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Protection from La Crosse Virus Encephalitis with Recombinant Glycoproteins: Role of Neutralizing Anti-G1 Antibodies

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La Crosse virus, a member of the California serogroup of bunyaviruses, is an important cause of pediatric encephalitis in the midwestern United States. Like all bunyaviruses, La Crosse virus contains two glycoproteins, G1 and G2, the larger of which, G1, is the target of neutralizing antibodies. To develop an understanding of the role of each of the glycoproteins in the generation of a protective immune response, we immunized 1-week-old mice with three different preparations: a vaccinia virus recombinant (VV.ORF) that expresses both G1 and G2, a vaccinia virus recombinant (VV.G1) that expresses G1 only, and a truncated soluble G1 (sG1) protein prepared in a baculovirus system. Whereas VV.ORF generated a protective response that was mostly directed against G1, VV.G1 was only partially effective at inducing a neutralizing response and at protecting mice from a potentially lethal challenge with La Crosse virus. Nevertheless, a single immunization with the sG1 preparation resulted in a robust immune response and protection against La Crosse virus. These results indicate that (i) the G1 protein by itself can induce an immune response sufficient for protection from a lethal challenge with La Crosse virus, (ii) a neutralizing humoral response correlates with protection, and (iii) the context in which G1 is presented affects its immunogenicity. The key step in the defense against central nervous system infection appeared to be interruption of a transient viremia that occurred just after La Crosse virus inoculation.

La Crosse virus, a member of the California serogroup of bunyaviruses, is an important cause of pediatric encephalitis in the midwestern United States (for a review, see reference 18). Around 75 to 100 cases of La Crosse encephalitis are reported annually, and an additional 300,000 less severe infections are estimated to occur each year (5). La Crosse virus is transmitted by the woodland mosquito *Aedes triseriatus*, and the distribution and activity of its vector explain the restricted epidemiology (2). However, *Aedes albopictus*, the tiger mosquito, recently introduced into the United States from Asia, can also serve as a vector for bunyaviruses, raising concerns about the potential spread of La Crosse virus and other California serogroup viruses beyond their confined ecosystems (11, 25, 30). This could increase the population at risk.

The La Crosse virus genome is composed of three single-stranded RNA segments of negative polarity, designated according to size as large (L), medium (M), and small (S) (6, 15). The L RNA segment (6.5 kb) encodes the large (L) protein, the viral RNA-dependent RNA polymerase (8, 31). The M RNA segment (4.5 kb) encodes a single open reading frame (ORF) that is processed into the viral glycoproteins G2 and G1, as well as a nonstructural protein (NSm) of unknown function (3, 10, 20, 35). The S RNA segment encodes the nucleocapsid (N) protein and another nonstructural protein (NSs) in overlapping reading frames (12, 13).

To develop a strategy for investigating the role of protective immunity against La Crosse virus encephalitis, we relied on our previous studies on this group of viruses (18). These experiments have demonstrated that following a peripheral (subcutaneous [s.c.]) inoculation that mimics the bite of a mosquito, the virus replicates in striated muscle; this is associated with the development an active plasma viremia (21). Invasion of the central nervous system (CNS) by the virus is associated with this viremia and occurs by an unknown mechanism. We surmised that interruption of this viremia with neutralizing antibodies would prevent CNS invasion, and this hypothesis provided a framework for our immunization studies.

In this study, we have addressed several questions regarding protective immunity against La Crosse virus. (i) Was there a plasma viremia under the conditions that we used for virus challenge? (ii) Would passive administration of neutralizing antibody directed against G1 (17, 23) protect against a lethal challenge? (iii) Would a recombinant vaccinia virus (rVV) expressing both glycoproteins, or the G1 protein alone, raise neutralizing antibodies and protect? (iv) What was the minimal requirement for protective immunity against La Crosse virus? The results of these experiments confirm the critical role of immunity against the G1 protein in protection against La Crosse virus infection.

MATERIALS AND METHODS

Animals. Outbred albino CD-1 Swiss mice (Charles River Laboratories, Wilmington, Mass.) were used in all experiments, which were conducted at University Laboratory Animal Resources at the University of Pennsylvania.

Viruses. La Crosse virus (original strain) was propagated in baby hamster kidney (BHK-21) cells as described previously (21). Preparation of the rVVs containing the entire La Crosse virus M RNA ORF frame (VV.ORF), the coding region for G1 alone (VV.G1), or a β-galactosidase-expressing vector alone (VV.tk−) have been described elsewhere (20, 26).

Briefly, rVVs were prepared by using the plasmid vaccinia virus vectors pSC11 (VV.ORF and VV.G1) and pSC65 (VV.tk−) (the vectors were a gift from B. Moss, National Institutes of Health). VV.ORF encodes the entire M RNA ORF and expresses G2, NSm, and G1 (20). VV.G1 contains an *Nco*I-*Bam*H I fragment

