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Humoral, Mucosal, and Cellular Immunity in Response to a Human Immunodeficiency Virus Type 1 Immunogen Expressed by a Venezuelan Equine Encephalitis Virus Vaccine Vector

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A molecularly cloned attenuated strain of Venezuelan equine encephalitis virus (VEE) has been genetically configured as a replication-competent vaccine vector for the expression of heterologous viral proteins (N. L. Davis, K. W. Brown, and R. E. Johnston, J. Virol. 70:3781–3787, 1996). The matrix/capsid (MA/CA) coding domain of human immunodeficiency virus type 1 (HIV-1) was cloned into the VEE vector to determine the ability of a VEE vector to stimulate an anti-HIV immune response in mice. The VEE-MA/CA vector replicated rapidly in the cytoplasm of baby hamster kidney (BHK) cells and expressed large quantities of antigenically identifiable MA/CA protein. When injected subcutaneously into BALB/c mice, the vector invaded and replicated in the draining lymphoid tissues, expressing HIV-1 MA/CA at a site of potent immune activity. Anti-MA/CA immunoglobulin G (IgG) and IgA antibodies were present in serum of all immunized mice, and titers increased after a second booster inoculation. IgA antibodies specific for MA/CA were detected in vaginal washes of mice that received two subcutaneous immunizations. Cytotoxic T-lymphocyte responses specific for MA/CA were detected following immunization with the MA/CA-expressing VEE vector. These findings demonstrate the ability of a VEE-based vaccine vector system to stimulate a comprehensive humoral and cellular immune response. The multifaceted nature of this response makes VEE an attractive vaccine for immunization against virus infections such as HIV-1, for which the correlates of protective immunity remain unclear, but may include multiple components of the immune system.

The development of a vaccine against human immunodeficiency virus type 1 (HIV-1) has proven to be more difficult than first anticipated (42). Recent studies have demonstrated that immunity to infection is possible; however, the mechanisms of this protection remain poorly defined (17). These studies suggest that protection may be mediated either by an individual component of the immune system or through a combination of humoral immunity in the form of neutralizing antibodies (15, 23, 37), mucosal immunity (40, 50), and cellular immunity (28, 47). Until the correlates of protection are better understood, development of vaccines that simultaneously stimulate multiple components of the immune system would be desirable (42).

One approach to the development of HIV vaccines is the use of live-virus or bacterial vectors for the in vivo expression of lentivirus gene products (1, 11, 19, 20, 26, 38, 40, 41, 43). For example, strategies priming with recombinant vaccinia virus or canarypox virus vectors expressing HIV immunogens followed by booster inoculations with glycoprotein or peptides have been tested in primate HIV or simian immunodeficiency virus (SIV) challenge models. Many of these approaches afforded protection against homologous viral challenge, but protection against heterologous viral challenge was more variable (3, 11, 12, 20, 49).

The suitability of alphaviruses as viral expression vectors has been described previously (21, 46). These positive-stranded RNA viruses infect a broad range of host cells and initiate a rapid replication cycle within the cytoplasm (51). Expression of the structural proteins is controlled by a subgenomic mRNA promoter which transcribes message at levels 10-fold that of the genome. Alphavirus expression vectors have utilized this strong viral subgenomic mRNA promoter, either by replacing the viral structural protein genes with a foreign gene resulting in a defective recombinant (2, 32, 38, 52–54) or by adding a duplicate copy of the promoter to express the foreign gene, as was done here, resulting in a fully replicative recombinant virus (14, 33, 34). We have previously engineered an attenuated vaccine candidate strain of the alphavirus Venezuelan equine encephalitis virus (VEE) for the expression of heterologous viral proteins (8).

There are a number of features of VEE which suggest it may be unusually well suited as a viral vaccine vector, especially against HIV-1. First, parenteral immunization of rodents, primates, and humans with live attenuated VEE vaccines results in protection against lethal VEE challenge, not only via a parenteral route, but also after intranasal or aerosol challenge (6, 7, 22, 27, 44). This suggests that an HIV vaccine delivery strategy using VEE-based vectors could induce protection against invasion across a mucosal surface. This hypothesis was tested by immunizing mice by subcutaneous (s.c.) inoculation with a VEE vector expressing influenza virus hemagglutinin (HA) (8). The VEE-HA vector afforded complete protection against disease after a subsequent intranasal challenge with influenza virus. Influenza virus replication in the nasal mucosa was reduced, demonstrating that this protection extended to the mucosal surface itself. Second, unlike vaccinia virus, poliovirus, adenovirus, herpesvirus, and influenza virus-based vaccine vectors, most of the human population has never been exposed to VEE. Therefore, immunization to HIV with a VEE-based vector would not be restricted by preexisting immunity to the vector itself. Finally, unlike other alphaviruses, VEE is lymphotrophic (24). After s.c. inoculation, replication

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is first detected in the lymph nodes draining that site. This attribute confers the ability to specifically target the expression of a heterologous protein to lymphoid tissues, where one might expect the initiation of a vigorous immune response (29, 31, 32).

To test the ability of a VEE-based vector to induce a complete immune response against an HIV immunogen, the matrix/capsid (MA/CA) coding region of the HIV-1 *gag* gene was cloned under the control of an additional VEE mRNA subgenomic promoter. The matrix and capsid proteins were chosen because they are major structural components of the HIV-1 virion, representing the N-terminal portion of the Gag precursor protein, and contain known murine cytotoxic T-lymphocyte (CTL) epitopes. Immunization with MA/CA alone might not be expected to provide protective immunity in humans, but MA/CA as a constituent of a vaccine together with gp160 and other HIV immunogens should elicit a broader immune response which may be sufficient to provide protection. Humoral and cellular immune responses directed against the matrix and capsid proteins are readily detectable during HIV infection in humans (17).

