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Antibody to the E3 Glycoprotein Protects Mice against Lethal Venezuelan Equine Encephalitis Virus Infection▽

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Six monoclonal antibodies were isolated that exhibited specificity for a furin cleavage site deletion mutant (V3526) of Venezuelan equine encephalitis virus (VEEV). These antibodies comprise a single competition group and bound the E3 glycoprotein of VEEV subtype I viruses but failed to bind the E3 glycoprotein of other alphaviruses. These antibodies neutralized V3526 virus infectivity but did not neutralize the parental strain of Trinidad donkey (TrD) VEEV. However, the E3-specific antibodies did inhibit the production of virus from VEEV TrD-infected cells. In addition, passive immunization of mice demonstrated that antibody to the E3 glycoprotein provided protection against lethal VEEV TrD challenge. This is the first recognition of a protective epitope in the E3 glycoprotein. Furthermore, these results indicate that E3 plays a critical role late in the morphogenesis of progeny virus after E3 appears on the surfaces of infected cells.

Viruses in the *Alphavirus* genus of the family *Togaviridae* are composed of an icosahedral nucleocapsid surrounded by a lipid envelope studded with a distinctive lattice of glycoprotein spikes. The structural proteins of alphaviruses arise through co- and posttranslational processing of a polyprotein encoded by a single 26S mRNA (22, 27) in which the order of the gene products is NH_2-capsid-PE2-6K-E1-COOH. The capsid (C) protein cleaves itself from the nascent polypeptide soon after emergence from the ribosome. The PE2 glycoprotein is a precursor containing the E3 glycoprotein fused to the amino terminus of the E2 envelope glycoprotein. The PE2 glycoprotein is followed by 6K, a small membrane-associated protein, and E1, the second polypeptide component of glycoprotein spikes. Trimerized heterodimers of the E1 and E2 viral glycoproteins form the surface spikes and contain determinants of viral tropism and virulence (1).

The E3 glycoprotein acts as a signal for transport of PE2 across the membranes of the rough endoplasmic reticulum (22) and may promote the formation and intracellular transport of E1-PE2 heterodimers (12, 23, 46) to the cell surface. The E2 glycoprotein promotes specificity of virus binding to the host cell surface and is a target of protective antibodies (7, 18–20, 39). The E1 glycoprotein mediates fusion of the virion envelope with the membranes of acidified endosomes, allowing release of the nucleocapsid into the cytoplasm and the onset of viral replication (21, 40, 43). Antibodies to the E1 glycoprotein do not typically neutralize virus infectivity *in vitro* but can protect against lethal challenge in animals (41, 42). During transport to the cell surface, PE2 undergoes a maturational cleavage event by a furin-like protease to produce E2 and E3. The E3 glycoprotein may be subsequently released into the extracellular space (26, 49) or incorporated into the virion (6, 13). At the plasma membrane, trimerized E1-E2 heterodimers are incorporated into the budding virus particle.

Mutations that block cleavage of PE2 of Venezuelan equine encephalitis virus (VEEV) are lethal mutations (3). However, transfection of RNA transcribed from cleavage site deletion genomic cDNA clones results in rescue of pseudorevertant virus due to the appearance of second site mutations arising at a variety of locations in the glycoprotein genes (17). As a consequence of the cleavage site mutation, the spikes of pseudorevertant virions are composed of PE2 and E1.

One cleavage site deletion mutant, V3526, was prepared by mutagenesis of a genomic cDNA clone of Trinidad donkey (TrD). The virus encoded by this clone contains a 12-nucleotide deletion of the sequence encoding the furin cleavage site, as well as a Phe-to-Ser change at position 253 of the E1 glycoprotein (8). V3526 is attenuated and induces a robust protective antibody response against VEEV TrD in rodents, equines, and nonhuman primates (5, 9, 14–16, 37). V3526 also elicits protection in animals against challenge by other VEEV subtypes (9, 15, 37). During the characterization of the immune response elicited by V3526 in mice, a collection of monoclonal antibodies (MAbs) was isolated that preferentially bound V3526 virions compared to VEEV TrD. We report here that these MAbs bind a previously unrecognized epitope on the E3 glycoprotein. In addition, we show that MAbs specific for the VEEV E3 glycoprotein inhibit production of subtype I VEEVs in cell culture and protect mice from lethal challenge with VEEV TrD.

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MATERIALS AND METHODS

Viruses. The plasmid encoding VEEV strain V3526 was obtained from N. Davis, University of North Carolina, Chapel Hill, NC, and virus was rescued by transfection of BHK-21 cells (8). VEEV subtype I-C strain 686, subtype I-D

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strain 6880, and subtype I-E strain 68U201 viruses were obtained from S. C. Weaver, University of Texas Medical Branch, Galveston, TX. VEEV TrD, Mucambo virus, Eastern equine encephalitis virus (EEEV) strain Fla91-4679 (28), and Western equine encephalitis virus (WEEV) strain CBA87 (4) were obtained from USAMRIID archives. Working stocks of each virus were prepared by a single passage in BHK-21 cells.

