JOURNAL OF VIROLOGY, July 2003, p. 7796–7803 Vol. 77, No. 14
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.14.7796–7803.2003


# Kunjin Virus Replicon Vectors for Human Immunodeficiency Virus Vaccine Development†

Tracey J. Harvey,[1,2] Itaru Anraku,[1,2,3] Richard Linedale,[1,2] David Harrich,[1] Jason Mackenzie,[1,2] Andreas Suhrbier,[3] and Alexander A. Khromykh[1,2]*

*Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital,[1] Clinical Medical Virology Centre,[2] and The Australian Centre for International and Tropical Health and Nutrition, Queensland Institute of Medical Research,[3] University of Queensland, Brisbane, Queensland, Australia*

Received 2 December 2002/Accepted 29 April 2003

**We have previously demonstrated the ability of the vaccine vectors based on replicon RNA of the Australian flavivirus Kunjin (KUN) to induce protective antiviral and anticancer CD8$^+$ T-cell responses using murine polyepitope as a model immunogen (I. Anraku, T. J. Harvey, R. Linedale, J. Gardner, D. Harrich, A. Suhrbier, and A. A. Khromykh, J. Virol. 76:3791-3799, 2002). Here we showed that immunization of BALB/c mice with KUN replicons encoding HIV-1 Gag antigen resulted in induction of both Gag-specific antibody and protective Gag-specific CD8$^+$ T-cell responses. Two immunizations with KUN*gag* replicons in the form of virus-like particles (VLPs) induced anti-Gag antibodies with titers of ≥1:10,000. Immunization with KUN*gag* replicons delivered as plasmid DNA, naked RNA, or VLPs induced potent Gag-specific CD8$^+$ T-cell responses, with one immunization of KUN*gag* VLPs inducing 4.5-fold-more CD8$^+$ T cells than the number induced after immunization with recombinant vaccinia virus carrying the *gag* gene (rVV*gag*). Two immunizations with KUN*gag* VLPs also provided significant protection against challenge with rVV*gag*. Importantly, KUN replicon VLP vaccinations induced long-lasting immune responses with CD8$^+$ T cells able to secrete gamma interferon and to mediate protection 6 to 10 months after immunization. These results illustrate the potential value of the KUN replicon vectors for human immunodeficiency virus vaccine design.**

A major requirement for an effective human immunodeficiency virus (HIV) vaccine is the induction of potent, broad, and durable anti-HIV CD8$^+$ T-cell immunity (11, 21, 28). Only a few vaccine modalities that safely and effectively induce CD8$^+$ T-cell responses in humans have emerged, and a substantial diversity of approaches are currently being tested in preclinical models (9, 21, 28). Replicon-based vaccine vectors derived from positive-strand RNA viruses have recently emerged as potentially valuable systems for the development of vaccines (17). Alphavirus-based replicon vaccines have been shown to be capable of inducing potent antibody and CD8$^+$ T-cell responses in mice and monkeys (22, 34) and have been applied to the design of HIV type 1 (HIV-1) vaccines (7, 39, 42).

Replicon vectors based on the flavivirus Kunjin (KUN) have recently been developed in our laboratories (18, 19, 40, 41) and show considerable potential for use as vaccine vectors for induction of protective CD8$^+$ T-cell responses (3). KUN replicon vectors have several potentially valuable characteristics for vaccine design. KUN replicons do not induce cytopathic effects, which allows immunogens to be expressed for extended periods both in vitro and in vivo (19, 40, 41), thus potentially generating long-lived immunity. Also, flaviviruses are not known to recombine in nature, thus precluding generation of potentially harmful recombinant viruses in KUN replicon-immunized individuals infected with other flaviviruses. Furthermore, the enzootic host of KUN virus appears to be mainly birds, with infections in humans nearly always asymptomatic. There is no preexisting immunity to KUN virus in the majority of the world's populations, except in northern parts of Australia and neighboring islands where KUN virus is endemic. KUN replicon-based vaccines can be delivered in the following two ways: (i) plasmid DNA carrying replicon cDNA, which in turn produces functional replicon RNA in vivo from the cytomegalovirus (CMV) promoter by cellular RNA polymerase II; or (ii) replicon RNA, which is transcribed in vitro and delivered either as naked RNA or packaged into virus-like particles (VLPs) before delivery by infection with these VLPs. KUN replicons are unable to generate infectious virus in vivo, and the design of the heterologous packaging system for production of KUN VLPs also precludes the generation of any infectious recombinant viruses during VLP preparation (18, 40).

HIV particle assembly and budding are directed by the pr55$^{gag}$ polyprotein, which is the precursor for the internal structural proteins (matrix, capsid, nucleocapsid, and p6) of the mature virion (6). HIV Gag proteins are relatively conserved and the target of cross clade immunity (8, 26). Both CD4 and CD8$^+$ T-cell immunity directed against Gag proteins are believed to be important for protection (10, 15). Although not believed to mediate protection, anti-Gag antibodies are raised in HIV-infected individuals and by experimental vaccines containing Gag, where the antibody responses may be viewed as one measure of vaccine performance (30). Here we show that immunization with KUN replicons expressing the complete HIV-1 *gag* gene induced potent Gag-specific CD8$^+$

* Corresponding author. Mailing address: Sir Albert Sakzewski Virus Research Centre, Royal Children's Hospital, Brisbane, Queensland, 4029 Australia. Phone: (617) 3636 1568. Fax: (617) 3636 1401. E-mail: a.khromykh@mailbox.uq.edu.au.

† Publication 148 of the Clinical Medical Virology Centre and the Sir Albert Sakzewski Virus Research Centre, Brisbane, Queensland, Australia.

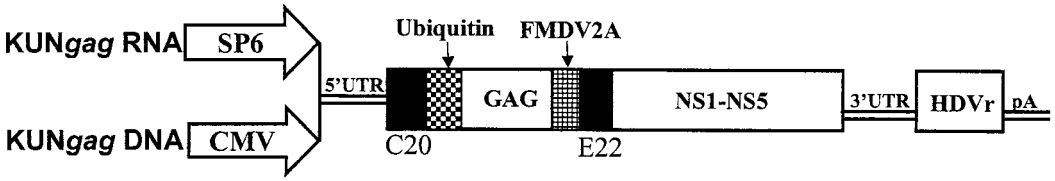


FIG. 1. DNA and RNA KUN replicon constructs carrying the HIV-1 *gag* gene. The DNA and RNA constructs are driven by the CMV and SP6 promoters, respectively. The constructs contain the following: (i) sequences required for KUN RNA replication (5′ and 3′ untranslated region [UTR]); (ii) sequence coding for the first 20 amino acids of the KUN C protein (C20); (iii) sequence coding for the last 22 amino acids of the KUN E protein (E22); and (iv) sequences coding for the nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (shown as NS1-NS5). Constructs also contain either an SP6 or CMV promoter upstream of the KUN 5′ UTR for in vitro or in vivo RNA transcription, respectively. The antigenomic sequence of the hepatitis delta virus ribozyme (HDVr) and the polyadenylation signal from simian virus 40 (pA) have been inserted downstream of the 3′ UTR to ensure production of KUN replicon RNA molecules with precise 3′ termini for efficient initiation of replication. Ubiquitin is the mouse ubiquitin gene, and FMDV2A is autoprotease 2A of the foot-and-mouse disease virus.

T-cell and antibody responses and protected mice from challenge with recombinant vaccinia virus expressing the *gag* gene.

