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Mucosal Immunity Induced by Parenteral Immunization with a Live Attenuated Venezuelan Equine Encephalitis Virus Vaccine Candidate

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Induction of a mucosal immune response is generally thought to require introduction of an immunogen directly onto the mucosal surface. It has been observed, however, that live, attenuated mutants of the alphavirus, Venezuelan equine encephalitis virus (VEE), induce protection from virulent challenge at the respiratory mucosa even after parenteral inoculation. In this report, we propose a mechanism by which subcutaneous immunization with a molecularly cloned, attenuated double mutant of VEE is able to stimulate the production of mucosal anti-VEE IgA. Our results showed that the immunizing virus spread to, and replicated within, lymphoid tissues throughout the mouse. Several tissues known to be inductive sites of the mucosal immune system were found to be positive for the presence of VEE RNA by 48 hr postimmunization. Moreover, this mucosal lymphotropism resulted in the production of virus-specific IgA antibody detectable in vaginal secretions of immunized mice.

INTRODUCTION

Venezuelan equine encephalitis virus (VEE) is a member of the alphavirus genus, family *Togaviridae,* a group of viruses that cause disease in humans and other vertebrates (Peters and Dalrymple, 1990). In the natural setting, alphaviruses are transmitted by arthropod vectors. An infectious dose is delivered by the bite of an infected mosquito, which can be mimicked experimentally by subcutaneous inoculation (Turrel and Spielman, 1992; Turrel *et al.,* 1995). VEE is unusual among alphaviruses in that it also can spread via aerosol transmission, which makes it a significant hazard to laboratory and veterinary workers. Therefore, one goal in the design of a VEE vaccine is to protect against both parenteral and aerosol routes of infection.

We have constructed a series of VEE-derived cDNA clones in which selected attenuating mutations, identified in biological isolates, were combined (Davis *et al.,* 1989, 1991; Johnston and Smith, 1988). Transcription of such clones *in vitro* yielded infectious RNA genome equivalents from which the cognate series of VEE vaccine candidates was derived. V3014 is one of these candidates. It contains two attenuating mutations in the surface glycoprotein genes, a change from the parental Glu at E2 209 to Lys, and a change from the parental Ala at E1 272 to Thr, as well as a third change, from Ile to Asn at E2 239, resulting in no known phenotypic change from wild type (Grieder *et al.,* 1995). As was the case for all of the recombinant vaccine candidates tested, V3014 showed greatly reduced virulence in mice, hamsters, and horses and induced solid protection against a high dose parenteral challenge with virulent VEE (Davis *et al.,* 1991; Smith and Johnston, unpublished observations).

Virulent VEE delivered to mice by aerosol or intranasal inoculation replicates within the nasal mucosa and within olfactory sensory neurons located there. The virus invades the brain rapidly by anterograde spread of virus down the axons of the chemosensory olfactory neurons (Charles *et al.,* 1995; Ryzhikov *et al.,* 1991, 1995). Vaccination with vaccinia vectors expressing VEE glycoproteins protects mice against parenteral, but not against intranasal challenge with virulent VEE (Kinney *et al.,* 1988). A formaldehyde-inactivated VEE vaccine also is protective against parenteral, but not against aerosol challenge in hamsters (Jahrling and Stephenson, 1984). Live, attenuated VEE vaccines, however, generally provide protection from aerosol and/or intranasal challenge (Davis *et al.,* 1995; Jahrling and Stephenson, 1984; Kinney *et al.,* 1988).

Antigen presentation within the mucosal associated lymphoid tissue is thought to be critical for the production of protective secretory IgA antibody (Lange *et al.,* 1980; Liang *et al.,* 1988; Nedrud *et al.,* 1987; Weisz-Carrington *et al.,* 1979, 1987). Once presented in an inductive site, IgA specific for the antigen is found at mucosal surfaces throughout the mouse. In most cases, delivery of the immunogen to a mucosal surface is a prerequisite for uptake into one of the mucosal-associated lymphoid tissues. At least one such site,

