table 1). Sera from 4GI and 3X7, and to a lesser extent from 4GP, reacted on immunoblots only with gp32 (Table 1). All sera tested at day 43 to 120 days after initiation of immunization could also precipitate the $^{35}$S-labeled native gp32 transmembrane protein and the envelope gp120 or its precursor gp160 polypeptide (Table 1). Sera from monkeys 3X7 and 4GP developed virus-neutralizing antibodies *in vitro* by day 43 (Table 1). In summary, after three initial immunizations of monkeys with the different SIVenv–βgal polypeptides, a SIV-specific humoral response was induced to the recombinant peptides and, more interestingly, to the native SIV envelope antigens. Following the three initial immunizations,

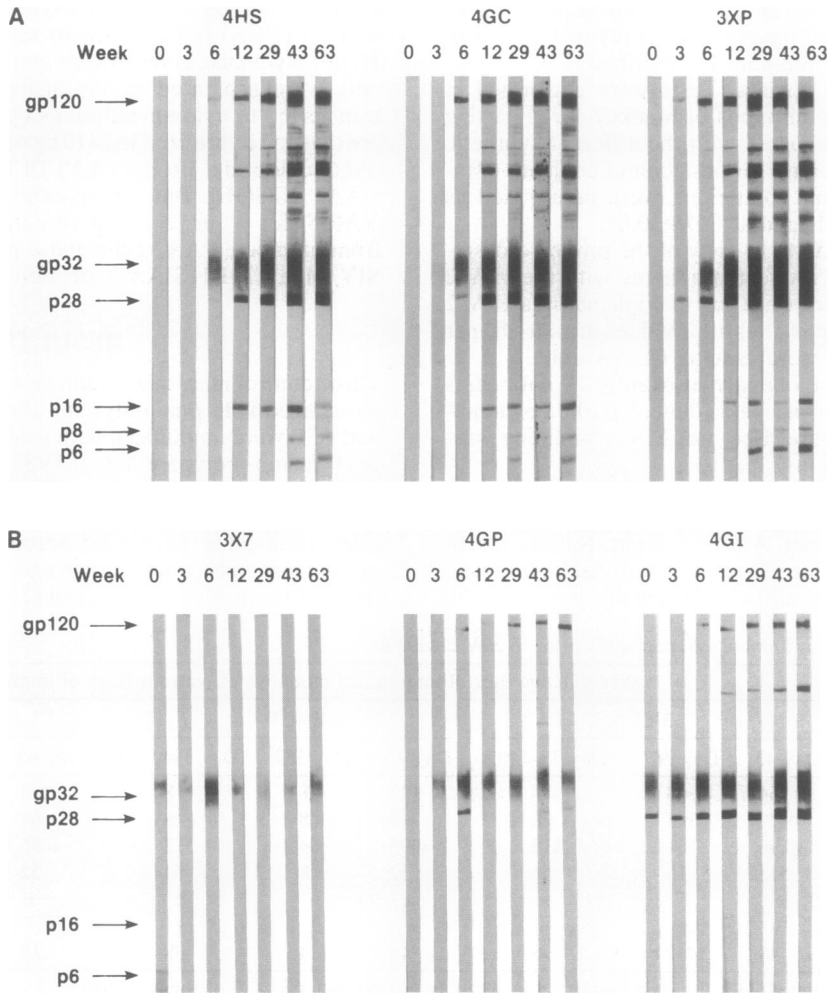


FIG. 1. Immunoblot analyses of antibodies to SIV/Mne in inoculated macaques. Antibodies to the envelope glycoprotein (gp120), the transmembrane protein (gp32), the major gag protein (p28), the amino-terminal gag protein (p16), the nucleic acid binding protein (p8), and the carboxyl-terminal gag peptide (p6) are identified. (*A*) Control macaques. (*B*) Immunized macaques.