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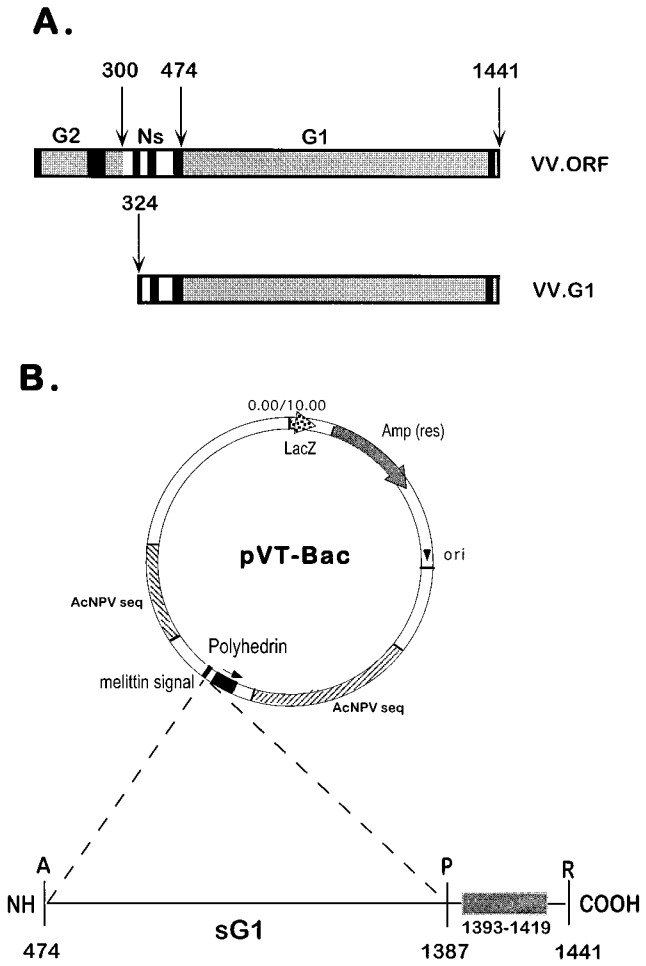

FIG. 1. Construction of recombinant viruses. (A) rVVs were prepared by using either the entire M RNA ORF or portions of NSm and G1, as indicated. (B) Recombinant baculovirus expressing a soluble form of the G1 protein was prepared by inserting the indicated G1 sequences (seq) downstream of the honeybee melittin signal sequence in pVT-Bac. The resulting sG1 does not contain the signal sequence, transmembrane, or cytoplasmic domain of the native G1.

(nucleotides 1044 to 4518 of the M RNA ORF) and generates a 1,117-amino-acid translation product that contains all 968 amino acids of the G1 protein and the carboxy-terminal 149 amino acids of the NSm protein (Fig. 1). The mobility of the G1 protein expressed in VV.G1 in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis is comparable to that of native G1 and to that of G1 expressed by VV.ORF (20).

All rVV working stocks were grown in HeLa and/or in 143 TK^- (thymidine kinase-deficient human osteosarcoma) cells and consisted of cytoplasmic extracts prepared by three freeze-thaw cycles; titers were determined in HeLa cells (20). Working stocks of La Crosse virus were prepared, and titers were determined in BHK-21 cells.

Recombinant baculovirus expression of sG1. To construct a recombinant baculovirus expressing a secreted form of G1 (soluble G1 [sG1]), sequences of the ORF between amino acids 474 and 1387, including all of G1 except for its putative signal peptide and transmembrane/cytoplasmic tail domains, were amplified from a plasmid (pM.ORF) by using PCR. The G1 sequences were then inserted in pVT-BAC, a baculovirus vector in which expression was directed with the honeybee mellitin signal sequence upstream of G1 (37) (Fig. 1). Transfection of this plasmid into *Spodoptera frugiperda* SF9 cells by using insect-specific liposomes (Insectin; Invitrogen, San Diego, Calif.) and linearized wild-type baculovirus (*Autographa californica* nuclear polyhedrosis virus [AcNPV]) DNA (BaculoGold; Pharmingen, San Diego, Calif.) led to production of polyhedron-negative recombinant baculoviruses that secreted G1 into the tissue culture media (designated rBac-sG1). Recombinant baculoviruses were plaque purified three times, and stocks of sG1 were harvested 72 h after infection of SF9 cells (multiplicity of infection of 10 PFU per cell).

Cells. BHK- 21 clone 13 cells were maintained in Earle's minimum essential medium (EMEM; GIBCO Life Technologies, Grand Island, N.Y.) supplemented with 5% heat-inactivated horse serum (Sigma Chemical Co., St. Louis, Mo.) and 5% heat-inactivated fetal bovine serum (FBS; GIBCO Life Technologies). NIH 3T3, HeLa, and 143 TK^- cells were propagated in Dulbecco's modified Eagle's medium (GIBCO Life Technologies) supplemented with 10% heat-inactivated FBS. *S. frugiperda* SF9 cells were grown in Grace's complete medium containing 10% FBS.

Viral pathogenesis. A standard protocol was used to monitor the replication and pathogenesis of La Crosse virus in 4-week-old mice inoculated intraperitoneally (i.p.) with 10 100% lethal doses (LD_{100}) of La Crosse virus and euthanized at predetermined intervals (7, 19, 21). After euthanasia, animals were perfused with 50 ml of phosphate-buffered saline (PBS; GIBCO Life Technologies) containing 0.1% heparin, and tissue samples (blood, hindleg thigh muscle, and cerebral cortex) prepared as a 10% suspension in EMEM with 2% FBS. Virus titers of the tissue homogenates and plasma were determined by standard plaque assay on BHK-21 cells, and the titers were recorded as PFU per milligram of tissue or PFU per microliter of plasma, with a limit of sensitivity of 1 PFU/2 mg of tissue or 1 PFU/20 μl of serum (19).