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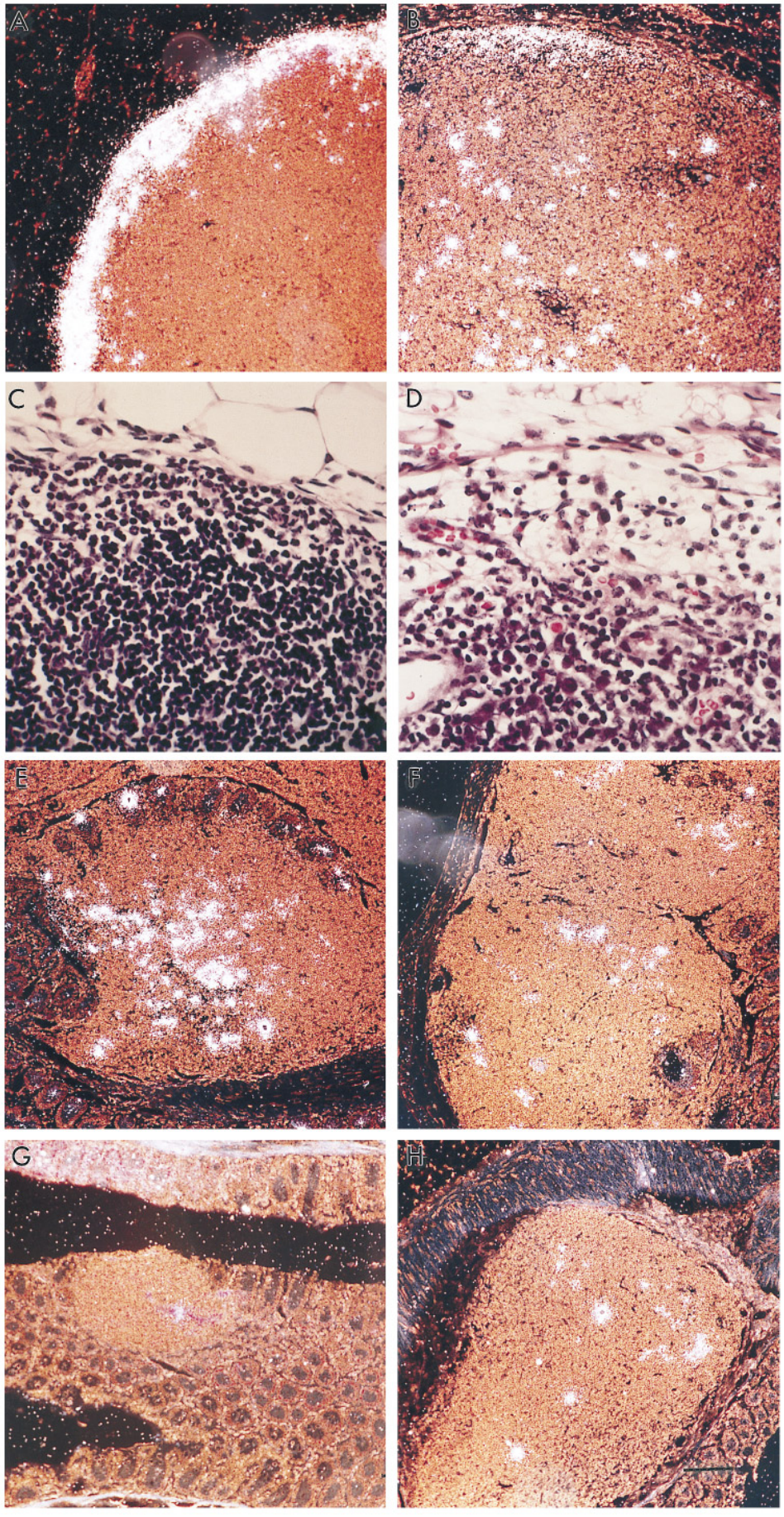
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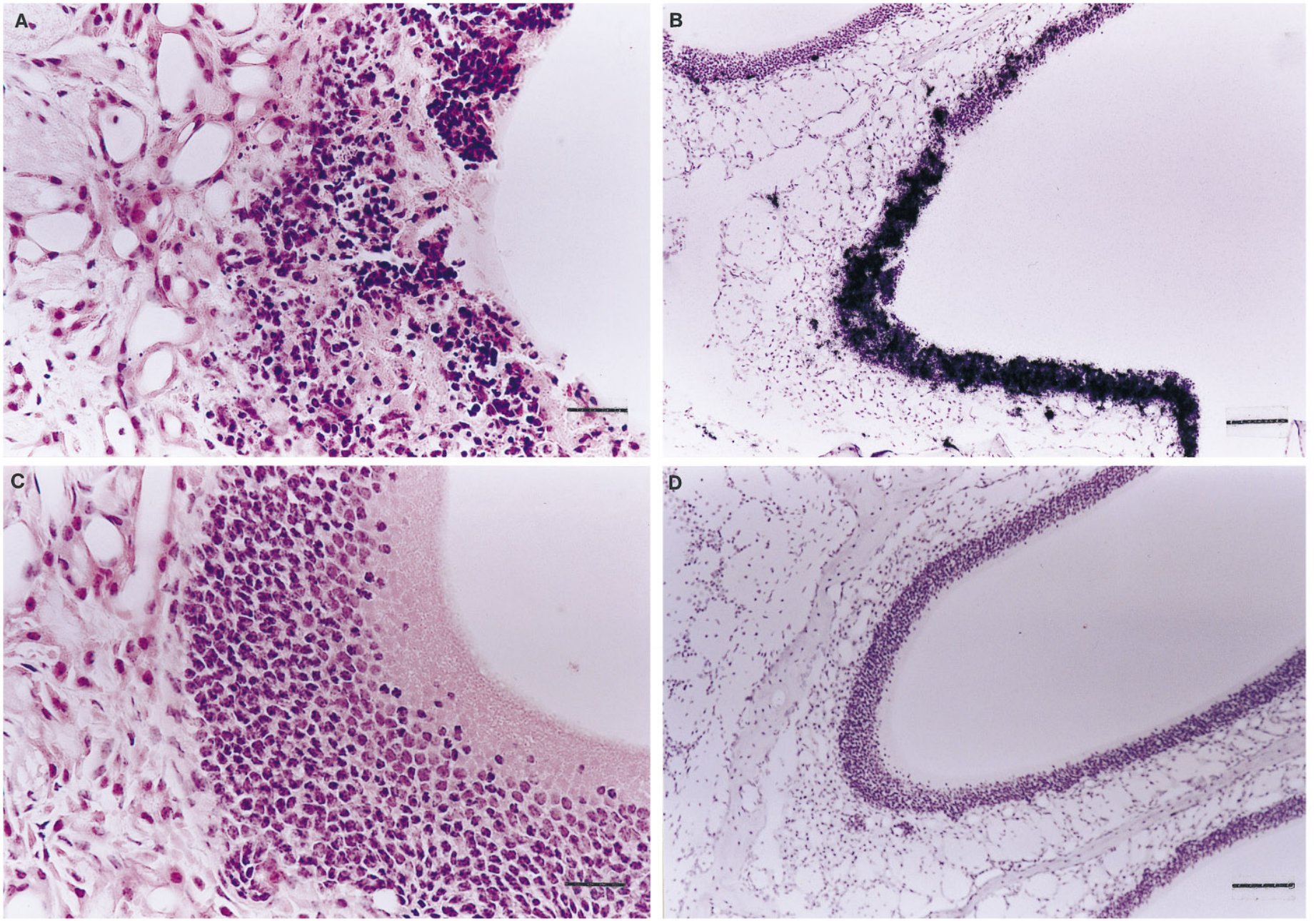
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the Peyer's patch, has been implicated in the pathology of VEE in hamsters (Gorelkin and Jahrling, 1975). Therefore, we tested the hypothesis that subsequent to subcutaneous immunization with V3014, the virus itself or V3014-infected cells are targeted to a mucosal IgA inductive site(s). In this report we demonstrate (1) that subcutaneous inoculation with a recombinant VEE vaccine candidate, V3014, results in targeted replication within inductive sites of the mucosal immune system, (2) that this mucosal lymphotropism results in the production of mucosal IgA specific for VEE, and (3) that the immune response to V3014 can protect mice against intranasal challenge with virulent virus. While it is not yet clear which specific elements of the immune response to V3014 are involved in the protection afforded to animals immunized with this virus, mucosal anti-VEE IgA is an appealing candidate.

