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During year 03, significant progress has been made in this project. Using the cost-effective mouse model involving Rauscher murine leukemia virus (RLV), we have demonstrated that DNA vaccination can induce protection against virus challenge, even when the animals were vaccinated only with *gag-pol* expression vectors, indicating that neutralizing antibodies are not required for vaccine protection.

A large DNA vaccine experiment involving neonatal rhesus macaques has been initiated at the Yerkes Regional Primate Research Center. The course of DNA inoculations has been completed, and all animals have been tested for humoral and cellular immune responses to the vaccines. Overall, 62.5% of the vaccinees were seropositive for Env by ELISA, although no neutralizing antibodies were detected. None of the vaccinees had specific cytotoxic T-cell responses. Given these results, boosting with either recombinant gp160 or additional DNA inoculations has been started.

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Ruth Ruppelt 4/14/98

PI - Signature Date

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INTRODUCTION

The overall goal of this project is to develop DNA vaccine strategies to treat and/or prevent primate lentivirus infection in infant rhesus macaques. As outlined in the year 02 project report, the original Specific Aims had been readjusted, and we had entered into a new affiliation with the Yerkes Regional Primate Research Center (Atlanta, Georgia). This primate center maintains a large rhesus monkey breeding colony, which has been shown to be free of simian immunodeficiency virus (SIV), simian retrovirus type D (SRV/D), and simian T-lymphotropic virus type I (STLV-I) as well as tuberculosis. The breeding colony has been surveyed with our newly developed and highly sensitive polymerase chain reaction (PCR) method for detection of low levels of SRV/D and STLV-I (Liska et al., 1997 a, b) and found to be clean.

Approximately a year and a half ago, a total of 28 newborn rhesus macaques were given various DNA vaccines or DNA control vector. This experiment is being conducted in parallel to a similar trial carried out in adult rhesus monkeys by Dr. Harriet Robinson, who is a collaborator on our study. Our experimental design gives us the unique opportunity to test whether DNA vaccines exhibit age-related differences in immunogenicity, as outlined previously. If the vaccines are found to be safe as well as immunogenic in neonatal primates, important information will have been generated to apply this new technology worldwide for childhood vaccination.

As discussed previously, we are also asking whether neutralizing antibodies, which need to be directed by necessity against epitopes on the virus glycoprotein envelope, are required for immune protection against retroviruses. Using DNA vectors expressing only virus gene products contained within the virion but not displayed on the viral envelope, vaccine trials have been conducted in a mouse model involving Rauscher murine leukemia virus (RLV) in mice and in parallel in newborn rhesus monkeys vaccinated with DNA expression vectors lacking *env*. The mouse DNA vaccine study has yielded a provocative result: partial protection was seen in animals vaccinated with DNA vectors expressing *gag-pol* as well as in mice vaccinated with vectors expressing *env.* The partial success of the former vaccine strategy implies that vaccine strategies can be developed that do not depend on the inclusion of envelope. Given the high mutation rate of *env* in primate lentiviruses, a vaccine strategy based on the more conserved *gag* and *pol* genes could result in a broader spectrum of vaccine protection.

The project is currently on a no-cost extension, which was requested for the following reason. Dr. Harriet Robinson's DNA vaccine study in adult macaques had revealed that the overall strategy of 3 DNA inoculations resulted only in low specific antiviral immune responses. Consequently, she and her colleagues boosted the DNA-primed vaccinees with purified recombinant gp160 twice. Eventually, the animals were challenged. Partial immune protection was seen only in animals primed with DNA vaccines and boosted with gp160 later on. Animals given only the three inoculations with DNA vectors were not resistant (Dr. Harriet Robinson, personal communication).

Given these results in the parallel study in adult macaques, we decided to boost our infant macaque vaccinees as follows: animals vaccinated with DNA vectors that included *env* expression vectors will receive protein boosts with the same recombinant gp160 used in Dr. Robinson's study. Animals vaccinated only with *gag-pol* and *nef* vectors will receive an additional two boosts with DNA. After the completion of this additional boosting, the animals will be challenged intravenously with 10 50% animal infectious doses (10 AID_{50}). We plan to complete the boosts and virus challenge in the rhesus macaques during a no-cost extension of this project. We also plan to conduct follow-up experiments in the low-cost RLV system, in which we will optimize DNA vaccine dose/schedules and elucidate mechanisms of immune protection. A more detailed description of the individual experiments is given below.

Publications in Year 03

1. Khimani AH, Lim M, Graf TG, Smith TF, Ruprecht RM. Phylogenetic relationship of the complete Rauscher murine leukemia virus genome with other murine leukemia virus genomes. Virology 1997; 238:64-67.
2. Liska V, Lerche NW, Ruprecht RM. Simultaneous detection of simian retrovirus type D serotypes 1, 2, and 3 by polymerase chain reaction. AIDS Research and Human Retroviruses 1997a; 13:433-437.
3. Liska V, Fultz PN, Su L, Ruprecht RM. Detection of simian T-cell leukemia virus type I infection in seronegative macaques. AIDS Research and Human Retroviruses 1997b; 13:1147-1153.
4. Ruprecht RM, Rasmussen RA, Hu Y. Induction of protective immunity by exposure to low doses of a live, pathogenic retrovirus. Manuscript submitted.

MATERIALS AND METHODS

A detailed description of the methodologies used has been included in the progress report for year 02. For easy reference, the key methods used during year 03 are summarized below.

1. Construction of RLV expression plasmids. The expression plasmids were constructed using vector pJW4303 (kindly provided by Dr. Jim Mullins, University of Washington School of Medicine, Seattle, Washington). This vector has a strong CMV immediate early promoter and bovine growth hormone polyadenylation signal. We have constructed the following RLV expression plasmids: pJW*gag*, pJW*gag-prot* (a plasmid containing functional *gag* and *protease*), pJW*gag-pol* and pJW*env*. The inserts were derived from the full length RLV genome, which we have sequenced completely (Khimani et al., 1997; please see enclosed reprint).

2. Transient transfection of RLV expression plasmids. Before vaccination of mice, the DNA expression vectors were tested in vitro by immunoprecipitation of Cos cell transfectants. Cos cells were transfected with each plasmid (1 mg DNA/ml) using DEAE-dextran. After 72 hrs, the cells were harvested, and cell lysates were prepared in a 1% NP-40 lysate buffer containing protease inhibitors. Nuclei and debris were pelleted, and lysates were then incubated with Protein A-Sepharose beads that had been preincubated with either anti-Env or anti-Gag monoclonal antibodies (mAbs). After washing, specific antigens were eluted from the beads by boiling in SDS-PAGE sample buffer and separated by gel electrophoresis. Proteins were transferred to nitrocellulose membranes, and the immunoprecipitated antigens were detected by incubating the membrane with goat anti-RLV antiserum followed by horseradish peroxidase-conjugated rabbit anti-goat IgG.

3. Stable transfection and generation of RLV-env-expressing murine cells. We have also used the pJW*env* plasmid to establish permanent transfectants of P815 cells. Cells were cotransfected with the RSV-2neo plasmid, and G418-resistant cells were selected in drug-containing medium. Transfected P815 cells were stained using an anti-Env IgG.

4. Mice and virus. Six-to eight-week-old female BALB/c mice (Taconic Farms, Germantown, NY) were used for all experiments. Rauscher murine leukemia virus (RLV), strain RV-B (Ruprecht et al., 1990), derived from the original stock, had been prepared by tail vein injection of 10^4 plaque-forming units (PFU) of RLV into mice. Single cell suspensions of spleens obtained from animals sacrificed on day 20 post-inoculation had been prepared in medium (2 ml/g spleen) supplemented with 20% fetal calf serum (FCS), and cell supernatants had been stored in liquid N_2. The number of plaque-forming units (PFU) in the

stock had been determined by XC plaque assay, and the stock had been titrated also in mice.

5. Immunoblot analysis for RLV. Total protein of serum samples was measured using Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Twenty μg of serum protein were separated by 10% SDS-PAGE and transblotted onto Immobilon-P membranes (Millipore, Bedford, MA). After blocking nonspecific binding sites with 0.1% Tween, the membranes were incubated first with goat anti-RLV antiserum (NIH Repository Number 75S000294) and, after washing, with horseradish peroxidase-conjugated rabbit anti-goat IgG (Bio-Rad, Hercules, CA). Specific binding was demonstrated after the blot was developed with the substrate 4-chloro-1-naphthol (Life Technologies, Inc., Gaithersburg, MD). Individual lanes were scored positive for RLV by the presence of p30 Gag, p15E and gp70 Env bands.

6. CTL responses in RLV-immune mice. A specific CTL assay has been developed to measure cytotoxicity of immunized BALB/c mice against RLV-infected target cells. Such cells were prepared by incubating P815 cells (BALB/c mastocytoma cell line) with RLV (5×10^4 PFU/10^6 cells) in the presence of Polybrene (Sigma Chemical; 8 mg/ml) overnight at 37°C in culture media (Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 10 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin). The cells were expanded and stained for RLV Env and Gag antigen expression using anti-gp70 Env mAb 48, and anti-p30 Gag mAb R187 (both from ATCC), which were developed against Friend MuLV but cross-react with RLV antigens. RLV-infected P815 cells were sorted by flow cytometry for Env expression, expanded and routinely checked for Env and Gag antigen expression, which was maintained at >90%. The RLV-infected P815 cells were termed P815-R.

   To measure specific cytotoxicity, P815-R or non-infected P815 cells were radiolabelled by incubation with $Na^{51}CrO_4$ (new England Nuclear; 100 μCi/10^6 cells) for 1 hr at 37°C, then washed and incubated with the indicated effector cells (obtained from mice inoculated with live, drug-"attenuated" virus) in 96-well round-bottom plates. After 5 h, supernatants were harvested onto SCS harvesting frames (Skatron Inc., Sterling, VA) and released ^{51}Cr measured in a gamma-counter. Assays were performed in triplicate, and the percent specific cytotoxicity was determined according to the formula:

$$\frac{\text{(experimental release - spontaneous release)}}{\text{(total release - spontaneous release)}} \times 100$$

   where spontaneous release is that from target cells cultured in medium alone and total release that from target cells cultured in 1% NP-40 detergent.

7. Vaccination of mice with anti-RLV DNA vaccines. After a one-week quarantine, the mice were started on the vaccine protocol as outlined on Table 1. On day 0, the indicated number of mice per group received the DNAs indicated intramuscularly (im). Booster injections of DNA were given as outlined in the legend to Table 1. By week 9, all mice were challenged by intravenous (iv) injection of RLV at 20 AID_{50}. By week 12, all mice were sacrificed, and their spleens were weighed.

8. Generation of an ELISA assay to measure anti-RLV antibodies. An assay to measure humoral responses in RLV-immunized mice using a cell ELISA employing P815-R cells (and their uninfected counterparts as controls) has been established.

9. DNA vaccination of neonatal rhesus monkeys. Groups of 4 rhesus macaque neonates were enrolled into a vaccine protocol that involves 7 groups. Prior to delivery, the pregnant dams were pre-screened by serology and PCR to rule out subclinical infection with simian retroviruses (SIV, SRV/D and STLV-I). The offspring were delivered by natural vaginal birth, and the dams were returned to their breeding colonies. The neonatal macaques were given the DNA vaccines as outlined (Table 2) within the first few days of life. Animals enrolled in groups A through E were entered into trial according to a computer-generated randomization schema. Because the births occurred over a period of several weeks, this strategy was adopted to avoid potential bias because of seasonal or other factors. Because the staff at the Yerkes Regional Primate Research Center needed to be trained specifically in the correct use of the gene gun by a member of Dr. Harriet Robinson's team, the animals in groups F and G were all vaccinated within a time span of eight days.

   The neonatal animals were hand-reared. Booster injections of DNA plasmids were administered at 4 weeks, and at 6 and 11 months. Blood samples were collected at birth, at 2 weeks, 6 weeks, and at 5 ½ months and at 6 ½, 10 ½ and 12 months. Antibody levels are being followed by ELISA, Western blot analysis and neutralization of autologous virus.

10. Western blot analysis of specific anti-SHIV immune responses. Commercially available HIV-1 Western blot strips were used to measure anti-HIV-1 Env responses. Because the Gag antigens are well conserved between HIV-1 and SIV, we expect to find anti-Gag responses by this Western blot analysis as well; however, the serum samples were also tested for anti-SIV responses also by commercially available HIV-2 strips, which we have used extensively for our previous SIV work (Baba et al., 1994; 1995).

11. SIV-specific CTL responses in macaques. CTL assays in the rhesus macaque system were established by evaluating adult macaques infected with SIVmac251.

11a. Recombinant virus vectors. Recombinant vaccinia viruses containing wild type vaccinia or *gag*, *pol*, or *nef* genes of SIV have been obtained from Dr. Panicali (Therion Biologics Corp., Cambridge, MA). The appropriate recombinant vaccinia viruses to measure CTL activity in SHIV-DNA-vaccinated animals have been obtained also.

11b. Preparation and labeling of B-lymphoblastoid cell lines (B-LCL). Peripheral blood samples were collected in sterile, preservative-free heparin and shipped to the Dana-Farber Cancer Institute on ice. Peripheral blood mononuclear cells (PBMC) were separated on Ficoll-hypaque gradients and infected with cell-free herpesvirus *papio*. Autologous B-LCL target cells were labeled with ^{51}chromium and infected overnight with wild-type vaccinia or recombinant vaccinia vectors containing either a single SIV *gag/pol/env* gene insert, individual SIV *gag-pol*, SIV *nef*, or HIVIIIB *env* gene inserts.

11c. CTL effector cell cultures. PBMC from the experimental monkeys were obtained by Ficoll-hypaque gradient centrifugation of blood. PBMC were cultured in RPMI-1640 media supplemented with 15% FCS, penicillin, streptomycin, and L-glutamine. Mitogen-stimulated PBMC effector cells were cultured in medium containing a 5 μg/ml Concanavalin A (ConA) for 3 days, washed and cultured for four days in medium containing 10% human IL-2. Other cultures were stimulated for 2 days with autologous paraformaldehyde-fixed B-LCL infected with a vaccinia vector containing an SIV *gag/pol/env* or an HIVIIIB *env* gene construct, and then cultured for five days in medium containing 10% human IL-2.

11d. SIV-specific cytotoxic CTL assays. SIV-specific CTL activity was determined using various effector-to-target cell (E:T) ratios in a standard 5 h ^{51}Cr release assay. The percentage of specific cytotoxicity was determined from the formula: 100 (experimental release - spontaneous release)/(maximum release - spontaneous release).

11e. CTL assays in the presence of cold targets. Because CTL assays with effector cells of some vaccinated infants revealed high backgrounds, the assays were repeated in the presence of unlabeled ("cold") target cells as follows. The cold-hot target cell ratio was 10:1. Cold targets were prepared from autologous B-LCL by incubation overnight with wt-vaccinia construct in the absence of ^{51}Cr.

RESULTS

This progress report covers year 03 of the overall proposal. Please note that the project is currently on a no-cost extension, as mentioned above. The results will be discussed under separate headings for easy review. First, we will briefly summarize our recent DNA vaccine studies conducted in the Rauscher murine leukemia virus (RLV) system. Subsequently, the large DNA vaccine study that is ongoing in infant rhesus macaques will be discussed. Lastly, future plans will be summarized.

1. Efficacy of DNA vaccination against RLV in mice.

In the previous progress report that covered year 02, we have summarized the generation of the necessary reagents as well as the establishment of key assays that enabled us to conduct a DNA vaccine study in the RLV/mouse system. Specifically, we have sequenced the entire RLV genome (Khimani et al., 1997), we have constructed a number of expression vectors and evaluated them in transient transfection assays, and we have established anti-RLV CTL assays as well as an ELISA to measure anti-RLV antibody levels. The investment into these technical aspects has paid off: we are now well positioned to determine optimal DNA vaccination strategies in this cost-effective retrovirus challenge model. Once effective protection is found, we will seek to identify the correlates and mechanisms of immune protection. Please note that the relevant assays are all established, including adoptive transfer of well defined cell populations. Much of the progress is due to the expertise provided by Dr. Robert Rasmussen, an experienced immunologist and retrovirologist.

Given our success in generating the DNA vectors that yielded high levels of expression in cell culture systems (please see report from year 02), a large DNA vaccine study was designed. We enrolled 7 groups of 10 mice each, which were given DNA inoculations as outlined in Table 1. The experiments were designed to ask the following questions: Is there a dose-response in DNA vaccine efficacy? Can vaccination with a DNA vector only expressing RLV *env* lead to protection? And lastly, can a DNA vaccine expressing only *gag-pol*, but no *env* sequences, lead to protection?

The animals were given 3 inoculations with DNA vectors at 3-week intervals. The DNA vaccines were given at doses that increased 10 fold. Three weeks after the last DNA boost, the animals were challenged intravenously with 200 AID_{50} of an RLV challenge stock. After an additional 3 weeks, they were euthanized, and the spleen weights were determined. Blood samples and spleen sections were collected for subsequent immunoblot analysis to determine the presence or absence of a viral antigens. In the RLV system, the degree of splenomegaly measured 3 weeks post-inoculation reflects the virus titer. Thus, inhibition of splenomegaly, a simple measurement, is an indirect reflection of vaccine efficacy. Serum samples from all

animals were subjected to immunoblot analysis. Animals with small spleens that tested negative on serum immunoblot analysis were tested further by immunoblot analysis of splenic protein extracts.

The results are depicted in Table 1. When compared to animals receiving only vector backbone DNA, vaccination with *env*-expressing DNA resulted in a statistically significant partial protection at a DNA dose of 100 μg per animal (Group D, Table 1, $p = 0.0002$). At this dose, two animals showed no evidence of infection by immunoblot analysis of serum as well as spleen extracts. A significant partial protection ($p = 0.0082$) was seen also with DNA vectors expressing only *gag-pol*. Interestingly, partial protection was seen in 2 out of 10 animals as a dose of only 25 μg of DNA. It should be noted that this dose is equivalent to 10 μg of pJWenv because of differences in the insert size. At the higher dose of 250 μg of DNA per animal, 4 animals tested negative by serum immunoblot analysis. One of these as well as one animal vaccinated with only 25 μg of pJWgag-pol (Group F, Table 1) also showed no evidence of infection by spleen immunoblot analysis.

Discussion: DNA vaccination using expression vectors encoding *env* or *gag-pol* has prevented splenomegaly after RLV challenge with 200 AID_{50}. In 2 animals vaccinated with pJWenv, no evidence of infection was found. Likewise, 2 animals vaccinated with pJWgag-pol were completely protected overall; 1 animal even at a dose of 25 μg of DNA. These results indicate that DNA vaccination can protect some animals completely against RLV viremia. Splenomegaly was prevented in a larger fraction of vaccinees. Most intriguingly, we have observed partial protection in animals vaccinated with pJWgag-pol. The absence of *env* in this vaccine precludes the formation of neutralizing antibody. We postulate that cellular rather than humoral immune responses are responsible for the vaccine effects seen in the animals vaccinated with *gag-pol* expression vectors.

The latter observation has implications for vaccine development against other retroviruses, most notably, the lentiviruses. If vaccine strategies can be found that do not depend on the highly variable envelope glycoproteins, but rather are based on more conserved gene products such as Gag and Pol, we would expect that more broadly reactive immunity against a wide spectrum of viral quasi-species can be generated.