Preparation of MAbs. BALB/c mice were inoculated intraperitoneally (i.p.) with 2×10^5 PFU of V3526. Four weeks later, an additional 40 μg of Co^{60}-irradiated V3526 were administered by tail vein infusion. Spleen cells were harvested 48 h later and fused with SP2/0-Ag14 myeloma cells as described previously (45). Hybridoma supernatants were screened by enzyme-linked immunosorbent assay (ELISA) for reactivity with purified V3526 and VEEV TrD. VEEV MAb 1A4A-1, specific for epitope $E2^C$ (39), was obtained from John Roehrig, Centers for Disease Control and Prevention, Fort Collins, CO. For passive immunization, antibodies were purified by MAbTrap column chromatography (Amersham Biosciences). Plaque reduction neutralization tests (PRNTs) on Vero cells were performed as described previously (36).

Competitive binding assay. Sucrose gradient purified virus was diluted to 4 μg/ml in sodium carbonate buffer (15 mM Na_2CO_3 and 35 mM $NaHCO_3$ in distilled water [pH 9.6]) and 50 μl/well were added to 96-well Flexplates, which were incubated at 4°C overnight. Plates were washed five times with wash buffer (1% fish gelatin, phosphate-buffered saline [PBS], 0.5% Tween 20). Residual binding sites were blocked with 300 μl of blocking buffer (5% fish gelatin, PBS, 0.5% Tween 20), and plates were incubated at 37°C for 1 h. Blocking buffer was aspirated from the wells. Unlabeled MAbs were serially diluted 3-fold from 2 μg/well to 0.001 μg/ml in bovine serum albumin (BSA)-PBS blocking diluents (KPL, Inc.). Diluted unlabeled MAbs were added to the plates at 50 μl/well, followed by incubation at 37°C for 1 h. Plates were washed five times with wash buffer as described above. Biotin-labeled MAbs were added at a concentration of 2 ng/well (50 μl) to all wells of a single 96-well plate, followed by incubation at 37°C for 1 h. After a washing step, 100 μl of a 1:2,000 dilution of peroxidase-labeled Strep-Avidin (Amersham) was added/well, followed by incubation at 37°C for 1 h. Plates were washed and developed with ABTS [2,2′azinobis(3-ethylbenzthiazolinesulfonic acid)] substrate (KPL, Inc.) for 30 min at room temperature. Absorbance was measured by utilizing dual wavelengths of 410 to 490 nm. Any dilution of unlabeled MAb showing an optical density at 410 nm (OD_{410}) value of <0.3 was considered to show competition with the biotin-labeled MAb. The mean OD_{410} values with no subtraction of background were obtained for duplicate wells at each concentration tested.

Plaque reduction neutralization assay. Aliquots (100 μl) of virus containing 100 PFU were combined with an equal volume of Dulbecco modified Eagle medium (DMEM) containing the serially diluted antibody indicated and held overnight at 4°C. The virus antibody mixtures were then added to confluent monolayers of Vero cells, followed by incubation at 37°C at 5% CO_2 for 1 h. The monolayers were then overlaid with 2 ml of 0.6% agarose in DMEM containing 10% fetal bovine serum (FBS). After 24 h, a second overlay of 0.6% agarose in DMEM containing 5% FBS and neutral red was added, and the plates were incubated at 37°C at 5% CO_2. Plaques were enumerated the following day. Virus infectivity was considered neutralized if the number of plaques was decreased by 80% compared to controls that contained FBS or an irrelevant MAb.

Virus infection and protein blotting. BHK-21 cells were infected at a multiplicity of infection (MOI) of 10. At 12 h after infection, lysates were analyzed on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels. The gels were electroblotted onto nitrocellulose and probed with primary antibody overnight at room temperature, followed by a peroxidase-conjugated anti-mouse IgG (KPL, Inc.). The membrane was developed with TMB membrane peroxidase substrate and membrane enhancer (KPL, Inc.) in a ratio of 9:1.

E3 glycoprotein expression. An expression cassette encoding amino acids 1 to 45 of the VEEV E3 glycoprotein fused to amino acids 18 to 565 of the hemagglutinin (HA) protein of influenza virus A/PR/8/34 (accession no. EF190974) was prepared by overlap extension PCR (48). Primer sequences are available on request. Flanking ClaI sites and an ATG codon were added to the cassette in the final amplification step, and the fragment was cloned into a VEE replicon, designated VRP-E3HA, for expression in BHK-21 cells (35). At 16 h posttransfection of VRP-E3HA RNA, the cells were collected, and lysates were analyzed by immunoblotting with MAb 13D4 or antisera to the HA protein of influenza virus A/PR/8/34 (Genetex, Inc.).