## MATERIALS AND METHODS

**Plasmids.** The RNA-based and DNA-based KUN replicon vectors C20UbHDVrep and pKUNrep1, respectively (41), were used for construction of plasmids containing the HIV-1 *gag* gene. Essentially, the complete HIV-1 *gag* gene was amplified by PCR from the pBRDH2-neo plasmid (an HIV-$1_{SF2/BH10}$ construct) (14) with primers *gag*BssHII-F (5′-ACCATGGGCGCGAGCATCG GTATTA-3′) and *gag*BssHII-R (5′-CTAAAGCGCGCCTTGTGACGAGGGG TC-3′). The PCR product was then digested with *Bss*HII and inserted into the *Asc*I sites of the two KUN vectors to produce plasmids KUN*gag*RNA and KUN*gag*DNA, respectively (Fig. 1).

**DNA and RNA transfections and immunofluorescence.** For DNA transfection, BHK21 cells in 60-mm-diameter dishes or on glass coverslips were transfected with 2 or 0.4 μg of plasmid DNA, respectively, using Lipofectamine Plus transfection reagent (Life Technologies, Melbourne, Australia), as described by the manufacturer. RNA was transcribed in vitro from the *Xho*I-linearized KUN*gag*RNA plasmid DNA using SP6 RNA polymerase and electroporated into BHK21 cells as described previously (19). Coverslips with transfected cells were fixed in 4% paraformaldehyde 28 to 48 h posttransfection and assayed for expression of KUN NS3 or E protein by indirect immunofluorescence (IIF) with anti-NS3 or anti-E antibodies, respectively, as described previously (43).

**Radioimmunoprecipitation assay (RIPA).** To metabolically label proteins, BHK21 cells electroporated with KUN*gag* RNA were seeded into a six-well plate, and at 32 h postelectroporation, the cells were radiolabeled for 4 h with ~100 μCi of [$^{35}$S]methionine/cysteine in the presence of actinomycin D (Sigma, Castle Hill, Australia). Tissue culture fluid was collected, and the cells were lysed in lysis buffer (phosphate-buffered saline [PBS] containing 0.5% Nonidet P-40). Samples were incubated with anti-pr55$^{gag}$ antibody (diluted 1:50) overnight at 4°C and then incubated for 1 h with 30 μl of 10% protein A-Sepharose beads at 4°C. Pelleted Sepharose beads were washed three times with PBS, resuspended in gel loading buffer, boiled for 5 min, and separated on a sodium dodecyl sulfate–10% polyacrylamide gel.

**Electron microscopy (EM).** BHK21 cells electroporated with KUN*gag* RNA or KUN vector RNA were seeded into a 60-mm-diameter dish, and the cells were then harvested at 24 and 48 h after electroporation. The cells were collected in PBS and fixed with 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4. The samples were treated with 1% osmium tetroxide, dehydrated with acetone, and embedded in Epon 612 resin as previously described (23). Sections collected on grids were stained with uranyl acetate and lead citrate. All specimens were examined on a JEOL 1010 transmission electron microscope at 80 kV.

**Preparation of VLPs.** VLPs were prepared essentially as described previously except that $3 \times 10^6$ BHK21 cells were electroporated with ~30 μg of in vitro-transcribed KUN*gag* RNA. At 32 h postelectroporation, the cells were trypsinized, subjected to a second electroporation using in vitro-transcribed noncytopathic Semliki Forest virus (SFV) replicon RNA containing the Leu713-to-Pro codon substitution in the SFV nsP2 gene and encoding KUN structural proteins (derivative of SFV-MEC105) (18) (details will be described elsewhere) and incubated for 48 to 60 h before harvesting secreted VLPs. The titer of infectious VLPs was determined by infection of Vero cells with 10-fold serial dilutions of the VLPs and counting the number of NS3-positive cells by IIF analysis at 30 to 40 h postinfection.

**Immunization of mice.** Female BALB/c (*H-2$^d$*) mice (6 to 8 weeks old) were supplied by the Animal Resources Centre (Perth, Western Australia). Mice were immunized as follows. (i) KUN*gag* DNA (100 μg) was diluted in 100 μl of PBS and injected intramuscularly (i.m.) into the quadriceps muscle of each hind leg (50 μl in each leg). (ii) In vitro-transcribed KUN*gag* RNA (~30 μg) was dissolved in 100 μl of diethyl pyrocarbonate-treated PBS and injected i.m. (50 μl into each leg). (iii) KUN*gag* VLPs in Dulbecco modified Eagle medium containing 5% fetal calf serum was injected intraperitoneally (i.p.) at ~$10^6$ infectious units (IU) per mouse. (iv) A KUN VLP encoding the murine polyepitope (KUNmptVLP) which contains four *H-2$^d$*-restricted epitopes, YPHFMPTML (YPH), RPQASGVYM (RPQ), TYQRTRALV (TYQ) and SYIPSAEKI (SYI) (2) was injected as for method iii. (v) Recombinant vaccinia virus (WR TK$^-$) carrying HIV-1 *gag* (rVV*gag*) (29) ($2 \times 10^7$ PFU in 200 μl of PBS) was injected i.p.

**HIV Gag protein determination.** BHK cells ($1.25 \times 10^5$ of $2 \times 10^6$) electroporated with 5 to 10 μg of KUN*gag* RNA were seeded into each well of a 24-well plate. Culture fluid and cell lysate samples were harvested at various time intervals. The total volume of culture fluid (500 μl) was clarified and stored at 4°C. For cell lysate, the cells were washed twice with PBS and lysed in 500 μl of PBS containing 0.5% Nonidet P-40 for 5 min at room temperature. Samples were then vortexed, clarified, and stored at −70°C. The level of pr55$^{gag}$ protein present in the sample was then quantified by using an indirect CAp24 enzyme-linked immunosorbent assay (ELISA) kit (Perkin-Elmer Life Sciences, Boston, Mass.). The assay was performed as recommended by the manufacturer. The total amount of cell-associated and secreted pr55$^{gag}$ protein produced per $10^6$ initially transfected cells was calculated by multiplying the amount of protein detected in the total volume of samples obtained from $1.25 \times 10^5$ of initially transfected cells by a factor of 8.

**Detection of antibodies to HIV-1 Gag protein by indirect ELISA.** Each well of the 96-well plates was coated with 1 μg of recombinant pr55$^{gag}$ protein (expressed as His-tagged protein in *Escherichia coli* and purified using a Ni-agarose column) overnight at 4°C in antigen coating buffer (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$ [pH 9.6]). The wells were then blocked by incubation with 50-μl portions of blocking buffer (PBS containing 0.25% gelatin and 0.1% Tween 20) for 1 h at 37°C and washed three times with wash buffer (PBS containing 0.05% Tween 20). Serum samples from immunized mice, diluted in blocking buffer, were placed in the wells and incubated for 1 to 2 h at room temperature, and the wells were washed three times. The secondary antibody, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G diluted 1:2,000 in blocking buffer, was placed in the wells and incubated for 30 min at room temperature. After the wells were washed three times, bound conjugate was developed by incubation with 50 μl of K-blue tetramethylbenzidine substrate (Graphic Scientific, Brisbane, Australia) for 10 min at room temperature in the dark or until the color developed. The reaction was then stopped by the addition of 50 μl of 2 M $H_2SO_4$, and the absorbance readings (optical density at 450 nm) were determined using an ELISA plate reader.