In mice inoculated parenterally, the VEE-MA/CA vector targeted lymphoid tissue, resulting in the generation of humoral and cellular responses specific for HIV MA/CA. In addition, anti-MA/CA immunoglobulin A (IgA) antibodies were detected in mucosal secretions from vaginal washes. These findings suggest the suitability of the VEE vector to elicit humoral, mucosal, and cellular immunity.

MATERIALS AND METHODS

Clones and viruses. The attenuated VEE vector has been described previously (8). Briefly, the full-length cDNA of the virulent Trinidad donkey strain of VEE was altered to contain two independently attenuating mutations (E2 Lys 209 and E1 Thr 272) (9, 13). This attenuated mutant (pV3014) was engineered further to express heterologous genes with the introduction of a duplicate copy of the 26S subgenomic mRNA promoter inserted immediately downstream of the E1 gene. The cloned cDNA of the complete MA/CA gene of HIV strain HXB2 (45) (nucleotides [nt] 336 to 1424) was amplified by PCR (Perkin-Elmer Cetus) for 25 cycles with *Taq* DNA polymerase (Gibco, BRL) with primer addition of *Sal*I restriction sites to the 5′ and 3′ ends to facilitate introduction into a shuttle vector multiple cloning site. The myristylation signal encoded at the 5′ end of the matrix coding domain was removed with the alteration of Gly 2 to Ala (nt 310 changed from G to C) encoded by the amplification primers. The upstream primer sequence was 5′ TCCCAACACT*GTCGAC*AGTCTAGTCCGCCAAG**A TG**GCTGCGAGAGCGTCAGTATTA 3′. The downstream primer sequence was 5′ CTTGGCTCAT*GTCGAC***TTA**CAAAACTCTTGCCTTATG 3′, where italics indicate the *Sal*I sites and the underlining indicates 16 nt of the 5′ untranslated region of the VEE subgenomic mRNA added upstream of the MA/CA AUG to retain the context of the subgenomic promoter. The boldface letters indicate the initiation and termination codons in the upstream and downstream primers, respectively.

The MA/CA coding domain was transferred from the shuttle vector into the full-length pV3014 background with *Cla*I. The insert and the additional promoter were sequenced (*Taq* DNA cycle sequencing procedure; U.S. Biochemical Corp.) to ensure no alterations were introduced during generation of the clone. Infectious transcripts were generated by in vitro transcription of cDNA clones linearized with *Not*I with T7 RNA polymerase (Gibco, BRL). Electroporation of baby hamster kidney (BHK) cells produced progeny virus stocks after 24 h of incubation at 37°C (10). The specific infectivity of the vector RNA transcripts, plaque morphology, and growth kinetics of the vector virions closely resembled those of the parental attenuated V3014 virus.

Immunization of mice. Six-week-old female BALB/c mice were obtained from Charles River Laboratories (Wilmington, Mass.). Mice were acclimatized for 1 week in the BL-3 laboratory with sterile bedding and cages prior to any procedure.

Mice were inoculated s.c. in both rear footpads with 10^4 PFU of VEE-MA/CA vector in diluent (phosphate-buffered saline [PBS] with 1% donor calf serum) under light Metofane anesthesia (Pitman-Moore). Control mice were mock immunized with diluent alone or VEE vector without insert. Booster immunizations were performed 3 weeks postpriming with an increased virus concentration of 10^5 PFU per rear footpad.

Polyacrylamide gel analysis of [^{35}S]methionine-labeled viral proteins. BHK cell monolayers (10^7 cells) were infected with VEE vector virus at 37°C at a multiplicity of infection of 10 and were incubated in methionine-free media between 6 and 10 h postinfection. Ten hours postinfection, cells were washed and placed in medium containing [^{35}S]methionine (20 μCi/ml) for 2 h. Twelve hours postinfection, cytoplasmic extracts were prepared by lysis in 1 ml of lysis buffer with protease inhibitors (0.05 M Tris-HCl [pH 7.5], 0.1 M NaCl, 0.001 M EDTA, 0.2% Nonidet P-40, 1 μM leupeptin, 1 μM pepstatin, and 0.2 mM phenylmethylsulfonyl fluoride). Nuclei were removed by centrifugation at 11,000 × *g* for 1 min. Labeled cytoplasmic proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% polyacrylamide; stock 30% [wt/vol] acrylamide, 0.8% [wt/vol] bisacrylamide; Protogel; National Diagnostics) under reducing conditions (50 mM 2-mercaptoethanol) (30). Protein bands on gels were visualized by Coomassie brilliant blue staining or fluorography with Amplify (Amersham). Expression levels of MA/CA protein were determined with an HIV p24 Core Profile enzyme-linked immunosorbent assay (ELISA) kit (Dupont; NEK-060A).

Western blotting. Cell lysates were prepared as detailed in the previous section. Western blotting was performed by standard methods (16). Briefly, proteins were separated by SDS-PAGE through 15% acrylamide gels. After transfer onto nitrocellulose, blots were blocked for 1 h in 2% bovine serum albumin in TBST (10 mM Tris base [pH 8.0], 150 mM NaCl, 0.05% Tween 20). Blots were incubated for 1 h with one of the following primary antibodies: mouse anti-HIV p17 monoclonal antibody (Cellular Products, Buffalo, N.Y.), HIV-positive patient serum (BB6) (25), or anti-VEE hyperimmune mouse ascites fluid (HMAF) diluted 1:2,000 in TBST. Secondary antibodies were anti-mouse or anti-human IgG alkaline phosphatase-conjugated antibodies (Promega) diluted 1:4,000 in TBST buffer. Blots were developed in 10 ml of bromochloroindolyl phosphate-nitroblue tetrazolium substrate (BCIP/NBT) in 0.1 M Tris (pH 9.5)–0.1 M NaCl–5 mM $MgCl_2$ (Promega).