Indirect immunofluorescence analysis. Lab-Tek chamber slides (Fisher) containing baby hamster kidney (BHK) cells were mock infected or infected with VEE-TrD or V3526 (MOI of 1, 50 μl) at 37°C. After 1 h, complete medium was added to the wells, followed by incubation at 37°C overnight. Approximately 18 h postinfection, cells were washed with PBS and fixed in 2% paraformaldehyde in PBS for 15 min. Slides were washed in several changes of PBS and allowed to air dry. Cells were incubated at room temperature for at least 1 h with primary antibody, MAb 13D4 or 1A4A-1, diluted 1:250 in PBS plus 5% fetal bovine serum (FBS). The slides were washed in several changes of PBS and incubated at least 30 min with goat anti-mouse fluorescein isothiocyanate serum (1:80; KPL, Inc.). The slides were then washed in PBS, and each coverslip was mounted with Vectashield (Vector Labs). Slides were viewed on a Nikon Eclipse E800 microscope, and images were captured with SPOT software (Diagnostic Instruments, Inc.).

Inhibition of virus yield by MAbs. Monolayers of BHK-21 cells were infected at an MOI of 0.1 at 37°C. One hour later, the monolayers were washed twice, and culture medium containing MAbs was added. The medium was harvested at the times indicated, and the virus yield was determined by using a plaque assay. As an alternate measure of virus particles released from the cells, viral genome equivalents were quantitated by reverse transcription-PCR (RT-PCR). RNA was extracted from a 0.25-ml sample of cell culture supernatant with TRIzol LS (Invitrogen). A 92-bp fragment representing nucleotides 7293 to 7383 of the nSP4 gene of the VEEV TrD genome (accession no. J04332) was amplified in 20-μl volumes using a R.A.P.I.D. system (Idaho Technologies). Each reaction contained 1× Invitrogen SuperScript One-Step RT-PCR with Platinum *Taq* PCR buffer, 25 μg of BSA/ml, 6 mM $MgSO_4$, 0.9 nM concentrations of each primer, and 0.06 nM probe. The amplification program for the LightCycler consisted of one cycle at 50°C for 15 min and one cycle at 95°C for 5 min, followed by 45 cycles of 95°C for 1 s and 60°C for 20 s. A fluorescence reading was taken at the end of each 60°C step. Each capillary was read in channel 1 (F1) at a gain setting of 8, with data being analyzed by using LightCycler data analysis software (version 3.5.3). Sample curves were analyzed by using the "Second Derivative Maximum" with the baseline adjustment set to "Arithmetic". The relative number of virus genomes in each sample is reported as the crossing point (Cp), indicating the number of cycles at which fluorescence rose above background levels.

Passive immunization and virus challenge. BALB/c mice were administered 2, 20, or 50 μg of purified MAbs in 0.2 ml of PBS by i.p. injection. The animals were bled 20 h later, and the level of serum antibody in each mouse was measured by ELISA. At 25 h after antibody administration, the mice were challenged by i.p. administration of 10^4 PFU of VEEV TrD. Mice were observed for signs of illness daily for 2 weeks, and serum was collected using retro-orbital bleed from surviving mice at 4 weeks postchallenge.

RESULTS

Specificity of the MAbs to V3526. A total of 138 MAbs prepared after vaccination with V3526 were tested for reactivity with VEEV by ELISA using purified VEEV TrD or V3526 as target antigens. Six exhibited significantly increased binding to immobilized V3526 compared to VEEV TrD. These antibodies belonged to the IgG2a (MAbs 13D4, 5E2, 5E4, and 3F2) or IgG2b (MAbs 10D6 and 10D7) isotype. Competitive binding assays established that four unlabeled anti-V3526 MAbs (3F2, 13D4, 10D6, and 10D7) effectively competed with all six labeled MAbs. Fig. 1A shows the competitive binding curve for MAb 13D4 as a representative of these four MAbs. Unlabeled MAbs 5E2 and 5E4 competed with themselves, and each other but much less effectively with the other four labeled anti-V3526 MAbs, especially 10D6 and 13D4. The decreased competition seen may have been due to differences in affinity. Figure 1B shows the competitive binding curve for MAb 5E2 as a representative of these two MAbs. MAb 13D4 was the most effective at competing with the other five MAbs. Thus, these six MAbs bind to the same or closely related antigenic determinant(s).

The specificity of these antibodies was investigated by probing immunoblots of V3526 and VEEV TrD viruses and lysates of virus-infected BHK-21 cells. Virions of VEEV TrD are composed of E2 and E1 envelope glycoproteins and C protein, as shown in Fig. 2A, lane 2. The cleavage site deletion in V3526 results in the incorporation of PE2 glycoprotein in the virus