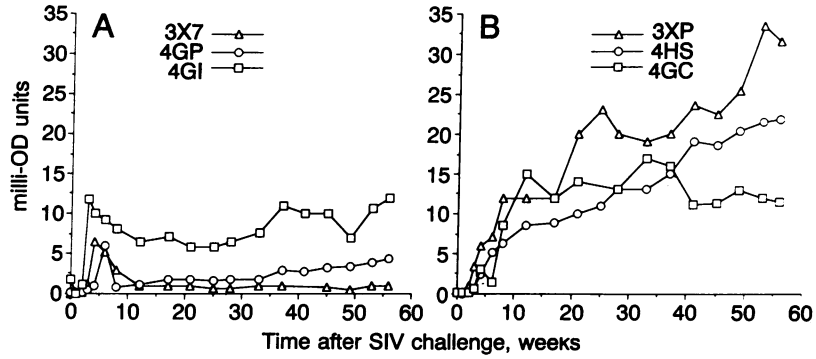


FIG. 2. Quantitative ELISA. Sera were run in duplicates at 1:400 dilution in a HIV-2 ELISA kit. Values of milli-OD units were used as ELISA units. (*A*) Immunized macaques. (*B*) Control macaques.

anti-SIV antibody levels gradually declined (Table 1). One to 3 weeks after the fourth and final immunization (day 387) a clear anamnestic antibody response was detected in all monkeys; however, ELISAs could detect only antibodies to the transmembrane 582 and 647 peptides (Table 1).

All immunized and control animals were challenged i.v. on day 409 (3 weeks after final immunization) with about 100–300 TCID of SIV/Mne CL E11S. The control group became infected as expected and virus could be isolated from these animals' lymphocytes from 6 weeks postchallenge (p.c.), and monkeys were still virus positive 63 weeks p.c. (Table 2). All control macaques seroconverted and developed increasing levels of antibodies to SIV structural proteins (Fig. 1). Quantitative ELISA performed on sera from control monkeys following challenge showed a gradual increase in titers from week 4 through week 25 (Fig. 2).

The response to challenge of the three macaques immunized with the mixture of SIVenv–βgal polypeptides was distinctly different from that of the control monkeys in patterns of virus isolation (Table 2) and antibody response (Figs. 1 and 2). One macaque, 3X7, was consistently negative for virus isolation; however, PCR on DNA from blood lymphocytes showed that all monkeys, including 3X7, had become infected (Fig. 1*C*). From the second immunized macaque, 4GP, SIV was isolated only once (6 week p.c.). Virus could be isolated from the third immunized macaque, 4GI, from 6 through 17 weeks p.c., but repeated virus isolation attempts after week 17 p.c. failed (Table 2).

Analyses of antibody responses p.c. in the immunized group were consistent with the virus isolation and PCR data (Figs. 1 and 3). Monkey 3X7, which was consistently virus negative, developed a detectable anamnestic response only to gp32 (Fig. 1). This was most evident 6 weeks p.c. and then gradually declined (Figs. 1 and 2). In comparison to 3X7, monkey 4GP developed antibodies to gp32 as well as to p28 and gp120 (Fig. 1*A*); this is consistent with the fact that 4GP was virus positive once (6 weeks p.c.). Quantitative titration of total anti-SIV antibodies for 3X7 and 4GP (Fig. 1*B*) showed a brief anamnestic response, peaking about 3–6 weeks p.c. followed by a decline. The third immunized macaque, which was virus isolation positive from 6 to 17 weeks p.c., showed antibody responses with some similarities to the controls but with distinct differences (Figs. 1 and 2). In 4GI an anamnestic peak titer of total anti-SIV antibodies was attained 3 weeks p.c., which then declined by 6–10 weeks p.c.; in contrast, the controls exhibited a gradual increase in antibody titers over a period of several months. Furthermore, anti-SIV antibodies by immunoblot to some SIV structural proteins were absent or present at low intensity (compared to controls) from sera of 4GI even 63 weeks p.c. These results together with the apparent inability to isolate virus from monkey 4GI after week 17 p.c. would be consistent with the notion that, even in this animal, SIV proliferation was suppressed.

## DISCUSSION

To assess the feasibility of a HIV peptide vaccine we used the SIV macaque model to test efficacy and develop an immunization strategy. Based on earlier studies of highly conserved HIV envelope peptides (21) we selected four SIVmac peptides (22) from gp160. These peptides were presented as recombinant fusion products of a βgal immunocarrier and were used for immunization. The study included three control rhesus macaques (4HS, 4GC, and 3XP) immunized with βgal only and three others (3X7, 4GP, and 4GI) immunized with a mixture of the four SIVenv–βgal fusion polypeptides.