MATERIALS AND METHODS

Mice

Five-week-old female CD-1 mice were obtained from Charles Rivers Laboratories (Wilmington, MA). Mice were maintained in a BL-3 laboratory in barrier cages with sterile bedding, water, and standard mouse chow (*ad libitum*). All mice were allowed to acclimatize in the BL-3 laboratory for at least 1 week prior to undergoing any procedure. Mice were inoculated by injection of virus diluted in PBS plus 1% donor calf serum (PBS-DCS) into the left rear footpad while under light Metophane anesthesia. Mice were sacrificed at the time points indicated in the figure legends and perfused transcardially with 100 ml of ice-cold 4% paraformaldehyde in PBS, pH 7.2. Ipsilateral popliteal lymph node, mandibular lymph node, mesenteric lymph node, spleen, thymus, five sections of ileum containing Peyer's patches, and sections of rectal tissue containing perirectal lymph nodes were dissected and processed for histological examination and ISH. Nasal tissues were examined by decalcification of whole heads, followed by sectioning and embedding, as previously described (Charles *et al.,* 1995; Davis *et al.,* 1996). All animal experimentation was performed in accordance with National Institutes of Health guidelines. All animal protocols were approved by the animal care and use committee of the University of North Carolina at Chapel Hill.

Cells

BHK-21 cells were obtained from ATCC in the 53rd passage and maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% donor calf serum, 10% tryptose phosphate broth, 0.29 mg/ml L-glutamine, 0.5 mg/ml streptomycin, and 100 units/ml penicillin. Cells were used from passage 55 to 65.

Virus

Stocks of VEE virus were produced as described previously (Davis, 1989; Grieder, 1995). Briefly, the plasmids pV3000 and pV3014, containing full-length viral cDNAs, were linearized with *Not*I, and runoff RNA transcripts were produced with T7 RNA polymerase. RNA transcripts were introduced into BHK-21 cells by electroporation (Davis *et al.,* 1996). Tissue culture supernatants were harvested at 24 hr when CPE was maximal, and virus was quantitated by standard plaque assay on BHK-21 cells. Working stocks of virus were prepared by dilution of supernatants into phosphate-buffered saline containing 1% donor calf serum (PBS-DCS), to give a final concentration of 10^5 PFU/ml. Working stocks were frozen in 0.20-ml aliquots at −70° until used.

FIG. 1. CD-1 mice were inoculated in the left rear footpad with either 10^4 PFU of V3014 or V3000 in 10 μl of diluent or diluent alone (mock immunized). Tissues for *in situ* hybridization were harvested at 24 and 48 hr. All controls for ISH were uniformly negative. Viral signal was detected in the draining (ipsilateral popliteal lymph node) of V3014- and V3000-infected mice at 24 hr (A and B, respectively). VEE signal in the draining lymph node was more widespread in the V3000-infected mice, being present in subcapsular cortical areas, medullary, and paracortical regions. Examination of hematoxylin- and eosin-stained serial sections revealed severe liquefactive necrosis in draining lymphnodes of V3000-infected animals (D), but which was not seen in the V3014-infected mice (C). At 48 hr, viral RNA was detectable in the Peyer's patches of both V3014 (E) and V3000 (F) infected mice, as well as in the perirectal lymph nodes (G, V3014; H, V3000). In contrast to draining lymph nodes (A and B), viral RNA was confined to paracortical and medullary areas of gut-associated lymphoid tissues. Original magnification 25× in A, B, E, F, G, H; 63× in C and D.