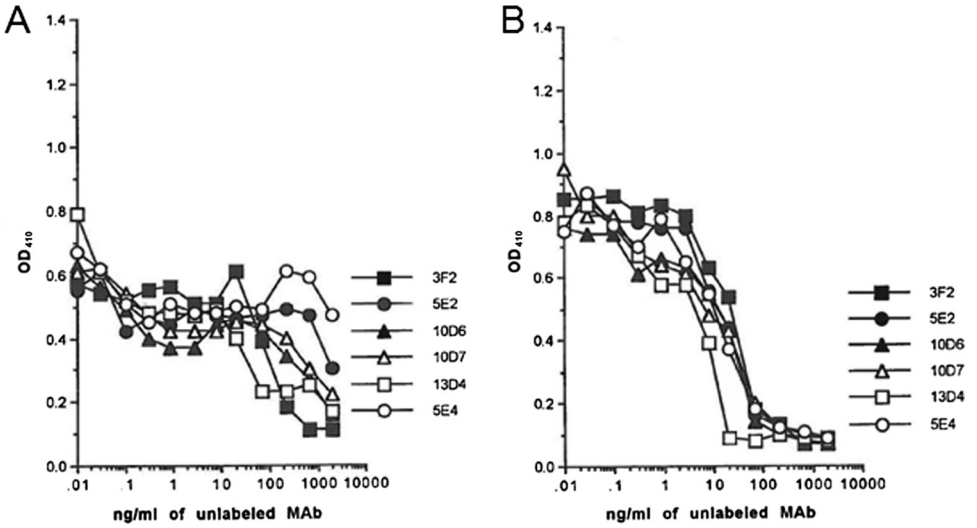

FIG. 1. Competitive binding curves for anti-V3526 MAbs. ELISA data obtained on V3526 virus are shown using unlabeled MAbs that were serially diluted 3-fold from 2 μg/well and biotin-labeled MAbs that were diluted to 2 ng/well. Binding curves are shown for the unlabeled MAbs against the biotin-labeled 13D4 (A) and 5E2 (B). The mean OD_{410} values (with no subtraction of background) obtained for duplicate wells at each tested concentration are shown.

particle (8) and a lack of detectable E2 (Fig. 2A, lane 3). In lysates of BHK-21 cells infected with VEEV TrD, MAb 1A4A-1, which is an $E2^c$-specific MAb, bound both PE2 and E2 (Fig. 2B, lane 3). In lysates of V3526-infected cells, 1A4A-1 bound only a single polypeptide corresponding to PE2 (Fig. 2B, lane 4). In the same lysates, MAb 13D4, one of six antibodies exhibiting preferential binding to V3526 virus, bound only PE2 of both V3526 and VEEV TrD (Fig. 2C, lanes 3 and 4). The remaining five MAbs exhibited the same specificity (data not shown). These findings demonstrated that MAb 13D4 recognized an epitope specific to the PE2 glycoprotein, presumably located in the E3 glycoprotein. Although equivalent amounts of protein were loaded onto the gels shown in Fig. 2B and 2C, the decreased amount of PE2 in VEEV TrD- versus V3526-infected cells was expected reflecting the continued furin protease processing of PE2 in TrD-infected cells. In addition, the slightly decreased mobility of PE2 of TrD compared to that of V3526 was likely due to the presence of the -RRTR- furin cleavage site which was absent from the PE2 of V3526.

We have been unable to consistently detect E3 glycoprotein by immunoblotting lysates of VEEV-infected BHK-21 cells with MAbs or polyclonal sera from vaccinated mice. Therefore, to definitively identify the target of the MAbs, the sequence encoding the E3 glycoprotein of VEEV was fused to the HA gene of influenza virus A/PR/8/34 in place of the HA signal sequence in the VEEV replicon system (35). This E3-HA fusion protein was expressed in BHK-21 cells. As shown in Fig. 3A, murine antiserum to the influenza virus HA bound both the influenza virus HA protein and the E3-HA fusion protein. In the same lysates, MAb 13D4 bound only the E3-HA fusion protein (Fig. 3B). These results confirmed that the epitope bound by MAb 13D4 is located in the E3 glycoprotein. Identical results were obtained with the remaining five V3526-specific MAbs (data not shown).

To further characterize the specificity of these MAbs, 13D4 was used to probe the lysates of BHK-21 cells infected with other subtypes of VEEV, EEEV, and WEEV. Lysates of virus-infected cells served as the source of PE2 because cleavage deletion mutants were not available for all viruses. As shown in

