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Semliki Forest Virus E_2 Envelope Epitopes Induce a Nonneutralizing Humoral Response Which Protects Mice against Lethal Challenge

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Along the 422 amino acids of the Semliki Forest virus (SFV) E_2 envelope glycoprotein, we identified 13 peptide cassettes (ranging in size from 15 to 25 amino acids and designated A through N) that contain hydrophilic sequences flanked by amino acid sequences conserved in the E_2 envelopes of the alphavirus family. Six peptide blocks containing either a single cassette or two to three contiguous cassettes (A, BC, DE, FG, HIK, and LMN) were produced in *Escherichia coli* as recombinant proteins fused to the N terminus of β-galactosidase. All of the SFV E_2 recombinant polypeptides except A–β-galactosidase were recognized on Western blots (immunoblots) by anti-SFV polyclonal antisera. In addition, these five recombinant proteins induced in mice antibodies that interacted specifically with SFV E_2 protein on Western blots as well as with the intact virions in an enzyme-linked immunosorbent assay. The six hybrid proteins were used to vaccinate mice and were tested for the ability to confer resistance against lethal doses of SFV. Peptides BC and HIK, located at amino acid positions 114 to 149 and 216 to 288, respectively, of E_2, protected partially (40 to 60%) against SFV challenge. A third peptide, LMN, located between amino acid positions 289 and 352, rendered mice totally resistant to an SFV challenge of 250 50% lethal doses. The partially protective effects of the BC and HIK cassettes and the high efficacy of the LMN cassette were consistently demonstrated, independent of the adjuvant (complete Freund or alum), immunization protocol, and strain of mice used. None of the antisera raised against any given cassette could neutralize the virus in an in vitro tissue culture assay or in a plaque reduction neutralization test. Nevertheless, passive transfer experiments demonstrated that in the case of LMN, the protective effect was mainly of a humoral nature.

Many of the alphaviruses are important human or veterinary pathogens. They differ in host range and in the pathological results of infection but are extremely similar in molecular architecture and in the pattern of events involved in viral replication. (For review, see reference 21). Despite this marked structural conservation, antibodies from different species in the group are not efficient in cross-protection, which indicates that the regions eliciting protective antibodies differ from one species to another (6). This phenomenon can be instrumental in delineating the potential immunogenic epitopes by comparison of the primary sequences of analogous structural proteins of the alphavirus family. Identification of such peptides could provide the basis for production of defined synthetic vaccines.

The site for neutralization of alphaviruses appears to be located on the E_2 envelope protein, since polyvalent antibodies to E_2 are generally neutralizing. Antibodies to E_1, on the other hand, are cross-reactive and generally not neutralizing (8, 17). This is in agreement with the observation that E_1 sequences are more conserved than those of E_2. It appears, therefore, that E_2 functions during virus evolution to generate strain diversity and is the primary target of the immune system in that it elicits the production of protective antibodies. We report here a systematic analysis for selection and evaluation of potential epitopes on the E_2 envelope of SFV. To evaluate the immunogenicity and protective efficacy of these epitopes, we chose to fuse them by DNA recombinant techniques to an immunocarrier, β-galactosidase. Use of the large bacterial β-galactosidase polypeptide as an immunocarrier was reported previously (19, 20). There is extensive genetic and immunological information on this protein: it can stabilize the synthetic product, allows manipulations of epitopes independent of their size, facilitates technical aspects of production and purification, and, above all, provides a completely uniform and defined peptide carrier system. A preliminary report on construction of Semliki Forest virus (SFV) E_2 peptide–β-galactosidase fusion proteins was presented recently (10).

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MATERIALS AND METHODS

Construction and identification of plasmids carrying SFV E_2 cassettes. An SFV cDNA clone derived from pSV_2-SFV (a kind gift from H. Garoff) (11) was digested with *Eco*RI-*Sca*I. The 750-base-pair (bp) band was isolated and further digested with *Sau*3A, producing five subfragments separable on acrylamide gel. These DNA fragments coded for the cassette groups designated BC (108 bp), DE (117 bp), FG (81 bp), HIK (217 bp), and LMN (192 bp) (Fig. 1). Each of these DNA fragments was ligated to one of the *trp*-regulated expression vectors pTZ_1 and pTZ_2. These vectors were designed to allow in-frame ligation of virus segments to the *lac*Z gene (10). Table 1 lists the various cassettes, their sizes and locations on the E_2 polypeptide, the cloning vectors used, and the appropriate cloning sites. Cassette A sequences were prepared by chemical DNA synthesis:

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   (Met)SerAlaGlnIleGlyIleAspLysSerAspAsnHisAspTyrThrLysIleArgTyrAla
C  ATG TCTGCACAAATTGGCATCGACAAGTCTGACAACCACGACTACACTAAGATCCGTTACCCG
     AGACGTGTTTAACCGTAGCTGTTCAGACTGTTGGTGCTGATGTGATTCTAGGCAATGCGCCTA G
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The synthetic DNA fragment was cloned into the *Nco*I-*Bam*HI sites of pTOZ, a derivative of pTZ_1 carrying an appropriate polylinker sequence. After transformation into competent *Escherichia coli* MC1060, [Δ(*lacIPOZYA*) X74 *galU galK* $StrA^r$ *hsdR*), cells were plated on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)-supplemented plates. Blue colonies, indicative of correct in-frame insertions, were selected and further analyzed by restriction enzymes to verify insertion of the SFV E_2 fragments in the correct orientation downstream from the *trp* operator-promoter region and at the 5′ end of the β-galactosidase gene. Finally, sequence analysis was done to verify the correct sequence of the inserted cassette-coding DNA. Additional information on the construction of these plasmids has been published elsewhere (10). Recombinant techniques were used as described by Maniatis et al (13). DNA synthesis was performed on an Applied Biosystem DNA synthesizer.

Expression, extraction, and partial purification of SFV E_2–β-galactosidase hybrid polypeptides. Transformed MC1060 cells were grown in M9 medium (14) supplemented with 0.5% Casamino Acids (Difco Laboratories) and a limited amount of tryptophan (2 μg/ml). The cells were harvested at an A_{550} of 3.0 (tryptophan is depleted at this stage). Frozen bacterial cells were suspended in 50 mM Tris hydrochloride buffer (pH 7.6) containing 30 mM NaCl, and lysozyme was added to a final concentration of 1 mg/ml. After 30 min of incubation at 0°C, the resulting spheroplasts underwent five consecutive freeze-thaw cycles and were then centrifuged for 30 min at 27,000 rpm in a Ti 45 rotor (Beckman Instruments, Inc.). The resulting supernatant was collected and assayed for β-galactosidase content (14). Finally, the hybrid protein was concentrated and partially purified by ammonium sulfate fractionation (40% saturation).

Mouse anti-SFV antibodies. A 10% (wt/vol) phosphate-buffered saline extract of SFV-infected mouse brain was inactivated by 0.5% Formalin, diluted threefold in saline, and injected intraperitoneally (i.p.) with 1 volume of complete Freund adjuvant (CFA). A month later, the same injection protocol was repeated; after an additional 20 days, ascitic fluid induced by i.p. injection of CFA was collected. The anti-SFV antiserum was used to immunostain the various SFV hybrid cassettes on a Western blot (immunoblot) (1). A 1:400 dilution of the antiserum was used. As a second antibody, goat anti-mouse alkaline phosphatase (Sigma Chemical Co.)-labeled immunoglobulin G (IgG) diluted 1:400 was used. Development was by the phosphatase-specific chromagenic reaction of Fast Blue RR salt and Naphthol As-Mx (Sigma). The same immunostaining technique was used with anti-hybrid SFV E_2 cassettes except for a lower dilution (1:40) for the first binding reaction.

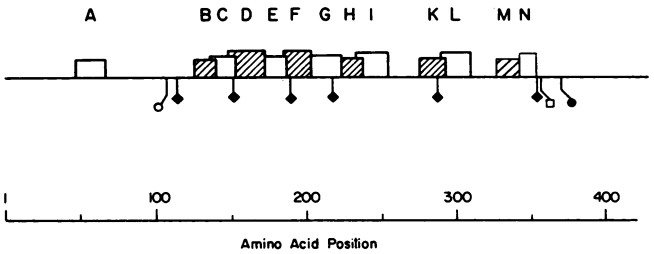

FIG. 1. Positions of SFV E_2 peptide cassettes on the E_2 polypeptide. Coding blocks (A through N) of cassettes are shown at the top. Restriction enzyme sites: ◆, *Sau*3A; ○, *Eco*RI; □, *Sca*I; ●, *Eco*RV.

Immunization protocols. In protocol I, 25 μg of each SFV-related hybrid polypeptide was injected intramuscularly (10- to 12-g ICR female mice) with CFA. This injection procedure was repeated 2 weeks later, followed by two injections of 25 μg of protein in phosphate-buffered saline at intervals of 2 weeks.