Passive immunization with MAb 807-31. Monoclonal antibody (MAb) 807-31, an anti-G1 neutralizing MAb, was prepared from mouse ascites fluid with staphylococcal protein A-Sepharose as described previously (16, 27). The undiluted stock contained 5.7 mg of antibody per ml and had an enzyme-linked immunosorbent assay (ELISA) titer against La Crosse virus of 1:6,400. Four-week-old animals were inoculated s.c. with 100 μl of 10-fold serial dilutions (10^0 to 10^{-3} in PBS) of MAb 807-31 or PBS alone. Twenty-four hours after s.c. injection of MAb 807-31, animals were inoculated i.p. with 10 LD_{100} of La Crosse virus, and mortality was recorded for 21 days. Blood samples were harvested at the time of challenge and analyzed for the presence of MAb 807-31 by ELISA and virus neutralization assay (see below). In a consecutive experiment, 100 μl of undiluted MAb 807-31 was inoculated s.c. 24 h after challenge with La Crosse virus (10 i.p. LD_{100}), and mortality was recorded.

Active immunization and challenge. Seven-day-old mice were immunized s.c. with 100 μl of 10-fold serial dilutions of VV.ORF, VV.G1, or VV.tk− prepared in EMEM with 2% FBS. Seven or 14-day-old animals were immunized i.p. with supernatants from SF9 cells that had been infected 72 h earlier with either rBac-sG1 or wild-type baculovirus (AcNPV). At 28 days after birth, animals were challenged by i.p. injection of 10 LD_{100} (10^8 PFU per animal) of La Crosse virus and were then observed for at least 21 days.

ELISA. The ELISA for the detection of La Crosse virus antibodies has been described in detail elsewhere (14). Briefly, La Crosse virus was grown in BHK-21 cells, purified by precipitation with polyethylene glycol (PEG 8000; Fisher Scientific, Pittsburgh, Pa.), and banded by ultracentrifugation on sucrose gradients. Protein concentration was measured by A_{280}, and ELISA plates (ICN Biomedicals Inc., Horsham, Pa.) were coated with 250 ng of purified virus per well. All samples were diluted in EMEM or ELISA buffer (PBS containing 6% bovine serum albumin) and incubated for 1 h at room temperature. After washing, the plates were incubated with peroxidase-conjugated goat anti-mouse immunoglobulin G (whole molecule) antibody (Cappel, Organon Teknika, Durham, N.C.) for 30 min at room temperature. The substrate (0.03% tetramethyl benzidine [T3405; Sigma] in 0.1 M sodium citrate buffer [pH 4.5] with 0.012% H_2O_2) was added, the reaction was stopped with sulfuric acid, and the optical density at 450 nm was read in a Titertek Multiscan MC. Titers were recorded as the reciprocal of the highest dilution measuring above twice the optical density obtained with normal mouse serum at a 1:4 dilution.

Western immunoblotting. The assay was performed as previously described (10). Supernatants from rBac-sG1-infected cells were resolved in a low-SDS (0.1%)–10% polyacrylamide gel. Following transfer to Immobilon P (Millipore, Bedford, Mass.), the filters were blocked in PBS with 0.5% Tween 20 and 3% dry milk powder for 1 h at room temperature. Hybridoma supernatant (MAb 807-15) was then diluted in blocking buffer and incubated with the membrane for 1 h at room temperature. Binding of the MAb was detected with anti-mouse immunoglobulin and an electrochemoluminescence system (ECL system; Amersham Life Science, Amersham, England). The membranes were exposed to Fuji RX medical X-ray film at room temperature for 1 to 5 min.

Virus neutralization assay. One hundred PFU of La Crosse virus in 50 μl of EMEM was mixed with an equal volume of twofold dilutions of either heat-inactivated serum or MAb 807-31 in EMEM supplemented with 2% FBS. Virus-antibody or control samples were incubated at 33°C for 1 h, following which the mixture was transferred to BHK-21 cells in 96-well plates and incubated at 33°C for 1 h. The plates were then washed twice with EMEM, further incubated at 33°C for an additional 72 h with 200 μl of EMEM supplemented with 2% FBS, and stained with Diff-Quik (Baxter Healthcare Corp., Miami, Fla.) to reveal cytopathology. Neutralization titers are reported as reciprocal of the highest dilution of serum that completely inhibited the cytopathic effect.

Radioimmunoprecipitation of viral proteins. Serum samples from 4-week-old mice and MAb hybridoma supernatants (both at final dilutions of 1:10) were used to immunoprecipitate [^{35}S]methionine/cysteine-labeled, sucrose gradient-purified La Crosse virus proteins or rVV-infected, [^{35}S]methionine/cysteine-labeled NIH 3T3 cell lysates (20).