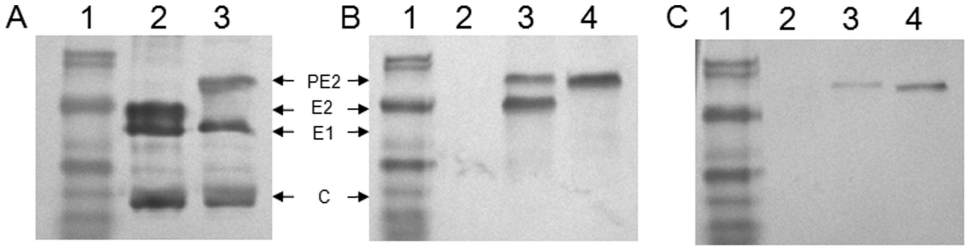

FIG. 2. V3526-specific MAbs bind PE2. Protein samples were separated by SDS-PAGE, and immunoblot analyses were performed with anti-VEEV ascites fluid or PE2-specific MAb 13D4. (A) Immunoblot of purified VEEV viruses probed with murine anti-VEEV ascites fluid. Lanes: 1, prestained molecular mass markers; 2, VEEV TrD; 3, V3526. (B) Immunoblot of lysates of VEEV-infected BHK cells probed with anti-$E2^C$ MAb 1A4A-1. Lanes: 1, prestained molecular mass markers; 2, uninfected BHK cells; 3, TrD-infected BHK cells; 4, V3526-infected BHK cells. (C) Immunoblot of VEEV-infected cells probed with MAb 13D4. Lanes: 1, prestained molecular mass markers; 2, uninfected BHK cells; 3, TrD-infected BHK cells; 4, V3526-infected BHK cells.

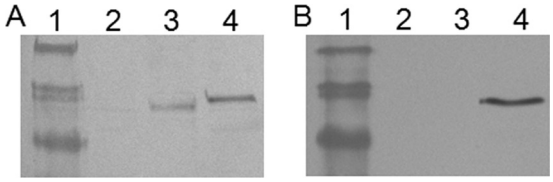

FIG. 3. MAbs specific for VEEV PE2 glycoprotein bind an epitope in the E3 glycoprotein. Lysates of BHK cells expressing influenza virus A/PR/8/34 HA or an E3-HA fusion protein were resolved by SDS-PAGE and blotted to nitrocellulose. (A) Reactivity with MAb to influenza virus HA. (B) Reactivity with MAb13D4. Lanes: 1, prestained molecular mass markers; 2, uninfected BHK cells; 3, BHK cells expressing influenza virus HA; 4, BHK cells expressing E3-HA fusion protein.

TABLE 1. Neutralization of VEEV by anti-E3 MAb

Antibody	Antibody concn (μg/ml)[a]	
	V3526	TrD
3F2	0.2	>10
5E2	0.6	>10
10D6	0.3	>10
10D7	0.3	>10
13D4	0.04	10
5E4	1	>10
1A4A-1	0.001	0.02
IgG2a and IgG2b[b]	>10	>10

[a] That is, the antibody concentration resulting in 80% plaque reduction.
[b] Isotype controls were individually tested, and all produced the same results.

Fig. 4, E2^c-specific MAb 1A4A-1 bound to denatured and reduced E2 and PE2 proteins of VEEV subtypes IA/B, IC, and ID (Fig. 4, lanes 3, 4, 5, and 8) but failed to bind either polypeptide in lysates of cells infected with subtype IE VEEV 68U201, Mucambo virus (previously classified as VEEV subtype IIIA), EEEV, or WEEV. MAb 13D4 was able to bind the PE2 protein of VEEV subtypes IA/B (TrD), IC, ID, and IE (Fig. 4B, lanes 3 to 6 and 8). These results indicate that although the VEEV IE strain 68U201 has diverged from the other subtype I viruses with respect to the E2^c-epitope recognized by 1A4A-1, the epitope bound by MAb 13D4 in E3 appeared to be conserved. In addition, 13D4 did not bind analogous targets in BHK-21 cells infected with Mucambo virus, EEEV, or WEEV (Fig. 4B, lanes 7, 9, and 10).

***In vitro* neutralization of infectivity by E3-specific MAbs.** In order to determine the effect of anti-E3 antibody binding on the infectivity of the virus, PRNTs were performed with all of the E3 specific MAbs. As reported previously (25), MAb 1A4A-1 neutralized VEEV TrD. Low concentrations of MAb 1A4A-1 also neutralized V3526 (Table 1). All six anti-E3 MAbs neutralized V3526 *in vitro* to various extents. However, 13D4 was the most efficient, requiring at least 7-fold less than the other MAbs. When tested against VEEV TrD, only 13D4 appeared to exhibit plaque-reducing ability at a concentration of ≥10 μg/ml. We subsequently found that if monolayers were washed twice with PBS before the addition of the first agarose overlay, plaque formation by VEEV TrD was not inhibited by 13D4 (data not shown). This indicated that the apparent neutralization of TrD VEEV plaque formation by MAb 13D4 was due to postinfection inhibition evident only when high concentrations of antibody remained in the agarose overlay during the course of the plaque assay. In contrast, washing of the monolayers prior to addition of the agarose overlay had no effect on the reduction of plaque formation by V3526, demonstrating that infectivity of V3526 was neutralized by preincubation with MAb 13D4.