2. DNA vaccines in neonatal macaques with IL-12 as adjuvant.

A large DNA vaccine study involving 7 groups of 4 neonatal rhesus monkeys has been started previously as discussed in the progress report for year 02. Our DNA vaccine experiment is being carried out with SHIV-vpu^+, a chimeric virus that encodes the HIVIIIB glycoprotein envelope. The experimental design is summarized in Table 2. Animals in Groups A and B were vaccinated with 5 different DNA expression vectors,

which led to the expression of all viral gene products but no replication-competent virus. Animals in Groups C an D were vaccinated only with expression vectors encoding either *gag-pol* or *nef*. We deliberately omitted including any *env*-expressing vectors, in analogy to the experiments described in the murine leukemia virus system (please see above). Monkeys in Group E serve as controls; they were given backbone vector DNA only. Lastly, monkeys in Groups F and G received the same 5 DNA vectors as animals in Groups A and B; however, instead of intradermal delivery, gene gun vaccination was used for Groups F and G.

Specific Antibody Responses: After the 3 planned DNA vaccinations were given, the animals were evaluated systemically for evidence of humoral immunity. The antibody responses obtained by week 52 are depicted in Table 2. By ELISA to HIV gp160, 3 out of 4 animals were positive in Group A, and 2 out of 4 animals in Group B. As expected, all animals in Groups C, G and E are negative, as they were not exposed to *env*-expressing DNA vectors. One of out 4 animals in Group F and all 4 monkeys in Group G were ELISA positive. For easy review, the ELISA results against HIV gp160 are depicted as bar graphs (Fig. 1). By Western blot analysis, only 5 of the vaccinated animals were clearly positive (Table 2). No significant levels of neutralizing antibodies were found in any of the vaccinated infants (data not shown).

Specific CTL Responses: At the conclusion of the 3 DNA inoculations, all animals were tested also for evidence of specific CTL responses against SIV Gag-Pol, Nef, or Env of HIVIIIB. PBMC were stimulated with paraformaldehyde-fixed, autologous B lymphocytic cell lines infected with SHIV antigen-encoding vaccinia virus constructs. Il-2 was added every 3 days and cultures tested on day 7 for CTL activity against autologous ^{51}Cr-labeled target cells infected with vaccinia constructs encoding *SIV gag-pol*, SIV *nef*, or HIVIIIB *env*.

To date, no specific CTL responses have been observed in any of the vaccinated animals. A high fraction of rhesus monkey infants revealed high backgrounds when tested with wild-type vaccinia virus only in the classical chromium release assay. To address the background problem, the CTL assays were repeated in the presence of a 10-fold excess of unlabeled "cold" targets. In general, background levels were diminished but did not decrease to $\leq$ 10% lysis in each case. The raw CTL data on each animal are included in Table 3 (which encompasses 4 pages). A few animals seemingly had target cell lysis that exceeded 10% of that observed with wild-type vaccinia virus-infected cells. For instance, animal RWt5 (vaccinated with DNA vectors expressing *gag-pol* and *nef* SIV; Table 3, page 2) showed 42% lysis against target cells infected with vaccinia expressing *nef*, whereas target cells infected with wild-type vaccinia virus exhibited 30% specific lysis. When the CTL assay was repeated in a 10-fold excess of cold target cells, the high background observed with target cells infected with wild-type vaccinia virus persisted. However, the lysis observed with *nef*-expressing target cells was now lower. Given these high backgrounds, we do not consider these results indicative of vaccine-induced CTL activity. Antigen-specific lymphocyte proliferation responses are now being assessed using PBMC from

inoculated animals as another measure of possible immunity. As a control, Gag-specific lymphocyte proliferation using PBMC from an SIVmac251-infected animal was tested.

It should be noted that for each CTL assay, a known CTL^+ control sample was included that revealed no background problems. Consequently, the high backgrounds observed in some of the vaccinated infants cannot be ascribed to technical problems. As an example of our proficiency in conducting CTL assays, Fig. 2 is enclosed. These results show clearly that our assay system can detect Gag-Pol and Nef-specific CTL activity that is MHC restricted.

These disappointing results were discussed with Dr. Harriet Robinson, our collaborator, who has conducted a parallel study in adult rhesus monkeys using the same DNA expression vectors. Her experience was not unlike our own, which prompted her to include various protein boosts to increase the specific immune responses.

Boosting Protocol: In the meantime, we have succeeded in obtaining purified gp160 of HIVIIIB (gift of Dr. Shiu-Lok Hu, University of Washington, Seattle). Animals primed with the 5 DNA expression vectors, which include HIVIIIB *env*-expressing plasmids, have been boosted already once with HIV GP160. Animals primed only with *gag-pol* and *nef* vectors were given an additional boost with the same DNA expression vectors. The control animals were given another dose of the vector backbone. This experimental approach is summarized in Table 4.

Please note that a new group of 4 age-matched rhesus macaques have been enrolled into the study. These animals have received no priming with DNA vaccines. They will only receive the gp160 protein boosts, followed by intravenous challenge with SHIV-vpu^+. Consequently, these animals represent a necessary control to evaluate the efficacy of the protein boosts alone. It should be noted that this particular preparation of HIV GP160 has shown some protection against SHIV challenge when used alone, in the absence of priming with other candidate vaccines (Harriet Robinson, personal communication).

Health Status of Vaccinated Macaque Infants: As stated previously, the infants have been hand reared at Yerkes Regional Primate Research Center. Some of the animals have developed chronic diarrhea which, in some cases, has led to delays in growth and development. Particularly, 1 infant that was vaccinated through the gene gun (animal REw5, Group F) has had diarrhea of such severity that we not only delayed our vaccine schedules but even contemplated euthanasia. Multiple diagnostic work-ups have been conducted; on occasion, enteropathogens have been isolated by stool culture. In such instances, the animal has been treated with appropriate antibiotics. However, on other instances, no specific diagnosis for the chronic diarrhea was made. According to Dr. Harold McClure, several animals of the Yerkes breeding colony have had similar problems. On occasion, no diagnoses have been obtained even at full necropsy. In

other cases, scleroderma-like conditions were found in untreated, colony-derived animals.

In case the diarrhea problems do not resolve, and in case 1 or more of the animals need to be euthanized, we will obtain a full histopathological examination. Furthermore, the animals will be tested for evidence of autoimmunity.

DISCUSSION AND FUTURE PLANS

During the no-cost extension of the project, we plan to complete experiments in the RLV/mouse system as well as the large ongoing DNA prime-boost vaccination in infant rhesus macaques. The experimental plans for both systems are given separately below.

1. DNA vaccination in the RLV/mouse system

A repeat experiment similar to the one outlined in Table 1 is ongoing currently. The major difference is an additional DNA boost, which has been administered already. Three weeks after the last boost, the mice will be challenged with 200 AID_{50} of RLV by the intravenous route. After an additional three weeks, the animals will be sacrificed. The spleens will be collected from all animals. A small portion of the splenocytes will be used to prepare protein extracts which will be tested for the presence of RLV antigens by our standard immunoblot assay. The remainder of the splenocytes will be used to test the vaccinated animals for specific CTL and proliferative responses.

We also plan to conduct an additional DNA vaccine experiment, in which we will either increase the DNA vaccine dose, the number of boosts, or both, depending on the outcome of the currently ongoing study. This new experiment will also include a group of animals that will be vaccinated with both *env* and *gag-pol*-encoding DNA vectors. The aim of the latter vaccine approach is to test whether complete protection against RLV challenge and subsequent viremia can be obtained. We will also re-test our hypothesis that protection can be achieved in the absence of *env*-encoding DNA plasmids.

Should we find complete protection, we will attempt to assess the correlates of immunity by the assays described above. Depending on the outcome of the next two DNA vaccine series in this systems, we may also include adoptive transfer of serum or purified T cells, followed by challenge of the recipients by cell-free RLV. However, given that the project currently under a no-cost extension, consideration of timing and finances will determine whether this last and highly important experiment can be included.

2. Completion of the DNA Vaccines Experiments in Infant Rhesus Macaques

During the no-cost extension, we plan to complete the scheduled protein boosts with purified recombinant HIV GP160 as discussed. We will continue to evaluate specific humoral and cellular immune responses in the vaccinees. At the completion of the boosts, the animals will be challenged intravenously with 10 AID_{50} of SHIV-vpu^+. After challenge, the animals will be followed prospectively for signs of viremia by co-cultivation, DNA PCR, RT-PCR, ELISA and Western blot analysis. If we find no

evidence of infection by these assays, we will also perform lymph node biopsies and test the specimens by co-cultivation for virus isolation, and DNA PCR.

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Liska V, Lerche NW, Ruprecht RM. Simultaneous detection of simian retrovirus type D serotypes 1, 2, and 3 by polymerase chain reaction. AIDS Research and Human Retroviruses 1997a; 13:433-437.

Liska V, Fultz PN, Su L, Ruprecht RM. Detection of simian T-cell leukemia virus type I infection in seronegative macaques. AIDS Research and Human Retroviruses 1997b; 13:1147-1153.

Ruprecht RM, Mullaney S, Bernard LD, Gama Sosa MA, Hom RC, Finberg RW. Vaccination with a live retrovirus: the nature of the protective immune response. PNAS 1990; 87:5558-5562.

Table 1. DNA Vaccination of BALB/c Mice Against RLV

Group #	DNA			Immunoblot (negative)		Mean Spleen Weight (mg)	*p* Value
	Vector DNA	Insert Size (bp)	Dose (μg)[1]	Serum IB	Spleen IB		
A	pJW[2]	-	100	0/10		1191 ± 318	-
B	pJW env	2039	1	0/10		1172 ± 272	0.9397
C	pJW env	"	10	0/10		1038 ± 418	0.4963
D	pJW env	"	100	2/10	2/2	483 ± 225	0.0002*
E	pJW gag-pol	5223	2.5	0/10		1489 ± 382	0.0588
F	pJW gag-pol	"	25	2/10	1/2	676 ± 416	0.0102
G	pJW gag-pol	"	250	4/10	1/4	547 ± 439	0.0082*

Legend:

Each group had 10 mice. The vaccination schedule was as follows:

first DNA inoculation	week 0,	0.1 ml, i.m.
second DNA inoculation	week 3,	0.1 ml, i.m.
third DNA inoculation	week 6,	0.1 ml, i.m.
virus challenge	week 9,	0.2 ml, 200 50% animal infectious doses (AID_{50}), i.v.
bleeding and sacrifice	week 12,	weigh the spleens; perform immunoblot analysis on serum and spleen extracts.

Virus stock: RLV stock, 2,000 plaque-forming units (pfu)/ml, and 10,000 AID_{50}/ ml, prepared 7/27/94.

[1] The DNA doses were normalized to yield equivalent concentrations of *env* and *gag-pol* sequences, respectively

[2] vector (pJW4303) size: 5085 bp

* statistically significant after Bonferroni correction for multiple comparisons ($p \leq 0.0083$)

Table 2. DNA Vaccines in Neonatal Macaques with IL-12 as Adjuvant

Group #	Animal #	DNA	Delivery	IL-12	HIVgp160 ELISA (week 52)	Western Blot (week 52)
A (1)	Reu5	5 DNAs	ID	none	2.05	
	RIv5	"	"	"	3.10	+
	RJu5	"	"	"	2.24	+
	Ruv5	"	"	"	0.48	
B (2)	(RSu5) died 7/7/96	5 DNAs	ID	+		
	RTv5	"	"	+	0.36	
	RDv5	"	"	+	0.06	
	RYv5	"	"	+	1.78	+
	RMw5 (replacement for RSu5)	"	"	+	2.92	+
C (3)	RWt5	*gag-pol/nef* DNAs	ID	none	0.44	
	RBv5	"	"	"	0.21	
	ROu5	"	"	"	0.53	
	ROv5	"	"	"	0.29	
D (4)	RTu5	*gag-pol/nef* DNAs	ID	+	0.49	
	RJv5	"	"	+	0.58	
	RSv5	"	"	+	0.23	
	RCw5	"	"	+	0.34	
E (5)	RIu5	control DNA	ID	none	0.33	
	RVv5	"	"	"	0.20	
	RWu5	"	"	"	0.45	
	RZu5	"	"	"	0.21	
F (6)	RAw5	5 DNAs	gene gun	none	0.34	
	RBw5	"	"	"	0.78	
	RDw5	"	"	"	0.38	
	REw5	"	"	"	1.52	
G (7)	RFw5	5 DNAs	gene gun	+	1.67	
	RGw5	"	"	+	2.10	
	RIw5	"	"	+	1.22	
	RJw5	"	"	+	1.37	+

Legend, Table 2. DNA vaccines in neonatal macaques with IL-12 as adjuvant.

Infant macaques were vaccinated as outlined. After 3 inoculations with DNA vaccines, serum was collected at week 52 and tested by ELISA for the presence of antibodies specific to HIVgp160. The same serum samples were also tested by Western blot analysis, using strips prepared with both HIV-1 and HIV-2. These experiments were conducted in collaboration with Dr. David Montefiori.

Table 3. CTL Data of All Vaccinated Rhesus Macaques (page 1)

			130:1	43:1	14:1	5:1
Control DNA	RIu-5	WT	35	28	13	1
	(yes)	Gag-Pol	30	18	10	6
		Nef	36	26	15	7
		Env	40	25	17	1

			100:1	33:1	11:1	4:1
	RVv-5	WT	7	4	3	0
	(no)	Gag-Pol	6	3	0	1
		Nef	8	3	2	0
		Env	7	2	2	0

			120:1	40:1	13:1	4:1	(yes) 200:1	67:1	22:1	7:1
	RWu-5	WT	38	28	18	12	15	6	3	2
	(no)	Gag-Pol	36	22	31	15	14	6	0	0
		Nef	53	43	37	17	11	10	4	0
		Env	38	31	17	12	11	5	3	4

			200:1	67:1	22:1	7:1	(no) 75:1	25:1	6:1	2:1
	RZu-5	WT	22	19	9	6	18	8	4	5
	(no)	Gag-Pol	21	13	8	8	20	10	3	1
		Nef	19	13	8	3	18	8	3	3
		Env	21	13	6	3	17	12	5	3

			70:1	23:1	7:1	2:1
5 DNA	REu-5	WT	10	6	2	3
	(yes)	Gag-Pol	16	3	4	2
		Nef	9	7	5	2
		Env	7	2	0	0

			200:1	67:1	22:1	7:1	(no) 70:1	23:1	8:1	3:1
	RJu-5	WT	45	39	23	17	20	11	8	3
	(no)	Gag-Pol	48	29	20	14	19	9	11	5
		Nef	49	36	20	14	21	16	7	2
		Env	49	31	24	15	18	14	10	3

			100:1	33:1	11:1	4:1
	RIv-5	WT	6	1	0	1
	(no)	Gag-Pol	10	7	1	2
		Nef	5	3	0	3
		Env	8	3	2	4

			75:1	25:1	6:1	2:1	(yes) 63:1	37:1	18:1	9:1
	RUv-5	WT	38	34	25	24	21	12	4	4
	(no)	Gag-Pol	33	29	16	16	11	10	8	5
		Nef	34	31	20	14	19	12	5	3
		Env	37	32	30	25	22	13	9	1

Table 3. CTL Data of All Vaccinated Rhesus Macaques (page 2)

5 DNA + IL-12

		200:1	67:1	22:1	7:1
RDv-5	WT	9	4	3	1
(no)	Gag-Pol	7	2	2	3
	Nef	4	4	0	0
	Env	16	6	0	0

		160:1	53:1	18:1	6:1
RTv-5	WT	20	11	3	1
(yes)	Gag-Pol	15	6	5	0
	Nef	18	8	2	0
	Env	22	12	2	0

		200:1	67:1	22:1	7:1
RYv-5	WT	5	1	2	1
(no)	Gag-Pol	4	0	2	0
	Nef	0	0	0	0
	Env	6	3	3	1

		100:1	33:1	11:1	4:1
RMw-5	WT	11	9	9	2
	Gag-Pol	15	16	7	8
	Nef	6	5	4	3
	Env	11	11	7	5

Gag-pol/nef

						(no)				(yes)			
		200:1	67:1	22:1	7:1	125:1	42:1	14:1	5:1	178:1	59:1	20:1	7:1
RWt-5	WT	21	18	9	3	30	27	13	8	43	20	5	3
(no)	Gag-Pol	22	19	10	7	41	33	20	10	25	16	3	1
	Nef	24	24	14	9	42	33	24	13	37	14	5	1
	Env	23	20	12	6	41	32	27	13				

		200:1	67:1	22:1	7:1
ROu-5	WT	29	15	3	0
(yes)	Gag-Pol	31	17	7	4
	Nef	27	17	5	2
	Env	29	17	6	0

		50:1	25:1	12:1	6:1
RBv-5	WT	4	1	0	0
(yes)	Gag-Pol	11	4	0	3
	Nef	6	1	0	1
	Env	7	0	0	0

						(yes)			
		200:1	67:1	22:1	7:1	108:1	36:1	12:1	4:1
ROv-5	WT	60	46	32	17	41	22	11	0
(no)	Gag-Pol	61	39	31	16	43	19	12	1
	Nef	61	47	24	13	47	30	11	0
	Env	54	39	17	9	46	31	14	4

Table 3. CTL Data of All Vaccinated Rhesus Macaques (page 3)

Gag-pol/nef + IL-12

		60:1	20:1	7:1	2:1
RTu-5	WT	10	2	2	2
(yes)	Gag-Pol	5	4	1	0
	Nef	5	3	1	1
	Env	5	0	0	0

		200:1	67:1	22:1	7:1	(no) 90:1	30:1	10:1	3:1	(yes) 45:1	15:1	5:1	1:1
RJv-5	WT	32	25	15	9	38	34	26	14	55	36	17	8
(no)	Gag-Pol	28	21	14	6	40	37	25	12	37	23	13	4
	Nef	43	29	19	15	46	42	19	16	56	37	19	6
	Env	34	26	20	11	53	43	35	23	50	35	14	9

		200:1	67:1	22:1	7:1
RSv-5	WT	17	5	0	1
(no)	Gag-Pol	15	4	4	0
	Nef	13	6	5	1
	Env	15	8	5	4

		200:1	67:1	22:1	7:1	(yes) 100:1	33:1	11:1	4:1
RCw-5	WT	17	14	7	0	6	2	1	2
(no)	Gag-Pol	40	21	17	8	4	0	1	0
	Nef	41	29	29	8	9	1	2	0
	Env	39	39	19	13	7	2	0	0

5 DNA Gene Gun

		200:1	67:1	22:1	7:1
RAw-5	WT	14	11	5	1
(no)	Gag-Pol	20	17	8	6
	Nef	25	15	6	7
	Env	26	20	9	3

		90:1	30:1	10:1	3:1	(yes) 60:1	30:1	15:1	8:1
RBw-5	WT	34	20	10	13	43	20	8	2
	Gag-Pol	40	27	16	8	31	19	6	0
	Nef	34	26	18	8	37	25	11	3
	Env	26	30	14	17	36	22	14	1

		100:1	33:1	11:1	4:1
RDw-5	WT	17	10	5	2
(yes)	Gag-Pol	21	10	6	1
	Nef	24	9	5	0
	Env	17	9	1	0

		200:1	67:1	22:1	7:1
REw-5	WT	9	7	4	0
(no)	Gag-Pol	3	0	0	0
	Nef	10	11	10	4
	Env	12	9	7	4

Table 3. CTL Data of All Vaccinated Rhesus Macaques (page 4)

5 DNA Gene Gun + IL-12

		166:1	55:1	18:1	6:1
RFw-5	WT	3	0	2	0
(no)	Gag-Pol	0	0	0	0
	Nef	0	0	0	1
	Env	1	0	0	3

		40:1	13:1	4:1	1:1
RGw-5	WT	29	19	10	5
(no)	Gag-Pol	15	15	5	4
	Nef	22	23	11	5
	Env	22	19	7	5

		133:1	67:1	33:1	17:1
RIw-5	WT	6	2	1	1
(yes)	Gag-Pol	6	3	1	0
	Nef	8	4	2	1
	Env	9	2	1	0

		200:1	67:1	22:1	7:1
RJw-5	WT	13	9	9	3
(no)	Gag-Pol	19	13	8	0
	Nef	18	13	7	3
	Env	20	10	1	2

Legend, Table 3. Raw CTL data on each individual rhesus monkey infant enrolled in the DNA vaccine study.