In protocol II, 25 μg of each SFV-related hybrid polypeptide was injected intramuscularly with *Bordetella pertussis* as the adjuvant (1.5 optical density units per mouse). This was followed by three consecutive injections in the presence of alum at 2-week intervals.

In both protocols, challenge with SFV was performed 8 days after the fourth injection. Antisera for monitoring immune response were collected 1 day before challenge.

Solid-phase enzyme-linked immunosorbent assay (ELISA). Each well of 96-well plates (Dynatech Laboratories, Inc.) was coated overnight with 100 μl of virus suspended in glycine buffer (pH 9.0). Plates were washed three times with TST buffer (0.05 M Tris [pH 7.6], 0.15 M NaCl, 0.05% Tween 20) and incubated for 1 h at 37°C with fourfold dilutions of the tested sera in TST containing 0.5% bovine serum albumin. To washed plates, 100 μl of alkaline phosphatase-labeled rabbit anti-mouse IgG (1:500 dilution; Sigma) was added; plates were incubated for 1 h at 37°C and developed by the phosphatase substrate 104 (Sigma). After 30 min, plates were read at 405 nm in a Microplate autoreader spectrophotometer (Bio-Tek Instruments).

Virus challenge. SFV strain B26146 (passaged 14 times in mice and twice in BHK cells) was received from the American Type Culture Collection. Virus stock used for challenge was produced in BHK cells (grown in Dulbecco modified medium supplemented with 10% fetal calf serum and 70 μg of gentamicin per ml) and collected from clear supernatants by centrifugation (27,000 rpm; Spinco rotor 30; 2 h). Resuspended (in phosphate-buffered saline) pellets were diluted to a titer of 1.5×10^8 PFU and kept at −70°C. The 50% lethal dose (LD_{50}) of the stock preparation was determined by i.p. injection of female ICR or BALB/c mice of the same age (~12 weeks) and weight as the vaccinated animals. The LD_{50}s were calculated (15) 14 days after injection of virus and found to be identical in both mouse strains. In most challenge experiments, 250 LD_{50} (10^{-5} dilution of the stock preparation) was used.

Virus neutralization tests. (i) $TCID_{50}$ inhibition. The tested sera were serially diluted (twofold) in 96-well flat-bottom

TABLE 1. Cloned SFV E2-related sequences

Cassette	Length (amino acids) of SFV E_2 peptide	Amino acid position on E2	Cloning vector (cloning site)	Plasmid
A	19	50–69	pTOZ (*Nco*I-*Bam*HI)	pT(A)Z
BC	35	114–149	pTZ_1 (*Bgl*II)	pT(BC)Z
DE	38	150–188	pTZ_1 (*Bgl*II)	pT(DE)Z
FG	26	189–215	pTZ_1 (*Bgl*II)	pT(FG)Z
HIK	72	216–288	pTZ_1 (*Bgl*II-*Bam*HI)	pT(HIK)Z
LMN	63	289–352	pTZ_2 (*Bgl*II)	pT(LMN)Z

tissue culture microdilution plates (Linbro). The dilution medium was Dulbecco modified medium containing 2% fetal calf serum and antibiotics. Then 1,000 to 3,000 50% tissue culture infective doses ($TCID_{50}$) of the virus (50 μl in the same medium) was added to each well. After the plates were incubated for 1 h at 37°C in the presence of 5% CO_2, 20,000 BHK-21 cells in Dulbecco modified medium containing 10% fetal calf serum was added to the wells (100 μl per well), and the plates were again incubated at 37°C. Cytopathogenic effect was monitored 24 to 28 h later. The last dilution of the serum at which no cytopathogenic effect could be detected was considered the titer of the serum. Duplicates were used, and positive and negative controls were included in each test.

(ii) Plaque reduction neutralization test. An SFV suspension of ~500 PFU/ml was mixed with an equal volume of antiserum at the appropriate dilution. After 1 h of incubation at 37°C, 0.2-ml portions of this mixture were absorbed (1 h at 37°C) to BHK cell monolayers in 50-mm-diameter plates; then 0.4% tragacanth (Sigma) in Eagle minimal essential medium containing 2% fetal calf serum was added to the plates. Plaque numbers were recorded 48 h later, after staining with neutral red.