FIG. 2. Six-week-old female CD-1 mice were immunized subcutaneously in the left rear footpad with a dose of 1×10^3 PFU of V3014 in 10 μl of PBS-DCS or PBS-DCS alone (mock immunized). Seven weeks after immunization, mice were challenged with virulent virus by instilling 10 μl of PBS-DCS containing 10^4 PFU of V3000 into each nostril. Mice were mock challenged by instillation of 10 μl of PBS-DCS alone into each nostril. At 24, 48, and 72 hr postchallenge, mice were sacrificed, perfused with 4% paraformaldehyde in PBS, pH 7.2, and nasal olfactory tissues were removed and prepared for histopathology and ISH. Negative controls included mock-immunized/mock-challenged and immunized/mock-challenged mice probed with the VEE-specific riboprobe, and mock-immunized/mock-challenged, mock-immunized/challenged, immunized/mock-challenged, and immunized/challenged mice probed with an irrelevant riboprobe (influenza HA glycoprotein specific). Two slides, each with tissue from five levels within the olfactory system, were examined for each group (3 mice per group). All controls were uniformly negative by ISH. Histopathologic appearance of hematoxylin and eosin (H/E) sections of all mock-challenged mice were normal. (A) Characteristic lesions were present within the olfactory neuroepithelium of mock-immunized/challenged mice at 72 hr postchallenge. Focal necrosis with concomitant structural disorganization across the full thickness of the olfactory mucosa was present at 24 hr postchallenge and became essentially confluent by 72 hr. (B) Areas of histopathology were positive for the presence of viral RNA as detected by ISH. (C) Immunized mice showed no evidence of histopathological change at 72 hr postchallenge. (D) There was no viral RNA detectable by ISH in immunized mice at 72 hr postchallenge. Bar represents 25 μm (A, C) or 100 μm (B, D).

TABLE 1

Immunization with V3014 Results in the Production of Both Serum IgG and Mucosal IgA Antibody

	Vaginal IgA				Serum IgG
	Day 10	Day 14	Day 21	Day 49	Day 49
Mock	<1:2	<1:2	<1:2	1:2.5 ± 0.86*	<1:50 ± 0**
Immunized	1:16	1:64	1:32	1:9 ± 4.4*	1:28,666 ± 15,216**

Note. Anti-VEE vaginal IgA and serum IgG ELISA titers in CD-1 mice immunized with V3014 or mock immunized with diluent as described in the legend of Fig. 1. Titers of anti-VEE vaginal IgA titers for Days 10, 14, and 21 were determined on pools of 16 mice. Day 49 anti-VEE IgA titer is the mean of 4 pools of 4 mice each. Day 49 titers for serum anti-VEE IgG are the mean of titers of 16 individual mice. Day 49 titers are expressed as mean titer ± standard deviation. Significance of Day 49 vaginal IgA and serum IgG as determined by Student's *t* test: *$P = 0.04$; **$P = 2.7 \times 10^{-9}$.

ELISA for anti-VEE antibody

Sucrose gradient-purified VEE virions were diluted into carbonate buffer (100 m*M* $NaHCO_3$, pH 9.6), and 250 ng of protein in a volume of 50 μl was evaporated in each well of an Immulon-4 microassay plate (Dynatech). Vaginal washes were performed by flushing the vaginal introitus with 75 μl of sterile PBS. Wash fluids for a given day were pooled in groups of 4 or 16 mice, concentrated 10-fold in Centricon-100 microconcentrators, and analyzed by ELISA as described (Olmstead *et al.,* 1986). Mouse serum or vaginal wash fluid was diluted into PBS/3% ovalbumin and analyzed for the presence of IgM, -G, -G_1, -G_{2a}, and -A class antibodies using horseradish peroxidase-conjugated, class-specific anti-mouse goat serum (Sigma). The substrate was *o*-Phenylenediamine (OPD, Sigma), and reactions were stopped by addition of 0.1 *M* NaF. The absorbance was read at 450 nm on a Dynatech MR4000 automated microplate reader. Titers are reported as the highest dilution to give an $OD_{A_{450}}$ of >0.200.