PMBC from each individual monkey were stimulated for 6 days in the presence of autologous fixed B-LCL which had been pre-infected with vaccinia virus constructs expressing SIV *gag-pol*, SIV *nef*, or HIVIIIB *env*. After 6 days in culture, the cells were tested for specific killing activity against autologous B-LCL, again pre-infected with vaccinia virus constructs expressing SIV *gag-pol*, SIV *nef*, or HIVIIIB *env*. The cells were tested at the indicated effector:target cell (E:T) ratios in a standard 5-hour chromium release assay. The assays were performed in the presence or absence of a 10-fold excess of cold targets, as indicated in parentheses by either (yes) or (no), respectively. Some animals gave initial slight indications of specific activity albeit in the presence of high backgrounds against autologous target cells infected with wild-type vaccinia virus only. When the assays were repeated in the presence of cold targets, such activity was not observed.

Table 4. DNA Vaccine Experiment in Neonatal Macaques with IL-12 as Adjuvant: Boosting Protocol

Group #	n	DNA	IL-12	DNA Vaccine Delivery	gp160 boost	DNA boost
1	4	5 DNAs	-	ID	IM	-
2	4	5 DNAs	+	ID	IM	-
3	4		-	ID	-	*gag-pol/nef* DNAs ID
4	4		+	ID	-	*gag-pol/nef* DNAs ID
5	4	control DNA	-	ID	-	control DNA ID
6	4	5 DNAs	-	gun	IM	-
7	4	5 DNAs	+	gun	IM	-
8	4	-	-	gun	IM	-

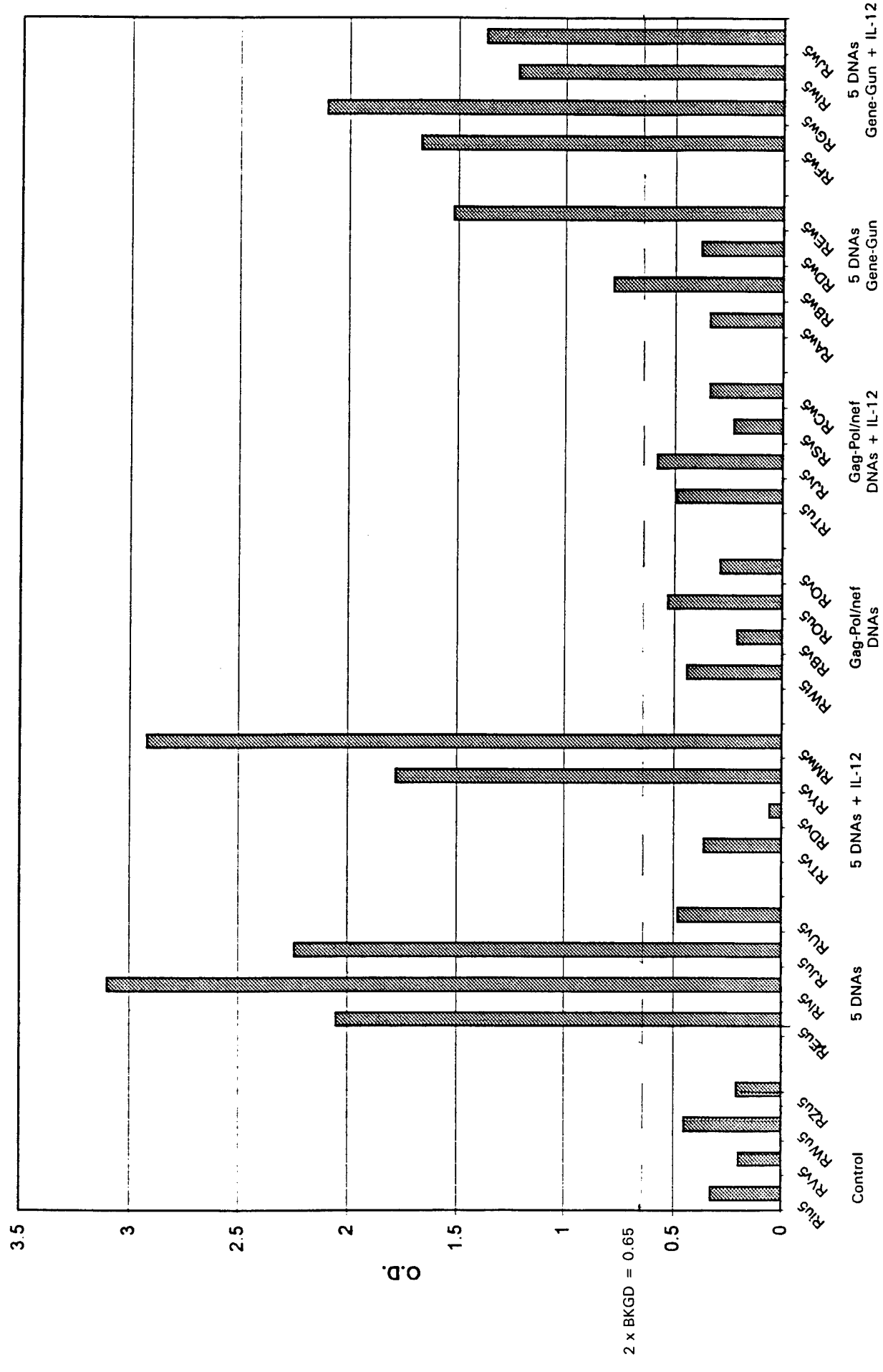
ID = intradermal

IM = intramuscular

Legend.

Because of the unexpected low immunogenicity of the DNA vaccines given as outlined in Table 1, animals primed with *env*-expressing DNA vectors (Groups 1, 2, 6, and 7) will be boosted with purified recombinant gp120 prepared from HIV-1 strain LAI (kindly provided by Dr. Shiu-Lok Hu).

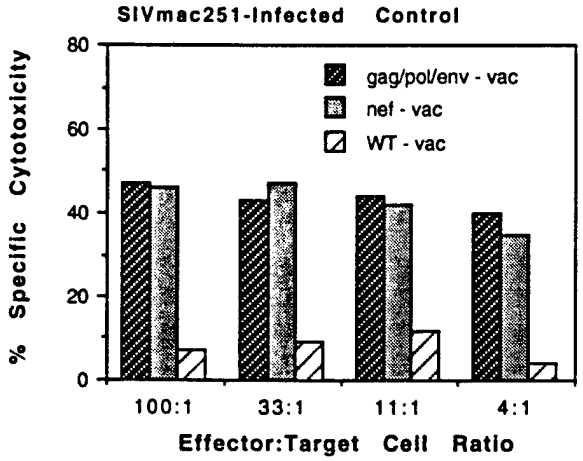
Figure 1

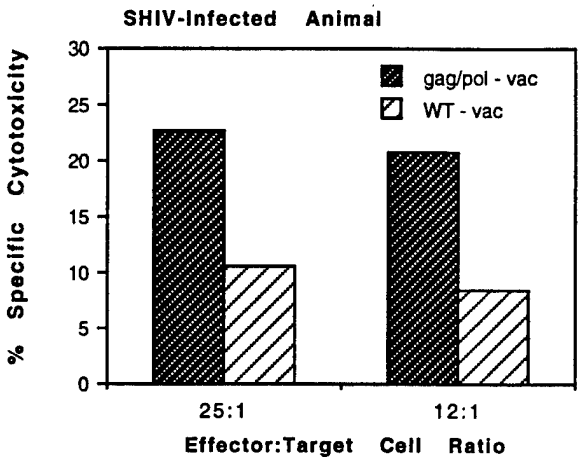

3.5
3
2.5
2
1.5
1
0.5
0
O.D.
2 x BKGD = 0.65
Control
5 DNAs
5 DNAs + IL-12
Gag-Pol/nef DNAs
Gag-Pol/nef DNAs + IL-12
5 DNAs Gene-Gun
5 DNAs Gene-Gun + IL-12

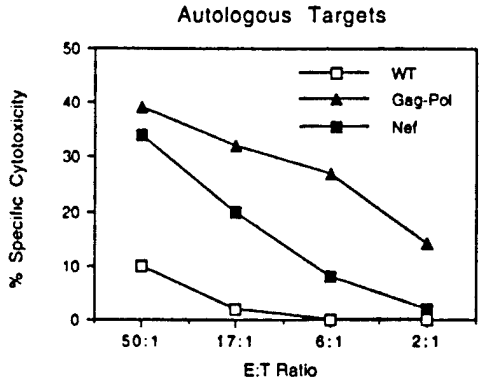
Legend, Fig. 1. Anti-HIV-1 gp160 antibody responses in the vaccinated infant macaques as measured by ELISA.

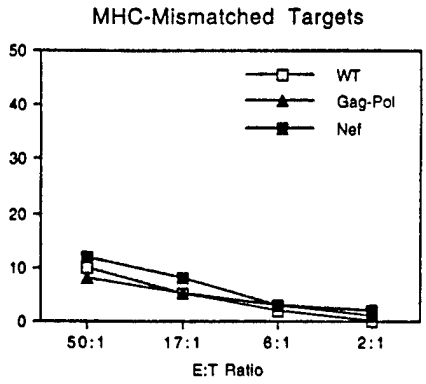
Serum samples were tested by a specific ELISA for antibodies against HIV-1 gp160. The data are expressed as optical density (OD) for each individual animal. Specific reactivity was seen only in the groups vaccinated with the 5 DNAs, which include HIVIIIB *env*-expressing plasmids.

Figure 2


SIVmac251-Infected Control
% Specific Cytotoxicity
80
60
40
20
0
gag/pol/env - vac
nef - vac
WT - vac
100:1
33:1
11:1
4:1
Effector:Target Cell Ratio


SHIV-Infected Animal
% Specific Cytotoxicity
30
25
20
15
10
5
0
gag/pol - vac
WT - vac
25:1
12:1
Effector:Target Cell Ratio


Autologous Targets
% Specific Cytotoxicity
50
40
30
20
10
0
WT
Gag-Pol
Nef
50:1
17:1
6:1
2:1
E:T Ratio


MHC-Mismatched Targets
50
40
30
20
10
0
WT
Gag-Pol
Nef
50:1
17:1
6:1
2:1
E:T Ratio

Legend, Fig. 2. CTL Data of All Vaccinated Rhesus Macaques.

Upper panel, left: PBMC isolated from an SIVmac251$^+$adult rhesus macaque were stimulated with paraformaldehyde-fixed, autologous B-LCL infected with SIV *gag, pol, env*-expressing vaccinia (gag/pol/env-vac) and *nef*-expressing vaccinia (nef-vac) for 6d. Activated cells were tested for CTL activity with ^{51}Cr-labeled autologous B-LCL infected with either wild-type vaccinia (WT-vac), gag/pol/env-vac, or nef-vac at effector to target cell ratios indicated. Percent specific lysis of target cells was measured 5 hr later. **Upper panel, right**: PBMC from an SHIV-vpu$^+$ infected animal were stimulated as above except using B-LCL infected with SIV *gag* and *pol*-expressing vaccinia (gag/pol-vac) for 6d prior to testing against identically infected or WT-vac infected autologous target cells. **Lower panel**: PBMC from an SIVmac251$^+$ adult were stimulated as above with gag/pol-vac infected autologous B-LCL and tested against autologous targets (left) and against MHC-mismatched targets (right) identically infected.

LIST OF APPENDIX ITEMS

1. Khimani AH, Lim M, Graf TG, Smith TF, Ruprecht RM. Phylogenetic relationship of the complete Rauscher murine leukemia virus genome with other murine leukemia virus genomes. Virology 1997; 238:64-67.

2. Liska V, Lerche NW, Ruprecht RM. Simultaneous detection of simian retrovirus type D serotypes 1, 2, and 3 by polymerase chain reaction. AIDS Research and Human Retroviruses 1997a; 13:433-437.

3. Liska V, Fultz PN, Su L, Ruprecht RM. Detection of simian T-cell leukemia virus type I infection in seronegative macaques. AIDS Research and Human Retroviruses 1997b; 13:1147-1153.

4. Rasmussen RA, Sharma PL, Hu Y, Ruprecht RM. Protective immunity generated by low doses of a live, pathogenic retrovirus. Manuscript submitted.

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Phylogenetic Relationship of the Complete Rauscher Murine Leukemia Virus Genome with Other Murine Leukemia Virus Genomes[1]

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We report the complete nucleotide sequence of the genome of Rauscher murine leukemia virus (R-MuLV), the replication-competent helper virus present in the Rauscher virus complex, and its phylogenetic relationship with other murine leukemia virus genomes. An overall sequence identity of 97.6% was found between R-MuLV and the Friend helper virus (F-MuLV), and the two viruses were closely related on the phylogenetic trees constructed from either *gag, pol,* or *env* sequences. Moloney murine leukemia virus (Mo-MuLV) was the next closest relative to R-MuLV and F-MuLV on all trees, followed by Akv and radiation leukemia virus (RadLV). The most distantly related helper virus was Hortulanus murine leukemia virus (Ho-MuLV). Interestingly, Cas-Br-E branched with Mo-MuLV on the *gag* and *pol* trees, whereas on the *env* tree, it revealed the highest degree of relatedness to Ho-MuLV, possibly due to an ancient recombination with an Ho-MuLV ancestor. In summary, a phylogenetic analysis involving various MuLVs has been performed, in which the postulated close relationship between R-MuLV and F-MuLV has been confirmed, consistent with the pathobiology of the two viruses.

INTRODUCTION

The Rauscher virus complex contains the replication-competent helper virus, termed Rauscher murine leukemia virus (R-MuLV), and the replication-defective Rauscher spleen focus-forming virus (R-SFFV); spleens of infected mice also contain a third component, Rauscher mink cell focus-inducing (R-MCF) virus (Pluznik and Sachs, 1964; Weiss *et al.,* 1985). This virus complex induces a clinical disease spectrum similar to that of the Friend virus complex, which is also composed of a replication-competent helper virus, the Friend murine leukemia virus (F-MuLV), and the defective anemia strain of the Friend spleen focus-forming viruses (Weiss *et al.,* 1985). Both the Rauscher and Friend virus complexes cause erythroleukemia in newborn and adult mice. Earlier studies from this laboratory have shown that live, attenuated Rauscher virus complex generated immune responses that protected against high-dose challenge with live virus (Ruprecht *et al.,* 1990a, 1990b). By adoptive transfer, we determined the correlates of immune protection in this system; immune T cells alone were able to confer protection (Ruprecht *et al.,* 1990a; Hom *et al.,* 1991). Presently, we are seeking to determine the epitopes that are recognized by cytotoxic T cells. To generate the necessary reagents, the complete nucleotide sequence of R-MuLV was needed.

The similar pathogenicity of the Friend and Rauscher virus complexes suggested a strong degree of homology at the primary nucleotide sequence level. The components of the Friend virus complex have been sequenced (Obata *et al.,* 1984; Remington *et al.,* 1992; Friedrich *et al.,* 1991; Perryman *et al.,* 1991a), whereas only the long terminal repeat (LTR) sequences of R-MuLV clone-9 (Van Der Feltz *et al.,* 1986), R-SFFV (Bestwick *et al.,* 1984), R-MCF (Vogt *et al.,* 1985), and sequences of the envelope (*env*) gene of R-SFFV (Bestwick *et al.,* 1984) and R-MCF virus (Vogt *et al.,* 1985) have been reported. The full-length genomes of other ecotropic, replication-competent helper murine leukemia viruses that have been characterized fully are: Moloney murine leukemia virus (Mo-MuLV) (Shinnick *et al.,* 1981), Akv (Herr, 1984; Etzerodt *et al.,* 1984), radiation leukemia virus (RadLV) (Merregaert *et al.,* 1987), and Cas-Br-E (Perryman *et al.,* 1991b). Here, we report the complete nucleotide sequence of R-MuLV, a comparison of this sequence with those of other ecotropic replication-competent helper MuLVs, and their phylogenetic relationships. As expected from the similar biological characteristics, we have found a high degree of sequence identity between R-MuLV and F-MuLV, and both viruses appeared on the same branch by phylogenetic analysis of the *gag, pol,* and *env* sequences.

MATERIALS AND METHODS

The integrated proviral DNA of R-MuLV clone RV-1, generated by Habara *et al.* (1982) using a bacteriophage

[1] The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned Accession No. U94692.

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0042-6822/97 $25.00

TABLE 1

Sequence Homology between the R-MuLV and Other Murine Leukemia Virus Genomes

Strain	U3	R	U5	5′ Leader[a]	*gag* nt[b]	*gag* a.a.[c]	*pol* nt	*pol* a.a.	*env* nt	*env* a.a.	Complete genome
F-MuLV											
PVC-211[d]	97.3	98.5	100	96.4	98.0	97.4	98.0	98.5	96.9	95.4	97.6
57[e]	97.3	92.6	100	96.8	97.8	96.8	97.5	98.0	96.9	96.3	97.3
FB29[f]	97.6	98.5	98.7	96.0	97.6	96.7	98.1	98.3	96.9	95.7	97.6
Mo-MuLV[g]	86.3	94.1	97.4	91.6	84.8	90.7	90.9	96.1	82.1	85.9	87.6
Cas-Br-E[h]	89.0	97.1	97.4	90.3	84.8	92.5	91.7	96.6	72.1	78.1	85.7
Akv[i]	74.8	95.6	89.5	74.2	79.2	85.3	84.7	89.8	75.9	80.7	80.8
RadLV[i]	69.4	94.1	90.8	73.9	79.5	84.5	84.7	90.2	75.5	80.6	80.5
Ho-MuLV[k]	79.8	92.6	90.8	80.2	79.8	85.3	ND[l]	ND	69.3	71.6	ND

[a] 5′ leader sequence from the 3′ end of U5 to the start codon of *gag*.
[b] nt, percentage nucleotide identity.
[c] a.a, percentage amino acid identity.
[d] Accession No. M93134 (Remington *et al.*, 1992).
[e] Accession No. X02794 (Friedrich *et al.*, 1991).
[f] Accession No. Z11128 (Perryman *et al.*, 1991a).
[g] Accession No. J02255 (Shinnick *et al.*, 1981).
[h] Accession No. X57540 (Perryman *et al.*, 1991b).
[i] Accession No. J01998 (Herr, 1984; Etzerodt *et al.*, 1984).
[j] Accession No. K03363 (Merregaert *et al.*, 1987).
[k] Accession No. M26527, M26528 (Voytek and Kozak, 1989).
[l] Not determined, sequence not available.

Charon 4A vector and further subcloned into the *Eco*RI site of the plasmid pBR322, was kindly provided by Dr. S. Aaronson. The resulting plasmid, p3028N, containing the R-MuLV provirus with flanking mouse genomic DNA, was used for the DNA sequence analysis. The primers used for sequencing the R-MuLV genome were designed based on the reported F-MuLV sequence (Remington *et al.*, 1992). DNA sequence analysis was performed on double-stranded p3028N DNA templates by the Sanger method (Sanger *et al.*, 1977), using dye-labeled dideoxy nucleotides as terminators. Samples were analyzed on an automated DNA sequencer (Applied Biosystems Model 373A automated DNA sequencer) (Smith *et al.*, 1986). Both strands of the entire genome were sequenced by using primers in both directions.