RESULTS

Selection of SFV E_2 cassettes. We based the selection of potential protective SFV epitopes on the E_2 envelope polypeptide on the following assumptions: (i) there is a common basic architecture in the E_2 envelopes of all alphaviruses, which is maintained by stretches of conserved amino acid sequences along the polypeptides; (ii) the specificity of immune protection, revealed by the lack of cross-neutralization among alphavirus subtypes, is directed by type-specific hydrophilic sequences embedded within the conserved backbone structure; and (iii) besides the spatial epitopes, there are some linear sequences, characterized by the pattern conserved–specific hydrophilic–conserved, that contribute to induction of virus-specific protection. Guided by these assumptions, one could identify potential protective linear epitopes. We first superimposed hydropathic plots (12) derived from Ross River virus (7), SFV (9), and Sindbis virus (16) E_2 sequences (SFV and Sindbis represent distal evolutionary branches of alphaviruses [2]). On these superimposed plots, we searched only for those regions that are hydrophilic in all of the three different E_2 sequences and are also bound by stretches of at least five conserved amino acid sequences. By applying this algorithm, 13 cassettes fulfilling these criteria were found and designated A to N. The distribution of these cassettes along the E_2 sequence of SFV is schematically shown in Fig. 1. Cassette length varied between 16 and 24 amino acids, with a hydrophilic core ranging from 6 to 10 amino acids.

To facilitate analysis of the immunogenicity of these SFV E_2 cassettes, we first tested pairs or triplets of cassettes consisting of 19 to 72 amino acids. cDNA fragments from pSV_2-SFV, coding for the relevant peptides, were isolated and cloned in vectors specifically designed to express SFV E_2–β-galactosidase fusion products. This strategy allows use of the bacterial product directly in immunization experiments, obviating the need to couple the various antigens to an immunocarrier. The sequences coding for cassettes BC, DE, FG, HIK, and LMN were isolated and cloned in appropriate vectors (Materials and Methods; Table 1). Cassette A of SFV E_2, which could not be conveniently isolated from the cDNA clone, was chemically synthesized as a DNA duplex and cloned in pTOZ. These six recombinant SFV E_2-derived antigens were used for the studies described below.

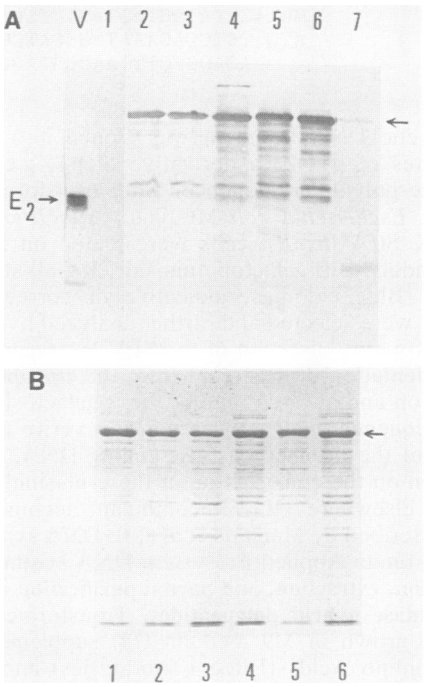

FIG. 2. Ability of various SFV E_2–β-galactosidase hybrids to interact with anti-SFV mouse polyclonal antisera. (A) Western blot of the SFV-related hybrid proteins (2 μg per lane) from a 10% polyacrylamide-sodium dodecyl sulfate gel. Blots were reacted with anti-SFV antibodies and immunostained as described in Materials and Methods. (B) Coomassie brilliant blue-stained gel of SFV E_2–β-galactosidase polypeptides. Lanes 1 to 6 in each gel contain A–, BC–, DE–, FG–, HIK–, and LMN–β-galactosidase polypeptides, respectively. Control lanes: V, SFV virion proteins from sucrose gradient-purified virus (position of the 52,000-molecular-weight E_2 polypeptide is indicated on the left); 7, *E. coli* β-galactosidase polypeptide (positions of the 135,000-molecular-weight β-galactosidase monomer and its hybrids are indicated on the right). All protein samples were boiled for 10 min in sodium dodecyl sulfate–β-mercaptoethanol (Laemmli sample buffer) before electrophoresis.