**KUN VLP neutralization assay.** KUN VLPs (200 μl) containing encapsidated KUN vector replicon RNA ($5 \times 10^5$ IU) were incubated for 1 h at 37°C with 20 μl of three different serum samples from KUN*gag* VLP-immunized mice which showed the highest titers of Gag antibodies by ELISA. VLPs were also incubated under the same conditions with a KUN anti-E monoclonal antibody (1) and with sera from naive mice as positive and negative controls for the assay, respectively. The titer of VLPs in each neutralization reaction mixture was determined from

the number of NS3-positive Vero cells detected by IIF analysis after infection with serial 10-fold dilutions of the neutralization reaction mixtures.

**ELISPOT and chromium release assays.** Epitope-specific $CD8^+$ T cells secreting gamma interferon (IFN-γ) were enumerated by an enzyme-linked immunospot (ELISPOT) assay using peptide epitopes (Mimotopes, Clayton, Australia) as described previously (20). The statistical significance of the different values found for the groups was determined using an unpaired Student's *t* test. $^{51}$Chromium ($^{51}$Cr) release assays were performed using splenocytes from mice sacrificed 2 to 3 weeks postimmunization, and splenocytes were restimulated in vitro for 6 days with irradiated lipopolysaccharide blasts (responder/stimulator ratio of 20:1) sensitized with the AMQMLKETI peptide (25 μg/ml for 1 h in 200 μl of medium at 37°C, followed by two washes). The resulting effector populations were split, and equal numbers were used in duplicate experiments against peptide-sensitized and unsensitized $^{51}$Cr-labeled P815 target cells at the indicated effector/target ratios.

**Vaccinia virus protection assay.** Immunized and control groups of 6- to 8-week-old female BALB/c mice (Animal Resource Center, Perth, Australia) were challenged with recombinant vaccinia virus ($TK^-$) carrying the HIV-1 *gag* gene (rVV*gag*) (29) or with the control rVV encoding the murine polyepitope (rVVcont) (17). At day 4 postinfection, both ovaries were removed, washed, and homogenized in 1 ml of PBS using aluminum mesh. The ovary vaccinia virus titers were then determined by plaque assay on confluent CV1 cells (3). The significance of the differences between the virus titers in the experimental and control groups was calculated using a nonparametric unpaired *t* test.

## RESULTS

**Expression and secretion of *gag* particles in cells transfected with KUN replicon vectors.** To determine that the KUN replicon-encoding *gag* gene constructs were able to efficiently produce $pr55^{gag}$ protein, plasmid KUN*gag*DNA was transfected into BHK21 cells and examined for expression of KUN NS3 protein and $pr55^{gag}$ protein by IIF analysis (data not shown). IIF analysis of transfected cells with KUN anti-NS3 antibodies or HIV-1 anti-$pr55^{gag}$ antibodies showed that ~40% of cells were strongly positive for both proteins 40 h after transfection with DNA-based replicons (data not shown). In vitro-transcribed KUN*gag* RNA was transfected into BHK21 cells by electroporation and examined for KUN NS3 expression by IIF analysis with anti-NS3 antibodies and for HIV-1 *gag* expression and secretion by RIPA with anti-$pr55^{gag}$ antibody. IIF analysis of RNA-electroporated cells demonstrated significantly greater transfection efficiency compared to cells transfected with the corresponding DNA-based replicon vector, i.e., 85 and 40%, respectively (data not shown). RIPA with anti-$pr55^{gag}$ antibody showed efficient Gag expression in KUN*gag* RNA-electroporated cells and its secretion into the culture fluid (Fig. 2A). A decrease in the amount of Gag protein in cells from 0.5 h to 4 h of the chase period (Fig. 2A, cell lysate lanes), correlated with the appearance of secreted Gag in the culture fluid 4 h after chase labeling (Fig. 2A, lane 2). The amount of Gag protein present in cells and in the culture fluid was quantitated using an indirect ELISA (Fig. 2B) with anti-CAp24 antibody-coated microtiter plates (Perkin-Elmer Life Sciences). Comparison with a purified p24 protein of known concentration showed that the total amount of Gag protein produced from $10^6$ of initially transfected BHK cells 60 h after electroporation of KUN*gag* RNA was equivalent to 632 ng of CAp24 protein, with 410 ng detected in the cell lysates and 222 ng detected in the culture fluid.

To examine whether Gag protein was assembled into secreted particles, we performed EM analysis of transfected cells. Characteristic 100- to 120-nm-diameter particles were present in cells transfected with KUN*gag* RNA (Fig. 3A) but

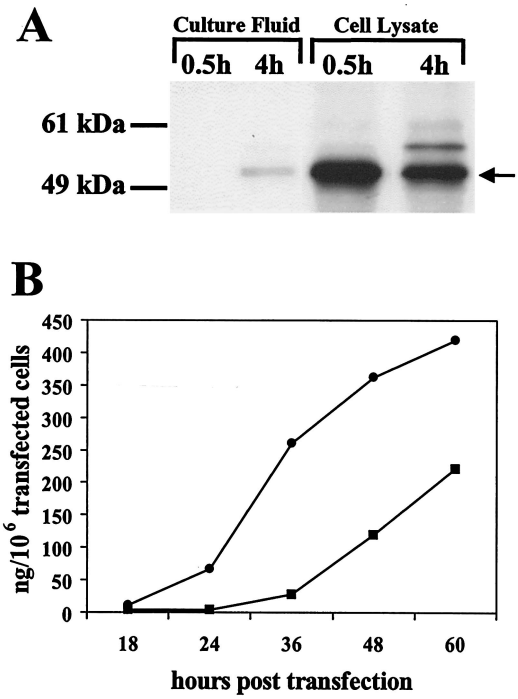


FIG. 2. Production and secretion of Gag protein. (A) Radiolabeled culture fluid and cell lysate, collected 0.5 and 4 h after labeling with [$^{35}$S]methionine/cysteine from KUN*gag*-electroporated cells, was immunoprecipitated with anti-$pr55^{gag}$ antibody and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The migration of a radiolabeled $pr55^{gag}$ protein in the gel is indicated by the arrow. (B) Culture fluid and cell lysate were collected from KUN*gag* RNA-electroporated cells at different time intervals, and the amount of produced Gag protein was determined by using a Perkin-Elmer p24 ELISA kit. The total amount of cell-associated (cell lysate [black circles]) and secreted (culture fluid [black squares]) $pr55^{gag}$ produced per $10^6$ initially transfected BHK cells at different times posttransfection was calculated as described in Materials and Methods.

not in cells transfected with the KUN vector replicon RNA (Fig. 3B). In addition, the assembled *gag* particles were observed to bud from the plasma membrane (Fig. 3C) and were released into the extracellular medium (Fig. 3D). Taken together, the results of IIF, RIPA, ELISA, and EM analyses demonstrate efficient expression of secreted *gag* particles in cells transfected with KUN replicon vectors carrying the HIV-1 *gag* gene.

**Induction of HIV-1 Gag-specific antibody responses.** Sera from mice immunized twice with KUN*gag* VLPs ($10^6$ IU per mouse) or once with rVV*gag* ($2 \times 10^7$ PFU per mouse) were examined for the presence of antibodies to Gag. The antibody responses were similar after immunization with KUN*gag* VLPs and rVV*gag* (Fig. 4).