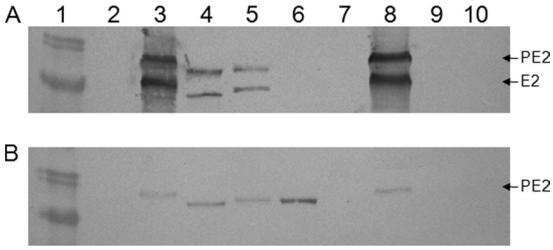

FIG. 4. Reactivity of lysates of *Alphavirus* virus-infected BHK cells with MAbs specific for E3 and E2. Infected cell lysates were subjected to SDS-PAGE and electroblotted to nitrocellulose. (A) Lysates probed with E2^c-specific MAb 1A4A-1. (B) The same lysates reacted with E3-specific MAb 13D4. Lanes: 1, prestained molecular mass marker; 2, uninfected BHK cells; 3, VEEV subtype IA/B (TrD); 4, VEEV subtype IC; 5, VEEV subtype ID; 6, VEEV subtype IE; 7, Mucambo virus; 8, TrD virus; 9, EEEV; 10, WEEV.

Inhibition of virus yield. Immunofluorescence analysis of TrD-infected BHK-21 cells with Mab13D4 demonstrated that the E3 protein, either in its free form or still in the form of PE2, was present on the surfaces of infected cells (Fig. 5). To determine whether anti-E3 antibody would impact progeny virus production, we measured the effect of 13D4 on the yield of VEEV TrD in cell culture. BHK-21 cells were infected with VEEV TrD at an MOI of 0.1 at 37°C to allow for binding and internalization of the virus. One hour later, medium was added containing 10 μg of purified 13D4/ml, 10 μg of E2-specific MAb 1A4A-1/ml, or an irrelevant MAb to Ebola virus glycoprotein. The supernatants were collected at increasing times after infection, and progeny virus was quantitated by plaque assay on Vero cells.

As shown in Table 2, VEEV TrD reached an extracellular titer exceeding 10^8 PFU/ml by 24 h after a low-multiplicity infection of BHK-21 cells. The presence of a MAb against the glycoprotein of Ebola virus had no effect on the production of progeny VEEV (data not shown). When the medium contained 10 μg of MAb 13D4/ml, the extracellular virus titer was reduced to ca. 10^5 PFU/ml, a 10^3-fold decrease. In repeated assays, equivalent levels of inhibition were evident at MAb 13D4 concentrations as low as 0.5 μg/ml (data not shown). The remaining E3-specific MAbs consistently inhibited virus production by 100-fold (not shown). Including MAb 1A4A-1, which neutralizes viral infectivity, also reduced virus production to 10^5 PFU/ml. Therefore, although MAb 13D4 exhibited little plaque-reducing activity against the parental VEEV TrD compared to MAb 1A4A-1 (Table 1), MAb 13D4 was as effective as MAb 1A4A-1 in reducing production of progeny virus. Similar results were obtained when the cells were infected at an MOI of 10, suggesting that the reduced yield of virus was due to inhibition of virus formation or release rather than an inability to disseminate the infection throughout the cell monolayer.

The observation that MAb 13D4 decreased the yield of

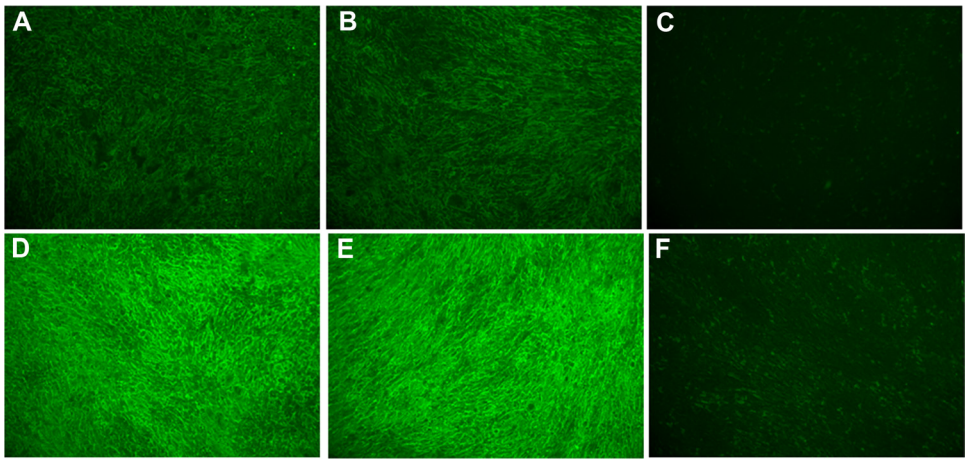

FIG. 5. The E3 protein is present on the surface of VEEV-infected cells. Indirect immunofluorescence assay performed with MAb 13D41 (A to C) or MAb 1A4A-1 (D to F) on BHK cells infected with VEE TrD (A and D), V3526 (B and E), or mock infected (C and F).

infectious virus did not exclude the possibility that noninfectious particles were produced in the presence of the antibody. Therefore, portions of the supernatant collected from infected BHK cells incubated with 13D4 or 1A4A-1 were analyzed by quantitative RT-PCR (qPCR) (Table 2). In the absence of antibody or in the presence of an anti-Ebola virus MAb (data not shown), the decreasing Cp values reflected the rising titer of virus in the culture supernatant. The decrease in extracellular titers in cultures incubated with MAbs 13D4 or 1A4A-1 was reflected by increased Cp values, confirming that the decrease in extracellular virus was not due to neutralization of extracellular virus by the MAbs or production of noninfectious viral particles. Taken together, these results demonstrate that the decrease in virus production mediated by MAb 13D4 was due to antibody-mediated inhibition of morphogenesis or release of infectious progeny.