In situ hybridization (ISH)

Probes for VEE structural genes and the influenza HA gene (negative control) were generated as previously described (Grieder *et al.,* 1995; Charles *et al.,* 1995). Briefly, a pGEM-3 clone containing the 993-bp *Pst*I–*Sac*I fragment (nt 9,493–nt 10,486) of pV3000 was linearized at the unique *Sma*I site. *In vitro* Sp6 polymerase transcription reactions containing α[^{35}S]UTP produced a 678-nt radiolabeled RNA probe complementary to the 3′ end of the virus message sense RNA (6K and E1 genes). The negative control construct produced an approximately 500-nt riboprobe complementary to the influenza (strain PR-8) hemagglutinin mRNA. Incorporation of α[^{35}S]UTP was typically 80–95% and was quantitated by liquid scintillation counting. Probes were hybridized to 3-μm paraffin sections that had been mounted on aminopropyl triethoxysilane-coated slides (ProbeOn Plus, Fisher Scientific). Hybridization and wash conditions were as previously described (Grieder *et al.,* 1995; Charles *et al.,* 1995). Slides were dipped in Kodak NTB-2 emulsion, dried, and exposed at −70° for 24 to 144 hr. Following development, slides were counter-stained with Gill's hematoxylin.

TABLE 2

Immunization with V3014 Protects against Intranasal Challenge with Virulent V3000

Route of V3000 challenge	Diluent inoculated	V3014 inoculated
Intraperitoneal	100% mortality (2/2) AST = 7.5 days	0% mortality (0/2)
Intranasal	100% mortality (6/6) AST = 7.7 ± 1.1 days	0% mortality (0/8)

Note. Deaths per total are shown in parentheses. AST represents the average survival time of mice that died.

RESULTS

Spread of V3014 to peripheral lymphoid tissue

The spread of V3014 and the virulent parent virus, V3000, to peripheral lymphoid tissues of the mouse following subcutaneous inoculation was examined. Following a single dose of 10^4 PFU of V3014 or V3000 delivered subcutaneously into the left rear footpad, peripheral lymphoid tissues were analyzed 24 and 48 hr later for the presence of VEE RNA by ISH. Both V3000 and V3014 were present in the draining popliteal lymph node at 24 hr (Figs. 1A and 1B). Replication of V3000 was more extensive and was accompanied by severe necrosis (Figs. 1C and 1D). Necrosis was primarily liquifactive, although apparently apoptotic cells were also present. These data agree well with previous experiments with these viruses (Grieder *et al.,* 1995). Hybridization signal was detectable first in the subcapsular, cortical areas of the lymph node, consistent with entry via the afferent lymphatics. By 48 hr, RNA from both V3000 and V3014 was detectable in spleen, Peyer's patches (Figs. 1E and 1F), mesenteric lymph nodes, mandibular lymph nodes, and the perirectal lymph nodes (Figs. 1G and 1H). RNA from V3000, but not V3014, was found in the thymus at 48 hr pi (data not shown). In all of the lymphoid tissues beyond the draining lymph node, viral RNA was confined

largely to paracortical and medullary areas, suggesting viral entry via the high endothelial venules or capillaries.

Anti-VEE antibody levels in V3014-immunized mice

Antibody response to V3014 vaccination is brisk and includes a mucosal component. Following immunization of mice with 10^4 PFU of V3014 in the left rear foot pad, antibody levels in the sera and vaginal secretions were determined by ELISA. Titers of anti-VEE serum IgG antibody ranged from 1:4,000 to 1:64,000 (mean, 1:28,666) at 21 days postimmunization and remained at these levels for at least 7 weeks (Table 1). Subtyping of antibody response to VEE revealed that essentially all of the anti-VEE IgG antibody produced by the mice was of the IgG_{2a} subtype (data not shown), indicating a predominantly TH-1 type response. No anti-VEE reactivity was detected in sera from mock-vaccinated mice at a minimum dilution of 1:50. ELISA for the presence of anti-VEE IgA antibody in mucosal secretions was performed on pooled, concentrated vaginal washes obtained at 10, 14, 21, and 49 days pi (Table 1). A strong mucosal antibody response was present on Days 10, 14, and 21 and peaked at a titer of 1:64. By 49 days postimmunization, vaginal IgA titers were still detectable but had fallen to a range of 1:4 to 1:16 (mean, 1:9). Mock-vaccinated controls showed background vaginal anti-VEE IgA titers of 1:2.5 or below, which remained unchanged throughout the observation period.