**Absence of neutralizing antibody response to the KUN envelope protein after VLP immunizations.** Effective booster immunization using a second inoculation with the same virus vector-based vaccine usually demands that the primary immunizations did not lead to induction of neutralizing antibodies (36). To determine whether there was any significant neutralizing antibody response to the KUN envelope VLP protein after two immunizations with KUN*gag* VLPs, a VLP infectivity neutralization assay was performed. In the absence of

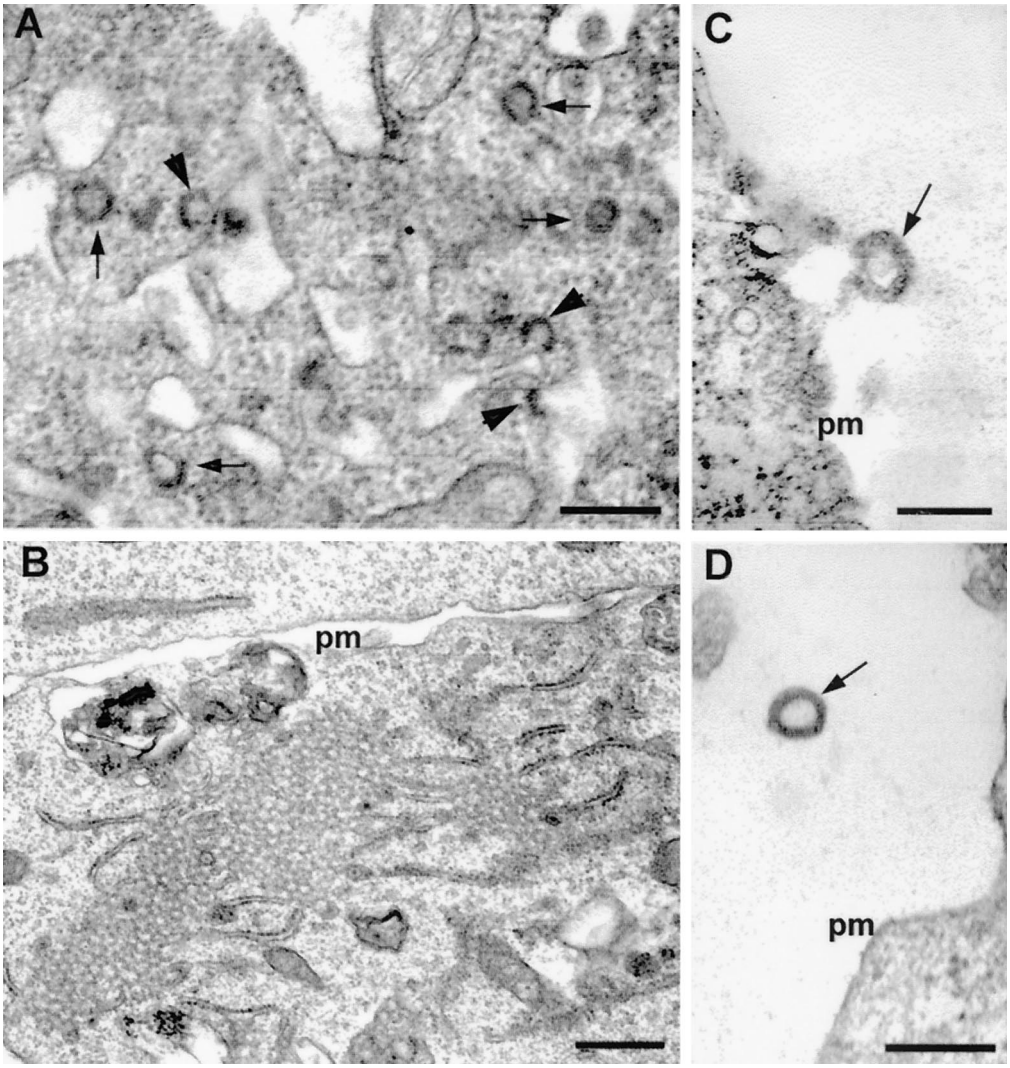


FIG. 3. EM of KUN*gag* RNA-transfected BHK21 cells. (A) 100- to 120-nm-diameter *gag* particles assembling and budding (indicated by arrows and arrowheads, respectively) from internal cytoplasmic membranes. (B) Cells transfected with control KUN RNA. (C) *gag* particle (indicated by arrow) budding from the plasma membrane (pm). (D) *gag* particle (indicated by arrow) secreted into the extracellular medium. Bars, 200 nm (A, C, and D) and 500 nm (B).

neutralizing antibodies, VLPs incubated with sera from naive mice showed VLP titers of $2.1 \times 10^6 \pm 3.8 \times 10^5$ IU/ml. As expected, in the presence of a neutralizing antienvelope monoclonal antibody, the VLP's infectivity was completely neutralized (data not shown). Sera from mice ($n = 3$) immunized twice with KUN*gag* VLPs showed no significant neutralizing activity, as the VLP titer was $6.3 \times 10^5 \pm 1.3 \times 10^5$ IU/ml.

**Induction of HIV-1 Gag-specific CD8$^+$ T-cell responses.** HIV-1 Gag protein contains a *H-2$^d$*-restricted CD8$^+$ T-cell epitope, AMQMLKETI (AMQ), which was used as a peptide to detect Gag-specific CD8$^+$ T-cell responses in immunized BALB/c mice using the IFN-γ ELISPOT assay. BALB/c mice were immunized once or twice with rrVV*gag* or KUN replicon vaccines encoding Gag (KUN*gag* DNA, KUN*gag*RNA, and KUN*gag* VLPs). Overall, KUN*gag* VLPs generated higher CD8$^+$ T-cell responses than KUN*gag* RNA did, which gave higher responses than KUN*gag* DNA, with responses from the latter two modalities broadly comparable to that of rVV*gag* (Fig. 5A). However, a single immunization with KUN*gag* VLPs induced a 4.5-fold-greater response than one immunization with rVV*gag* (KUN*gag* VLP versus rVV*gag*, $P = 0.012$) (Fig. 5A).

Mice immunized twice with KUN*gag*RNA, KUN*gag* VLPs, and rVV*gag* were also assayed for the induction of cytotoxic T-cell responses by $^{51}$Cr release assay (Fig. 5B). Significant cytotoxic activity specific for the AMQ epitope was observed (Fig. 5B). The response induced by immunization with KUN*gag* VLPs was comparable to that observed in mice immunized with rVV*gag*.

These data illustrate that KUN replicon-based vaccines can efficiently induce Gag-specific CD8$^+$ T cells, generating responses greater or similar in magnitude to those induced by recombinant vaccinia virus.

**KUN*gag* RNA or KUN*gag* VLP immunization protects mice from experimental viral challenge.** To determine whether the

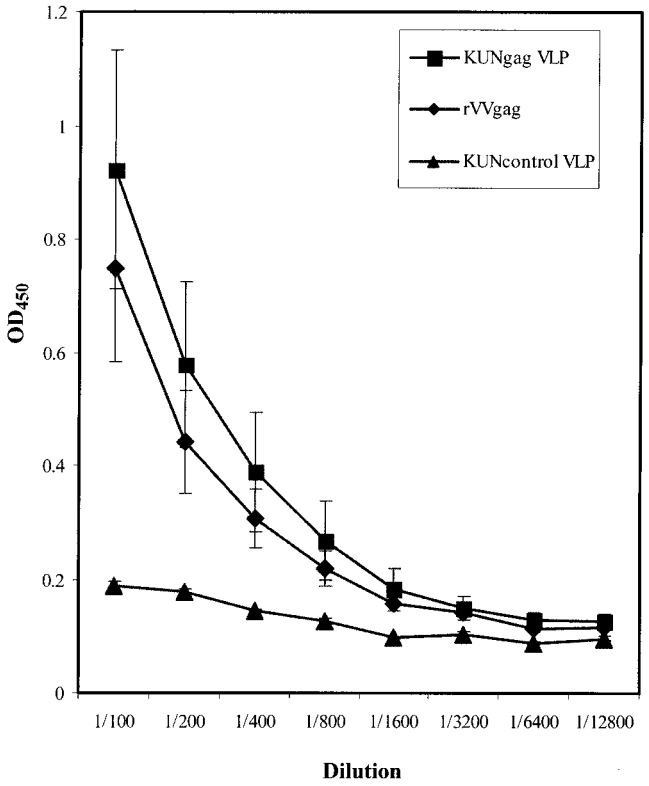


FIG. 4. Anti-pr55$^{gag}$ antibody levels in mice after immunization with KUN*gag* VLPs and rVV*gag*. Mice were immunized twice with $10^6$ IU of KUN*gag* VLPs or control KUN VLPs or once with $2 \times 10^7$ PFU of rVV*gag*. Two to three weeks after the last immunization, mouse sera were analyzed for anti-pr55$^{gag}$ antibodies using an ELISA against purified recombinant pr55$^{gag}$ protein, as described in Materials and Methods. Optical density at 450 nm ($OD_{450}$) is shown on the *y* axis, while the dilution of the serum sample is shown on the *x* axis.