The criteria for selecting murine leukemia viruses for sequence alignments and phylogenetic analyses were: (1) ecotropic, replication-competent helper viruses; and (2) at least fully sequenced *gag* and *env* genes. Sequence alignments were performed by using the GAP alignment program in GCG (Genetics Computer Group, Madison, WI). Phylogenetic trees were constructed by using the PIMA (Pattern-Induced Multi-Sequence Alignment) algorithm (Smith and Smith, 1992) and PAUP (Phylogenetic Analysis Using Parsimony) software (Swofford, 1985). Based on the amino acid sequence alignments of the Gag, Pol, and Env regions and the third codon position variation in these genes of R-MuLV and other murine leukemia viruses, phylogenetic trees were constructed. The use of the third codon position eliminates any selective bias. The protein sequences were aligned initially with PIMA. Subsequently, the protein alignments with the corresponding nucleotide sequences were entered into the PAUP program to generate the phylogenetic trees. Bootstrap analysis using the PHYLIP software package (Felsenstein, 1989, 1990) was also performed to generate phylogenetic trees based on the third codon position and the complete sequence of the *gag, pol,* and *env* genes of all MuLVs.

RESULTS AND DISCUSSION

The Genbank accession number of the complete nucleotide sequence is U94692. The entire R-MuLV genome is 8282 bases in length, which is the same length as that of the F-MuLV strain PVC-211 (Remington *et al.*, 1992). This full-length helper R-MuLV sequence was aligned with the sequences of other murine leukemia viruses at the nucleotide and amino acid levels (Table 1). The nucleotide sequence alignment showed an overall identity between R-MuLV and F-MuLV of 97.6% and of 87.6% with the T-cell tropic Moloney murine leukemia virus (Mo-MuLV), respectively. R-MuLV is closely related to F-MuLV in each region of the genome, whereas the greatest sequence divergence between R-MuLV and other MuLVs was seen in the U3 and *env* regions. GAP

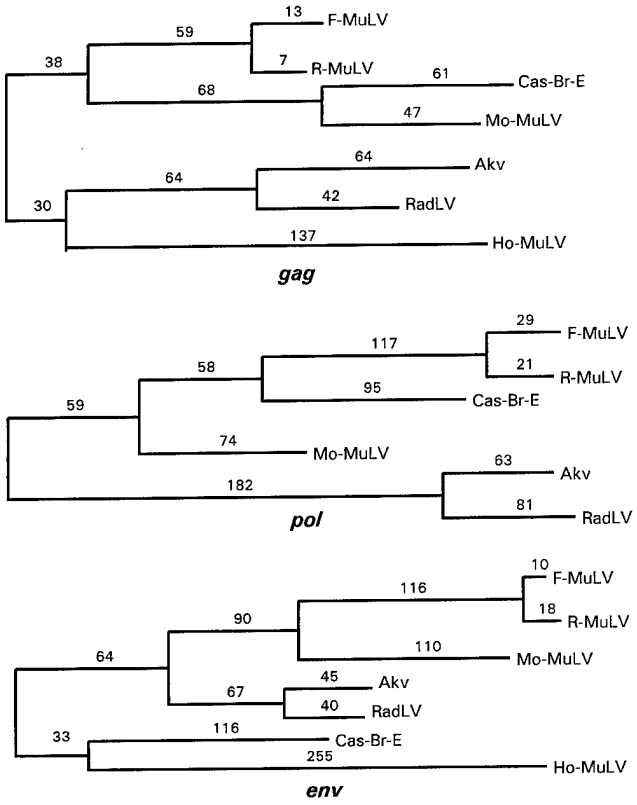

FIG. 1. Phylogenetic relationship of murine retroviruses based on *gag, pol,* and *env* sequences. The sequences for each virus were obtained from the Genbank by the accession numbers listed in Table 1. The phylogenetic analysis was carried out by initial amino acid sequence alignment of the Gag, Pol, and Env sequences followed by imposition on the nucleotide sequence of the respective *gag, pol,* and *env* genes using the PIMA algorithm (Smith and Smith, 1992). The three trees were constructed using the PAUP software (Swofford, 1985) based on the PIMA alignments. The numbers on the tree branches represent the number of implied third codon position substitutions.

analysis of the R-MuLV *env* region with the *env* sequence of R-SFFV showed 55% homology in the 5′ half and 98% homology in the 3′ half, which is consistent with the known origin of the R-SFFV *env* from MCF-like sequences at the 5′ end and truncated ecotropic *env* sequences at the 3′ end (Bestwick *et al.,* 1984). R-MuLV *env* and R-MCF (Vogt *et al.,* 1985) *env* sequences showed an overall identity of 78%; again, the 3′ region was highly homologous (99.2%) to ecotropic sequences (not shown). Furthermore, the overall sequence identity between the LTR region of the R-MuLV sequence reported here and the published LTR sequences of R-MuLV clone-9 (Van Der Feltz *et al.,* 1986), R-SFFV (Bestwick *et al.,* 1984), and R-MCF (Vogt *et al.,* 1985) was >98%.

The three phylogenetic trees shown were constructed by the PAUP program (Swofford, 1985) based on codon position 3 variation in the *gag, pol,* and *env* regions of all the murine leukemia viruses (Fig. 1). The Doolittle amino acid trees (Feng and Doolittle, 1987) were the same as those obtained from the third codon position analysis (data not shown). Phylogenetic trees, which were generated by Bootstrap analysis (Felsenstein, 1989, 1990) from the third codon position and the complete nucleotide sequence of the *gag, pol,* and *env* genes also showed nearly identical branching of each MuLV as that obtained by the other methods (data not shown).

R-MuLV and F-MuLV fall into one very closely related subgroup in all trees. The *gag* genes of these two viruses appear to have evolved more slowly than those of all other MuLVs based on the shorter branch lengths on the *gag* tree. This may be due to some selective constraint on the *gag* gene of R-MuLV and F-MuLV. Mo-MuLV, a virus that causes T-cell leukemias/lymphomas in neonatal mice, is the next closest relative to R-MuLV and F-MuLV on all trees, followed by Akv and RadLV. The latter two viruses form a separate subgroup and cluster together on all three trees. The two viruses are similar biologically; both are present as integrated proviruses in the germ line of their inbred host mouse strains, and both cause thymic leukemias (Weiss *et al.,* 1985). The most distantly related helper virus is Hortulanus murine leukemia virus (Ho-MuLV), a virus isolated from wild European *Mus hortulanus* that causes various hematological malignancies (Voytek and Kozak, 1988, 1989). Another wild mouse isolate, the neurotropic Cas-Br-E (Gardner *et al.,* 1976), branches with Mo-MuLV on the *gag* and *pol* trees. In contrast, Cas-Br-E exhibits the highest degree of relatedness on the *env* tree to Ho-MuLV. The different topology of Cas-Br-E on the *env* tree relative to its *gag* and *pol* positions is also supported by the consensus tree generated by the Bootstrap analysis of the third codon position and the complete sequence of the three genes (data not shown). The unexpectedly close phylogenetic relationship between Cas-Br-E and Ho-MuLV *env* sequences may be explained by a recombination event that may have occurred with a putative ancestor of Ho-MuLV.

In summary, we have performed an extensive phylogenetic analysis of ecotropic, replication-competent MuLVs. This analysis confirms the postulated close relationship of the complete R-MuLV nucleotide sequence with the F-MuLV genome.

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Short Communication

Simultaneous Detection of Simian Retrovirus Type D Serotypes 1, 2, and 3 by Polymerase Chain Reaction

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ABSTRACT

Asymptomatic infection of macaques with simian retroviruses type D (SRV/D), the etiologic agents of one form of retrovirus-induced simian immunodeficiency disease, can confound experiments with the simian immunodeficiency virus (SIV), which also induces immunodeficiency disease in macaques. The SIV/macaque model is the preferred nonhuman primate model for AIDS-related research. Serological screening for SRV/D alone is insufficient because not all infected animals seroconvert, and virus isolation by cocultivation may require 4 to 6 weeks. We have established a DNA polymerase chain reaction (PCR) assay. One set of nested primers allows detection of SRV/D serotypes 1, 2, and 3 and distinguishes SRV-2 from the other two serotypes. The PCR assay is sensitive; a single proviral copy of SRV/D could be detected in 150,000 to 210,000 macaque peripheral blood mononuclear cells (PBMCs). When applied to a panel of virus isolation-positive macaque samples, the PCR assay was positive in 100% of the tests. No false-positive results were seen when known specific-pathogen-free (SPF) macaques were examined. We propose that macaques be screened with a combination of SRV/D serology and this DNA PCR assay prior to enrollment in experiments with SIV.

INFECTION WITH SIMIAN TYPE D retroviruses (SRV/D) is prevalent in wild as well as in colony-born macaques.[1–3] Five neutralization types have been identified,[3] three of which, SRV-1, SRV-2, and Mason–Pfizer monkey virus (MPMV, SRV-3), have been molecularly cloned and sequenced.[4–6]

Macaque models provide an opportunity to develop vaccines against simian immunodeficiency virus (SIV), which induces a disease similar to human AIDS. SIV experiments in macaques can be affected adversely by inapparent SRV/D infection. Tests to screen for SRV/D include enzyme-linked immunosorbent assays (ELISA) for antibody or antigen,[7,8] fluorescent antibody assays,[9] and Western blot analysis (WB).[8] Overall, SRV/D seroprevalence ranges from 1 to 4%, but may be much higher in some hyperendemic macaque colonies. Indeterminate SRV/D serological results, characterized by reactivity only to the products of a single viral gene on Western blot (WB), can be as high as 30%.[10] For these reasons and because of the existence of seronegative SRV/D carriers,[11] virus isolation by cocultivation is performed.[12] We have developed a DNA PCR assay to detect a conserved nucleotide sequence in the *env* genes of SRV/D serotypes 1–3.

Heparinized blood samples originated from 15 cynomolgus monkeys (*Macaca fascicularis*) collected during an outbreak of SRV/D, type 2 in a colony (California Regional Primate Research Center, Davis, CA), and 7 specific pathogen-free (SPF) rhesus monkeys (*Macaca mulatta*) (M. D. Anderson Cancer Center, Bastrop, TX). Genomic DNA from macaque peripheral blood mononuclear cells (PBMCs), Raji cells infected with SRV/D serotypes 1 (R-20216), 2 (R-10867), or 3 (R-23200), and CEMx174 cells was isolated as described.[13] Plasmid DNA containing cloned SRV-1 (pSRV-1),[4] SRV-2 (D2C/Oregon),[14] and SRV-3 (pSHRM15),[15] respectively, was isolated using Qiagen plasmid kits (Qiagen, Chatsworth, CA). For titrations, 10-fold dilutions of plasmid DNA (1×10^{-1} to 1×10^{-10} ng) were prepared in the presence of 1 μg of CEMx174 DNA.

Sequences of external primers (SRV*env*1E: 5′ GCC CGT GGT AAA GAA AAA ATT G 3′; SRV*env*2E: 5′ ACT TCT GCT AGA GAG TCT AC 3′) and nested primers (SRV*env*3N:

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5′ GAA GAT TGC TGG CTG TGC TT 3′; SRV*env*4N: 5′ ATA GCT GGA ATG GGG ACA GG 3′) were derived from the *env* genes of SRV/D serotypes 1–3[4–6] (NCBI-GenBank, Bethesda, MD; Accession Nos. M11841, M16605, and M12349, respectively). The primer sequences are located at the following positions: SRV*env*1E: 6500–6522 (type 1), 6468–6490 (type 2), and 6884–6906 (type 3); SRV*env*2E: 7208–7228 (type 1), 7149–7169 (type 2), and 7589–7609 (type 3); SRV*env*3N: 6593–6613 (type 1), 6558–6578 (type 2), and 6974–6994 (type 3); and SRV*env*4N: 6995–7015 (type 1), 6936–6956 (type 2), and 7376–7396 (type 3). Corresponding sequences of SRV/D (type 1 and 3) served for designing of both sets of SRV/D-specific primers. All DNA samples were analyzed for amplifiable cellular DNA with primers for human β-actin[16] (XAHR 17 and XAHR 20; Research Genetics, Huntsville, AL). Bacteriophage λ DNA and primers PCO1 and PCO2 (GeneAmp PCR Reagent kit; Perkin-Elmer Cetus, Branchburg, NJ) were used as control.

Reaction conditions for both sets of SRV/D-specific primers consisted of 10 mM Tris-HCl, pH 9.2; 1.5 mM $MgCl_2$; 25 mM KCl (Opti-Prime buffer #9; Stratagene, La Jolla, CA); 100 μmol of each dNTP (Amplitaq; Perkin-Elmer Cetus); 50 pmol of each primer (Research Genetics); and 1.25 U of *Taq* polymerase (Amplitaq; Perkin-Elmer Cetus) in 50 μl. Typically, 1 μg of macaque genomic DNA was analyzed. For nested polymerase chain reaction (PCR), 1 μl of product from the first PCR round was added to 50 μl of the second-round reaction mixture. Alternatively, "hot start" PCR, in which *Taq* polymerase was inhibited with TaqStart antibody (Clontech, Palo Alto, CA), was used. Reaction mixtures were overlaid with 20 μl of mineral oil (Sigma, St. Louis, MO). The PCR was performed in a Perkin-Elmer GeneAmp PCR System 9600 (Perkin-Elmer Cetus, Norwalk, CT). Cycling conditions for the first PCR round were as follows: initial denaturation (98°C, 15 sec) followed by six cycles of "touchdown"[17] amplification, which started with a melting step (97.5°C, 15 sec), annealing (58°C, 40 sec), and extension (72°C, 55 sec). In each subsequent cycle, the melting temperature was lowered by 0.5°C, but melting times remained constant; the annealing temperature was lowered by 1°C, and the annealing time was shortened by 5 sec; extension temperatures stayed constant but the extension time was shortened by 5 sec. Then, cycling continued (94°C for 15 sec; 53°C for 15 sec; 72°C for 30 sec) for 40 cycles, and was finished at 72°C for 6 min. Nested PCR began with denaturation (94°C, 1 min) followed by 40 cycles of annealing (55°C, 15 sec), extension (72°C, 30 sec), and denaturation (94°C, 15 sec) per cycle and terminal extension (72°C, 6 min). Negative controls consisted of genomic CEMx174 and uninfected Raji cell DNA, H_2O without added template, and CEMx174 cell DNA without *Taq* polymerase. The PCR products were analyzed by agarose gel electrophoresis.

Antibodies to SRV/D in macaque serum were measured by enzyme immunoassay (EIA), and EIA-reactive sera were confirmed by WB as previously described.[10] The EIA results were considered positive if the optical density (OD) was ≥2.5 times value of negative controls. Results for WB were considered positive if reactivity against the product of two viral genes was observed. Blots showing reactivity against the product of a single viral gene were considered "indeterminate."

SRV/D isolations were performed by cocultivation of

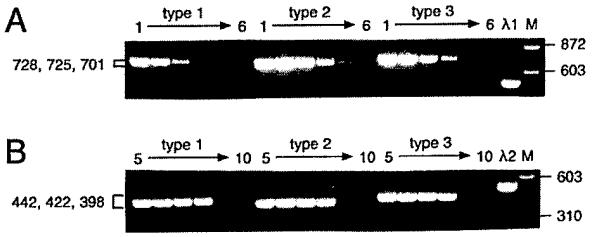

FIG. 1. Titrations of SRV/D, serotypes 1–3. Ten-fold dilutions of plasmid DNA (1×10^{-1} to 1×10^{-10} ng, lanes 1–10) were amplified in the presence of 1 μg of CEMx174 DNA. λ_1 and λ_2, independent PCR controls (Perkin-Elmer Cetus). Amplified products were resolved on 1.5% agarose gels and stained with ethidium bromide. (A) The first PCR round of cloned SRV/D plasmid DNAs yielded specific products of 728 bp (type 1), 701 bp (type 2), and 725 bp (type 3), respectively. (B) The second (nested) PCR round yielded specific bands of 422 bp (SRV-1), 398 bp (SRV-2), and 442 bp (SRV-3). The last positive dilution of plasmids DNA (1×10^{-8}, types 1–3) is equal to one proviral copy in 150,000–210,000 cells.

macaque PBMCs (2×10^6/ml) with the permissive cell line Sup-T1, as previously described.[10] Briefly, macaque PBMCs were separated from heparinized whole blood by Ficoll gradient centrifugation. The PBMC suspensions of 1×10^6 cells/ml were stimulated with *Staphylococcus* enterotoxin A (SEA) for 48–72 hr, then cocultivated with an equal volume of Sup-T1 cells at a concentration of 5×10^5 cells/ml. Levels of reverse transcriptase (RT) in culture supernatants were monitored at regular intervals for 6 weeks. Cultures were considered positive if RT levels were ≥five times the background in three samples.

Our PCR assay could amplify *env* sequences of SRV/D serotypes 1–3 and allowed the distinction of SRV/D serotype 2 from the other two (Fig. 1A and B). Plasmids or genomic DNA from infected cells could be amplified equally well (Figs. 1A and B and 2A and B). Even though primers SRV*env*3N and SRV*env*4N displayed four or three mismatches, respectively, when compared to SRV-2 *env* (Table 1), target DNA could be

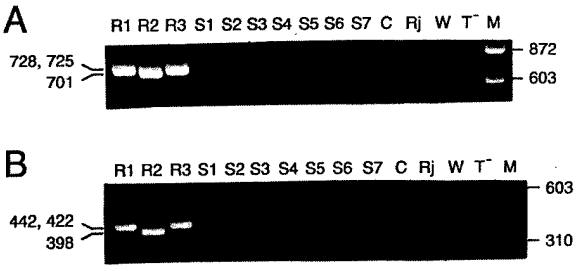

FIG. 2. Controls and standards. First (A) and second (B) PCR rounds are shown of genomic DNA from SPF rhesus monkeys (S1-S7), CEMx174 cell DNA (C), uninfected Raji cell DNA (Rj), H_2O without added template (W), and CEMx174 cell DNA without *Taq* polymerase (T^-). Raji cells infected with SRV1-3 (lanes R1–R3) were used as positive controls. Agarose gels (1.5%) were stained with ethidium bromide. Marker: ϕX174 DNA digested with *Hae*III.

TABLE 1. NESTED PRIMER AND SRV-2 DNA SEQUENCES

Primer name	*Virus*	*Primer[a] and/or viral sequence[b]*
SRV*env*3N	SRV-1	5′ GAA GAT TGC TGG CTG TGC TT 3′[a,b]
	SRV-2	a·c ··g ··· ··· ··a ··· ··[c]
	SRV-3	··· ··· ··· ··· ··· ··· ··[a,b]
SRV*env*4N	SRV-1	ATA GCT GGA ATG GGG ACA GG[a,b]
	SRV-2	··· ··· ··· ·ca ··t ··· ··[b]
	SRV-3	··· ··· ··· ··· ··· ··· ··[a,b]

The primer sequences listed above were derived from identical DNA sequences of SRV-1 and SRV-3, respectively. Internal mismatches of the primers, when compared to the SRV-2 DNA sequence, are noted in lower case. Because the mismatches were not located close to the 3′ end of the nested primers, the sensitivity of the second round of the PCR assay was not affected when tested with plasmid DNA encoding SRV-2 (see text). The outer primers, used in the first round of the PCR assay, had no mismatches.

Superscript *a* indicates a primer sequence; superscript *b* indicates a viral sequence.

amplified effectively. The PCR sensitivity was determined by end-point titration of cloned SRV/D in the presence of carrier DNA. The first PCR round detected 1×10^{-4} to 1×10^{-5} ng of plasmid DNA, representing 8.5×10^3 (SRV-1), 8.6×10^2 (SRV-2), and 6.9×10^2 (SRV-3) proviral copies (Fig. 1A). The first PCR round became more sensitive using "hot start" PCR[18] (not shown). The second PCR round could detect 1×10^{-8} ng of plasmid DNA (Fig. 1B), which represents from 0.7 to 0.9 proviral copies in 150,000 cells.