Antigenicity of the β-galactosidase fusion peptides. The hybrid SFV E_2 polypeptides were partially purified and tested by Western blotting with anti-SFV mouse antisera obtained from mice immunized with inactivated SFV particles. Cassettes BC, DE, FG, HIK, and LMN (Fig. 2A, lanes 2 to 6) were recognized by the anti-SFV antiserum used, whereas cassette A (lane 1) and the carrier β-galactosidase protein (lane 7) were not labeled by this antiserum. These differences in immunostaining cannot be attributed to variations in amounts of hybrid proteins applied to the gel, as indicated by the Coomassie brilliant blue staining pattern (Fig. 2B). The ladder of immunostained bands detected in each positive lane under the main band of β-galactosidase probably resulted from partial degradation of the hybrid polypeptide rather than from cross-reaction with bacterial contaminants. This conclusion is suggested by the fact that these bands did not appear in the control β-galactosidase preparation (lane 7). Using 14 different antisera from mice immunized with either live or inactivated SFV, we were unable to demonstrate interaction with the SFV A–β-galactosidase hybrid protein. Cassettes BC, HIK, and LMN were

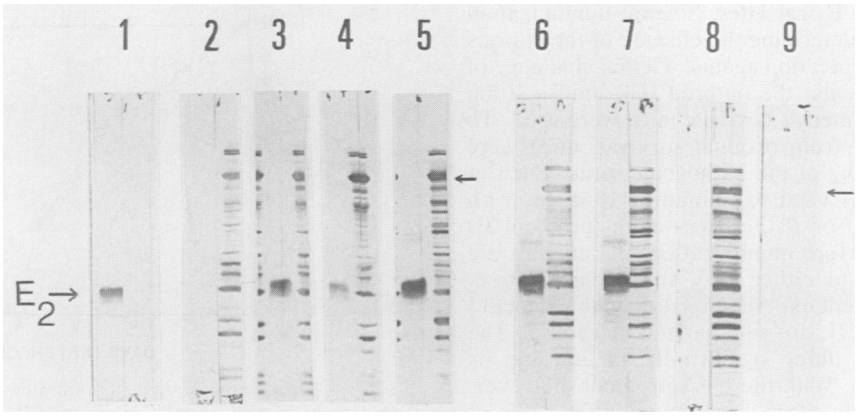

FIG. 3. Immunostaining of the SFV E_2 polypeptide and of bacterial β-galactosidase by the anti-cassette–β-galactosidase antibodies. Each antiserum (diluted 1:40) was reacted with a pair of nitrocellulose strips carrying SFV proteins (left strip) and partially purified *E. coli* β-galactosidase (right strip) as follows: 1, with anti-SFV antiserum; 2, with anti-β-galactosidase antibodies; 3 to 8, with antisera to cassettes BC, DE, FG, HIK, LMN, and A, respectively; 9, with serum of a normal mouse. Arrows indicate positions of the SFV E_2 polypeptide (left) and β-galactosidase (right).

recognized by all 14 sera tested, although with varied intensities, and cassettes DE and FG were recognized by 10 of the 14 sera. The results indicate that the β-galactosidase hybrids have antigenic properties of SFV. The nonreactivity of cassette A with any of the antisera tested implies either that cassette A is a nonantigenic epitope on the SFV protein or that fusion of the A sequence to β-galactosidase alters the structure of the A epitope.

Immunogenicity of SFV E_2 hybrid polypeptides. The ability of the bacterial SFV–β-galactosidase hybrid proteins to induce in mice a virus-specific humoral immune response was evaluated by Western blotting (Fig. 3) and ELISA (Table 2). Sera from mice injected with the various cassette–β-galactosidase hybrids were used to immunostain SFV structural proteins and β-galactosidase-containing bacterial extracts. All of the recombinant polypeptides triggered an efficient antibody response, as indicated by production of antibodies against the carrier moiety (β-galactosidase) (Fig. 3). Each cassette except A could elicit antibodies specific to the E_2 protein. Thus, all of the β-galactosidase hybrid cassettes that interacted with anti-SFV antiserum (Fig. 2, lanes 2 to 6) were also effective immunogens (Fig. 3, lanes 3 to 7).

Since the immunoblots were performed on totally denatured protein, we were interested in determining whether the anti-SFV–β-galactosidase antibody could also interact with native E_2 on an intact virion. This was tested by ELISA, using complete virions as the first layer. ELISAs were performed on sera from all of our immunization experiments. Geometric mean ELISA titers from individual animals in a single experiment are provided in Table 2 (standard deviations of the $\log_{10}$ transformed titers ranged between 0.5 and 0.6). All of the cassette blocks produced antibodies that could interact with the complete virion. Again, cassette A induced only background titers. All animals were also tested for anti-β-galactosidase antibody titers. Titers were above 1:400,000 in all animal groups, including the A–β-galactosidase groups. This result and the immunoblot in Fig. 3 (lane 8) suggest that the inability to detect antibody to cassette A was not due to faulty immunization protocol. There was a good correlation between the antibody titers (Table 2) and the intensity of the response in the immunoblots (Fig. 3). It appeared that LMN was the most immunogenic SFV E_2 block. HIK, FG, and BC had somewhat lower immunogenic potential, and DE was the least immunogenic block. These results demonstrated that the SFV E_2–β-galactosidase fusions can represent in an authentic manner the native configuration of sequential epitopes of the E_2 protein on SFV virions.