T-cell responses induced by immunization with KUN*gag* replicon vectors can protect against viral challenge, groups of mice were vaccinated twice with KUN*gag* VLP or a control VLP not encoding Gag. The mice were then challenged with either rVV*gag* or a control rVV not encoding Gag (rVVcont). Mice immunized with KUN*gag* VLPs showed an average reduction of ~30-fold ($P = 0.0003$) in rVV*gag* titers compared to mice immunized with a control VLP (Fig. 6, top graph). In a parallel experiment, the mice immunized with KUN*gag* VLP or control VLPs were challenged with rVVcont, and no significant difference in ovary rVVcont titers was observed ($P = 0.9$) (Fig. 6, bottom graph). This illustrates that Gag-specific immunity was responsible for the KUN*gag* VLP-mediated reduction in rVV*gag* titers. The rVV titers in naive animals were not significantly different from those of control VLP-immunized animals in both experiments (data not shown). In a separate experiment, two immunizations with 30 μg of KUN*gag* RNA also resulted in a ~95% reduction in ovary rVV*gag* titers ($P = 0.01$) (data not shown).

**Long-term CD8$^+$ T-cell responses elicited by KUN replicon VLP vaccines.** To analyze the long-term CD8$^+$ T-cell immunity induced by KUN VLP vaccines, mice were immunized with a single inoculation of KUN*gag* VLPs and the AMQ-specific responses were analyzed by an ELISPOT assay after 2 weeks and after 6 months. The responses at 6 months were not significantly different from those seen at 2 weeks ($P = 0.75$) (Fig. 7A), illustrating the induction of long-lasting CD8$^+$ T-cell responses by KUN*gag* VLP immunization. A similar long-term maintenance of epitope-specific CD8$^+$ T cells capable of secreting IFN-γ was observed after immunization with a KUN VLP encoding the murine polyepitope (KUNmptVLP), a vaccine encoding four *H-2$^d$*-restricted CD8$^+$ T-cell epitopes. We have previously published the responses to these four epitopes 2 to 3 weeks after immunization with KUNmptVLP (3), and we now show that after 6 months these responses have not significantly diminished (Fig. 7B), illustrating that long-term maintenance of CD8$^+$ T-cell responses is not restricted to AMQ-specific CD8$^+$ T cells. Furthermore, when mice, which had been immunized with KUNmptVLP 10 months previously, were challenged with a recombinant vaccinia virus encoding the same four epitopes, substantial protection was still apparent (Fig. 7C). KUNmptVLP-immunized mice showed an average reduction of ~100-fold in ovary virus titers from that of mock-immunized control mice ($P = 0.015$).

## DISCUSSION

We describe here the generation of KUN replicon vaccine vectors carrying the complete HIV-1 *gag* gene and show that they were able to produce secreted *gag* particles in vitro. Mice immunized with KUN*gag* DNA, RNA, or VLPs induced significant Gag-specific CD8$^+$ T-cell responses, with one immunization of KUN*gag* VLP inducing 4.5-fold-more CD8$^+$ T cells than those induced after immunization with rVV*gag*. KUN*gag* VLP and RNA immunization also mediated substantial protection of mice against challenge with rVV*gag*. Importantly, KUN VLP vaccines were able to induce enduring CD8$^+$ T cells capable of secreting IFN-γ and mediating protection against vaccinia virus challenge 6 to 10 months postimmunization. These results significantly extend our previous data obtained with murine polyepitope (3) and demonstrate the utility of KUN-based vectors for HIV vaccine design.

The ability to induce HIV Gag-specific immune responses effectively appears to depend on the vector's ability to produce Gag protein in the form of secreted, self-assembled *gag* particles (31). The assembly of secreted *gag* particles requires expression of Gag with the native amino-terminal glycine residue (12). To achieve this, the HIV-1 *gag* gene was inserted into the KUN replicon vector downstream of the mouse ubiquitin gene (Fig. 1). Cleavage by cellular proteases at the carboxy terminus of ubiquitin allows the release of Gag with native amino-terminal glycine residue. In order to release the Gag protein from the remaining Gag-KUN polyprotein, the foot-and-mouse disease virus 2A autoprotease sequence was inserted at the C terminus of Gag (Fig. 1). The results of EM (Fig. 3) and of RIPA (Fig. 2A) demonstrated that the Gag protein produced from the KUN replicon RNA vector was correctly processed and assembled into secreted *gag* particles. Quantitative analysis of Gag expression, using a commercial anti-p24 ELISA kit, in cells transfected with KUN*gag* RNA showed a high level of total Gag expression of approximately 600 ng per $10^6$ initially transfected cells by 60 h posttransfection, with at least one-third of the protein being secreted into the culture fluid (Fig. 2B). These very high yields compared to other systems (14, 27, 31) are