Genomic DNAs from 15 macaques infected with SRV/D (type 2), and 7 DNAs from specific pathogen-free (SPF) macaques were screened by PCR, and the results were compared to those obtained by EIA, WB and virus isolation monitored by RT (Table 2). All cellular DNA samples analyzed were amplifiable as evidenced by β-actin-specific PCR (not shown). The PCR results correlated well with those obtained by virus isolation. Fifteen of 15 macaque samples shown to be RT positive were PCR positive (Table 2 and Fig. 3B). By simultaneous serological screening, only one animal was positive and three others had indeterminate WB (Table 2). All DNA samples from seven SPF monkeys were PCR negative (Fig. 2A and B). Taken together, these results indicate a sensitivity of 100% and a specificity of 100%.

In summary, we have developed a sensitive DNA PCR assay to detect proviral DNA of SRV/D serotypes 1–3, using conserved *env* sequences, which allowed detection of a single

TABLE 2. CYNOMOLGUS MONKEYS NATURALLY INFECTED WITH SRV/D, TYPE 2: STATUS AND SIMULTANEOUS SEROLOGY (EIA, WB), VIRUS ISOLATION (RT) AND PCR

Animal No.	*Sex*	*Age (months)*	*Clinical signs*	*Serology*	*Virus isolation*	*PCR* (env)
1	F	49	Diarrhea (colitis)	+	+	+
2	M	26	None	IND, p20 only	+	+
3	M	45	None	−	+	+
4	M	45	None	−	+	+
5	F	42	None	−	+	+
6	M	44	None	−	+	+
7	F	42	Diarrhea (colitis), lymphadenopathy, splenomegaly	IND, p20 only	+	+
8	F	40	None	IND, p20 only	+	+
9	M	40	Diarrhea (enterocolitis), lymphadenopathy	−	+	+
10	M	20	None	−	+	+
11	M	7	None	−	+	+
12	M	42	None	−	+	+
13	M	45	None	−	+	+
14	M	41	None	−	+	+
15	F	42	None	−	+	+

Sex, age, clinical status, serology, virus isolation and PCR *(env)* results of cynomolgus monkeys infected naturally with SRV/D, type 2. All animals were infected during an outbreak of SRV/D, type 2, in the colony. Thus, the exact time of infection for each animal is unknown. Following recognition of early clinical cases, the entire colony was screened. Many animals were found to be infected without clinically apparent disease. Serology: IND, indeterminate pattern on Western blot.

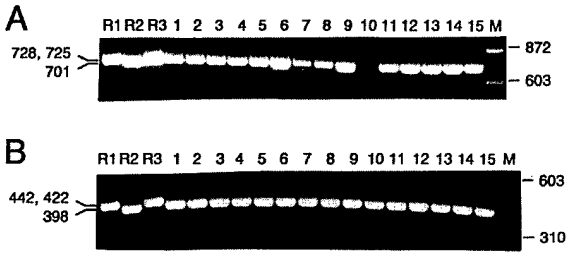

FIG. 3. Screening of 15 cynomolgus monkey DNA samples (lanes 1–15) for the presence of specific proviral SRV/D (type 2) DNA in the first (A) and second (B) PCR round. Raji cells infected with SRV/D, types 1–3 (lanes R1–R3), were used as positive controls. Agarose gels (1.5%) were stained with ethidium bromide. Marker: ϕX174 DNA digested with *Hae*III.

proviral copy in 150,000–210,000 cells. Apparently, the internal mismatches (none of them located within the last five bases of 3′ oligomer ends) of the nested primers with SRV-2 DNA did not affect the yield of PCR product. The SRV/D outbreak among University of California Davis cynomolgus monkeys was due to SRV-2. Our assay was 100% accurate in identifying infected samples. These results agree with those of Kwok *et al.*,[19] who analyzed the effects of various primer–template mismatches on DNA amplification. The fact that 14 RT-positive macaques were seronegative underscores that antibody testing alone is insufficient to detect all infected animals. Of these, 14 animals were PCR positive.

Cocultivation of rhesus PBMCs and sequential screening for virus-associated RT is effective in identifying retrovirus-infected animals, but a 6-week wait for results can be problematic.[10] SRV/D-specific PCR analysis, using either generic or serotype-specific *gag* primers followed by hybridization with radiolabeled oligoprobes, was employed previously to search genomic human PBMC DNA for the presence of SRV/D sequences.[20] This assay could distinguish serotypes 1 and 3 from serotype 2. A recently published PCR approach for SRV/D DNA detection[21] used three different sets of primers specific for each serotype and generic radiolabeled DNA probes. The sensitivity of this assay, although appearing significantly lower, has not been evaluated fully. Our PCR assay uses one set of nested primers for the simultaneous detection of all three serotypes and does not depend on the use of radiolabeled probes. Furthermore, this assay is quantitative since plasmid DNA for each serotype is titrated. Combined with serological testing, PCR represents a rapid, sensitive, and reliable diagnostic tool for colony management and for laboratories using macaque models to study AIDS-related viruses.

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Detection of Simian T Cell Leukemia Virus Type I Infection in Seronegative Macaques

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ABSTRACT

Simian species of Asian and African origin are naturally infected with the simian T cell leukemia virus type I (STLV-I). Like the closely related human T cell leukemia virus type I (HTLV-I), STLV-I is primarily cell associated, and typical infections exhibit low viral burdens. Four macaques experimentally inoculated with a new STLV-I strain isolated from a sooty mangabey monkey were examined over extended periods of time for signs of infection by (1) commercial enzyme immunoassay and immunoblot assay for cross-reactive serum antibodies to HTLV-I, (2) commercial HTLV-I $p24^{gag}$ antigen-capture assay on supernatants from cocultures of macaque peripheral blood mononuclear cells (PBMCs) with human PBMCs, and (3) nested PCR amplification of proviral sequences in macaque PBMC DNA. The nested PCR assay was 100% specific and detected a single STLV-I copy in 150,000 PBMCs. In addition, our data show that experimental infection of macaques with STLV-I can be serologically silent for more than 43 months.

INTRODUCTION

SIMIAN T CELL LEUKEMIA VIRUS TYPE I (STLV-I), an exogenous retrovirus closely related to human T cell leukemia virus type I (HTLV-I),[1–4] has been isolated from numerous genera of Old World nonhuman primates originating in Asia and Africa.[2,3,5,6] STLV-II, a simian counterpart of HTLV-II, has been identified in New World monkeys.[7] As a group, these viruses are referred to as primate T cell lymphotropic viruses (PTLVs). Although infection with STLV-I has been linked etiologically to malignant lymphomas and lymphoproliferative diseases in several primate species,[8–12] most STLV-I-infected animals, like HTLV-I-infected humans, remain healthy and asymptomatic.[5,13] Among different simian populations, the STLV-I seroprevalence varies from 4 to 60%, depending on species, age, and sex of the animals tested.[2,5,6,9,11,12,14,15] The prevalence of STLV-I infection in nonhuman primate populations has been determined primarily by screening for the presence of serum antibodies to HTLV-I using commercial enzyme immunoassay (EIA) and/or Western blot kits.[11–14] However, inconsistencies between virologic and serologic results have been reported, not only for HTLV-I and -II, but also for STLV-I.[2,16,17] These discordant results might be explained either by low sensitivity of the assays, perhaps owing to antigenic differences, or by the observation that asymptomatic infections with these retroviruses are characterized by low viral burdens,[18,19] which could lead to transient or low levels of antibodies as a result of insufficient antigenic stimulation.

Asian macaque species are used routinely in biomedical research. It is imperative, therefore, to rule out clinically silent infections with other retroviruses, such as the simian immunodeficiency virus (SIV), simian retrovirus type D (SRV/D), or STLV-I, prior to study entry. Little is known about the natural history of STLV-I infection because the time of exposure in naturally infected animals is unknown, and the information available on experimentally infected nonhuman primates is scarce.[20,21] In addition, the ability to detect antibodies has not been correlated with viral status at different stages of infection.

To define these parameters in macaques experimentally infected with STLV-I, a sensitive STLV-I-specific polymerase chain reaction (PCR) assay was developed, which was used subsequently to analyze DNA samples from peripheral blood mononuclear cells (PBMCs) collected from four animals at different times after STLV-I inoculation. The PCR results were compared to those obtained by EIA and immunoblot for STLV-I-specific serum antibodies and by antigen capture for $p24^{gag}$

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antigen in cultured macaque PBMCs. The results revealed that STLV-I-infected macaques can remain seronegative for extended periods of time, lasting more than 43 months.

MATERIALS AND METHODS

Animals

Adult rhesus (*Macaca mulatta*) and pigtail macaques (*Macaca nemestrina*) were housed in biosafety level 2 facilities at the University of Alabama at Birmingham (UAB) according to standard practices, as outlined in the *Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, D.C.). Two pigtail macaques (16C, 60C) and one rhesus macaque (R8119) had been inoculated intravenously with a sooty mangabey (sm) (*Cercocebus atys*)-transformed cell line that was productively infected with both SIVsmmPBj14 and a new strain of STLV-I, designated STLV-I(sm). The cell line, FEd-P14, was established by continuous culture after infection with cell-free SIVsmmPBj14 of resting PBMCs from a sooty mangabey that was naturally infected with STLV-I(sm). One pigtail macaque (12-274) was inoculated with a cloned cell line, FEd-H11, that was obtained by limiting dilution of FEd-P14 cells. The FEd-H11 cell line was infected with and expressed only STLV-I(sm), as shown by PCR analysis of proviral DNA and radioimmunoprecipitation of lysates of cells grown in the presence of [^{35}S]cysteine–methionine. Full descriptions of these cell lines and STLV-I(sm) will be published elsewhere.[21a]

Serology

Serum samples from all macaques were tested for antibodies to SIV using the HIV-2 EIA kit (Genetic Systems, Seattle, WA) and to STLV-I using the Veronostika HTLV-I Microelisa kit (Organon Teknika, Durham, NC) or the Genelabs Diagnostics HTLV Blot 2.4 kit (Cellular Products, Buffalo NY). All three commercial kits that detect the human viruses are highly cross-reactive with the simian counterparts.

Isolation and culture of macaque peripheral blood mononuclear cells

Periodically, blood samples were collected from the macaques under ketamine anesthesia. PBMCs were isolated from heparin-treated whole blood processed on Ficoll–sodium diatrizoate gradients (LSM; Organon Teknika). Approximately 5–10 $\times$ 10^6 macaque PBMCs were cultured with 10^7 human PBMCs that had been stimulated 3 days prior with phytohemagglutinin (PHA), as described.[22] Aliquots of cell-free culture supernatants were removed every 4 to 5 days and tested for reverse transcriptase (RT) activity and STLV-I p24^{gag} antigen using an HTLV-I/II antigen assay kit (Coulter, Miami, FL) according to the manufacturer's directions. Some PBMCs were cryopreserved in liquid nitrogen vapor. Prior to the nested PCR assay, PBMCs were thawed, and cellular DNA was isolated using a standard phenol–chloroform method at the UAB. Each sample was coded and shipped to Dana-Farber Cancer Institute for blinded PCR assays. As negative controls, PBMC DNA samples obtained from specific pathogen-free (SPF) rhesus macaques housed at the Department of Veterinary Sciences (University of Texas M.D. Anderson Cancer Center Science Park, Bastrop, TX) were tested.

Construction of Plasmid pBC$_{3\text{-}6}$

On the basis of the complete nucleotide sequence (L. Su and P.N. Fultz, unpublished) of the STLV-I(sm) provirus in a continuous mangabey cell line (FAi/FEd-M9), oligonucleotide primers were designed for amplification of the 3′ half of the STLV-I(sm) genome (Table 1). Using the two primer pairs, FEd-C3 and -C4 or Fed-C5 and -C6, two PCR fragments of 2.3 and 2.8 kb, respectively, were generated. The 100-μl reaction mixture contained 50 mM KCl, 10 mM Tris-HCl (pH 8), 1.5 mM $MgCl_2$, 0.01% gelatin, a 0.2 mM concentration of each dNTP, 40 pmol of each primer, 2 μg of genomic DNA, and 2.5 U of *Taq* polymerase and was overlaid with 60 μl of mineral oil. The PCR was carried out in a PTC-100 programmable thermal controller (MJ Research, Watertown, MA) with denaturation at 96°C, 5 min, and then 35 cycles of 94°C, 60 sec; 60°C, 45 sec; and 72°C, 3 min. After electrophoresis of the PCR products in 0.8% agarose gels, DNA bands of the expected size were excised, purified with the GeneClean kit (Bio 101, Vista, CA), and cloned into the TA cloning vector (TA cloning kit; Invitrogen, San Diego, CA). The ligated products were used to transform JM109 competent cells. Two clones with the correct inserts, pC$_{3\text{-}4}$ and pC$_{5\text{-}6}$, were digested with *Kpn*I and *Hin*dIII, resulting in the 2.3-kb insert from pC$_{3\text{-}4}$ and a linear pC$_{5\text{-}6}$ plasmid containing both the vector and 2.8-kb insert. These two fragments were then ligated at the *Kpn*I site, forming plasmid pC$_{3\text{-}6}$ with a 5.2-kb insert encoding the 3′ half of STLV-I(sm); this insert was then transferred into the Bluescript vector to generate plasmid pBC$_{3\text{-}6}$. The plasmid was sequenced across the junction of the original two STLV-I(sm) fragments to confirm that the ligation did not introduce mutations around that site. Before performing the PCR assay (described below), plasmid pBC$_{3\text{-}6}$ was isolated using a Qiagen plasmid kit (Qiagen, Chatsworth, CA).

Primers and polymerase chain reaction conditions for screening assay

The nested set of primers for DNA PCR was derived from the *env* gene of an STLV-I strain of pigtail macaque (Ptm) origin, STLV-I(Ptm3), and is located in regions that are also conserved in HTLV-I.[4] Sequences of the external and internal primers and their locations relative to the first nucleotide in the *env* gene are as follows: STLV*env*1 (100–123), 5′ TCCTCATATCATTCTAAACCCTG 3′; STLV*env*2 (1161–1141), 5′ CCAAAACAGGAGATCAAGGCC 3′; and the internal nested primers STLV*env*3 (306–327), 5′ CTCTTATTCAGACCCTTGTTC 3′; and STLV*env*4 (782–762), 5′ GCTAACGATGGGTAAAGGAGG 3′. The sequence of STLV*env*4 is identical to the sequence of STLV-I(sm), but the other three primers have a 1-bp mismatch relative to STLV-I(sm). The reaction mixture for the set of external primers, STLV*env*1 and STLV*env*2, consisted of 10 mM Tris-HCl (pH 9.2), 1.5 mM $MgCl_2$, 25 mM KCl (Opti-Prime buffer 9; Stratagene, La Jolla, CA), 100 μmol of each dNTP, 50 pmol of each primer, and 1.25 U of *Taq* polymerase (Amplitaq; Perkin-Elmer Cetus, Branchburg, NJ) in a 50-μl volume. Nested primers STLV*env*3 and STLV*env*4 were used similarly with a modified PCR buffer:

TABLE 1. OLIGONUCLEOTIDE PRIMERS FOR POLYMERASE CHAIN REACTION AMPLIFICATION OF STLV-I(sm) TO GENERATE PLASMID pBC_{3-6}

Primer	*Sequence*[a]	*Primer position*[b]
FEd-C3	5′ TCACTCTTTCCCCGGTGATCATAGACGC 3′	3872–3899
FEd-C4	5′ CGGAGACAAGCCAGACCGCCACC**GGTACC** 3′	6149–6121
FEd-C5	5′ TTCCCGCCGAGC**GGTACC**GGTGGCGGTC 3′	6109–6136
FEd-C6	5′ TGTATACTAAATTTCTCTCCCGGAGAGT 3′	9037–9010

[a]The positions of the *Kpn*I restriction site in primers FEd-C4 and -C5 that were used to ligate the two PCR-amplified fragments are shown in boldface and underlined.
[b]Nucleotide positions are relative to the STLV-I(sm) genomic sequence (L. Su and P.N. Fultz, unpublished).

10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 75 mM KCl (Opti-Prime buffer 6; Stratagene). The *Taq* polymerase was blocked with TaqStart antibody (Clontech, Palo Alto, CA), according to the manufacturer-specified conditions. Typically, 1 μg of macaque genomic DNA, representing about 1.5×10^5 cells, was added just prior to amplification. For nested PCR, 1 μl of product from the first-round PCR was added to 50 μl of the second-round mixture. When amplification of a single sample was negative, five parallel independent reactions for each DNA sample (representing a total of 7.5×10^5 cells) were performed. The PCR was performed in a Perkin-Elmer GeneAmp PCR system 9600 (Perkin-Elmer Cetus). Cycling conditions for the first PCR round were as follows: initial denaturation (98°C, 15 sec) followed by six cycles of "touchdown" amplification, which started with a melting step (97.5°C, 15 sec), annealing (64°C, 40 sec), and extension (72°C, 55 sec). In each subsequent cycle, the melting temperature was lowered by 0.5°C, but melting time remained constant; the annealing temperature was lowered by 1°C, and the annealing time was shortened by 5 sec; extension temperatures stayed constant but the extension time was shortened by 5 sec. Cycling continued (94°C, 15 sec; 50°C for 15 sec; 72°C for 30 sec) for 40 cycles, and ended at 72°C for 6 min. Nested PCR began with denaturation (94°C, 1 min) followed by 40 cycles of annealing (59°C, 15 sec), extension (72°C, 30 sec), and denaturation (94°C, 15 sec) per cycle and terminal extension (72°C, 6 min). The PCR products were analyzed by agarose gel electrophoresis in the presence of ethidium bromide. To determine the sensitivity of the assay, 10-fold serial dilutions of plasmid DNA (1×10^{-1} to 1×10^{-10} ng) were amplified by PCR in the presence of 1 μg of genomic DNA from CEMx174 cells.