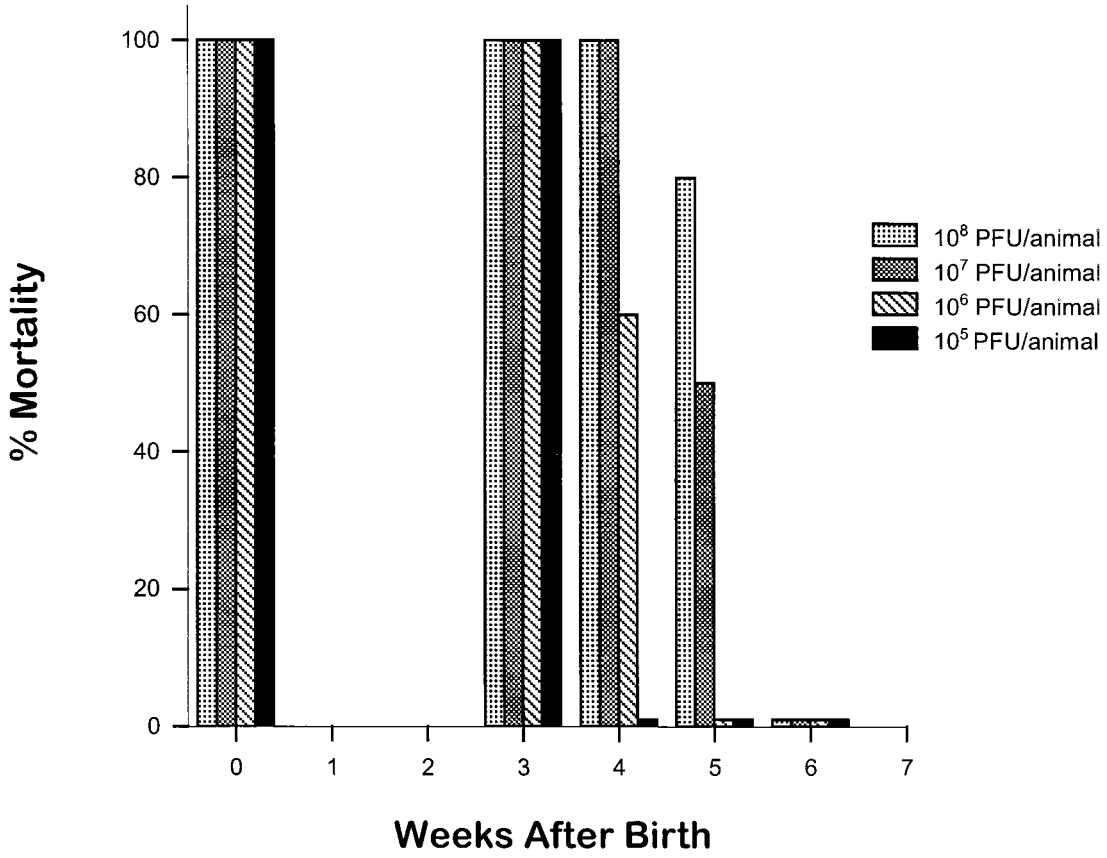

FIG. 2. Age-dependent response of CD-1 Swiss outbred mice to La Crosse virus. At the indicated age, mice were inoculated i.p. with La Crosse virus and observed for mortality over the next 21 days. Among the 4-week-old mice, the LD_{100} was 10^7 PFU, and a dose of 10^8 PFU resulted in uniform mortality. Approximately five to six animals (half litter) were inoculated with each dilution.

RESULTS

Age-dependent susceptibility of CD-1 Swiss outbred mice to La Crosse virus. Because the natural route of infection with La Crosse virus is by peripheral inoculation, we selected the i.p. route for challenge. With increasing age, mice rapidly lose their susceptibility to peripheral injection with La Crosse virus, and we first determined the oldest age at which 100% mortality could be obtained with a stock of virus available routinely. When mice up to 4 weeks old were each inoculated i.p. with $\geq 10^7$ PFU of La Crosse virus, they developed neurological symptoms, and all died within 5 to 7 days after inoculation (LD_{100}). By 5 weeks of age, the LD_{100} was $>10^8$ PFU (Fig. 2). Consequently, all of the immunizations were designed to generate a response by the time mice reached 4 weeks of age, at which time they were challenged with 10^8 PFU, or 10 LD_{100} for that age.

Pathogenesis of La Crosse virus in adult mice. Previous pathogenesis experiments in newborn and suckling animals have demonstrated that following s.c. inoculation, La Crosse virus replicates vigorously in striated muscle; this is associated with a plasma viremia and invasion of the CNS (7, 18, 19, 21, 22). To determine the sites of viral replication in 4-week-old mice, a group was challenged i.p. with 10 LD_{100}; at various times, blood, muscle, and brain tissue samples were harvested, and the viral content was determined. Figure 3 shows that La Crosse virus was detected in the blood only within the first 24 h, peaking at 1 h after inoculation and disappearing after 12 h. Virus was detected in striated muscle and in the brain after 5 days, and as expected, all of the animals died within 7 days. Since virus was not detected in any other tissues before its appearance in the brain, it is likely that the initial passive viremia introduced the virus into the CNS.