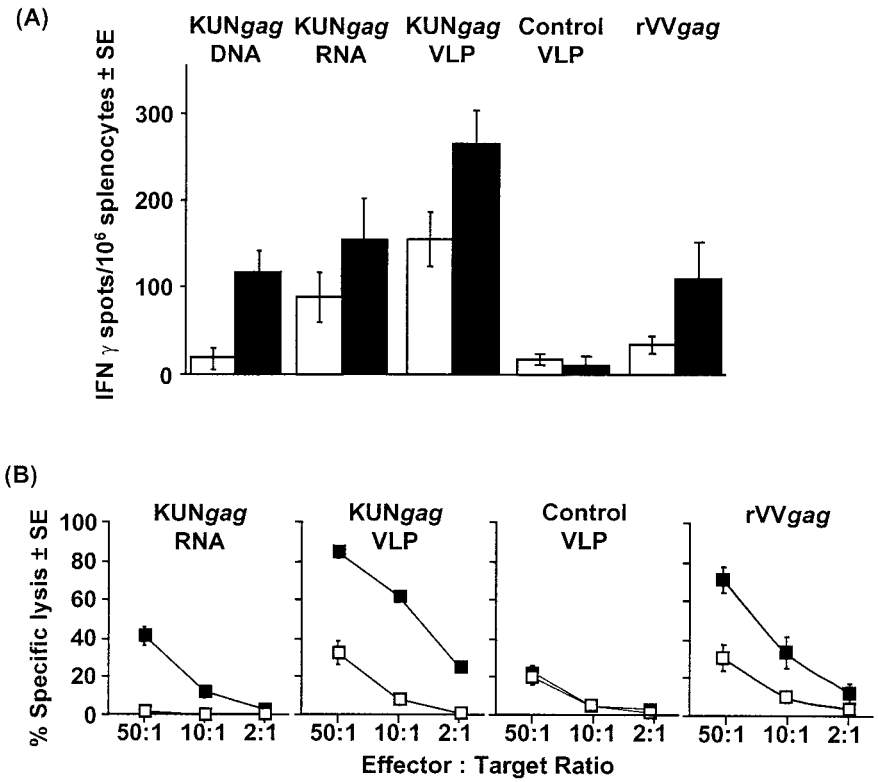


FIG. 5. T-cell responses specific for the $CD8^+$ T-cell epitope AMQMLKETI (AMQ) induced by different delivery modalities of KUN replicons carrying the HIV-1 *gag* gene or with recombinant vaccinia virus carrying the HIV-1 *gag* gene (rVV*gag*). (A) ELISPOT analysis of BALB/c mice (four mice in each group) responses after immunization with the indicated vaccine modalities. Mice received a single immunization (white bars) or two immunizations (2 weeks between the two immunizations) (black bars). (B) Standard 6-h $^{51}Cr$ release assay using restimulated splenocytes from BALB/c mice (four mice in each group) immunized twice with KUN*gag* RNA, KUN*gag* VLPs, or rVV*gag*. $^{51}Cr$-labeled target cells were sensitized with (black squares) or without (white squares) AMQ peptide. Values are means ± standard errors (SE). Control VLP, control KUN VLPs not encoding Gag.

likely to reflect the ability of the transfected cells to divide within the 60-h incubation period, with daughter cells retaining replicating KUN*gag* RNA and Gag protein production (41).

A major problem for efficient expression of native HIV structural genes when plasmid DNA-based expression vectors are used is the presence of multiple inhibitory sequences, which are responsible for poor transport of mRNAs from the nucleus to the cytoplasm (32, 35). The advantage of cytoplasmic virus-based vectors like KUN is that they do not require modification of HIV inhibitory sequences to facilitate Gag,

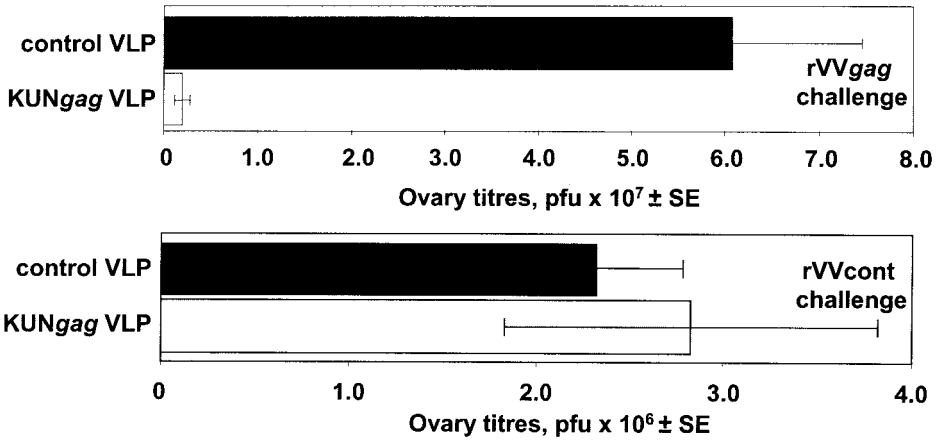


FIG. 6. Protection of BALB/c mice immunized with KUN*gag* VLPs against challenge with recombinant vaccinia virus carrying the *gag* gene. Mice (10 mice in each group) were immunized i.p. twice with $10^6$ KUN*gag* VLPs or the same dose of a control VLP not encoding Gag. The mice were then challenged intravenously 14 days after the last vaccination with $5 \times 10^6$ PFU of rVV*gag* (top graph) or a control rVV not encoding Gag (rVVcont) (bottom graph), and the ovary vaccinia virus titers on day 4 postchallenge were determined.

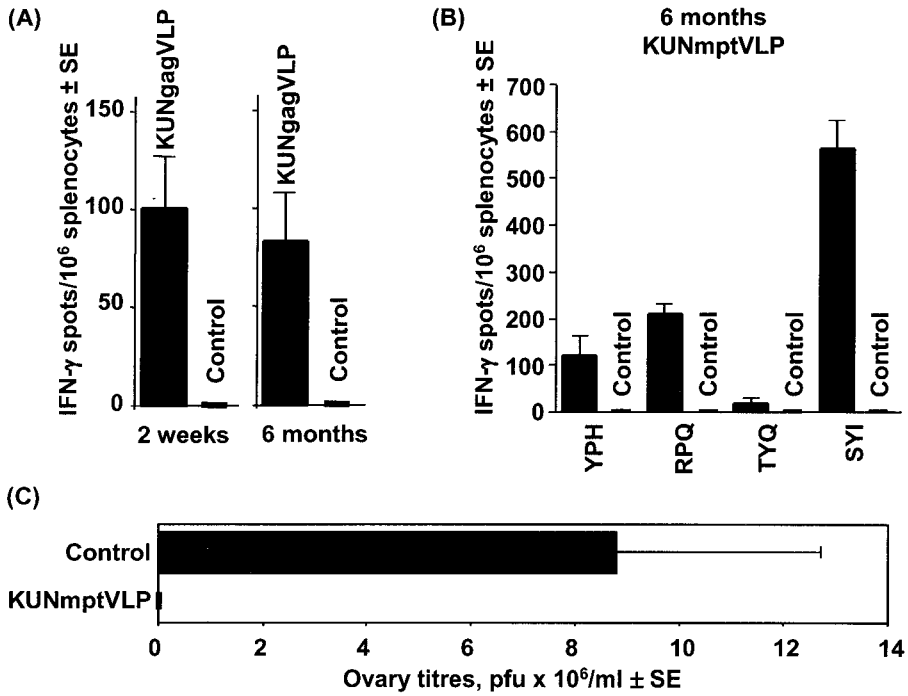


FIG. 7. Long-term immune responses elicited by KUN replicon VLP vaccines. (A) ELISPOT response to the AMQ epitope in BALB/c mice (four mice in each group) 2 weeks and 6 months after a single immunization with $10^6$ IU of KUN*gag* VLP. (B) ELISPOT analysis of splenocytes from BALB/c mice (eight mice in each group) detected at 6 months after two immunizations (4 weeks between the two immunizations) with $10^6$ IU of the KUN replicon VLPs encoding murine polyepitope immunogen (KUNmptVLP). $CD8^+$ T-cell responses are shown for individual epitopes encoded by murine polyepitope, i.e., YPHFMPTML (YPH), RPQASGVYM (RPQ), TYQRTRALV (TYQ), and SYIPSAEKI (SYI). (C) BALB/c mice (six mice in each group) were vaccinated twice with $10^6$ IU of KUNmptVLP (4 weeks between the two immunizations), and 10 months after the second immunization, mice were challenged with $10^6$ PFU of recombinant vaccinia virus encoding murine polyepitope. Vaccinia virus titers in ovaries were measured at day 4 after infection. All control mice were mock immunized with PBS. Values are means ± standard errors (SE).

Gag-Pol, and Env protein expression. This also appears to be true for KUN*gag* DNA, since HIV Gag was efficiently expressed by KUN*gag* DNA transfected cells, as judged by $CD8^+$ T-cell induction (Fig. 5A). Only a few KUN*gag* replicon RNA molecules probably need to escape from the nucleus into the cytoplasm to establish a replicating pool of replicon RNA capable of efficient protein production.