RESULTS AND DISCUSSION

First, we determined the sensitivity and specificity of the nested PCR assay. pBC_{3-6} plasmid DNA was amplified using the nested set of primers; the expected first- and second-round PCR products of 1061 and 476 bp, respectively, were observed after agarose gel electrophoresis (Fig. 1A and B). A 476-bp band was also observed after nested PCR amplification of 1 μg of genomic DNA isolated from the STLV-I-infected baboon cell line, X1713 (not shown). To determine the sensitivity of the PCR assay, serial dilutions of pBC_{3-6} plasmid DNA were added to 1 μg of genomic CEMx174 cell DNA. Nested PCR amplification detected 1×10^{-8} ng of plasmid DNA, which corresponds to approximately 1.14 proviral copies in 150,000 cells (Fig. 1A). In addition, this PCR assay amplified the target *env* sequences from cloned HTLV-I (pMT-2[23]), but it did not gen-

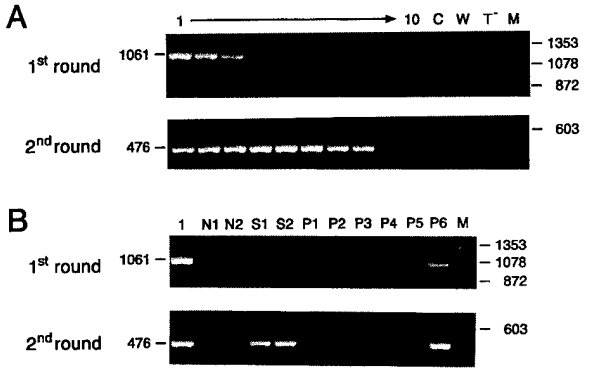

FIG. 1. (A) Sensitivity of STLV-I PCR. Tenfold dilutions of plasmid pBC_{3-6} DNA (1×10^{-1} to 1×10^{-10} ng, lanes 1–10), respectively) were amplified in the presence of 1 μg of CEMx174 cell DNA. Amplified products were resolved on 2% agarose gels and stained with ethidium bromide. The first PCR round of pBC_{3-6} DNA yielded a specific product of 1061 bp, and the second (nested) PCR round yielded a specific product of 476 bp. The last positive dilution of plasmid DNA (1×10^{-8} ng of DNA) is equal to 1.14 proviral copies in 150,000 cells. Controls: CEMx174 cell DNA (C), H_2O without added template (W), and CEMx174 cell DNA without *Taq* polymerase (T^-). Marker: ϕX174 DNA digested with *Hae*III. (B) Controls and standards. Amplicons of first and second (nested) PCR rounds are shown: genomic DNA from two SPF rhesus monkeys (N1, N2), and from two STLV-I-positive rhesus monkeys (S1, S2), respectively. Lanes P1–P6, PCR amplification of plasmid DNAs. Plasmid DNA (1 ng) was amplified in the presence of 1 μg of CEMx174 cell DNA: pSRV1 (SRV-1) (P1), D2C/Oregon (SRV-2) (P2), pSHRM15 (SRV-3) (P3), pSVIIIenv (*env* of HIV-1) (P4), p239SpE3′ (3′ half of SIVmac239) (P5), pMT-2 (HTLV-I) (P6), and pBC_{3-6} (STLV-I) (1) as positive control. Agarose gels (2%) were stained with ethidium bromide. Marker: ϕX174 DNA digested with *Hae*III.

erate products using 1 ng of molecularly cloned HIV-1 *env* sequences (pSVIIIenv[24]), SIVmac239 *env* sequences (p239-SpE3[25]), or simian retrovirus type D, serotypes 1 (pSRV-1[26]), 2 (D2C/Oregon[27]), and 3 (pSHRM15[28]) proviral sequences (Fig. 1B). DNA samples from PBMCs of four uninfected control macaques as well as two samples obtained from SPF rhesus macaques were repeatedly negative (Fig. 1B and not shown). Together, these results indicate that the PCR assay was 100% specific for STLV-I/HTLV-I.

Next, cryopreserved PBMCs obtained at different times after experimental inoculation of four macaques with a simian cell line expressing STLV-I(sm) were subjected to PCR analysis. For two animals (16C, 12-274), PBMCs were available either before or at the time of virus exposure and for an extended period thereafter. For the other two animals (60C and R8119), PBMCs from late times were tested to confirm the persistence of STLV-I(sm)-infected cells in the periphery. Although sera from all macaques were tested regularly for antibodies to STLV-I by ELISA and PBMC cocultures were analyzed for the release of STLV-I(sm) $p24^{gag}$ antigen into culture supernatants, results are shown only for times at which PBMCs were also available for STLV-I(sm) DNA PCR or when sera were tested by immunoblot assay (Table 2).

The most striking observation was the failure to detect antiviral antibodies by ELISA at any time in sera from macaques R8119 and 16C, both of which were clearly infected with STLV-I(sm) by the other assays. To verify these results, selected serum samples from all four animals were tested by immunoblot assay (Fig. 2). With the exception of the 2-month sample from 16-C, which showed reactivity to a recombinant epitope specific for both HTLV-I and -II (GD21) and faint reactivity to rgp46-I and the gp21/p19 band, none of the other sera from these two animals were unequivocally seropositive. Although all serum samples from all macaques reacted with the

TABLE 2. SUMMARY OF ANALYSIS OF SAMPLES FROM MACAQUES INFECTED WITH STLV-I(sm)

Macaque	*Months after infection*	*HTLV-I Ag⁺ culture*[a]	*Serology*[b]		*PCR positive*[c]
			Blot	*ELISA*	
60C	5	+++	+	+	ND
	9	++	ND	−	1/1
	23	−	+/−	−	ND
	34	+++	+	+	1/1
	43	+++	+/−	−	ND
R8119	2	ND	−	−	ND
	6	−	−	−	ND
	12	−	−	−	ND
	23	++	ND	−	1/1
	32	++	−	−	ND
	37	−	ND	−	1/1
	43	++	−	−	2/2
12-274	0	−	ND	−	0/1
	1.5	++	+	+	1/1
	4	−	ND	+/−	1/5
	8	++	+	+	3/5
	13	++	+	+	5/5
16C	−1	ND	ND	−	0/1
	2	ND	+/−	−	0/5
	6	++	−	−	0/5
	12	++	ND	−	3/5
	20	++	−	−	0/5
	32	+++	−	−	1/4
	41	+++	ND	+/−	5/5
	43	+++	−	−	ND
	44.5	+	ND	−	1/5

[a]Macaque PBMCs were cocultured with human PBMCs that had been stimulated with PHA for 3 days. Cocultures were maintained for at least 6 weeks, with fresh human PBMCs added every 10 days. Periodically, samples of cell-free culture medium were tested for the presence of STLV-I(sm) $p24^{gag}$ antigen. The number of + signs reflects relative OD values of medium samples. ND, Not done.

[b]Macaque serum samples were tested for antibodies to STLV-I using the Genelabs Diagnostics HTLV Blot 2.4 kit or the Vironostika HTLV-I Microelisa kit. For the immunoblot results: +, reactivity to one Gag protein and two Env bands (GD21 and rgp46-I); +/−, reactivity to GD21 and very faint reactivity to Gag and rgp46-I; −, reactivity only to GD21.

[c]Values reflect number of independent nested PCR amplifications that were positive for STLV-I(sm) provirus divided by the total number of PCR assays attempted with each DNA sample.

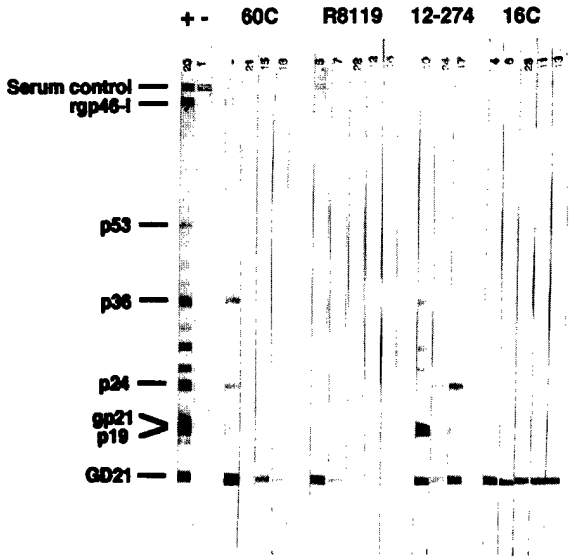

FIG. 2. Immunoblot reactivity of serum samples from STLV-I(sm)-infected macaques. Serum samples were thawed, heat inactivated, and 20 μl of each sample was tested for antibodies to specific proteins, using the HTLV Blot 2.4 kit (Genelabs Diagnostics) according to manufacturer instructions. Sera from individual animals, as indicated above each group of antigen strips, are placed together; the positive (+) and negative (−) control sera are shown to the left. HTLV-specific proteins are indicated by their molecular weights. The times after STLV-I(sm) inoculation at which serum samples were collected from individual animals are as follows (from left to right): 60C—5, 23, 34, and 43 months; R8119—2, 6, 12, 32, and 43 months; 12-274—1.5, 8, and 13 months; 16C—2, 6, 20, 32, and 43 months.

GD21 polypeptide, because no other virus-specific proteins were recognized, the results can only be interpreted as indeterminate. If other commercial or laboratory-generated immunoblot strips in which the GD21 epitope was not present were used, these sera would clearly be considered seronegative. Thus, R8119 and 16C consistently were seronegative (by immunoblot or ELISA) for anti-STLV-I antibodies throughout more than 3.5 years of infection. The serological results for macaque 60C were variable, but, in general, this animal had low levels of antibodies that decreased significantly with time. Such a phenomenon would be consistent with that observed by Nakamura *et al.*,[20] who reported loss of virus-specific antibodies in a macaque experimentally infected with HTLV-I.

Virus isolation by PBMC cocultivation followed by detection of STLV-I(sm) $p24^{gag}$ antigen in culture supernatants and detection of proviruses by the nested PCR assay yielded comparable results. In only one instance did the antigen capture assay fail to detect virus in the culture supernatant when the PCR assay was positive (R8119, 37 months). The reverse was true with two samples from macaque 16C (6 and 20 months). However, for the nested PCR assay to detect proviruses in some PBMC samples, multiple independent reactions were required, which is consistent with low numbers of PBMC harboring STLV-I(sm) proviral DNA. Because the sensitivity of the PCR assay is 1 copy in 150,000 cells, these results indicate that the viral burdens in these animals at different times after infection ranged from one or more proviruses in fewer than 150,000 cells (all PCR assays were positive) to fewer than 1 provirus in 750,000 cells (none of 5 PCR assays was positive). Finally, for macaque 12-274, after the first few weeks of the acute phase of infection, the percentage of positive PCR assays decreased (Table 2; macaque 12-274, 4 months) and then increased with time, suggesting an increasing viral burden in PBMCs. These results are consistent with those obtained by both serologic assays with serum samples from this animal.

Because Asian macaques and some monkeys of African origin are used routinely as models for human infectious diseases, such as those caused by HIV and HTLV-I, it is critical that animals infected with the simian counterparts of these viruses be identified prior to study enrollment. Most frequently, commercial reagents generated with HTLV-I antigens have been utilized to assess the seroprevalence of STLV-I among different simian populations. PCR studies have shown that some humans and monkeys harboring PTLVs do not have detectable antibodies in their sera.[2,16,17] In the examples reported here, failure to seroconvert (as in animals 16C and R8119) or having variable antibody responses (e.g., macaque 60C) strongly suggests that serologic assays underestimate the true prevalence of infection. In agreement with our study, Saksena *et al.*[2] reported that experimental transmission of STLV-I to African green monkeys (AGMs) by blood transfusion resulted in seroconversion as early as 1 month and as late as 1 year. In one case, at 3 years after transfusion, one AGM had developed only weak reactivity to HTLV-I antigens by immunoblot, which is similar to our observations with macaque 60C. That all serum samples from all four animals reacted with the recombinant GD21 band on immunoblots indicates that infection might have elicited a limited humoral immune response against STLV-I(sm); however, it should be emphasized that reactivity only with GD21 is not diagnostic of infection. As indicated above, any serum samples similar to those described here that are tested with immunoblot strips into which this recombinant polypeptide has not been incorporated would appear to be negative.

Although one might argue that the insensitivity of the serologic assays could be ascribed to the use of HTLV-I antigens, this is unlikely to have influenced the results reported by us and others for two reasons. First, a dominant immunogenic and neutralizing antibody domain in gp46 is highly conserved in multiple STLV-I and HTLV-I strains.[29,30] Second, nucleotide sequence and phylogenetic analyses of STLV-I(sm) and its comparison to HTLV-I and other STLV-I strains show that STLV-I(sm) branches with cosmopolitan HTLV-I and STLV-I strains of African origin. In fact, of the PTLV type I strains, the one most distantly related to STLV-I(sm) is the prototype STLV-I(Ptm3), which is of Asian origin (P.N. Fultz, L. Su, and E. Lefkowitz, unpublished). Despite any genetic diversity that might exist among PTLVs, the sensitivity of the nested PCR assay described herein is not compromised. Furthermore, because the four primers used in this assay are in regions highly conserved in both HTLV-I and STLV-I isolates, the assay is not specific for either PTLV subfamily.

Dezzutti *et al.*[31] previously described an HTLV-I-specific PCR assay for detection of STLV-I proviral sequences in PBMCs from infected monkeys. Their PCR assay used primers specific for *tax* and *pol* genes and was followed by Southern

blotting and hybridization with radiolabeled HTLV-I-derived oligonucleotide probes. Using this procedure, 1 infected cell among 100,000 cells could be detected. In comparison, our PCR assay can detect a single target DNA sequence among 150,000 cells and, more importantly, eliminates the hybridization step as well as the use of radiolabeled probes. Although the PCR and coculture assay results were comparable, cultures must be maintained for several weeks. Thus, the PCR assay is a more rapid method for screening captive and feral monkeys and should be useful for investigators using simian models to study T lymphotropic retroviruses and for colony management.

In summary, our results and those of others indicate that one cannot rely on serologic assays to identify STLV-I-infected nonhuman primates with accuracy. False-negative results can be obtained because some infected monkeys (and humans) do not develop antibodies until several months or years after infection. Alternatively, antibody responses may be transient, and some infected primates may never develop antibodies. Thus, a combination of assays should be used to monitor monkeys for natural infection or to follow experimental infection with STLV-I.

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Protective Immunity Generated by Low Doses of a Live, Pathogenic Retrovirus

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Running title: **Protective Immunity from Low Dose Retrovirus Inoculation**

SUMMARY

Mice inoculated with high, lethal doses of Rauscher Murine Leukemia virus (RLV) and treated with antiviral drugs cleared the virus. After cessation of treatment, they resisted virus rechallenge. Adoptive transfer demonstrated that cellular immunity is key for this protection. DNA PCR revealed that virus replicated to low levels despite antiviral therapy. Eventual provirus clearance coincided with the emergence of specific CTL responses. To test whether host cell immunity alone could protect against low doses of live virus, we titrated an RLV stock in normal and nude BALB/c mice; the apparent infectivity was 10-fold greater in the T cell deficient mice. Normal mice without viremia after exposure to low doses of live virus were rechallenged with fully infectious doses; 30% were immune. We conclude that protective immunity can be generated by exposure to subthreshold doses of live, fully pathogenic virus.

INTRODUCTION

We have postulated that following retrovirus infection, disease will only develop if a certain threshold of virus load is surpassed in a given host (Baba et al., 1995, Ruprecht et al., 1996). No disease will occur if overall viral burden is maintained below such a threshold, even if the retrovirus encodes all genes necessary for virulence. Furthermore, we hypthesize that host cellular defense mechanisms are key determinants for this threshold effect.

Previous results suggest that, indeed, host factors play a major role in maintaining viral loads below pathogenic levels. For example, inoculation of adult macaques with a replication-impaired simian immunodeficiency virus (SIV) mutant, deleted in either *nef* alone or in both the *nef* and *vpr* genes and the negative regulatory element of the long terminal repeat, (SIVmacΔ3), caused no disease and induced a degree of protection against subsequent challenge with wild-type SIV (Daniel et al., 1992; Wyand et al., 1996). However, we and others demonstrated that SIVΔ3 itself is pathogenic in neonatal macaques (Baba et al., 1995; Wyand et al., 1997). Differences in the pathogenicity of SIVΔ3 in adults and neonates were not due to changes in the innate virulence of the virus nor to the route of inoculation (Baba et al., 1995; Ruprecht et al., 1995). Neither neutralizing nor infection-enhancing antibodies correlated with attenuation in adults or AIDS in infants (Montefiori et al., 1996). We believe that cellular host immune defenses in the adults, but low or lacking in the neonates, kept viral replication below threshold levels in adults.

For many types of virus infections, specific cytolytic T lymphocyte (CTL) activity is clearly associated with viral clearance and/or protection from disease. Examples include infection with hepatitis B (Penna et al., 1991; Nayersin et al., 1993), hepatitis C (Spenglar et al., 1996), lymphocytic choriomenigitis virus (LCMV) (Matloubian et al., 1994; Zinkernagel et al., 1985), influenza (Yap et al., 1978; Taylor and Askonas, 1986) and Sendai virus (Hou et al., 1992). In addition, many studies have now suggested the importance of virus-specific CTL responses for the control of viremia in persons with human immunodeficiency virus type-1 (HIV-1) infection, especially during the initial stages of primary infection (Carmichael et al., 1993; Borrow et. al., 1994; Koup et al., 1994).

Evidence for cell-mediated immune defenses as a mechanism for clearance of retrovirus infection was demonstrated in our previous studies using the Rauscher murine leukemia virus (RLV) model. Protective immunity could be generated by inoculating mice with a potentially lethal dose of RLV whose replication was blocked by combination antiretroviral drug therapy (3'-azido-3'-deoxythymidine (AZT) and interferon-α (IFN-α)). Mice were inoculated with high-dose virus and 4 hrs later, treatment was started. Drug treatment was continued for 3 weeks. After this time, animals showed no evidence of infection (Ruprecht et al., 1988). These animals were then rechallenged with a fully infectious dose of RLV and >95% of the animals developed neither viremia nor disease (Ruprecht et al., 1990a). In effect, a live pathogenic virus actually acted as an attenuated strain vaccine because antiretroviral drug therapy restricted its

replication. Viral loads were kept at levels which did not exceed pathogenic thresholds yet allowed the generation of protective immune responses. Adoptive transfer experiments indicated that protective immunity was mainly cellular and required both $CD4^+$ and $CD8^+$ T lymphocytes (Ruprecht et al., 1990b; Hom et al., 1991).

One aspect of our study was to test if CTL activity constituted part of the protective cellular immune response observed in mice receiving post-exposure chemoprophylaxis following high-dose RLV inoculation. We found RLV-specific CTL activity coincided with clearance of a transient, low-level infection. We also tested if similar sub-pathogenic, immunizing levels of virus could be attained by exposing mice to low-dose, pathogenic RLV without antiviral drug therapy. Thirty percent of mice which cleared low doses of definite live virus were protected from subsequent challenge with higher dose RLV.

RESULTS

CTL activity in RLV-inoculated, drug-treated animals.

We first asked if RLV-specific CTL responses constituted part of the cellular immune response which plays a key role in preventing viremia in mice inoculated with RLV and receiving antiviral drug treatment. Mice were injected with a high dose of RLV (5000 50% animal infectious doses; AID_{50}), and drug therapy was started 4 hr later. At various time points after RLV inoculation, *ex vivo* RLV-specific CTL activity in spleen cells was measured. CTL activity was observed first on day 11, peaked on day 21 and dropped off rapidly by day 24 post-infection (Figure 1). No CTL activity was present in spleen cells of control mice that received the drugs alone.

Negative selection experiments indicated that the bulk of the RLV-specific CTL activity in spleen cells from RLV-inoculated, drug-treated mice resided in the $CD8^+$ T cell population (Figure 2). In some animals, RLV-specific CTL responses could not be detected (Figure 1; data not shown), and in 1 of 6 mice tested, CTL activity was still observed despite $CD8^+$ T cell depletion (data not shown).

One group of RLV-inoculated, drug-treated animals was rechallenged with 20 AID_{50} of virus after demonstrating clearance of the initial RLV infection by day 21. Interestingly, only a small RLV-specific *ex vivo* CTL response was found in

the spleen cells of these animals on day 1 after challenge (11-12% specific cytotoxicity) and little, if any, CTL activity was seen thereafter (data not shown).

Temporal clearance of virus infected cells.

To measure the extent of virus infection after inoculation with high dose RLV in conjunction with antiviral therapy, semi-quantitative polymerase chain reaction (PCR) analysis of DNA from splenic isolates using oligonucleotide primers specific for proviral RLV sequences was performed. Since the mouse genome contains multiple copies of integrated retroviral sequences, primers specific for the exogeneous RLV needed to be found. Among several sets of primers derived from *env* sequences and from the U3 region of the 3' LTR of cloned Rauscher Spleen Focus-Forming Virus (R-SFFV) (pBC10) (Bestwick et al., 1984) tested, those described here were specific for an exogeneous RLV sequence as no signal was seen in genomic DNA obtained from naive BALB/c mice (Figure 3).