In situ hybridization. For in situ hybridization mice were inoculated s.c. in the left rear footpad with 10^4 PFU of VEE-MA/CA vector in 10 μl of PBS–1% donor calf serum or diluent only for controls. At 24 and 48 h postinoculation, two mice per group were sacrificed, and draining popliteal lymph node, spleen, and thymus tissues were harvested and fixed in 4% paraformaldehyde in PBS. Riboprobes for the VEE structural genes (678 nt) and influenza virus (PR8 strain) HA gene (500 nt) (negative control) were generated as previously described (8, 13). To detect HIV MA/CA-specific RNA, a pGEM-3 (Promega) clone containing the 627-nt *Hin*dIII fragment of HXB2 (nt 631 to 1258) was linearized at the unique *Pvu*II site. In vitro Sp6 polymerase transcription reaction mixtures containing [α-^{35}S]UTP generated a 550-nt radiolabeled RNA probe complementary to MA/CA message sense RNA. Probes were hybridized to paraffin-embedded sections mounted on aminopropyl triethoxysilane-coated slides (ProbeOn Plus; Fisher Scientific). Wash conditions were as previously described (13). Slides were dipped in Kodak NTB-2 emulsion, dried, and exposed at −70°C for 144 h. After development, slides were counterstained with Gill's hematoxylin.

Antibody detection and titration. Serum samples were obtained from tail vein bleeds 3 weeks after primary or secondary inoculation. ELISAs for the detection of anti-VEE antibody used 250 ng of gradient-purified VEE virus per well as antigen. The HIV MA/CA ELISA used 250 ng of bacterially expressed MA/CA protein (the kind gift of H. Ke, Department of Biochemistry, University of North Carolina, Chapel Hill) per well as an antigen. Secondary antibody was either horseradish peroxidase-conjugated goat anti-mouse IgG or IgA (Sigma Immunochemicals). Positive control antibodies were IgG and IgA anti-p24 mouse monoclonal antibodies (Cellular Products) for MA/CA, and for the anti-VEE ELISA, an anti-VEE HMAF. The ELISA titer was defined as the inverse of that serum dilution giving an optical density at 450 nm greater than 0.2 above the background (wells lacking serum). In all assays, normal sera and sera taken prior to immunization were never positive at a 1:40 dilution, the lowest dilution tested. Geometric mean titers were calculated for serum antibody titers from sets of four mice.

Vaginal washes were assayed by the same ELISA protocols. Washes were performed with 50 μl of PBS-D (Mg^{2+} and Ca^{2+} free) under light Metofane anesthesia 7, 10, and 14 days after the booster immunization. Washes from four individual mice were pooled and concentrated to a 50-μl final volume in Centricon columns (30-kDa exclusion limit; Amicon).

CTL culture and cytotoxicity assays. One week post-booster immunization, splenocytes were harvested from VEE-MA/CA, vector alone, or PBS-inoculated BALB/c (*H-2^d*) mice. Spleen cells were stimulated in vitro with syngeneic feeder cells infected with a recombinant vaccinia virus expressing the entire HIV-1 *gag* gene (vv-gag). Splenocytes were cultured at 4.5×10^6/well in RPMI 1640 medium (Mediatech, Washington, D.C.) containing 5% fetal calf serum (HyClone) and 2 mM L-glutamine (GIBCO). Syngeneic feeder cells were prepared by infection with either vv-gag or vaccinia virus-alone controls (vv-sc11) at a multiplicity of infection of 5 for 1 h. Excess virus was removed from the cells before further incubation for 2 h at 37°C. The cells were washed, UV treated for 30 min, and irradiated with 20 Gy from a cesium source. Feeder cells were added to splenocyte cultures at a 1:3 cell ratio, and 25 ng of human interleukin 7 per ml was added (Endogen, Cambridge, Mass.).

Cultures were incubated for 7 days, after which dead cells and debris were removed by centrifugation through a lympholyte M gradient (Cedarlane). Cultures were restimulated on day 7 with feeder cells in RPMI complete media and 30% supernatant from concanavalin A-treated splenocytes. At day 14, cells were purified through lympholyte M, and chromium release assays were performed. Major histocompatibility complex (MHC)-matched P815 mastocytoma target