Protection of V3014-immunized mice against intranasal VEE challenge

Mice were immunized with a single dose of 10^3 PFU of V3014, inoculated subcutaneously into the left rear footpad in a volume of 10 μl, or mock immunized with 10 μl of diluent. Daily observation of the mice for the first 14 days postimmunization revealed no signs of illness. Three weeks after immunization, the animals were challenged with 10^4 PFU of virulent V3000 intranasally or intraperitoneally (challenge dose represents approximately 500 lethal doses of virus intranasally and 10,000 lethal doses subcutaneously). No mortality was observed among the V3014-immunized mice after either challenge, nor did any of these mice display clinically detectable signs of illness (Table 2). As in numerous prior experiments, the mock-immunized, challenged mice displayed the characteristic signs of murine VEE disease, including ruffled fur and lethargy on Day 3, with hind limb paralysis progressing to prostration and death between the sixth and eighth days postchallenge.

Replication of challenge virus in immunized mice

Sensitive techniques for measurement of virus growth *in vivo* were used to determine the step in challenge virus replication that was inhibited in the immune mice. Mice were immunized or mock immunized as previously described and challenged intranasally 7 weeks later with 10^4 PFU of V3000. Challenged mice were sacrificed at 24, 48, and 96 hr postchallenge, and viral spread was determined by plaque assay of serum, olfactory, and neural tissue. Additionally, paired tissue samples were harvested to test for evidence of viral replication by ISH and histopathology.

The outcome of the virulent virus challenge was dramatically different in the V3014- and mock-immunized mice. Mock-immunized mice had challenge viral titers exceeding 10^5 PFU of virus per gram of tissue for serum and all tissues examined, consistent with previous experiments (Charles *et al.,* 1995; Ryzhikov *et al.,* 1991, 1995). However, in the V3014-immunized animals, the challenge virus was not detectable by plaque assay at any time point in the olfactory neuroepithelium, olfactory bulbs, brain, or serum of any of the V3014-immunized mice (level of detection was 170 PFU/g of tissue). In the mock-immunized mice, histopathologic examination revealed extensive necrosis across the full thickness of the olfactory mucosa (Fig. 2A), consistent with previously obtained results (Charles *et al.,* 1995; Ryzhikov *et al.,* 1991, 1995). In tissue sections of these mice, degenerative changes also were seen in the olfactory bulbs and lateral olfactory tracts, indicative of viral spread to the central nervous system (data not shown). *In situ* hybridization analysis showed that necrotic areas of the olfactory neuroepithelium were strongly positive for VEE RNA (Fig. 2B). In contrast, tissues harvested from V3014-immunized mice revealed no evidence of VEE-induced histopathology (Fig. 2C) and no evidence of viral replication by ISH (Fig. 2D). These results indicate that in immunized mice, minimal if any replication of the challenge virus occurred at the mucosal site of inoculation, and suggest that the challenge virus may have been neutralized at the mucosal surface by the action of secretory IgA antibody and/or transudated humoral antibody.

DISCUSSION

The demonstration of V3014 replication in sites associated with the induction of a secretory IgA response provides a likely explanation for the ability of V3014 to induce VEE-specific mucosal IgA after a parenteral inoculation. Induction of secretory IgA is thought to require the direct introduction of antigen onto a mucosal surface, either respiratory, digestive, or reproductive (Holmgren, 1991; Liang *et al.,* 1988). Once antigen has been presented at one of these sites, secretory IgA antibody production ensues at most mucosal surfaces, implying the presence of a common mucosal immune system (Holmgren, 1991; Svennerholm *et al.,* 1980; Weisz-Carrington *et al.,* 1979, 1987). Conversely, introduction of immunogens at sites other than the mucosal surface usually does not induce a protective mucosal response. For example, intraperitoneal inoculation of mice with an inactivated influenza vaccine failed to protect the nasal mucosa against replication of challenge influenza virus administered intrana-