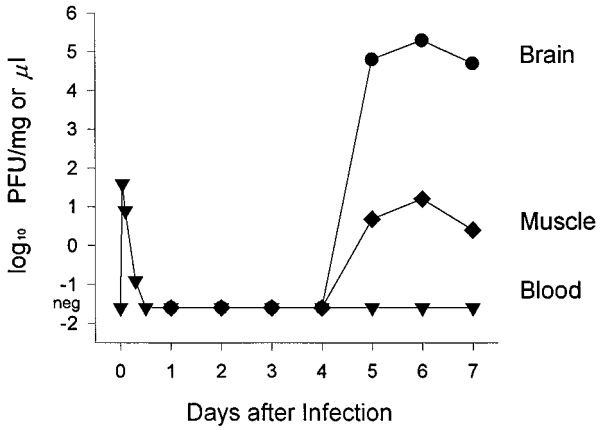

FIG. 3. Pathogenesis of La Crosse virus in 4-week-old mice. Swiss outbred mice were inoculated i.p. with 10^8 PFU (10 LD_{100}), and the quantities of virus in blood, muscle, and brain were determined at regular intervals by plaque assay. The titers are recorded as PFU per milligram of tissue or microliter of blood, with a limit of sensitivity of 1 PFU/2 mg of tissue or 1 PFU/20 μl of serum. Each datum point represents a pool of three animals. The passive viremia that was detected only at 3 to 12 h after injection is shown.

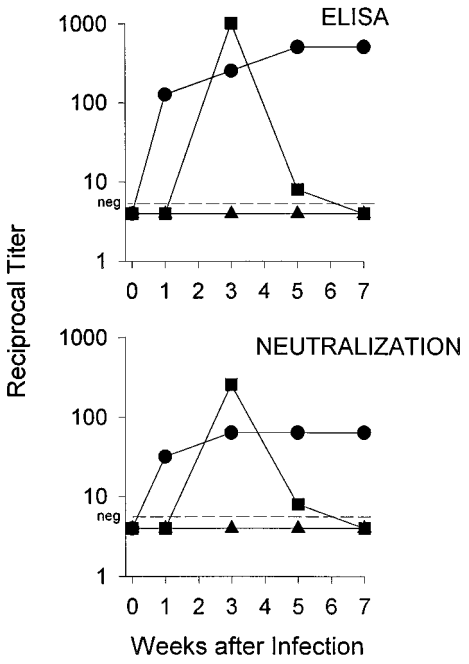

FIG. 4. Humoral responses to rVVs. One-week-old Swiss outbred mice were inoculated with VV.ORF, encoding G1, G2, and NSm of La Crosse virus (●), VV.G1, encoding the G1 glycoprotein (■), or control VV.tk− (▲). At the indicated time points, the pooled sera from two to three mice were assayed in triplicate for total antibody (ELISA) or for the development of a neutralizing response. Whereas animals inoculated with VV.ORF developed a strong immune response, inoculation with VV.G1 led to only a minimal and variable response. The positive titer at 3 weeks may represent a single mouse.

Passive protection from a lethal challenge with an anti-G1 MAb. Since in this model virus is delivered to the CNS by blood, it appeared plausible that circulating neutralizing antibody would prevent viremia and protect against encephalitis. To test this hypothesis, we used MAb 807-31 (16). A total of 0.1 ml of affinity-purified antibody was administered s.c. to 4-week-old mice, which were then bled after 24 h to determine the titer of antibody in the plasma. An equivalent group of animals was challenged by i.p. injection of La Crosse virus. Undiluted MAb 807-31 produced a neutralization titer of 1:16 at 24 h, after administration and protected seven of eight mice, while a 1:10 dilution failed to produce a measurable neutralization titer and offered no protection from the challenge (not shown). When undiluted MAb 807-31 was inoculated 24 h after challenge, the animals were not protected. These results indicated that neutralizing antibody, by itself, was capable of protection in this model.

Induction of anti-La Crosse virus neutralizing antibodies by rVVs. We then determined whether active immunization prior to virus challenge would protect the mice. We used two rVVs previously constructed in this laboratory: VV.ORF, which expresses the entire ORF of the M RNA segment of La Crosse virus, and VV.G1, which expresses the G1 glycoprotein but not G2 (20). To analyze the humoral immune response of animals inoculated with these rVVs, 1-week-old mice were injected with the most concentrated preparations available, 10^5 PFU per animal for VV.ORF or 10^6 PFU per animal for VV.G1 and control VV.tk−. At various times after infection, two or three mice were tested for anti-La Crosse virus antibodies. As Fig. 4 illustrates, inoculation with VV.ORF induced a brisk humoral response in mice, and anti-La Crosse virus antibodies were detected as early as 1 week after inoculation; these titers remained constant for up to 7 weeks after immunization. In contrast, the response to VV.G1 was variable, with very little detectable antibody at 7 weeks after inoculation. The positive response at 3 weeks probably reflects variability in immune responses among different animals. As expected, there was no detectable anti-La Crosse virus humoral response in the animals inoculated with the control rVV (VV.tk−).

TABLE 1. Individual mouse responses to immunization with VV.ORF or VV.G1

Immunogen[a]	Dose (PFU/animal)	ELISA[b]	Neutralization[c]
VV.ORF	$\leq 10^3$	32	16
		64	16
		1,024	128
	$\geq 10^4$	64	8
		128	64
		256	256
		512	16
		1,024	128
		1,024	256
VV.G1	$\leq 10^3$	<4	<4
		<4	<4
	$\geq 10^4$	<4	<4
		<4	<4
		16	<4
		128	32
		256	32
		256	64

[a] One-week-old mice were inoculated s.c. with 10-fold dilutions of either recombinant virus. The responses 3 weeks later of individual animals inoculated with $\leq 10^3$ PFU and those inoculated with $\geq 10^4$ PFU are grouped.
[b] Reciprocal dilution of serum resulting in a positive titer (see Materials and Methods).
[c] Reciprocal dilution of serum neutralizing La Crosse virus (see Materials and Methods).