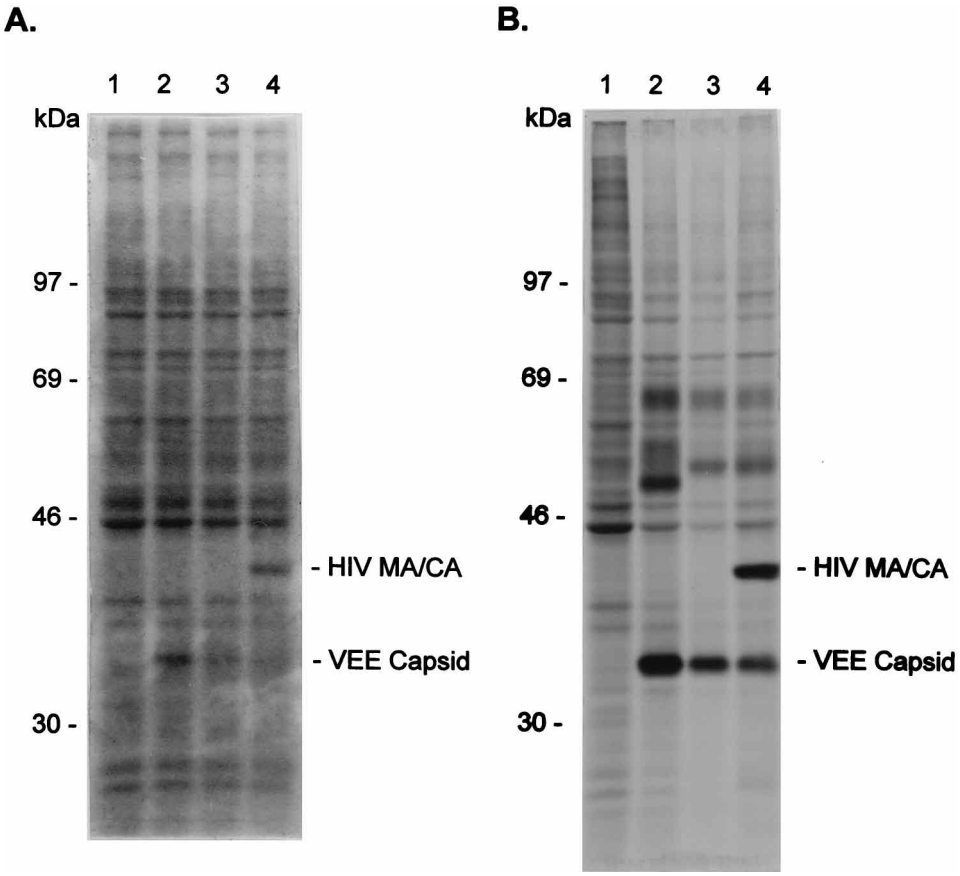

FIG. 1. PAGE of [^{35}S]methionine-labeled cytoplasmic proteins from VEE-MA/CA vector-infected BHK cells. (A) Coomassie brilliant blue-stained gel. (B) Autoradiograph of the same gel. Coelectrophoresed protein markers are indicated by molecular masses to the left. Proteins migrating at the position predicted for HIV MA/CA and the VEE capsid are labeled. Mock-infected cells (lane 1), VEE (V3000) wild-type-infected cells (lane 2), VEE vector-alone-infected cells (lane 3), and VEE-MA/CA-infected cells (lane 4) are shown.

cells were infected for 1 h with vv-gag. P815 cells infected with vaccinia virus alone (vv-sc11) or noninfected cells were used as negative controls. Targets were incubated for 18 h to allow *gag* expression, labeled with ^{51}Cr for 1 h, washed, and used in a standard 4-h chromium release assay (36).

RESULTS

Expression of HIV MA/CA protein in BHK cells. The MA/CA coding domain of HIV strain HXB2 was cloned into a VEE double-promoter expression vector (8) derived from a highly attenuated, infectious VEE clone, pV3014 (13). The myristylation site encoded in the MA/CA domain was mutated during cloning to retain the protein in the cytoplasm to potentiate processing and presentation by MHC class I proteins. To determine if HIV MA/CA was expressed from the inserted sequence, BHK cells were infected with the VEE-MA/CA vector or VEE vector alone and radiolabeled with [^{35}S]methionine, and cytoplasmic extracts were analyzed by PAGE. Both the Coomassie brilliant blue-stained gel (Fig. 1A) and autoradiograph (Fig. 1B) showed high-level expression of a protein which migrated with the same apparent molecular weight as MA/CA protein (lane 4 in Fig. 1A and B). The level of MA/CA protein synthesis was comparable to that of the VEE viral capsid, as well as the levels of some of the most predominant cellular proteins. The retarded mobility of the VEE E1 glycoprotein in lanes 3 and 4 (apparent molecular mass of 56 kDa) compared to that in lane 2 in the autoradiograph (apparent molecular mass of 51 kDa) (Fig. 1B) is due to the addition of a glycosylation site at E1 position 272 in the attenuated mutant vector background (V3014) which is absent in the wild-type virus (V3000).

To identify the protein expressed by the VEE vector as HIV MA/CA, cell lysate preparations were characterized by Western immunoblotting with either anti-p17 monoclonal antibody (Fig. 2A) or BB6 HIV-positive patient serum (Fig. 2B). A protein of the correct molecular mass was detected in lysates from cells infected with the VEE vector containing the MA/CA coding domain in the sense orientation (lanes 1), but a reactive protein was not detectable in cells infected with the antisense orientation of the MA/CA domain insert (lanes 2), or after infection with the VEE vector alone (lanes 3), or in mock-infected cells (lanes 4). As a positive control, a Western blot probed with anti-VEE polyclonal antibody stained reactive VEE capsid and glycoproteins in all three VEE-infected but not mock-infected cell lysates, as expected (Fig. 2C).

The quantity of MA/CA protein was estimated to be approximately 40 μg/10^7 BHK cells by the HIV p24 Core Profile ELISA standardized kit (Dupont). This was equivalent to 6×10^7 copies of MA/CA polypeptide/cell at 12 h postinfection. The cytoplasmic levels of the heterologous protein were comparable to those of the VEE structural proteins.