As indicated previously (Fig. 4B), MAb 13D4 bound the PE2 glycoprotein of all of the VEEV subtype I viruses when analyzed by immunoblotting. Coincident with the specificity for the PE2 proteins of subtype I VEEVs, as shown in Fig. 4, MAb 13D4 also significantly inhibited the production of virus from BHK-21 cells infected with subtype IC (by 10^3) and IE (by 10^4) viruses and had a modest effect on the production of 1D virus (Table 2).

***In vivo* activity of anti-E3 antibody.** The dramatic inhibition of virus production from infected cells by anti-E3 antibody suggested that MAb 13D4 might impact the course of disease in an infected host. Therefore, BALB/c mice were passively immunized i.p. with purified MAb 1 day before i.p. challenge with 10^4 PFU of VEEV TrD. ELISA measurements of serum antibody demonstrated that all mice were successfully passively immunized, and the geometric mean titers for each group are indicated in Table 3. Passive transfer of 20 or 50 μg of 13D4 to BALB/c mice protected the mice from lethal challenge (Table 3). The other E3 MAbs, all of which exhibited no plaque-reducing activity against TrD virus at 10 μg/ml and were less effective at reducing the number of progeny viruses, provided much less protection to the recipient mice, even when 50 μg of antibody was administered before challenge. However, in each case, the mean time to death was extended significantly ($P < 0.001$) compared to the control mice. MAb 1A4A-1 protected BALB/c mice as previously reported (33), and irrelevant iso-

TABLE 2. Inhibition of virus production by MAb to VEEV glycoproteins

Antibody and time postinfection (h)	TrD VEEV (IA/B)				VEEV subtypes			
	MAb concn (μg/ml)		PFU/ml	Cp[a]	MAb 13D4 concn (μg/ml)	PFU/ml		
	13D4	1A4A-1				IC	ID	IE
No antibody								
0			10^2	28.84		3.4×10^2	3.6×10^2	5×10^2
24			7×10^8	9.97		3.6×10^9	5×10^8	2×10^9
13D4								
0	10		$<10^2$	29.79	10	2.7×10^2	4×10^2	5×10^2
24	10		4×10^4	26.59	10	1.4×10^6	1.2×10^8	2.3×10^5
1A4A-1								
0		10	10^2	30.35	NA	NA	NA	NA
24		10	4×10^4	28.98	NA	NA	NA	NA

[a] Cp, crossing point.

TABLE 3. Protection of BALB/c mice by passive transfer of MAbs to VEEV glycoproteins

Antibody	Protection of BALB/c mice (GMT)[a] at an antibody concn of:			MDD (*P*)[b]
	2 μg	20 μg	50 μg	
13D4	1/9 (ND)	9/9 (5,432); 7/7 (2,400)	5/5 (8,778)	NA
5E2		1/9 (2,208)	0/5 (1,949)	10.56 (0.0002)
5E4		1/9 (2,208)	6/6 (1,949); 1/5 (2,208)	11.0 (0.006)
10D6		1/9 (1,808)	1/5 (5,653)	9.78 (0.001)
10D7		2/9 (1,998)	2/5 (6,400)	11.89 (0.004)
3F2	0/9 (ND)	8/9 (9,897); 0/8 (1,258); 0/5 (1,596)	1/5 (1,600)	NA
1A4A-1		13/13	5/5 (2,441)	NA
IgG2a and IgG2b[c]		0/18 (<100)	0/5 (<100)	7.0

[a] Protection is expressed as the number of protected mice/number of mice tested. The geometric mean titer (GMT) prior to challenge is indicated in parentheses. ND, not determined.

[b] The mean day of death (MDD) for each antibody was evaluated for significant differences from the results obtained with irrelevant isotype-matched antibody. NA, not applicable.

[c] Isotype-matched irrelevant antibody.

type-matched MAb at either 20- or 50-μg doses failed to provide protection. Serum collected from MAb 13D4 passively immunized mice surviving 1 month after challenge contained high titers of VEEV-specific antibodies of all four IgG isotypes. In contrast, mice passively immunized with 50 μg of MAb 1A4A-1 showed no serological evidence of infection (data not shown). These results indicate that anti-E3 MAbs did not protect mice against infection but suggests that they moderated disease progression until the adaptive response was able to resolve the infection.