To determine if analysis of pooled sera was obscuring strong individual responses to VV.G1, 1-week-old mice were inoculated with 10-fold serial dilutions of either VV.ORF (10^5 to 10^1 PFU) or VV.G1 (10^6 to 10^1 PFU) and bled 3 weeks later, and individual sera were assayed (Table 1). All of the animals inoculated with VV.ORF had antibody detectable by ELISA or neutralization assays. Some of the animals inoculated with the higher doses ($\geq 10^4$ PFU) of VV.G1 had a low-level neutralizing response, whereas none of the animals inoculated with the lower doses had any measurable response. These data indicated that VV.G1 is not as efficient at eliciting anti-La Crosse virus neutralizing antibodies as VV.ORF, even though neutralizing activity is usually directed against G1 (see below).

Protection of rVV-immunized mice against La Crosse virus challenge. To assess the ability of the rVVs to protect mice against La Crosse virus encephalitis, 10-fold dilutions of rVVs were used to immunize 1-week-old mice. Each animal was challenged i.p. with 10^8 PFU (10 i.p. LD_{100}; Fig. 2) of La Crosse virus 3 weeks later and monitored for 21 days. As shown in Table 2, VV.ORF was able to protect almost all of the mice at a dose of $\geq 10^3$ PFU per animal. In a separate experiment (not shown), 70% of the animals were protected at 10^2 to 10^3 PFU per animal. In contrast, VV.G1 could not protect all of the animals even at a maximal dose of 10^6 PFU per animal. No protection was observed in mice receiving either VV.tk− or tissue culture medium, indicating that the protection was specific for rVVs expressing the La Crosse glycoproteins. This result suggested that efficient protection from La Crosse encephalitis requires the expression of the entire M RNA ORF.

TABLE 2. Protection of mice against a lethal challenge with La Crosse virus by preexposure immunization with rVVs[a]

rVV	rVV inoculum (PFU/animal)	Survival after challenge with La Crosse virus	
		No. alive/total	%
VV.ORF	10^5	5/5	100
	10^4	8/8	100
	10^3	7/8	88
VV.G1	10^6	3/8	38
	10^5	5/7	71
	10^4	4/8	50
VV.tk−	10^6	0/5	0
Mock[b]		0/11	0

[a] Mice were immunized with a single s.c. injection of rVV at 1 week of age. The animals were then challenged with 10 LD_{100} at 4 weeks of age, and survival was recorded 21 days later. A repeat experiment gave similar results.
[b] Mice injected with medium (EMEM) only.

Comparison of antibodies raised in mice immunized with VV.ORF and VV.G1. To determine the relative immunogenicities of the proteins encoded by the M RNA ORF (G2, NSm, or G1), immunoprecipitations were performed with sera from immunized animals. Figure 5 shows that the G1 glycoprotein was immunoprecipitated by sera from mice immunized with either VV.ORF or VV.G1. These results confirm that VV.G1 raised anti-G1 antibodies.

Recognition of G1 by MAbs. To determine whether the G1 glycoprotein expressed alone is folded correctly, we performed immunoprecipitation experiments with various MAbs that are directed against conformation-specific epitopes of La Crosse virus G1 (Fig. 6). Following ^{35}S labeling, the G1 glycoprotein

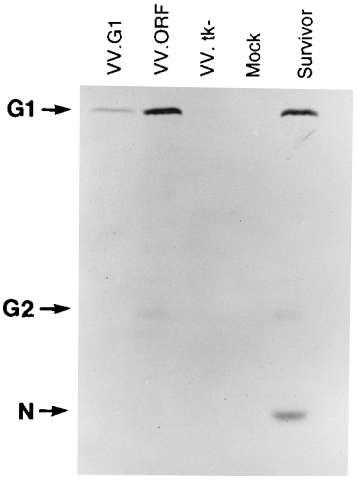

FIG. 5. Immmunoprecipitation of La Crosse virus proteins by sera from mice immunized with rVVs. One-week-old Swiss outbred mice were inoculated with VV.ORF, VV.G1, or control virus VV.tk−. Sera were obtained from two to three mice at 3 weeks after inoculation, pooled, and used in immunoprecipitation experiments with ^{35}S-labeled, gradient-purified La Crosse virus. Animals immunized with either VV.ORF or VV.G1 had antibodies that immunoprecipitated G1; animals immunized with VV.ORF also had variable levels of antibody that immunoprecipitated G2. Serum from a single surviving animal was also used to immunoprecipitate La Crosse virus, and this serum demonstrated reactivity against G1, G2, and N. In other experiments in which the sample was boiled, the quantity of G1 protein precipitated by serum from VV.G1-immunized mice was more equivalent to that precipitated by VV.ORF-immunized mice.