In most mice injected with RLV, virus-specific sequences were detected immediately after inoculation, even after 15 min (Figure 3). These signals were present in the virus inoculum and may have resulted from intravirion cDNA contamination, as has been seen with other viruses (Zhang et al., 1993). In untreated, RLV-exposed mice, the band intensity greatly increased beginning on day 4 and continued to do so through the time course of the experiment. In virus-exposed, drug-treated mice, the band intensity also increased on day 4 but

began to decrease by day 8. On day 20, either a faint band or no signal at all was observed in drug-treated, virus-exposed mice. Numerous negative control DNA samples were included in the analysis; 95% of 60 known negative control samples proved to be negative. Thus, the faintly positive scoring spleen DNA samples of animals given post-exposure chemoprophylaxis are not likely due to random contamination. It is of interest that the timing of the decrease in band intensity on day 8 precedes the onset of observable CTL activity (day 11) yet peak CTL activity coincides with the disappearance.

Immunosuppression after acute virus exposure accelerates disease progression.

To further explore the role of cellular immunity during acute RLV infection, groups of mice were treated with cyclosporin A (CsA), with vehicle only or left untreated after virus inoculation. CsA-treated mice had a significantly shorter survival as compared to control animals (Figure 4).

Apparent infectivity of an RLV stock in normal and immunodeficient animals.

Next, we asked whether cellular immunity could keep viral replication below threshold if animals were inoculated with a low-dose of RLV in the absence of antiviral drug treatment. To test this, the apparent infectivity of a stock of RLV was compared between normal, immunocompetent BALB/c mice

and isogenic, T cell-deficient nude mice. Both sets of animals were inoculated with decreasing concentrations of an RLV stock and tested for viremia 21 days later. Individual sera were tested for the presence of RLV antigens by immunoblotting, and results were confirmed by XC assay. The results of this virus titration are shown in Figure 5. In both sets of animals, a dose-dependent response was seen. The AID_{50} in normal mice was approximately a $\log_{10}$ higher than the AID_{50} for BALB/c nude mice.

The data in Figure 5 showing the infectivity of low doses of RLV in nude mice indicate the presence of live virus in these low dose inocula. Thus, the finding that 100% of the nude mice became infected if they received inoculations of $\geq$ 0.4 AID_{50} confirmed the presence of live virus at these dosages.

Protection against lethal challenge following low-dose RLV exposure.

Next, we tested whether inoculation with low-dose, live RLV could induce protective immunity in the absence of drug therapy. Normal mice that tested negative for RLV infection by immunoblotting and XC assay 3 weeks after low dose inoculation (Figure 5) were challenged iv with 20 or 200 AID_{50} of RLV. Animals were sacrificed 21 days after challenge, the degree of splenomegaly was measured, and sera and spleen cells were analysed for RLV antigens by immunoblot analysis. Partial protection was observed in all groups of animals challenged with 20 AID_{50} (Table I). However, only the two highest initial doses (0.4 and 2.0 AID_{50}) were 100% infectious in the nude mice (Figure 5). Therefore,

live virus was present with certainty at these doses. Overall, 30% of the mice that definitely received live RLV in the initial low-dose inocula remained virus free after rechallenge with higher doses of RLV. In all cases, the presence or absence of virus by immunoblot analysis was confirmed by XC assay (data not shown). Surprisingly, no *ex vivo* RLV-specific CTL activity was detected at any time after low-dose RLV inoculation (data not shown).

Protection is long term.

Mice which were initially inoculated with low-dose RLV and found to be virus free 21 days after challenge with 20 or 200 AID_{50} of virus were reanalyzed 8 months after challenge for signs of viremia. Serum from each mouse was assayed for RLV antigens and for infectious virus by XC assay. Among the 4 animals which were virus free after challenge with 200 AID_{50}, 3 remained free of RLV and 4 of 5 animals challenged with 20 AID_{50} were still virus free at this time. Sera from the other two mice were positive for RLV antigens (data not shown).

DISCUSSION

The key findings of this study are: 1) mice inoculated with high-dose RLV and given antiviral drug therapy have a transient infection with clearance of proviral DNA, 2) there is a temporal association of RLV-specific $CD8^+$ CTL activity with clearance of provirus, 3) the AID_{50} of a given stock of RLV is approximately 10-fold lower in nude mice as compared to isogenic, immunocompetent mice, and 4) approximately 30% of the immunocompetent mice which cleared low doses of live virus were protected against subsequent challenge with a potentially lethal dose of RLV.

The results present a seeming paradox – inoculation with a live pathogenic virus can act as a vaccine against subsequent infection. Mice inoculated with high-dose RLV and given drug treatment had a transient level of infection which generated a protective immune response. Mice inoculated with low-dose RLV also demonstrated both viral clearance and protective immunity but the percentage of animals which were protected was lower than was observed among high-dose, drug-treated mice. Mice inoculated with low virus doses without drug treatment clearly were exposed to live virus, as evidenced by 100% infectivity of the same doses in nude mice. The mechanism underlying the difference in protection between these two sets of animals is not understood but may be related to the initial number of virus-infected target cells subsequently presenting viral antigens.

For many virus infections, including influenza, hepatitis B, and LCMV, virus-specific CTL responses are associated with virus clearance (Doherty et al., 1992, Ada and Jones, 1986). After high-dose RLV inoculation plus chemoprophylaxis in mice, we observed a strong temporal correlation between virus specific CTL activity and proviral clearance. Immune protection generated by high-dose inoculation plus antiviral therapy required both $CD4^+$ and $CD8^+$ immune T cells, and no neutralizing antibodies were found after this live-virus vaccination (Hom et al., 1991; Ruprecht et al., 1990a). It is intriguing to speculate that the greater incidence of protection observed in the mice exposed to high doses of live, pathogenic virus under drug coverage is due to a high level of RLV-specific CTL activity. It is unclear why direct RLV-specific CTL activity following low-dose inoculation was not observed as part of the cellular immune response. Experiments are ongoing to assess other immune correlates of protection after low-dose inoculation.

Low-dose infection can induce immune protection against certain other pathogens. In one model, normally susceptible mice inoculated with low-dose *Leishmania major* are resistant to subsequent, normally pathogenic challenge doses (Bretcher et al., 1992). Protection is associated with cell-mediated immunity while *L. major*-specific antibody responses are diminished in a manner similarly described in earlier studies as "low-dose paralysis" (Mitchison, 1964; Stumpf et al., 1977). Low-dose exposure to hepatitis B virus or LCMV can also evoke protection against disease (Peters et al., 1991, Buchmeier et al., 1980). In

these cases, the protective immune response is apparently but one element of a complex, dynamic balance between virus and host immunity which can potentially include immunopathologic consequences at higher virus doses. (Zinkernagel and Hengartner, 1994).

Previous studies of SIV infection in rhesus macaques also examined whether low-dose inoculation with live, pathogenic retrovirus can induce immune protection against subsequent high-dose challenge. In some cases, resistance to high dose challenge was observed (Salvato et al, 1994; Clerici et al, 1994; Dittmar et al., 1995). In those studies, however, it was not possible to distinguish live virus from dead virus particles in the low dose inoculation preparations. Retroviral stocks are known to harbor only a small fraction of infectious virus particles (Klement and Nicolson, 1977). By directly comparing the apparent infectivity of a given virus dose in normal versus isogenic, nude mice, we were certain that the immunocompetent animals received live virus. At low doses in other systems, inoculation with infectious virus is not necessarily excluded but complete assurance of giving live virus is lacking. In addition, the involvement of cellular immune responses could not be addressed directly in the primate studies. Our finding that the apparent infectivity of a given RLV stock is 10-fold greater in nude mice as compared to normal mice offers a clear demonstration that cell-mediated immunity is required to achieve viral clearance after low-dose inoculations.

The low-dose protection model in this study is similar to those cases of HIV-exposed individuals who neither seroconvert nor show signs of persistent HIV infection, yet harbor HIV-1-specific T-cell immunity. These include infants born to HIV-1-infected mothers, health care workers exposed to HIV-infected blood or body fluids, prostitutes, i.v. drug users, and individuals who have had unprotected sexual intercourse with HIV-1 seropositive partners (reviewed in Shearer and Clerici, 1996; Rowland-Jones and McMichael, 1995). In many cases, these individuals have HIV-1 specific CTL or Th1-type immune responses and these may represent instances of a low-dose exposure to HIV-1 or defective virus which was of sufficient magnitude to induce a protective immune response. This would appear to be the case in individuals who have had multiple exposures and remain virus free (Paxton et al., 1996). In these clinical studies, however, it cannot be determined if the observed immune responses were truly protective and had cleared a transient, potentially pathogenic retrovirus infection. Transient retroviral infection has been described in children born to HIV-1-positive mothers (Bryson et al., 1995, Roques et al., 1995, Newell et al., 1996); however, the mechanism of viral clearance is unknown. Our current results in a murine leukemia virus system show a temporal association of RLV-specific CTL activity and clearance of RLV provirus. Furthermore, our results indicate that protective cellular immunity is, indeed, possible after a single, low-dose exposure to a live retrovirus. Apparent resistance to retroviral infection could also be due to a variety of genetically defined determinants, including, in

the case of HIV-1 infection, a homozygous deletion in the gene encoding the CCR5 receptor (Huang et al., 1996). However, our findings indicate that resistance could alternatively be ascribed to a protective vaccine effect induced by prior transient infection with low virus doses that were unrecognized.

Our data are consistent with a general hypothesis, outlined schematically in Figure 6, which describes potential short-term and long-term outcomes following retrovirus exposure. Central to this hypothesis is the idea that disease will occur only if viral loads exceed a certain disease threshold level. If viral load is maintained below this level, no disease will occur, even if the virus is fully replication competent and pathogenic. This concept was initially postulated to explain the observed differences in pathogenicity between adult and neonatal rhesus macaques after exposure to SIVΔ3 (Baba et al., 1995). We also postulate the existence of both a vaccine threshold – a level of viral load which must be attained to induce protective immunity in the host animal, and a virus detection threshold, below which it cannot be determined whether a host animal harbors virus. In our mouse model, animals inoculated with high-dose RLV and left untreated are represented by Pathway (1) which shows viral load exceeding the disease threshold. Greater than 95% of mice given high-dose RLV inoculation plus drug treatment, and 30% of mice exposed to low dose, live virus are represented by Pathway (2). Here, a transient level of viral load was induced, high enough to surpass a putative vaccine threshold level and generate a protective cellular immune response, yet kept below disease threshold levels.

The remaining 70% of low-dose, RLV-inoculated animals that cleared virus but did not resist challenge are represented by Pathway (3). In these animals, transient viral load was not sufficient to generate protective immunity. Importantly, the 10-fold difference in the amount of RLV necessary to establish persistent infection in immunocompetent mice as compared to nude mice provides direct evidence for the threshold effect in a host animal.

Measurements of retrovirus load in a host are restricted by detection methods and below the virus detection threshold, it cannot be determined if a host has truly cleared virus infection. As such, detectable levels of virus may appear in host animals at later time points from previously undetectable latent infection (Figure 6, Pathway 4). An example of this was observed in a long-term follow up of mice which received low-dose inoculation and then appeared to clear a challenge dose of RLV. Eight months after challenge, 2 mice had become RLV positive. Furthermore, if at any time point after exposure virus production is not kept in check by immune surveillance mechanisms, viral loads can eventually exceed disease thresholds (Pathway 5).

The results of this study clearly demonstrate that inoculation with a live, pathogenic retrovirus can induce protective immunity against subsequent challenge with a potentially lethal dose of virus. The next step will be to elucidate some of the correlates of this immune protection. Though our results have clear relevance towards understanding retrovirus-specific immunity, it is not a realistic possibility to directly use this approach for vaccine development. A

successful vaccine must demonstrate both efficacy and safety. Inoculation with a live, pathogenic virus at any dose does not satisfy the latter requirement.

EXPERIMENTAL PROCEDURES

Mice and virus. Six- to eight-week-old female BALB/c and BALB/c nu/nu mice (Taconic Farms, Germantown, NY) were used for all experiments. RLV (strain RVB3), derived from the original stock, was prepared by tail vein injection (i.v.) of 10^4 plaque forming units (PFU) of RLV into mice. Single-cell suspensions of spleens obtained from animals sacrificed on day 20 were prepared in RPMI-1640 medium (2 ml/g spleen) supplemented with 20% fetal calf serum and cell supernatants were prepared and stored in liquid N_2. The number of PFU in the stock was determined by XC plaque assay (Rowe et al., 1970).

RLV inoculation and drug treatment. For the first sets of experiments, mice were inoculated with RLV and treated with AZT and IFN-α as described (Ruprecht et al., 1990a, Hom et al., 1991). Mice were inoculated i.v. with 10^4 PFU of RLV in a volume of 0.2 ml. Four hr later, 10^4 Units of recombinant human IFN-α A/D (Hoffman-LaRoche, Nutley NJ) (IFN-α), formulated in 0.1 mg/ml mouse albumin (Sigma Chemical, St Louis, MO) were administered intraperitoneally (i.p). Oral administration of AZT (0.1 mg/ml in drinking water) was also started at this time and continued for 21d. For experiments without antiviral drug therapy, RLV inoculation doses were as shown.

Cyclosporin A (CsA) Treatment. The CsA stock solution (100 mg/ml in olive oil) (Sandoz Pharmaceuticals, Hanover, NJ) was diluted further in light mineral oil (Sigma). A dose of 25 mg/kg/day in 50 µl was given i.p. for 21 d. Control mice received either 50 µl of a mixture of olive oil + mineral oil (1:10) or nothing.

Spleen cell populations. At the indicated time points after RLV inoculation, mice were sacrificed, the spleens were removed and teased apart with forceps. Spleen cells were isolated by centrifugation over Lympholyte-M (Accurate Chem. Westbury, NY), washed in RPMI-1640 with 10% FCS (assay medium), and tested either immediately for CTL activity or fractionated further.

Highly enriched $CD4^+$ or $CD8^+$ T cells were prepared by negative selection using Cellectplus immunocolumns (Biotex Laboratories, Edmonton, Canada) according to the manufacturer's instructions. For each depleted population, <1% stained positive for respective CD4 or CD8 antigens as assessed by flow cytometry.

Cytotoxic T Lymphocyte Assays. RLV-infected target cells were prepared by incubating P815 cells (BALB/c mastocytoma cell line) with RLV (5×10^4 PFU/10^6 cells) in the presence of Polybrene (Sigma Chemical; 8 mg/ml) overnight at 37°C in culture medium (Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 10 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin). Cells were expanded and stained for

RLV Env and Gag antigen expression using anti-gp70 Env mAb 48, and anti-p30 Gag mAb R187 (both from ATCC), which had been developed against Friend MuLV but cross react with RLV antigens (Chesebro et al. 1983). RLV-infected P815 cells were sorted by flow cytometry for Env expression, expanded and checked routinely for Env and Gag antigen expression which was maintained at >90%.

To measure specific cytotoxicity, RLV-infected or noninfected P815 cells were radiolabeled by incubation with $Na^{51}CrO_4$ (New England Nuclear; 100 $\mu Ci/10^6$ cells) for 1 hr at 37°C, washed and incubated with the indicated effector cells in 96-well round bottom plates. After 5 h, supernatants were harvested onto SCS harvesting frames (Skatron Inc., Sterling, VA) and released ^{51}Cr measured in a γ-counter. Assays were performed in triplicate and the percent specific cytotoxicity was determined according to the formula:

$$\frac{\text{(experimental release - spontaneous release)}}{\text{(total release - spontaneous release)}} \times 100$$

where spontaneous release is that of target cells cultured in medium alone and total release that of target cells lysed in 1% NP-40.

Primer Design and PCR. Isolation of mouse spleens, DNA preparation and all PCR reactions were carried out in a separate room wearing surgical gown, cap, and mask to avoid contamination. Each animal was dissected with a separate

set of instruments and DNA isolated from single-cell suspensions as published All PCR reactions with the exception of positive controls were assembled in a SterileGuard™ hood. Nested primer pairs were derived from the *env* region for the plus strand and from the U3 region of the 3'LTR for the minus strand. The first round primers were 5'-CGTTGATATTCACCATCA-3' (nt 1444 to nt 1461); 5'-CGTTACTGCGGCTATCA-3' (nt 1679 to nt 1663) and the second round primers were 5'-GCATTCTTAATCAGGATCTCA-3' (nt 1508 to nt 1528); 5'-GCAACTTGGTGGGGTCGTTCA-3' (nt 1656 to nt 1636). One to 4 µg genomic DNA were amplified according to the manufacturer's instructions using DNA amplification kits from Cetus (Perkin Elmer, Newton, MA) and an ErikompTwin Block™ thermocycler. To determine PCR sensitivity, different copy numbers of cloned Rauscher Spleen Focus-Forming Virus (R-SFFV) DNA (plasmid pBC10; Bestwick et al. 1984) were amplified in the presence of 1 µg or 4 µg of genomic spleen DNA of naive mice. PCR reactions were covered using the Ampliwax™ system (PerkinElmer) in addition to hot start method to increase sensitivity. The temperature cycle program was as follows: 94°C for 1 min, 55°C for 90 sec, 72°C for 90 sec, all for a total of 35 cycles, followed by final extension for 10 min at 72°C. In comparison to light mineral oil which allowed detection of 1 copy per 10^4 cells using nested primers, the Ampliwax system with hot start resulted in a several-fold increase in sensitivity; during one round of PCR, 1-5 copies of

pBC10-plasmid DNA could be detected in the presence of 4 μg of genomic DNA, corresponding to approximately 6 x 10^5 cells.

XC plaque assay. Sera from RLV-exposed animals, diluted with phosphate-buffered saline (PBS) 1:10 and filter sterilized, were tested for infectious virus by XC plaque assay (Rowe et al., 1970). In brief, on day -1, SC-1 cells (mouse fibroblast line) were cultured in 6 well plates (5 x 10^4 cells /well using cell culture media) and the following day, Polybrene was added to a final concentration of 8 μg/ml. One hr later, test sera or stock RLV were added. On day 1, wells were washed 2x, and fresh medium was added. On day 5, cultured SC-1 cells were killed by UV irradiation and XC cells were added (5 x 10^5/well). Medium was replaced on days 6 and 7 and the XC cells were stained on day 8 with methyleneblue (0.33%)/carbol fuchsin (0.17%) stain to visualize plaques.

Immunoblot analysis for RLV. Spleens were homogenized in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride), and total protein of both spleen and serum samples was measured using Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA). Twenty μg of total protein from sera or spleen samples was separated by 10% SDS-PAGE and transblotted onto Immobilon-P membranes (Millipore, Bedford, MA). After blocking nonspecific binding sites with 0.1% Tween, membranes were incubated first with goat anti-RLV antiserum

(NIH Repository Number 75S000294) and, after washing, with horseradish peroxidase-conjugated rabbit anti-goat IgG (Bio-Rad, Hercules, CA). Specific binding was demonstrated after the blot was developed with the substrate 4-chloro-1-naphthol (Life Technologies, Inc., Gaithersburg, MD). Individual lanes were scored positive for RLV by the presence of p30 Gag, p15, and gp70 Env bands.

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Figure 1. *Ex vivo* RLV-specific CTL activity in AZT plus IFN-α treated, RLV-inoculated mice. Animals were given RLV inoculations (5000 AID_{50}) and started on combination anti-viral drug regimen as described in Materials and Methods. On the days indicated, mice were sacrificed and the CTL activity in purified spleen cells was measured immediately without stimulation or culture using RLV-infected target cells at an effector:target ratio of 200:1 The results show the CTL response from individual mice at each time point. Killing of control uninfected target cells was always <3% at the same effector to target ratio.