Protection of mice against lethal challenge with SFV by

TABLE 2. Immunization of mice with recombinant cassette–β-galactosidase hybrids: anti-SFV antibody titers (ELISA) and survival after lethal SFV challenge[a]

Antigen	Protocol I				Protocol II					
	Expt 1		Expt 2		Expt 3		Expt 4		Expt 5	
	Survial ratio	Ab titer	Survival ratio	Ab titer	Survival ratio	Ab titer	Survival ratio	Ab titer	Survival ratio	Ab titer
β-Galactosidase	0/10	100	0/10	100	1/10	100	0/10	100	0/10	100
A			0/10	100			0/10	100	0/10	100
BC	4/9	2,700	4/10	1,200	6/10	2,200	3/10	820	3/10	680
DE	1/10	175			3/10	510			1/10	150
FG	0/10	680	1/9	1,200	2/10	3,280	0/10	1,500	1/9	200
HIK	6/9	5,400	6/10	3,200	6/10	6,900	8/10	9,600	5/10	3,400
LMN	10/10	18,800	9/9	7,100	10/10	13,000	9/10	5,100	10/10	3,200

[a] Protocol I, CFA in the first two injections and no adjuvant in the next two injections; protocol II, *B. pertussis* adjuvant in the first injection and alum in the next three injections (see Materials and Methods). Antibody (Ab) titers (reciprocal geometric mean titer values) of sera collected 24 h before challenge were determined by ELISA, using virions as antigens. BALB/c mice were used in experiment 2; ICR mice were used in all other experiments.

immunization with SFV E_2 cassettes. Several immunization protocols were used to determine the efficacy of the various blocks of cassettes in protection against a lethal challenge of virus. In most experiments, the outbred ICR mouse strain was used; in two experiments, BALB/c mice were used. To avoid possible "noise" from residual survival, mice were challenged with 250 LD_{50} of the pathogenic virus. Each of the hybrid proteins was used to immunize 10 mice, with either CFA (protocol I) or *B. pertussis*-alum (protocol II) used as an adjuvant. Before immunization, the animals did not exhibit antibodies to either SFV or β-galactosidase. Results of five independent experiments, two with protocol I and three with protocol II, are summarized in Table 2. The two protocols did not differ significantly in efficacy, as judged by survival ratio. With the CFA protocol, however, higher anti-SFV antibody titers were obtained for some of the hybrid proteins. Immunization with β-galactosidase or with the nonantigenic and nonimmunogenic cassette A failed to protect mice against lethal challenge. Cassette FG, which was antigenic and in most experiments was also an efficient immunogen (antibody titers of 1:1,000 to 1:3,000), had a poor protective effect. Cassette DE was neither a potent immunogen nor an efficacious vaccine. Cassettes BC and HIK conferred protection of about 40 and 60%, respectively, consistent with the higher anti-SFV antibody titers induced by the HIK than by the BC cassette. The most striking protective effect was that induced by cassette LMN, which in four of five experiments gave 100% protection. Cassette LMN was also an effective antibody inducer. In some animals, the titers reached values of 1:25,000 to 1:100,000, comparable to those obtained by immunization with the complete SFV virion.

The general pattern of protection and the variability in antibody titers among animals were independent of whether outbred ICR or inbred BALB/c mice were used (Table 2). This finding suggested that the partial protective effects of cassettes BC and HIK did not reflect the variability in genetic background of the wild-type strain. An attempt to improve the protective effects of the recombinant cassettes by using mixtures of various blocks was unsuccessful. It appeared that protection induced by a mixture of BC, DE, FG, and LMN antigens was poorer than that obtained with the LMN antigen alone (data not shown).

Another attempt to improve the efficacy of the partially protective BC cassette by an increase in antigenic dose from 25 μg of BC–β-galactosidase to 100 μg led to a significant increase (fourfold) in antibody titer and to a 20% increase in survival ratio. The increase in the survival of mice immunized with the higher dose of BC antigen may not be statistically significant. An increase of four- to sevenfold in antibody titer was also obtained when the amount of the LMN–β-galactosidase cassette was increased from 25 to 100 μg.