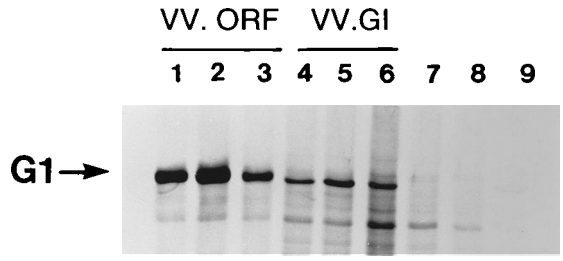

FIG. 6. G1 glycoprotein from rVVs is recognized by MAbs. NIH 3T3 cells were infected with VV.ORF (lanes 1 to 3) or VV.G1 (lanes 4 to 6) and labeled with [^{35}S]methionine/cysteine, and the lysates were then immunoprecipitated with MAb 807-31 (lanes 1 and 4), 807-22 (lanes 2 and 5), or 807-25 (lanes 3 and 6) (16) as described in Materials and Methods. The G1 protein produced by either VV.ORF or VV.G1 was recognized by each of the MAbs, which are directed against conformational epitopes (data not shown). Control lysates from cells infected with VV.tk− (lane 7), mock infected (lane 8), and infected with vaccinia virus WR (lane 9) showed no G1 signal when immunoprecipitated with a mixture of the three antibodies.

was immunoprecipitated from cell lysates of both VV.ORF and VV.G1-infected NIH 3T3 cells by three MAbs representing separate epitope groups (27). These results indicate that a portion of the G1 protein expressed by VV.G1 is not grossly misfolded and that at least some is recognized by MAb 807-31, which defines an epitope that was previously determined to be important in protection.

Localization of intracellular G1 expressed from different rVV constructs. Previous experiments with respiratory syncytial virus and rotaviruses have indicated that surface expression of recombinant antigens is important in generating a humoral immune response with rVVs (28). To determine whether the La Crosse virus glycoproteins were expressed at the cell surface following infection with VV.ORF or VV.G1, we performed indirect immunofluorescence assays on NIH 3T3 cells infected with rVVs at 18 h postinfection (data not shown). In paraformaldehyde-fixed cells, both G1 and G2 were detected at the cell surface of VV.ORF-infected cells as early as 12 h and were obvious at 18 h. In contrast, there was very little G1 detected at the cell surface of VV.G1-infected cells in spite of adequate production of G1 protein (20). These results suggest that G1 when expressed alone may be modified or targeted to different cellular compartments. This could have an effect on the generation of an effective immune response.

Preparation of sG1. These results suggested that the G1 protein is the key immunogen for protection but implied that its expression together with expression of G2 and NSm might be important for stimulation of a robust response. To investigate this question further, we prepared recombinant G1 by using a baculovirus expression system (see Materials and Methods and Fig. 1). The recombinant baculovirus, rBac-sG1, secreted sG1 into the supernatant, as demonstrated with a Western blot (Fig. 7). The sG1 was also immunoprecipitated by conformation-dependent MAbs in an experiment similar to that depicted in Fig. 6 (not shown).

Mice were then immunized with two doses of rBac-sG1 supernatant, 100 μl, corresponding to approximately 300 to 500 ng of G1, or twice that amount, at 7 and/or 14 days after birth. As shown in Table 3, a strong neutralizing response was generated in the animals immunized by two i.p. injections and in older (14 day) animals immunized with a single i.p. injection. This neutralizing response was associated with protection from a challenge with La Crosse virus. A single dose of sG1 at 7 days old was insufficient to induce neutralizing antibodies and was not protective.

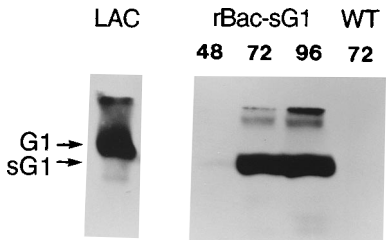

FIG. 7. rBac-sG1 produces a soluble form of the G1 glycoprotein. SF9 cells were infected with rBac-sG1 or wild-type baculovirus at a multiplicity of infection of ≈10, and the supernatant was harvested at various time points. Twenty-five microliters was resolved in a low-SDS gel, and G1 was detected with a MAb followed by electrochemoluminescence (see Materials and Methods). The leftmost lane contained gradient-purified La Crosse (LAC) virus, to mark the position of G1. WT, supernatant from SF9 cells infected with AcNPV.

To determine if conformational antibodies are important in the protection of mice from La Crosse virus infection, we immunized some mice with sG1 that had been denatured by heating at 95°C for 10 min. While an antibody response was detected by ELISA, there was no neutralizing activity and no protection of challenged mice. These results are congruent with prior evidence that most of our MAbs with neutralizing activity recognize conformation-sensitive epitopes (9).

DISCUSSION

These experiments have shown that recombinant La Crosse virus glycoproteins can generate a protective immune response in mice. As predicted from the virus neutralization experiments (17, 23), the G1 glycoprotein appeared to be the dominant immunogen, as mice inoculated with the protective recombinant VV.ORF developed only low levels of antibody against G2. Nevertheless, an rVV expressing full-length G1 (without G2) was unable to protect animals from a lethal challenge, and the neutralizing response it elicited was concomitantly weaker. From these data, it appeared that expression of the two glycoproteins in concert was necessary for appropriate presentation of the G1 molecule to the relevant arm of the immune system. Although immunoprecipitation experiments demonstrated that the G1 protein expressed alone was recognized by MAbs, a more quantitative assay might demonstrate that only a small fraction of the VV.G1 protein was folded correctly. One potential clue may lie in the observation that in contrast to cells infected with VV.ORF, in which G1 could be easily detected at the cell surface, VV.G1-infected cells demonstrated minimal surface expression in paraformaldehyde-fixed preparations (not shown). Although surface expression of recombinant proteins has been implicated in stimulation of an immune response with rVVs in other systems (1, 28), the precise relationship between intracellular localization and the immune response is unclear.