Figure 2. RLV-specific CTL activity of purified $CD4^+$ and $CD8^+$ T cells from AZT plus IFN-α treated, RLV-inoculated mice. Separate populations of $CD4^+$ and $CD8^+$ T cells were isolated by negative selection as described in Experimental Procedures from spleens of mice 21 d after RLV inoculation and the start of combination drug therapy. CTL activity against RLV-infected target cells was then measured for each cell population at the indicated effector to target ratios.

Figure 3. Time course of RLV proviral sequence levels in splenic isolates following RLV inoculation and post-exposure anti-viral drug therapy. **A** - PCR profile of representative spleen genomic DNA obtained from mice sacrificed at various times after RLV inoculation. In each case, 4 μg of genomic DNA was amplified and 10 μl of PCR products were run on 2% agarose gels. *Virus only*: no drug therapy was given after RLV inoculation; *Drug (+4 h) and (-3 d)*:

combination therapy (AZT + IFN-α) was initiated 4 hr after and 3d before RLV inoculation, respectively. **M**: ϕx174 DNA digested with *HaeIII* as molecular marker. **B** - Determination of PCR sensitivity. Different copy numbers of plasmid pBC10 (R-SFFV) were amplified in the presence of 1 μg and 4 μg of genomic DNA obtained from uninfected spleens. Lanes 1, 2, 9, and 16: uninfected control spleen DNA samples; Lanes 3-8: increasing copy numbers of pBC10, starting at 1, 5, 10, 20, 100, 1000 copies of plasmid DNA in the presence of 1 μg genomic DNA (about 1.5×10^5 cells); Lanes 10-15: same as 3-8 but in the presence of 4 μg of genomic DNA. Arrow indicates expected size of the 235 bp PCR product.

Figure 4. Survival analysis of CsA-treated RLV infected mice. The probability of survival was analysed by Kaplan-Meier statistics and is given as a function of time (in days) post inoculation of mice (10 per group). Control groups received RLV (20 AID_{50}) only or RLV + oil.

Figure 5. Rauscher Murine Leukemia Virus (RLV) stock titration in normal and athymic nude mice. Either normal BALB/c or BALB/c nu/nu mice were inoculated i.v. with increasing concentrations of a stock of RLV and, 21 d post-infection, serum from individual animals was analysed for RLV antigens by immunoblotting. The values at each dosage point indicate the number of infected mice in each group over the number of animals receiving the indicated

dose. For the middle 3 doses, differences in infectivity between nude mice and normal mice were statistically significant (p-values $\leq .05$) as analysed by exact logistic regression.

Figure 6. Threshold Hypothesis of Retrovirus Infection. According to this model, 3 threshold levels of virus load exist; the lowest level which can be assigned with any certainty is the virus detection threshold. In any system, viral loads below this level are unknown quantities. Following virus exposure, 3 potential pathways are described: (1) unchecked retrovirus replication results in a viral load exceeding pathogenic levels; (2) viral load is kept below disease threshold levels through drug intervention, replication defects, or by natural host factors, yet transient levels of infection are high enough to generate protective immunity; or (3) virus is detected yet is kept too low to initiate a protective immune response. After infection, a state of viral latency may exist with periodic virus production which is suppressed by immune surveillance mechanisms (4). Reactivation of virus production, in the absence of adequate immune protection, will result in disease progression (5)

Table I

Vaccination with low-dose, live pathogenic RLV

	Group	Initial RLV Dose (AID_{50}) [a]	Fraction Virus Free [b]	RLV challenge (AID_{50}) [c]	Fraction Virus Free
Expt 1	1	2	3/10	200	2/3
	2	0.4	7/10	200	2/7
	3	0.2	10/10	200	0/10
	4	0.04	9/10	200	0/9
Expt 2	1	2	3/10	20	1/3
	2	0.4	7/10	20	1/7
	3	0.2	9/10	20	2/9
	4	0.04	10/10	20	1/10

a - Animals (10 mice per group) were inoculated iv with the indicated amounts of RLV on day 0 and serum was tested on day 21 for RLV antigens by immunoblot analysis.

b - The presence or absence of RLV in serum was confirmed by XC plaque assay.

c - Mice shown to be virus free following initial inoculations were challenged with acute doses of RLV and serum was reanalysed for RLV antigens 21 days afterwards.

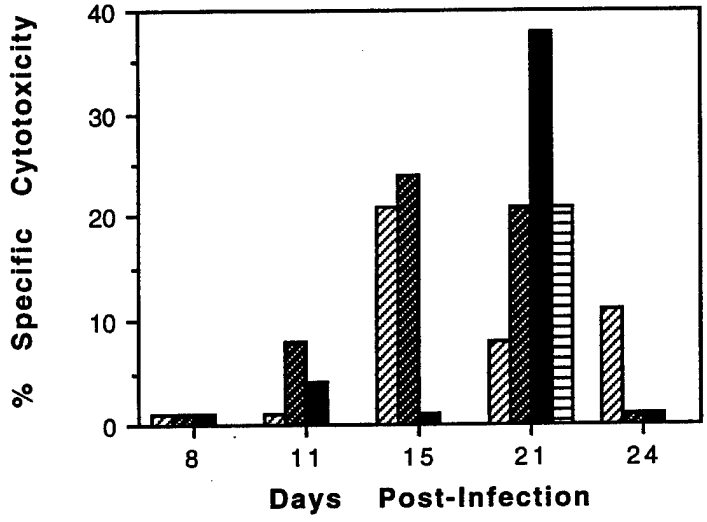
% Specific Cytotoxicity
40
30
20
10
0
8
11
15
21
24
Days Post-Infection

Figure 1

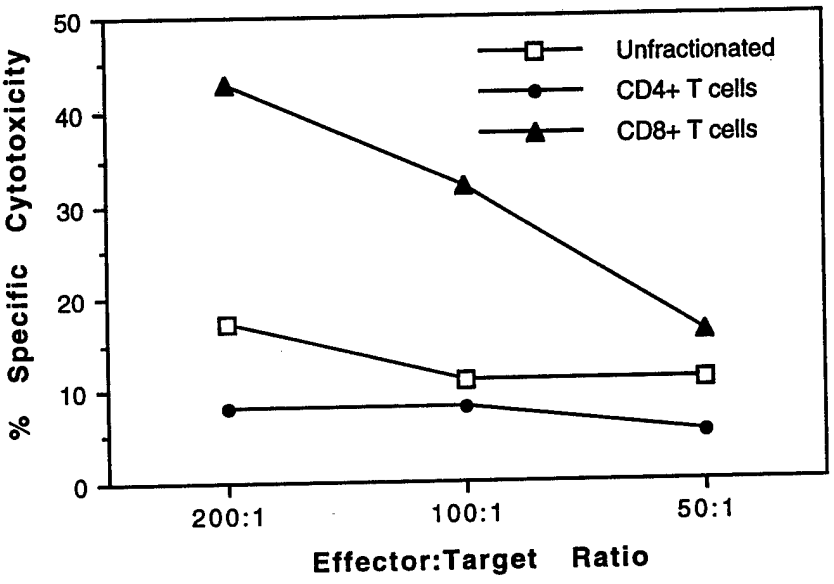
50
40
30
20
10
0
% Specific Cytotoxicity
Unfractionated
CD4+ T cells
CD8+ T cells
200:1
100:1
50:1
Effector:Target Ratio

Figure 2

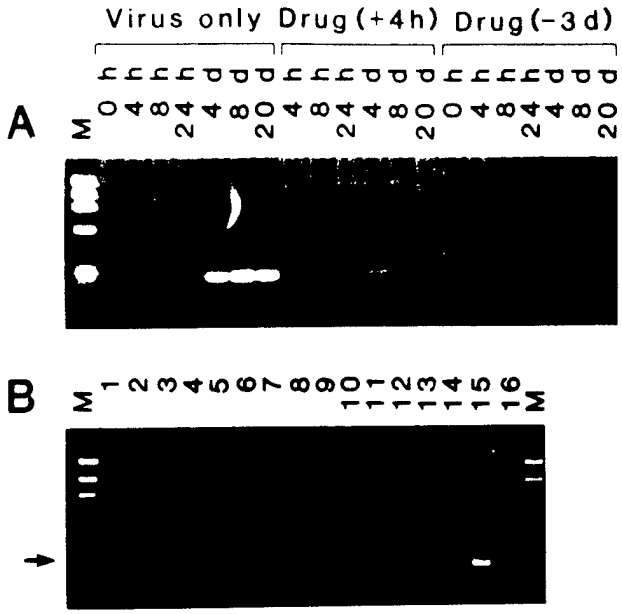

Figure 3

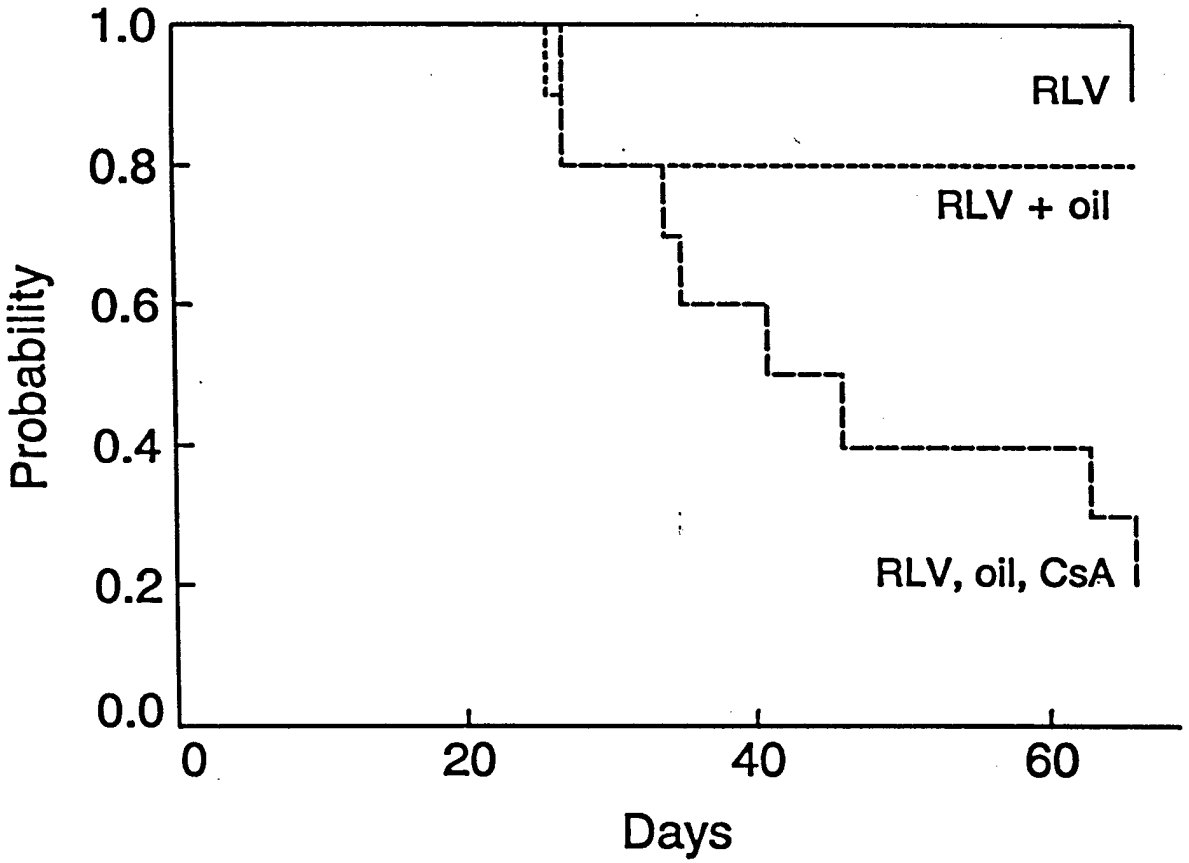
1.0
0.8
0.6
0.4
0.2
0.0
Probability
RLV
RLV + oil
RLV, oil, CsA
0
20
40
60
Days

Figure 4

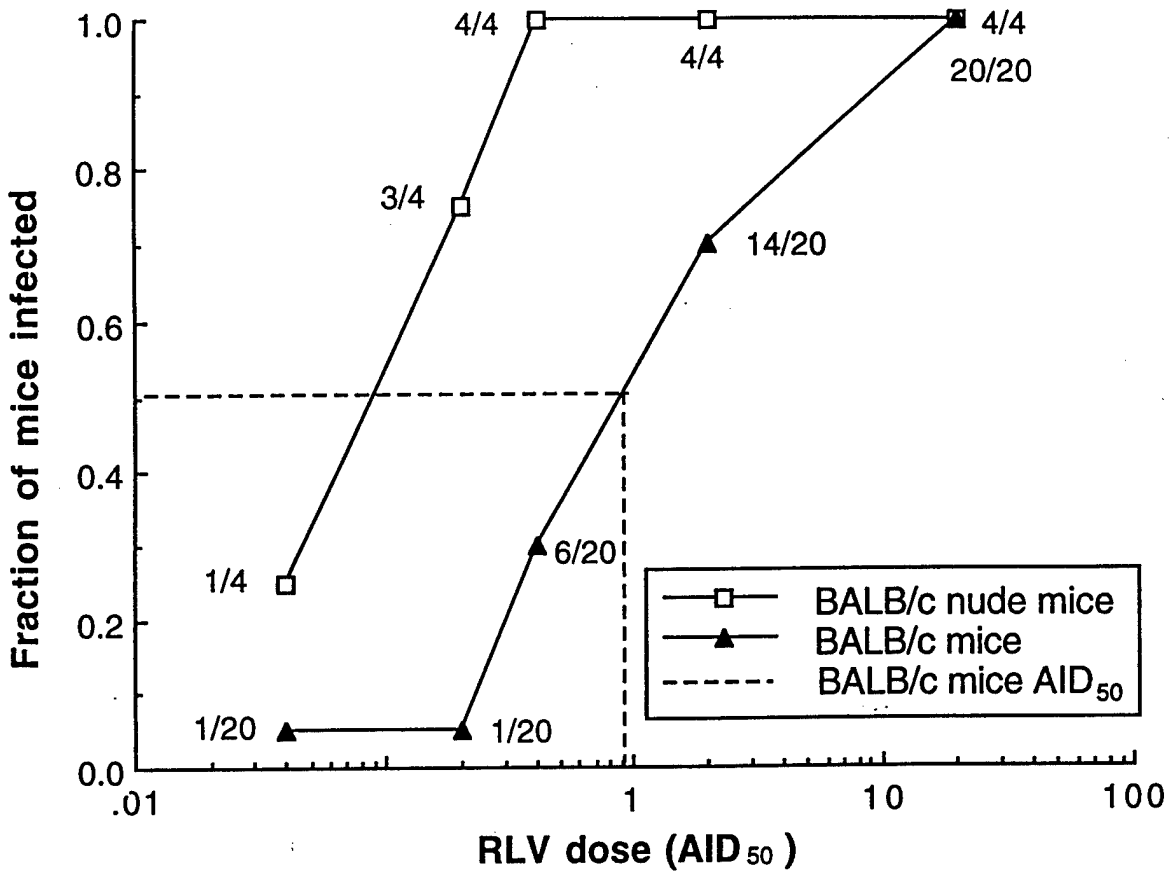
1.0
0.8
0.6
0.4
0.2
0.0
Fraction of mice infected
.01
1
10
100
RLV dose (AID50)
4/4
4/4
4/4
20/20
3/4
14/20
6/20
1/4
1/20
1/20
BALB/c nude mice
BALB/c mice
BALB/c mice AID50

Figure 5

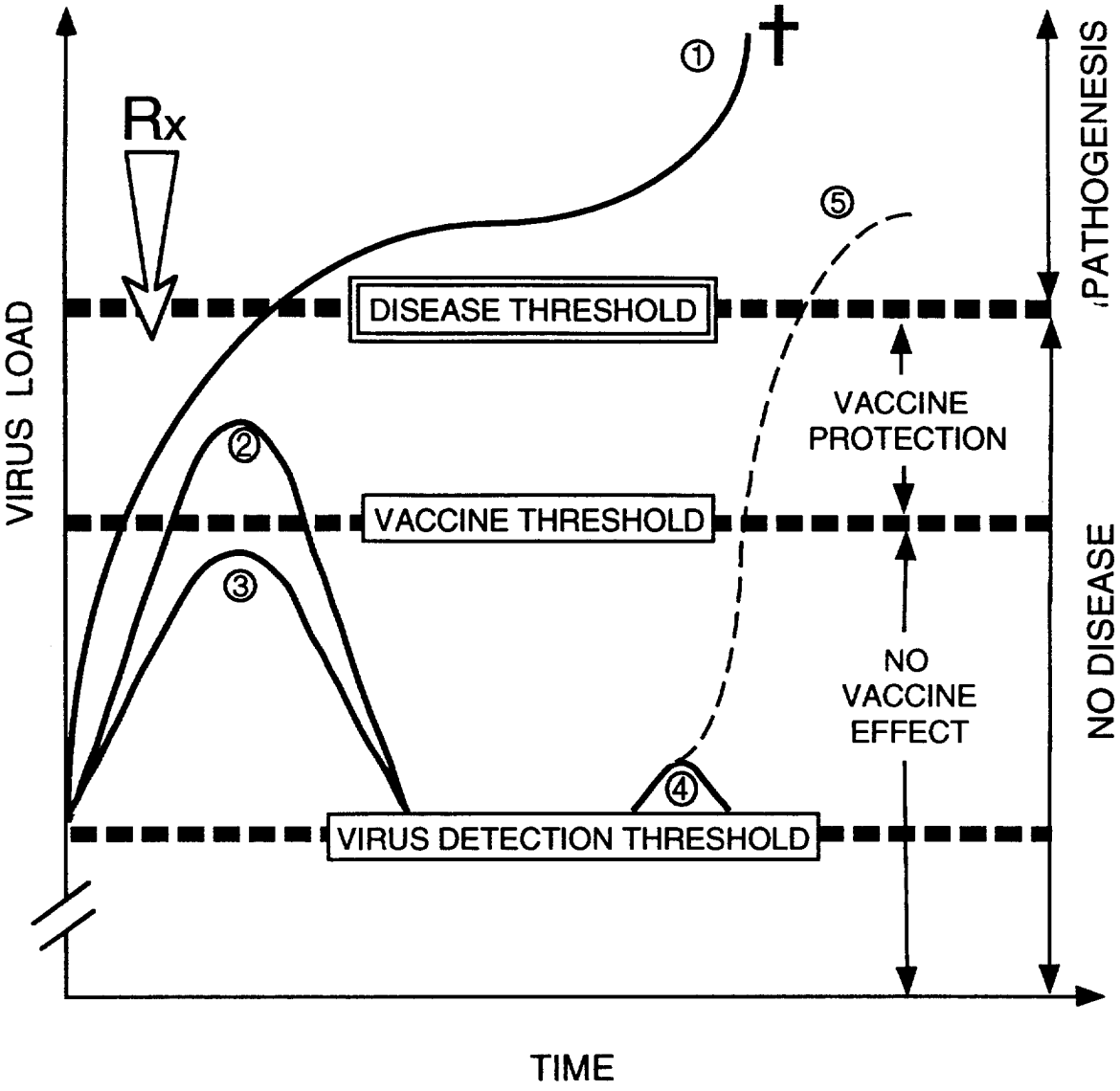
VIRUS LOAD
Rx
①
†
⑤
DISEASE THRESHOLD
PATHOGENESIS
VACCINE
PROTECTION
②
VACCINE THRESHOLD
③
NO
VACCINE
EFFECT
NO DISEASE
④
VIRUS DETECTION THRESHOLD
TIME

Figure 6