In conclusion, of the six cassette blocks representing 50% of the total amino acid sequence of the E_2 protein, three (BC, HIK, and LMN) conferred resistance against lethal doses of virus. Of these, LMN, which represented only 15% of the amino acid sequence of the E_2 protein, was the dominant linear immunogenic region, and it was also able to induce complete protection against a challenge of 250 LD_{50}.

Anti-cassette antibodies do not neutralize SFV in vitro. The correlation observed between the anti-SFV antibody titer induced by the various cassettes and the survival ratio after challenge (with the exception of FG) indicated that antibodies may play a major role in the acquired immunity of vaccinated mice. Since the E_2 envelope protein is considered

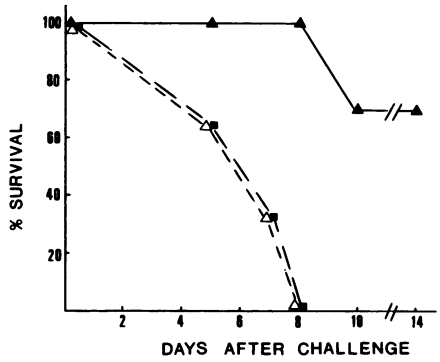

FIG. 4. Protection of mice by immune-transferred anti-LMN-β-galactosidase antiserum. Groups of ICR mice were injected with either anti-LMN antibody (0.7 ml, i.p.) (▲), anti-β-galactosidase antibody (■), or antibody from mice injected with adjuvant only (△). Antisera prepared from mice immunized by protocol I were pooled, and the antibody fraction was obtained by ammonium sulfate precipitation. Pools prepared from about 20 sacrificed animals in each group were injected i.p. into seven recipients. The anti-SFV titer of the anti-LMN–β-galactosidase antibody pool was 1:6,400, and that of its anti-β-galactosidase antibody was 1:400,000. At 24 h posttransfer, mice received 100 LD_{50} of SFV. Survival was monitored for 14 days.

to be a major target for neutralizing antibodies in the alphavirus family, we were interested in determining whether the antibodies induced by the linear cassettes had neutralizing activity in vitro. This was tested by measuring the ability of the sera collected before challenge to inhibit the cytopathic effect of virus in tissue culture ($TCID_{50}$) and in a plaque reduction neutralization test. None of the sera induced by the various SFV E_2 cassette blocks exhibited neutralizing activity in vitro in either of the two tests. The $TCID_{50}$ assay was repeated several times, using individual sera reaching titers as high as 1:100,000 (LMN), with no positive results. In contrast, polyvalent antiserum produced by mice immunized with inactivated SFV virions (ELISA titers of 1:50,000 to 1:100,000) always demonstrated high antivirus neutralization titers (1:25,000). In the plaque reduction neutralization test, antisera directed against any of the cassettes showed no reduction in plaque number at a dilution of 1:100, and their reactivities were identical (±5%) to that of normal mouse serum or anti β-galactosidase antiserum. On the other hand, polyclonal antibodies raised against complete inactivated SFV led to 95 and 65% reductions in plaque numbers at dilutions of 1:100 and 1:2,500, respectively. We therefore assume that the observed immunity acquired by mice injected with recombinant vaccine LMN, HIK, or BC resulted from either a cellular immune response or humoral immunity other than that correlated with in vitro neutralization.

Passive transfer of anti-SFV immunity with antibodies from immunized mice. To determine whether the anticassette antibodies produced by immunized mice played a major role in protection against lethal doses of SFV, 8-week-old ICR mice were injected with an antibody preparation collected from LMN–β-galactosidase-immunized mice. Each animal received 0.7 ml of a pool of ammonium sulfate-fractionated sera with an anti-SFV titer of 1:6,400 and was challenged 24 h later with 100 LD_{50} of SFV. The recipient mice acquired passive immunity (Fig. 4). Five of seven mice resisted the lethal challenge; for two mice, death was delayed by several days in comparison with the control groups. The similarly

treated control groups, injected either with a fractionated anti-β-galactosidase antibody preparation or with antibodies obtained from mice injected with adjuvant and phosphate-buffered saline alone, did not acquire such an immunity. These results strongly indicated that anti-LMN SFV antibodies induced by our recombinant vaccine were effective in protection, although they could not neutralize SFV in vitro. Moreover, it appears that the main avenue of protection induced by the LMN recombinant vaccine is humoral in nature, since the amount of passively transferred antibodies needed to rescue animals from lethal challenge was comparable to that present in challenge-resistant immunized mice.