HIV-1 Gag has been used as the immunogen in a number of murine studies evaluating different vaccine vectors (5, 13, 27, 30, 33, 39). For instance, three DNA immunizations that targeted Gag to the secretory pathway (pSc-Gag) followed by a rVV*gag* booster immunization was reported to induce 50 AMQ-specific IFN-γ spots/$10^6$ splenocytes at the peak of the $CD8^+$ T-cell response on day 5 (31), whereas more than 150 AMQ-specific IFN-γ spots/$10^6$ splenocytes were seen after 3 weeks following a single immunization with KUN*gag* VLP (Fig. 5). After two immunizations with KUN*gag* VLPs, a challenge with rVV*gag* resulted in an average reduction of ~30-fold in ovary vaccinia virus titers, a protective activity known to be mediated by Gag-specific CD4 and $CD8^+$ T cells (24). These results are broadly comparable with related studies measuring protection against rVV*gag* in mice (31, 33, 39). However, comparisons are complicated by the use of (i) different schedules and routes of immunization, (ii) vaccinia viruses with different virulence characteristics, and (iii) different routes of vaccinia virus challenge (31, 33, 39).

Two immunizations with KUN*gag* VLPs induced antibody responses to the replicon-encoded Gag protein with end point titers of approximately 1/10,000, similar to those reported for mice immunized with 10 μg of $p55^{gag}$ protein emulsified in the highly effective MF59 oil-in-water adjuvant (30). In contrast, the induction of neutralizing anti-KUN VLP vector antibodies by KUN VLP vaccines appeared to be inefficient as evidenced by (i) the ability to effectively boost KUN*gag* VLP-induced responses with KUN*gag* VLPs (Fig. 5 and 6), and (ii) the failure to detect significant levels of neutralizing antibodies to the KUN envelope protein after two immunizations with KUN*gag* VLPs (see Results). The latter is certainly not the case after two immunizations with recombinant vaccinia viruses (4). Although the majority of the world's populations have no anti-KUN neutralizing antibodies, cross-reactive flavivirus-specific $CD8^+$ T cells recognizing nonstructural proteins may be widespread (38). However, current evidence suggests that such preexisting anti-vector $CD8^+$ T-cell responses need to be extremely high before they effectively interfere with a vaccine vector's ability to raise $CD8^+$ T-cell responses specific for the vaccine antigen (37).

Perhaps the most impressive outcome of KUN VLP immunization is the maintenance for 6 to 10 months of $CD8^+$ T cells that are capable of immediate IFN-γ secretion and of mediating protection within the 4 days of the rVV challenge assay (Fig. 7). Such long-term maintenance of effector $CD8^+$ T cells capable of immediate protective activities may emerge as an important feature of effective vaccination against HIV, since the postchallenge generation of new effectors from a vaccine-induced memory $CD8^+$ T-cell pool may simply be too slow to

deal effectively with the explosive retroviral infection (2, 16, 25). We are currently evaluating whether KUN vaccine's unique noncytopathic persistence in vivo is responsible for the long-term maintenance of effector $CD8^+$ T cells.

## ACKNOWLEDGMENTS

T. J. Harvey and I. Anraku contributed equally to the experimental work and should be considered joint first authors. A. Suhrbier and A. A. Khromykh contributed equally to the intellectual input and planning and should be considered joint last authors.

This work was funded in part by grant RA21 AI48420-01 from the National Institute of Health and grant 142911 from the National Health and Medical Research Council of Australia.

We thank A. G. M. Bustra, University of Groningen, Groningen, The Netherlands, for help with the project.