HIV MA/CA expression in lymphoid tissue. Groups of four BALB/c mice were inoculated s.c. in the left rear footpad with 10^4 PFU of VEE-MA/CA vector or VEE vector alone. In VEE-MA/CA-inoculated mice sacrificed 24 h after inocula-

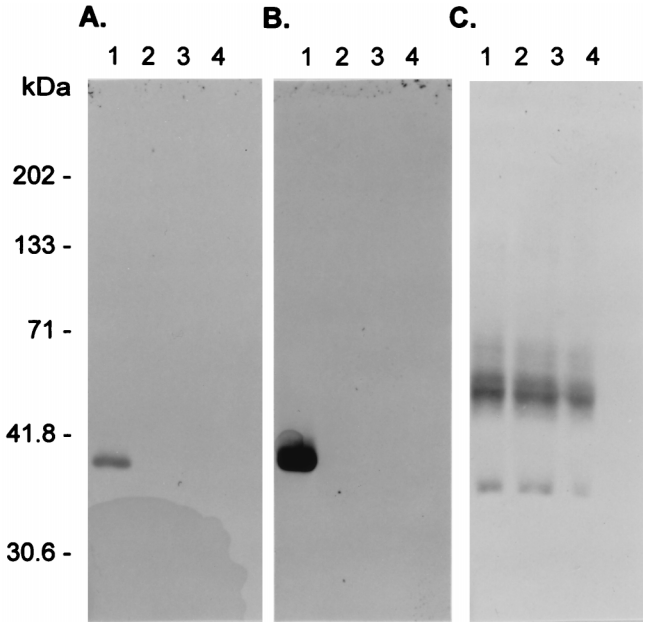

FIG. 2. Western blots of BHK cell lysates infected with the VEE-MA/CA vector. Blots were probed with a mouse anti-HIV p17 monoclonal antibody (A), HIV-positive patient serum (BB6) (B), and anti-VEE HMAF polyclonal antibody (C). In each panel, BHK cells were infected with VEE-MA/CA vector with the MA/CA coding domain in the sense orientation (lane 1), VEE-vector with the MA/CA coding domain in the antisense orientation (lane 2), VEE vector alone (lane 3), and mock-infected cell lysates (lane 4).

tion, a dense in situ hybridization signal was detected in the draining left popliteal lymph node, indicating both MA/CA and VEE mRNA expression (Fig. 3A and B, respectively). The MA/CA- and VEE-specific mRNAs were colocalized to the same foci, suggesting that the majority of infected cells expressed the MA/CA mRNA. No signal was detected in any tissue with the HA-specific negative control probe (Fig. 3C). Only VEE-specific signal was detected in mice infected with the VEE vector without the insert or with the MA/CA probe on tissues from mock-infected negative controls (results not shown). Low levels of signal also were detected in the spleen, but none were detected in the thymus of VEE MA/CA-inoculated mice. Mice sacrificed 48 h after inoculation showed a similar pattern, with slightly decreased levels of signal in the draining lymph node and very low levels in the spleen (results not shown). This demonstrates that the VEE-MA/CA vector, like its parental VEE strain, retained the ability to target replication, and hence expression of MA/CA, to lymphoid tissues.

Anti-MA/CA antibody responses in VEE-MA/CA vector-immunized mice. The ability to target expression of HIV MA/CA to lymphoid tissue might be expected to result in a strong immune response against the protein. To determine the ability of the VEE-MA/CA vector to elicit a humoral immune response, serum antibody levels were measured. Three weeks post-primary inoculation and 3 weeks post-booster inoculation, significant serum IgG and IgA anti-MA/CA levels were detected (Fig. 4). Control animals inoculated with the VEE vector alone had no detectable anti-MA/CA titer at the lowest dilution (1:40) after either the primary or boost inoculation. All animals inoculated with the VEE-MA/CA vector mounted significant anti-MA/CA serum IgG and IgA responses. These responses were significantly increased after the booster inoculation; individual mice all showed a significant increase in titer. The geometric mean titers after the booster inoculation were 12,800 and 2,265 for serum anti-MA/CA IgG and IgA, respectively. After the booster inoculation, increases in anti-VEE IgG and IgA responses also were detected. This demonstrates the ability to boost previously primed antibody responses to