sally, while intranasal or intrajejunal immunization with a vaccinia construct expressing the HA gene did afford protection of the nasal mucosa (Meitin *et al.,* 1991, 1994). It has been shown, however, that boosting from a parenteral site is capable of stimulating an anamnestic sIgA response in humans with preexisting mucosal immunity (Svennerholm *et al.,* 1980). In the case of parenterally administered VEE, a replicating virus is delivered to inductive mucosal sites from the bloodstream rather than from the mucosal surface. Although VEE administered subcutaneously enters the inductive pathway for a secretory IgA response from a different direction, the downstream events in the pathway, e.g., the induction of IgA itself, appear to be similar to those observed with immunogens delivered to mucosal surfaces. Indeed, when another live attenuated VEE vaccine candidate, V3526 (Davis *et al.,* 1995), was administered by aerosol to BALB/c mice, 100% of the animals had detectable anti-VEE IgA in vaginal washes and were completely protected against aerosol challenge with virulent virus (Hart, unpublished observations).

In the present study we demonstrate the ability of this vaccine candidate to provide protection of the airway mucosa of the mouse from virulent virus challenge. Whether this protection is due to the presence of secretory IgA within the upper respiratory tract is not yet known. In preliminary studies, we have been unable to detect anti-VEE IgA antibody in nasal washes from V3014-immunized animals (data not shown). Whether this represents a true failure of the animals to produce IgA within the respiratory tract or simply a lack of sensitivity of the assay is unknown at this point. However, the finding that the immunizing virus invades and replicates within the Peyer's patch, perirectal, and submandibular lymph nodes makes it likely that a generalized mucosal immunity is stimulated by parenteral inoculation with V3014. The fact that anti-VEE IgA is being produced at at least one mucosal site indicates that the level of replication of the virus within the mucosal-associated lymphoid tissue is of a sufficient magnitude that B cell activation is occuring. Secretory IgA production at one mucosal site is usually accompanied by production at most other mucosal sites (Holmgren, 1991; Svennerholm *et al.,* 1980; Weisz-Carrington *et al.,* 1979, 1987). It is unlikely that the production of serum IgG class antibody is sufficient to protect the nasal mucosa from aerosol challenge with VEE, in as much as experiments demonstrating that selective production of serum IgG by immunization with killed virus (Jahrling and Stephenson, 1984) or adoptive transfer of anti-VEE IgG (Rhyzikov *et al.,* 1991) fail to protect animals against aerosol challenge. What role T cells play in the generation of protective immunity to VEE is still unclear.

The parameters governing movement of V3014 to the tissues of the mucosal immune system are unknown at this point. The spread of V3014 to distant lymphoid tissue in the absence of a detectable serum viremia (Grieder *et al.,* 1995), and the localization of viral replication around high endothelial venules where circulating lymphoid and myeloid cells may enter the lymphoid tissues, suggest that the virus may move to these tissues in a cell-associated form. The identity of a putative homing cell type, and whether V3014 infects such cells or merely adsorbs to them, are active areas of inquiry.

The targeting of attenuated VEE vaccines to lymphoid tissue generally, and to mucosal-associated lymphoid tissue in particular, suggests that these viruses may serve as excellent vaccine expression systems for immunization against mucosal pathogens such as human immunodeficiency virus. To that end, V3014 has been configured as a replication-competent vector for expression of heterologous antigens by inserting a duplicate subgenomic promoter downstream of the structural genes (Davis, 1996; N. L. Davis, K. W. Brown, and R. E. Johnston, unpublished observations). Subcutaneous inoculation of a VEE vector containing the influenza hemagglutinin (HA) gene resulted in the expression of HA in the draining lymph node and protection of mice from disease following intranasal challenge with virulent strains of either influenza virus or VEE (Davis *et al.,* 1996). Significant reduction of influenza challenge virus replication in the nasal epithelia of HA vector-immunized mice suggested an active anti-influenza immunity at the mucosal surface. Therefore, the demonstration of protection against both homologous and heterologous mucosal challenge suggests that VEE vaccine vectors will be an important alternative vaccination strategy against mucosal pathogens when killed or subunit vaccines are ineffective or when the use of a live attenuated vaccine might be unsafe.

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