TABLE 3. Mortality and antibody responses of mice immunized with recombinant soluble G1

Immunogen[a]	Inoculum[b] (μl/animal, route)	Age at immunization (days)	No. surviving/ total[c]	Antibody response at challenge[d]	
				ELISA	NT
sG1	100, s.c.	7	0/7	4	<8
	100, s.c.	7, 14	1/5	64	8
	200, i.p.	7	2/8	16	<8
	200, i.p.	**14**	**7/7**	**128**	**16**
	100, i.p.	**7, 14**	**6/6**	**1,024**	**32**
	200, i.p.	**7, 14**	**6/6**	**1,024**	**128**
sG1, HI	200, i.p.	7, 14	0/8	32	<8
AcNPV	200, i.p.	7, 14	0/6	<4	<8
Grace's	200, i.p.	7, 14	0/7	<4	<8
None			0/6	<4	<8

[a] sG1, supernatant from recombinant baculovirus expressing G1; sG1, HI, sG1 preparation heat inactivated by treatment at 95°C for 10 min; AcNPV, wild-type baculovirus; Grace's, medium only. Lines in boldface represent inoculations that protected the animals from challenge. This experiment is representative of two similar immunizations.

[b] Each 100 μl of supernatant from rBac-sG1 contained approximately 300 to 500 ng of sG1.

[c] Mice were challenged at 4 weeks of age by i.p. injection of 10 LD_{100} of La Crosse virus. Survival was measured 21 days later.

[d] Reciprocal of pooled serum dilution giving a positive signal in either ELISA or neutralization (NT) assays.

Because these results made the role of G1 in protection somewhat ambiguous, we prepared a soluble form of this protein in a baculovirus system. Immunization of animals with this recombinant protein, even at a single dose, led to a robust neutralizing response and was completely protective from a La Crosse virus challenge. Furthermore, mild denaturation of the sG1 by heating abolished the neutralizing response, in spite of the presence of antibody as detected by ELISA (Table 3). (In other experiments not shown, the denatured sG1 was not recognized by some MAbs.) Although we do not have detailed information regarding the three-dimensional structure of G1, it contains 44 cysteines, and it probably has numerous intramolecular disulfide bonds. These findings with sG1 confirm the importance of a properly folded G1 in initiating an immune response with these bunyaviruses. Taken together with the results from studies with MAbs generated during sublethal La Crosse virus infections (16, 23), these data support the primary importance of G1 in the prevention of La Crosse virus replication and demonstrate its importance as an immunogen should vaccination ever become important from a public health standpoint.

Recombinant proteins have been used in immunization experiments in viruses representing other genera of the family *Bunyaviridae* (29, 32–34, 36, 38). Although all of the members of the *Bunyaviridae* contain two glycoproteins encoded in a single ORF, the sizes of the individual proteins and their positions in the ORF are variable. This makes it difficult to compare the immunogenicities of the La Crosse virus proteins with those of other members of the *Bunyaviridae*. Still, joint expression of the Hantaan virus G1 and G2 in either vaccinia virus or baculovirus resulted in higher titers of neutralizing antibody and better protection from a nonfatal challenge than individual expression of either of the proteins (32). In that system, antibodies against either G1 or G2 may demonstrate neutralizing activity. These studies also investigated the preventive role of passive humoral immunity and, similar to our results, suggested that the antibody response is sufficient for protection (32). Baculovirus recombinants expressing Rift Valley fever virus glycoproteins have also been prepared (34, 36). Recombinants expressing the two glycoproteins were effective immunogens in mice and protected from a lethal challenge. In contrast, a recombinant baculovirus expressing G2 alone did not protect the animals from challenge (34).

The role of cell-mediated immunity in preexposure immunization is unclear for any of the bunyaviruses. Immunization with a recombinant baculovirus expressing the N protein of Hantaan virus prevented antigen expression in the lungs of hamsters challenged intramuscularly (32). As antibodies

against the N protein are not involved in neutralization, these findings may be indirect evidence of cell-mediated immunity. However, since our pathogenesis experiments indicated that neutralizing antibody alone is enough to prevent the lethal complications of this infection, we did not address this aspect of the immune response.

Finally, it is important to emphasize that pathogenesis studies play a critical role in designing vaccine strategies. rVVs have been used with great success in some systems, for example, rabies, for which there is at least a modest understanding of the key steps in pathogenesis and the role of the immune response in interrupting them (4). In contrast, rVV expression of the E1 and E2 glycoproteins of Venezuelan equine encephalitis virus protected animals from i.p. but not intranasal challenge with the virus (24), calling into question the efficacy of this vaccine in protecting laboratory workers from aerosolized virus infection. In this simple bunyavirus model, the viremia is responsible for CNS penetration, and its interruption with neutralizing antibodies appears to be sufficient for protection. Thus, while recombinant viruses and proteins provide a powerful tool for vaccine construction, targeting the immune response to block at least one key step in the in vivo life cycle is an essential and often overlooked aspect of vaccine design.

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A.P. and C.G. contributed equally to this study.

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