After the four immunizations, SIV specific humoral responses were induced to the synthetic SIV peptides but more importantly also to native SIVenv antigens (Table 1). It is interesting to note that the rank-order of antibody titers to the SIV peptides was parallel to that found (22) in monkeys experimentally infected with SIV/Mne (i.e., 582 > 647 > 500 ≥ 88). It is noteworthy that the highly conserved linear envelope peptides were also able to elicit relatively high neutralizing antibody titers (Table 1). Unexpectedly, sera collected prior to challenge from macaque 4GI crossreacted in immunoblots with the gag p28 polypeptide (Fig. 1). The same sera did not react in a RIP assay with the gag protein (Table 1). The reactivity of these sera with the denatured p28 may be explained by the presence of an identical four-amino acid sequence peptide in SIV-582 (LNAW) and in the amino-terminal region of gag SIV/Mne (amino acid positions 154–157). Consistent with this explanation is the fact that in immunodot assays, sera from 4GI had the highest prechallenge antibody titers to SIV-582 (data not shown).

As challenge virus we used SIV/Mne clone E11S (13, 14). Previously we demonstrated the immunoreactivity of the SIVmac–βgal polypeptides with SIV/Mne-infected macaque sera (22). The complete sequence of a molecular clone derived from SIVMne clone E11S is now known (R.E.B., unpublished data); it differs from SIVmac envelope amino acid sequence (10, 11) by 8%. Thus, unlike previous HIV or SIV vaccine studies (15–18) in the present study, the vaccine and the challenge are not completely homologous [the 500 and 647 SIVmac sequences are identical to the SIV/Mne sequence, but there is a single amino acid difference in 88 (Val → Ile) and in 582 (Asp → Lys at position 5)].

The response to virus challenge of the immunized macaques differed from controls in SIV-specific antibodies

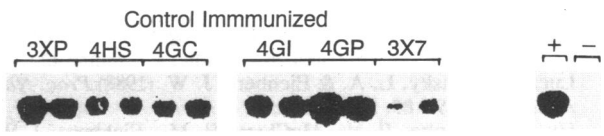


FIG. 3. PCR amplification performed in two replicates on DNA from blood lymphocytes 6 weeks p.c. +, Positive; −, negative.

and virus isolation profiles. Immunoblots (Fig. 1) and quantitative titration of total anti-SIV antibodies (Fig. 2) of sera from immunized macaques showed an anamnestic response peaking 3–6 weeks p.c. followed by a decline, whereas controls exhibited a gradual increase in titers over several months. These results and virus isolation data (Table 2) suggest the following: (*i*) Initially immunized and control macaques were susceptible to SIV replication. This conclusion is supported also by PCR analysis carried out 6 weeks p.c. (Fig. 3). (*ii*) Immunized but not control macaques were able to suppress SIV replication. The frequency of virus isolation in vaccinated macaques corresponded inversely to prechallenge titers of neutralizing antibody (Table 2). It is worth noting that prechallenge neutralizing antibody titers in the vaccinated macaque 3X7, which was consistently virus isolation negative, were within the maximal range of titers developed months after infection of control macaques. A similar correlation between prechallenge neutralizing antibody titers and extent of protection from HIV infection was observed in chimpanzees vaccinated with gp120 and gp160 (18). Interestingly, differences in prechallenge ELISA titers to the 647 peptide correlated with extended survival. Recently it was also reported that peptide sequences located in HIV-647 and HIV-582 are recognized by antibodies involved in antibody-dependent cell cytotoxicity (25). Although these correlations are interesting, the mechanism for suppression of virus replication in the immunized macaques remains unknown. Since control animals are expected to develop disease within the next few months, it remains to be seen if vaccine-induced protection prevents or only delays onset of disease in immunized macaques. The average $CD4^+$ cell counts remained stable in the immunized group, whereas control macaques showed a progressive decrease in $CD4^+$ cells beginning about 55 weeks p.c. At present (80 weeks p.c.), the immunized macaques have a mean $CD4^+$ cell count of $>800$ per $mm^3$, whereas control macaques have $<300$ per $mm^3$.

The possible success of our SIV peptide vaccine may be compared to three recent studies with whole inactivated SIV vaccines and homologous virus challenge. One study (13), using a SIV challenge of 200–1000 infectious doses (id), found four of six immunized animals became virus positive, with a delay in disease onset. Another study (14), using a challenge of 10 id, found none of six immunized macaques became virus positive. The antibody response and virus isolation data in our macaques suggest a different mechanism of protection. Whether these immunized macaques survive indefinitely or begin to circulate detectable virus and ultimately die may require years of observation. However, the results during the first year are encouraging, and, to our knowledge, an efficacious peptide vaccine against SIV or any other retrovirus has not been reported previously. Given the immunologic and structural parallelism between selected SIV peptides and the corresponding, conserved HIV peptides, the data suggest an analogous HIV peptide vaccine may be broadly protective in humans.


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