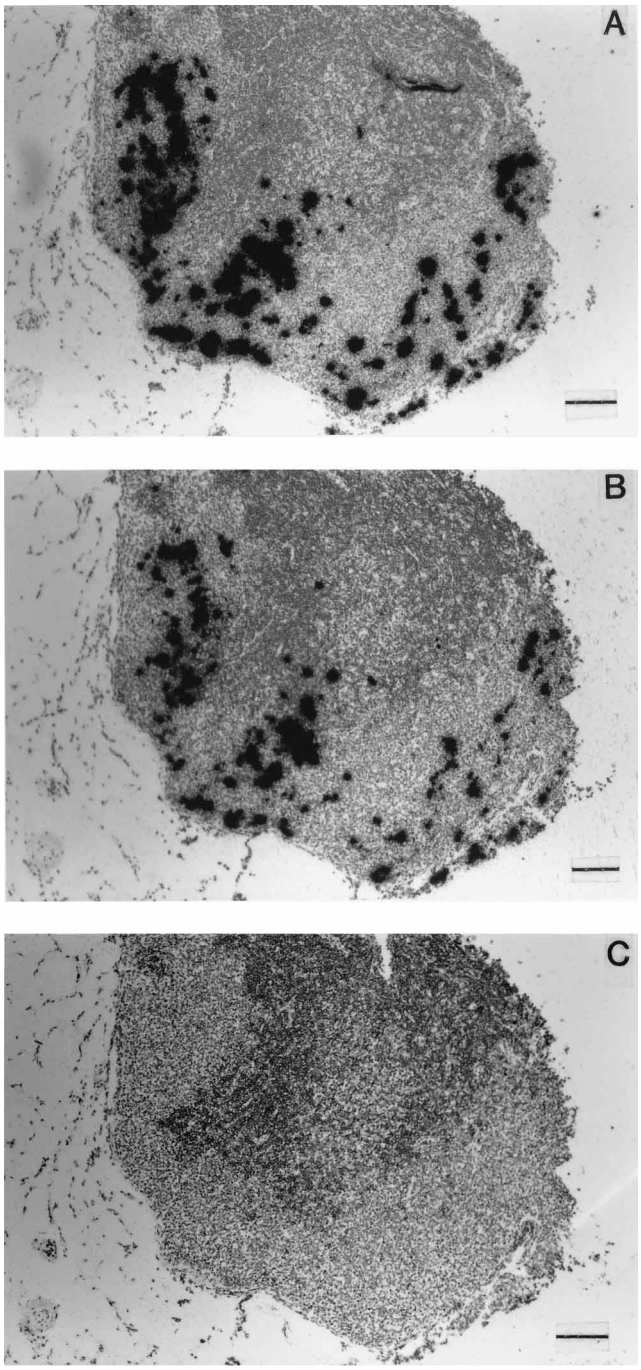

FIG. 3. Detection of HIV MA/CA mRNA expression in lymphoid tissue after footpad inoculation of BALB/c mice. Popliteal lymph nodes were harvested from mice 24 h postinoculation with the VEE-MA/CA vector. Serial sections of the lymph nodes were subjected to in situ hybridization to detect specific mRNA expression. HIV MA/CA mRNA was detected with negative-sense ^{35}S-UTP-labeled MA/CA-specific riboprobe (A). This signal was colocalized with VEE mRNA detected in an adjacent section with a VEE-specific riboprobe (B). As a negative control, an influenza virus HA-specific radiolabeled riboprobe hybridized to an adjacent tissue section detected no message (C). Magnification, ×35. Bars, 200 μm.

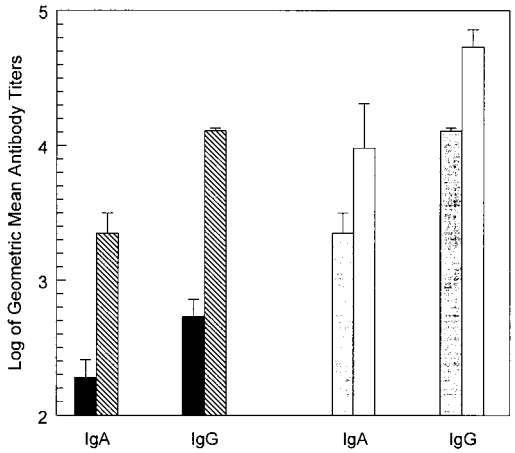

FIG. 4. Geometric mean serum antibody titers detected against HIV MA/CA or VEE. BALB/c mice received a primary s.c. inoculation and were given a booster inoculation 3 weeks later with the VEE-MA/CA vector. There were four mice per sample group. Antibody titers were measured by ELISA as described in Materials and Methods. Solid bars, anti-MA/CA serum IgA or IgG levels 3 weeks after primary inoculation with the VEE-MA/CA vector; hatched bars, anti-MA/CA levels 3 weeks after booster inoculation; shaded bars, anti-VEE titers after primary inoculation; open bars, anti-VEE titers after the booster inoculation. Error bars indicate 1 standard deviation.

both MA/CA and VEE in the presence of a preexisting immune response against the vector.

Anti-MA/CA and anti-VEE IgG levels were measured 3 and 6 months after the booster inoculation to determine the longevity of the humoral response. No decrease in titer was detected, suggesting that a long-lived immunity had been induced (results not shown).

Detection of anti-MA/CA-specific IgA antibodies in vaginal washes. The most common route of natural infection for HIV-1 is across a mucosal surface (50). A vaccine candidate which could stimulate mucosal immunity against HIV might prevent infection by this route. Mice were inoculated by the s.c. route and given booster inoculations 3 weeks later. Vaginal washes from each group of four mice (VEE-MA/CA or vector alone) were pooled and concentrated. HIV MA/CA-specific IgA antibodies were detected in vaginal washes from mice inoculated with the VEE-MA/CA vector, but not in controls (Fig. 5). This indicates the ability of the parenterally administered VEE-MA/CA vector to generate detectable IgA levels against an HIV antigen at the mucosal surface.

CTLs specific for HIV MA/CA. A likely requirement of a successful HIV vaccine candidate would be the ability to elicit a complete response, stimulating both humoral and cellular arms of the immune system. To evaluate this in the VEE system, we determined the ability of infected mice to generate CTL responses specific for MA/CA. BALB/c mice were inoculated and given booster inoculations with VEE-MA/CA or vector alone as described above. One week post-booster inoculation, splenocytes from each group were harvested, pooled, and stimulated twice in vitro with syngeneic feeder cells infected with either vaccinia virus alone (vv-sc11) or vaccinia virus expressing HIV Gag (vv-gag). CTL activity was measured with vaccinia virus-infected P815 target cells. A strong anti-MA/CA CTL response was detected in splenocytes from mice inoculated with the VEE-MA/CA vector. Cells from these mice lysed vv-gag-infected targets, but failed to lyse vv-sc11-infected or uninfected targets (Fig. 6A). Splenocytes from mice inoculated with the VEE-MA/CA vector and stimulated in vitro with feeder cells infected with vaccinia virus alone did not kill any targets (Fig. 6B). Splenocytes from control mice inoculated with the VEE vector alone showed no activity after in vitro stimulation (results not shown).