## REFERENCES

1. **Adams, S. C., A. K. Broom, L. M. Sammels, A. C. Hartnett, M. J. Howard, R. J. Coelen, J. S. Mackenzie, and R. A. Hall.** 1995. Glycosylation and antigenic variation among Kunjin virus isolates. Virology **206:**49–56.
2. **Altes, H. K., D. A. Price, and V. A. Jansen.** 2001. Effector cytotoxic T lymphocyte numbers induced by vaccination should exceed levels in chronic infection for protection from HIV. Vaccine **20:**3–6.
3. **Anraku, I., T. J. Harvey, R. Linedale, J. Gardner, D. Harrich, A. Suhrbier, and A. A. Khromykh.** 2002. Kunjin virus replicon vaccine vectors induce protective $CD8^+$ T-cell immunity. J. Virol. **76:**3791–3799.
4. **Belyakov, I. M., B. Moss, W. Strober, and J. A. Berzofsky.** 1999. Mucosal vaccination overcomes the barrier to recombinant vaccinia immunization caused by preexisting poxvirus immunity. Proc. Natl. Acad. Sci. USA **96:** 4512–4517.
5. **Bojak, A., J. Wild, H. Wolf, and R. Wagner.** 2002. Efficiency of a myogenic DNA vaccine is strictly dependent upon cellular localization of HIV-1 $Pr55^{gag}$. Vaccine **20:**1980–1984.
6. **Coffin, J. M., S. H. Hughes, and H. E. Varmus (ed.).** 1997. Retroviruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
7. **Davis, N. L., A. West, E. Reap, G. MacDonald, M. Collier, S. Dryga, M. Maughan, M. Connell, C. Walker, K. McGrath, C. Cecil, L. H. Ping, J. Frelinger, R. Olmsted, P. Keith, R. Swanstrom, C. Williamson, P. Johnson, D. Montefiori, and R. E. Johnston.** 2002. Alphavirus replicon particles as candidate HIV vaccines. IUBMB Life **53:**209–211.
8. **Durali, D., J. Morvan, F. Letourneur, D. Schmitt, N. Guegan, M. Dalod, S. Saragosti, D. Sicard, J. P. Levy, and E. Gomard.** 1998. Cross-reactions between the cytotoxic T-lymphocyte responses of human immunodeficiency virus-infected African and European patients. J. Virol. **72:**3547–3553.
9. **Edgeworth, R. L., J. H. San, J. A. Rosenzweig, N. L. Nguyen, J. D. Boyer, and K. E. Ugen.** 2002. Vaccine development against HIV-1: current perspectives and future directions. Immunol. Res. **25:**53–74.
10. **Gandhi, R. T., and B. D. Walker.** 2002. Immunologic control of HIV-1. Annu. Rev. Med. **53:**149–172.
11. **Gandhi, R. T., and B. D. Walker.** 2002. Promises and pitfalls in the reconstitution of immunity in patients who have HIV-1 infection. Curr. Opin. Immunol. **14:**487–494.
12. **Gottlinger, H. G.** 2001. The HIV-1 assembly machine. AIDS **15**(Suppl. 5)**:**S13–S20.
13. **Haglund, K., I. Leiner, K. Kerksiek, L. Buonocore, E. Pamer, and J. K. Rose.** 2002. High-level primary $CD8^+$ T-cell response to human immunodeficiency virus type 1 Gag and Env generated by vaccination with recombinant vesicular stomatitis viruses. J. Virol. **76:**2730–2738.
14. **Harrich, D., C. Hsu, E. Race, and R. B. Gaynor.** 1994. Differential growth kinetics are exhibited by human immunodeficiency virus type 1 TAR mutants. J. Virol. **68:**5899–5910.
15. **Imami, N., A. Pires, G. Hardy, J. Wilson, B. Gazzard, and F. Gotch.** 2002. A balanced type 1/type 2 response is associated with long-term nonprogressive human immunodeficiency virus type 1 infection. J. Virol. **76:**9011–9023.
16. **Kaul, R., S. L. Rowland-Jones, J. Kimani, T. Dong, H. B. Yang, P. Kiama, T. Rostron, E. Njagi, J. J. Bwayo, K. S. MacDonald, A. J. McMichael, and F. A. Plummer.** 2001. Late seroconversion in HIV-resistant Nairobi prostitutes despite pre-existing HIV-specific CD8+ responses. J. Clin. Investig. **107:** 341–349.
17. **Khromykh, A. A.** 2000. Replicon-based vectors of positive strand RNA viruses. Curr. Opin. Mol. Ther. **2:**555–569.
18. **Khromykh, A. A., A. N. Varnavski, and E. G. Westaway.** 1998. Encapsidation of the flavivirus Kunjin replicon RNA by using a complementation system providing Kunjin virus structural proteins in *trans*. J. Virol. **72:**5967–5977.
19. **Khromykh, A. A., and E. G. Westaway.** 1997. Subgenomic replicons of the flavivirus Kunjin: construction and applications. J. Virol. **71:**1497–1505.
20. **Le, T. T., D. Drane, J. Malliaros, J. C. Cox, L. Rothel, M. Pearse, T. Woodberry, J. Gardner, and A. Suhrbier.** 2001. Cytotoxic T cell polyepitope vaccines delivered by ISCOMs. Vaccine **19:**4669–4675.
21. **Letvin, N. L., D. H. Barouch, and D. C. Montefiori.** 2002. Prospects for vaccine protection against HIV-1 infection and AIDS. Annu. Rev. Immunol. **20:**73–99.
22. **Lundstrom, K.** 2002. Alphavirus-based vaccines. Curr. Opin. Mol. Ther. **4:**28–34.
23. **Mackenzie, J. M., M. K. Jones, and P. R. Young.** 1996. Improved membrane preservation of flavivirus-infected cells with cryosectioning. J. Virol. Methods **56:**67–75.
24. **Mata, M., Z. J. Yao, A. Zubair, K. Syres, and Y. Paterson.** 2001. Evaluation of a recombinant Listeria monocytogenes expressing an HIV protein that protects mice against viral challenge. Vaccine **19:**1435–1445.
25. **Mateo, L., J. Gardner, and A. Suhrbier.** 2001. Delayed emergence of bovine leukemia virus following vaccination with a protective cytotoxic T cell-based vaccine. AIDS Res. Hum. Retrovir. **17:**1447–1453.
26. **McAdam, S., P. Kaleebu, P. Krausa, P. Goulder, N. French, B. Collin, T. Blanchard, J. Whitworth, A. McMichael, and F. Gotch.** 1998. Cross-clade recognition of p55 by cytotoxic T lymphocytes in HIV-1 infection. AIDS **12:**571–579.
27. **McGettigan, J. P., S. Sarma, J. M. Orenstein, R. J. Pomerantz, and M. J. Schnell.** 2001. Expression and immunogenicity of human immunodeficiency virus type 1 Gag expressed by a replication-competent rhabdovirus-based vaccine vector. J. Virol. **75:**8724–8732.
28. **McMichael, A. J., and S. L. Rowland-Jones.** 2001. Cellular immune responses to HIV. Nature **410:**980–987.
29. **Nixon, D. F., A. R. Townsend, J. G. Elvin, C. R. Rizza, J. Gallwey, and A. J. McMichael.** 1988. HIV-1 gag-specific cytotoxic T lymphocytes defined with recombinant vaccinia virus and synthetic peptides. Nature **336:**484–487.
30. **O'Hagan, D., M. Singh, J. Kazzaz, M. Ugozzoli, M. Briones, J. Donnelly, and G. Ott.** 2002. Synergistic adjuvant activity of immunostimulatory DNA and oil/water emulsions for immunization with HIV p55 gag antigen. Vaccine **20:**3389–3398.
31. **Qiu, J. T., B. Liu, C. Tian, G. N. Pavlakis, and X. F. Yu.** 2000. Enhancement of primary and secondary cellular immune responses against human immunodeficiency virus type 1 Gag by using DNA expression vectors that target Gag antigen to the secretory pathway. J. Virol. **74:**5997–6005.
32. **Qiu, J. T., R. Song, M. Dettenhofer, C. Tian, T. August, B. K. Felber, G. N. Pavlakis, and X. F. Yu.** 1999. Evaluation of novel human immunodeficiency virus type 1 Gag DNA vaccines for protein expression in mammalian cells and induction of immune responses. J. Virol. **73:**9145–9152.
33. **Rayevskaya, M. V., and F. R. Frankel.** 2001. Systemic immunity and mucosal immunity are induced against human immunodeficiency virus Gag protein in mice by a new hyperattenuated strain of *Listeria monocytogenes*. J. Virol. **75:**2786–2791.
34. **Schlesinger, S.** 2001. Alphavirus vectors: development and potential therapeutic applications. Expert Opin. Biol. Ther. **1:**177–191.
35. **Schneider, R., M. Campbell, G. Nasioulas, B. K. Felber, and G. N. Pavlakis.** 1997. Inactivation of the human immunodeficiency virus type 1 inhibitory elements allows Rev-independent expression of Gag and Gag/protease and particle formation. J. Virol. **71:**4892–4903.
36. **Sharpe, S., N. Polyanskaya, M. Dennis, G. Sutter, T. Hanke, V. Erfle, V. Hirsch, and M. Cranage.** 2001. Induction of simian immunodeficiency virus (SIV)-specific CTL in rhesus macaques by vaccination with modified vaccinia virus Ankara expressing SIV transgenes: influence of pre-existing anti-vector immunity. J. Gen. Virol. **82:**2215–2223.
37. **Sherritt, M. A., J. Gardner, S. L. Elliott, C. Schmidt, D. Purdie, G. Deliyannis, W. R. Heath, and A. Suhrbier.** 2000. Effect of pre-existing cytotoxic T lymphocytes on therapeutic vaccines. Eur. J. Immunol. **30:**671–677.
38. **Spaulding, A. C., I. Kurane, F. A. Ennis, and A. L. Rothman.** 1999. Analysis of murine $CD8^+$ T-cell clones specific for the dengue virus NS3 protein: flavivirus cross-reactivity and influence of infecting serotype. J. Virol. **73:** 398–403.
39. **Vajdy, M., J. Gardner, J. Neidleman, L. Cuadra, C. Greer, S. Perri, D. O'Hagan, and J. M. Polo.** 2001. Human immunodeficiency virus type 1 Gag-specific vaginal immunity and protection after local immunizations with Sindbis virus-based replicon particles. J. Infect. Dis. **184:**1613–1616.
40. **Varnavski, A. N., and A. A. Khromykh.** 1999. Noncytopathic flavivirus replicon RNA-based system for expression and delivery of heterologous genes. Virology **255:**366–375.
41. **Varnavski, A. N., P. R. Young, and A. A. Khromykh.** 2000. Stable high-level expression of heterologous genes in vitro and in vivo by noncytopathic DNA-based Kunjin virus replicon vectors. J. Virol. **74:**4394–4403.
42. **Villacres, M. C., J. Zuo, and C. C. Bergmann.** 2000. Maintenance of $CD8^+$ T-cell memory following infection with recombinant sindbis and vaccinia viruses. Virology **270:**54–64.
43. **Westaway, E. G., J. M. Mackenzie, M. T. Kenney, M. K. Jones, and A. A. Khromykh.** 1997. Ultrastructure of Kunjin virus-infected cells: colocalization of NS1 and NS3 with double-stranded RNA, and of NS2B with NS3, in virus-induced membrane structures. J. Virol. **71:**6650–6661.