DISCUSSION

The alphavirus E3 glycoprotein is a multifunctional component of the process of synthesis and maturation of the viral surface glycoprotein spikes. In addition to acting as a signal to initiate translocation of viral glycoproteins across the membranes of the rough endoplasmic reticulum, E3 also promotes the heterodimerization and intracellular processing of E1 and PE2 glycoproteins (23, 46). Furin cleavage of PE2 into E2 and E3 activates the spikes to a metastable state capable of initiating infection (52). By analogy with the prM protein of dengue *Flavivirus* (51), furin cleavage of PE2 may begin after transit of an acidic late component of the Golgi body, where E3 is thought to suppress the acid pH-triggered activation of glycoprotein E1 fusion capability. Although the pr fragment produced by furin cleavage of prM of dengue is released after return to neutral pH, in the case of alphaviruses E3 may be released (2, 49) or retained on the mature virus particles (32).

Although elimination of the furin cleavage site from PE2 in VEEV is a lethal mutation (8), pseudorevertant viruses were rescued after transfection of transcripts of cleavage deletion genomic cDNA clones due to second-site suppressor mutations in the E1, E2, or E3 glycoprotein genes (17, 44, 47). The stability of the cleavage deletion mutation (24) and attenuated phenotype of the virus prompted the evaluation of V3526 as a vaccine for animals and humans (34, 37, 52, 53). In the course of preclinical evaluation of V3526 as a live vaccine, a series of MAbs were generated to further characterize the immune response elicited by vaccination with V3526.

The MAbs described in this report bound a previously unrecognized epitope on the E3 glycoprotein. Reconstructions of a cleavage-site deletion mutant of Sindbis virus revealed that the E3 portion of the PE2 molecule in the virus forms a prominent lobe on the distal portion of the glycoprotein spike (32). The potent neutralization of V3526 infectivity by MAb 13D4 was likely due to interference with cell surface binding or penetration as previously reported for the $E2^c$-specific MAbs (38).

The furin cleavage of alphavirus PE2 glycoprotein begins in the *trans*-Golgi network (29). The binding of MAb 13D4 to wild-type virus-infected cells indicates that E3 is present on the cell surface, but we were unable to determine whether the target is E3 or residual PE2. The observation that furin cycles between the Golgi body and the cell surface (30, 31) provides for the possibility that some processing occurs after PE2 appears on the plasma membrane. If true, this suggests several possible mechanisms by which anti-E3 antibodies may inhibit virus production. First, MAb 13D4 bound to PE2 might interfere with furin cleavage (10, 11). However, the incorporation of PE2 into nascent *Alphavirus* spikes does not prevent the budding of particles (8, 40), and the qPCR analysis indicates that 13D4 reduces particle formation to very low levels, as shown in Table 2. Alternatively, antibody binding of PE2 on the cell surface may inhibit formation of the glycoprotein spike matrix and coalescence of the envelope required for viral budding. Finally, if MAb 13D4 is bound to E3 after its cleavage from PE2, the potent inhibition of wild-type virus production by 13D4 would suggest the existence of an undescribed role for E3 in a critical late event preceding release of the virion from the cell. Efforts are ongoing to identify the nature of the E3 entity present on the cell surface.

Protection against lethal *Alphavirus* challenge by passive immunization with antibodies that do not inhibit plaque formation was demonstrated previously (41, 50). The ability of the six E3-specific MAbs described here to inhibit plaque formation by V3526 virus is not directly relevant to protection against wild-type viral challenge. However, it does correspond to the level of protection afforded by passive immunization. The expanded repertoire of antibody isotypes detected after challenge indicates that a significant level of viral replication occurs in passively immunized mice. These observations indicate anti-E3 MAb likely protects animals against wild-type VEEV by inhibiting the amplification of the challenge virus, subsequently moderating viremia and the progression of disease until the

adaptive immune response is capable of resolving the infection. In contrast, the $E2^c$ MAb provided apparent sterile immunity when passively transferred to mice, based on the lack of *de novo* antibody responses following challenge.

This study is the first in which the E3 protein has been demonstrated to contain protective epitopes. These findings are directly relevant to ongoing efforts toward developing licensable equine encephalitis virus vaccines and therapeutics. TC-83 is a live VEEV vaccine currently available for limited use in humans. The vaccine has a variable reactogenicity rate but also fails to induce a detectable serum neutralizing antibody response in ca. 20% of recipients. Because a neutralizing antibody response is currently accepted as the surrogate marker of protection against laboratory infection by VEEV, TC-83 nonresponders receive subsequent vaccinations with C-84 vaccine, an inactivated vaccine also available for use in at-risk laboratory investigators. However, C-84 is prepared by formalin inactivation of TC-83 and therefore, is unlikely to contain detectable E3 glycoprotein. Although the lack of E3 is unlikely to be responsible for the failure rate, an inactivated vaccine developed from a cleavage mutant virus, such as V3526, might be expected to elicit a broadened antibody response and provide an additional level of protection due to antibody to the E3 epitope. In addition, development of inactivated vaccines from cleavage-site mutants of WEEV and EEEV may likewise increase their effectiveness, since we found that an anti-E3 MAb specific for the WEEV E3 glycoprotein also affords protection in mice (unpublished observation). In addition to vaccine considerations, MAbs to this protective epitope on the E3 glycoprotein could be a promising component of an antibody-based therapeutic for alphaviruses.

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