To examine the variability of the CTL response, individual mice were also tested. Three of three mice which received the VEE-MA/CA vector mounted a CTL response specific for MA/CA (results not shown). These results indicate the ability of the VEE vector to generate a consistent CTL response directed against MA/CA-encoded epitopes.

DISCUSSION

The use of live-virus vaccine vectors for the expression of HIV proteins is an active area of investigation. A number of studies with vaccinia virus, picornaviruses, adenoviruses, herpesviruses, or influenza virus, as well as alphaviruses, as expression vectors all have shown the ability to elicit some degree of immunity to HIV (46). Each virus vector has intrinsic advantages and disadvantages, such as feasibility for human use, preexisting immunity to the vector itself, stability and size of heterologous insert, as well as the strength, breadth, and longevity of the immune response elicited. It seems unlikely that one vector will be optimal in all of the properties described above. Therefore, a case can be made for the development and testing of multiple vaccine vector candidates against HIV.

The experiments reported here demonstrate (i) that VEE vaccine vectors are efficient expression systems for in vivo delivery of HIV immunogens, (ii) that the response to the expressed gene product can be enhanced by booster inoculation, and (iii) that these vectors stimulate the cellular as well as the humoral and mucosal arms of the immune system.

Expression levels of the foreign protein generated by the VEE vector are comparable to levels of expression achieved in Sindbis virus, vaccinia virus, and adenovirus vector systems (52). We have found that heterologous genes approximately 1 kb in size are relatively stable and are retained during serial tissue culture passage. Larger genes on the order of 2.5 kb, such as those expressing the HIV and SIV envelope glycoproteins, can also be expressed by the vector, although these proteins are not tolerated quite as well by the vector. This may be

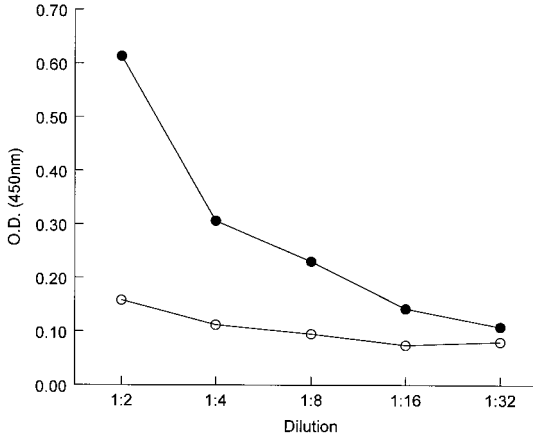

FIG. 5. Detection of anti-MA/CA IgA-specific antibodies in vaginal washes of mice immunized and given booster inoculations with the MA/CA vector or vector alone. Vaginal wash samples were pooled and analyzed by ELISA to detect MA/CA-specific IgA antibodies at the mucosal surface. Mice were immunized (primary and booster) with the MA/CA vector (●) or with VEE vector alone (○). Preimmunization and pre-booster antibody levels (not shown) were the same as those in the vector-alone group. O.D., optical density.

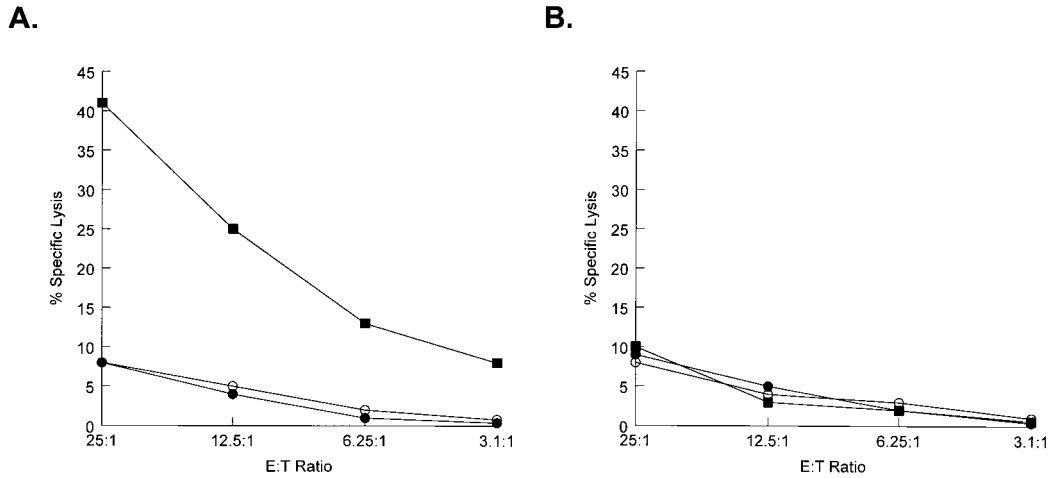

FIG. 6. CTL response specific for HIV MA/CA elicited in mice primed and given booster inoculations with the VEE-MA/CA vector. Splenocytes harvested from BALB/c mice 1 week post-booster inoculation with the MA/CA vector were stimulated twice in vitro with vv-gag (A)- or vv-sc11 (B)-infected syngeneic feeder cells. Cr^{51}-release CTL assays were performed to detect HIV MA/CA CTLs. P815 target cells were infected with vv-gag (■) or vv-sc11 (●) or were mock infected (○). The percent specific lysis for each effector/target (E:T) ratio was calculated as described in Materials and Methods.

due to size of the insert or the nature of the protein being expressed (5).

We previously have demonstrated the efficacy of a VEE-based vaccine vector expressing influenza virus HA in a mucosal challenge model (8). Mice immunized parenterally with the VEE-HA vector not only survived an otherwise lethal intranasal inoculation of virulent influenza virus, but were totally protected from disease. Protection was evident at the mucosal surface, as shown by reduced replication of influenza virus in the nasal respiratory epithelium. This study suggested that VEE could be a useful vaccine vector for pathogens such as HIV which enter across a mucosal surface.

To test the potential of VEE-based vaccine vectors to stimulate an immune response to a lentivirus immunogen, we expressed the matrix/capsid region of the HIV *gag* gene. Both humoral and cellular responses are mounted against the Gag protein in a natural infection, and core antigens may be an important component of a vaccine (18, 35). The VEE vector expressed high levels of HIV MA/CA protein in cell culture. In vivo, it produced no signs of infection upon s.c. inoculation because of the presence of two independently attenuating mutations in the glycoprotein genes (13).

Previous studies have shown that specifically targeting the iliac lymph node with an SIV envelope and core subunit vaccine provides protection against SIV challenge in macaques (31, 32). Here we show that the VEE-MA/CA vector was specifically targeted to the draining lymph node, where expression was readily detected by in situ hybridization. This suggests that the lymphoid targeting demonstrated by VEE may be a highly desirable characteristic for a vaccine vector, in that foreign protein expression is concentrated at a site of high immune activity. The molecular mechanisms underlying this lymphoid targeting are under active investigation.

The VEE vector efficiently elicited a humoral antibody response to HIV MA/CA in all mice tested. Both serum IgG and IgA antibodies were detected, indicating class switching had occurred. The humoral immune response elicited in mice given booster inoculations with the VEE-MA/CA vector was long lived, because serum IgG levels were undiminished at 6 months post-booster inoculation. The addition of a second inoculation significantly increased antibody titers in all mice. This was somewhat surprising, considering the preexisting levels of anti-VEE neutralizing antibodies elicited during the primary vector immunization (5). This may reflect the ability of the vaccine vector, when inoculated s.c., to efficiently traffic to the local lymph nodes by a route not exposed to high levels of circulating neutralizing antibody. Alternatively, achieving a secondary response to MA/CA in the presence of anti-VEE neutralizing antibodies may require much lower expression than that required to prime the response initially. Experiments are in progress to test the ability of these vectors to prime a response to a heterologous immunogen in a VEE immune host. These considerations suggest that booster inoculation strategies will be important in the design of VEE vaccines and VEE vaccine vectors.

In addition to the strong serum antibody response detected in the sera of mice inoculated with the vector, we detected anti-MA/CA IgA antibodies at the mucosal surface in vaginal washes. The mucosal immune system may play an important role in protective vaccination against HIV (50), and the ability of a vaccine candidate to generate antibodies both in the serum and at mucosal surfaces is a desirable characteristic. However, the mechanism by which these antibodies reached the vaginal mucosa is not understood. Two possibilities are transudation of IgA from the serum across the mucosal epithelium or secretion of IgA at the mucosal surface itself (39, 50). An s.c. inoculation of the parental attenuated virus, upon which the vector is based, provides protection against virulent VEE challenge at a mucosal surface, and the immunizing virus replicates in lymphoid tissue, including Peyer's patches, in the gut-associated lymphoid tissue (6). Whether VEE vectors also target lymphoid tissues in the gut and genital mucosa remains to be determined.

The cellular immune system is thought to play an integral role in the initial response to HIV infection. Cellular immune responses are believed to reduce viral load and may be responsible for viral clearance or immune protection in a small number of cases (17). We have shown the ability of the VEE vaccine vector to raise a cellular immune response specific for HIV MA/CA protein. Every animal inoculated with the vector raised a cellular response, with significant lysis at effector/target ratios as low as 6:1. The longevity of the cellular response elicited by the VEE vector is currently under investigation. The cellular immune response to MA/CA is probably

the more relevant response to this antigen, since matrix and capsid are present either inside the infected cell or inside the viral particle and are thus not exposed to neutralizing antibodies.

Safety issues are a major concern in the rational design of any vaccine. A VEE-based vaccine system has good potential for human use, as judged by a number of studies evaluating the use of live attenuated VEE vaccines. TC-83 is the only alphavirus vaccine tested extensively in humans to date (4). More recently, live attenuated strains of VEE with improved efficacy and safety in rodents (9), horses and primates (44), have been developed. These candidates (including the strain used for the VEE vector described here) provide improved protection compared to the existing TC-83 vaccine strain. They have the added benefit of not producing a serum viremia in rodents sufficient to support spread by mosquito vectors (13) and are unlikely to produce such a viremia in humans (48).

In conclusion, we have shown that a VEE-based vaccine vector expresses high levels of HIV MA/CA. The vector targeted expression of MA/CA protein to the lymph nodes, eliciting a strong humoral and cellular immune response in the periphery. The vector also generated MA/CA-specific IgA antibodies in the genital mucosa. This suggests that VEE-based vaccine vectors can stimulate multiple arms of the immune system to elicit a comprehensive immune response against HIV immunogens. Future studies will include expression of HIV glycoproteins and SIV immunogens, to allow the efficacy of VEE vectors to be tested in a primate challenge model.

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ADDENDUM IN PROOF

After this article was accepted for publication, the humoral and cellular responses elicited against HIV MA/CA were determined at 11 months post-booster inoculation. HIV MA/CA-specific serum IgG and IgA levels were found to be undiminished, and bulk CTL assays detected MA/CA-specific lysis in splenocytes from 4 of 4 mice primed and boosted with the VEE-MA/CA vector.

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