DISCUSSION

On the basis of overlapping hydrophilic patterns and conserved amino acid sequences on the E_2 envelope proteins of members of the alphavirus family, we selected 13 peptide cassettes (designated A through N) as possible candidates for synthetic vaccines against SFV. Each of the cassettes is composed of an SFV-specific hydrophilic sequence, flanked by sequences conserved in the envelope of the alphavirus family. An average cassette contains a peptide sequence of 20 amino acids, of which 6 to 10 constitute a hydrophilic SFV-specific core. To simplify the analysis of these numerous cassettes, we cloned five regions containing contiguous blocks of two to three cassettes in vectors that allow their expression as N-terminus extensions of the β-galactosidase subunits. Five blocks of cassettes were isolated from a cDNA coding for SFV E_2, and an additional region, coding for a single cassette (A), was prepared by chemical DNA synthesis.

We have demonstrated that the block of cassette BC, DE, FG, HIK, or LMN, when fused to β-galactosidase, conferred on the hybrid protein antigenic properties of SFV, as judged by the reactivity with anti-SFV antiserum (Fig. 2). The A–β-galactosidase hybrid did not manifest this antigenic property. DNA sequence analysis of a plasmid expressing the A–β-galactosidase fusion protein proved that the inability of this hybrid molecule to interact with the anti-SFV antiserum was not due to an erroneous coding sequence for the A region. We have indirect evidence that the lack of antigenicity of the A–β-galactosidase cassette results from structural constraints imposed on the SFV A sequence via its covalent linkage to β-galactosidase, since an identical, chemically synthesized A peptide of 16 amino acids did interact efficiently with most of the anti-SFV sera tested (data not shown).

All of the SFV E_2 chimeric proteins induced in mice a very high antibody titer (greater than 1:400,000) to the β-galactosidase carrier moiety. Specific anti-SFV E_2 antibodies were produced only by the five hybrids (Fig. 3) that could interact with anti-SFV antiserum (Fig. 2). Moreover, antibodies to these five hybrids (BC–, DE–, FG–, HIK–, and LMN–β-galactosidase proteins) could interact with complete virions in ELISA. We interpret these results as suggesting that at least in some of the SFV E_2–β-galactosidase polypeptides, a conformation mimicking that present on intact virions is maintained. Among these recombinant antigens, the most immunogenic region is included within the LMN block, located between amino acids 289 and 352 of E_2. The LMN–β-galactosidase antigen can induce specific SFV E_2 antibody levels similar to those induced by the complete, inactivated virions. The immunogenic potency of the various SFV E_2 peptides is not a simple reflection of size. HIK, which is the largest (72 amino acids), is less effective than the shorter LMN block; similarly, BC, which is 35 amino acids long, is comparable in potency to the 26-amino-acid-long FG block. Furthermore, similar titers are induced in animals injected with HIK and with BC. The nature of the protective effects of the partially protective antigens composed of the BC and HIK blocks awaits further elucidation. It appears that an increase in the antibody response against BC, brought about by higher doses of antigen, leads to higher protection.

Because of our method of cloning, the recombinant E_2 fragments represent a stretch of sequence on E_2 between amino acid positions 114 and 353, which is more than 50% of the total length of the E_2 polypeptide. It could therefore be expected that within such a significant portion of the neutralization-related envelope protein, we would be able to find epitopes eliciting antibody with neutralizing activity. As demonstrated by $TCID_{50}$ and plaque reduction neutralization tests, all antibodies induced with the linear cassettes were nonneutralizing in vitro. Boere et al. (4), using nine different monoclonal antibodies, described five distinct antigenic determinants located on the E_2 glycoprotein, two of which were involved in neutralization. Our inability to identify sequences involved in neutralization may be a consequence of the truncation of the E_2 sequence, which may lead to disruption of the neutralizing epitopes; alternatively, these epitopes may not be represented by linear contiguous amino acid sequences.

Although none of the cassette blocks induced antibodies with in vitro neutralizing activity, at least three of the five immunogenic blocks could induce a protective response in mice. Most striking was the effect of the LMN block, which could fully protect animals against a lethal challenge of 250 LD_{50}, independent of the adjuvant used, regimen, of immunization, and strain of mice. We are not aware of a similar case in which a short segment of viral envelope induces such effective protection against lethal doses of virulent virus. The high efficacy of this potential synthetic vaccine is suggested by the fact that as little as 1 μg of SFV E_2-specific sequence, with alum as the adjuvant, is needed to confer 100% protection. We have further demonstrated by a passive transfer experiment that the humoral response induced by the LMN block is responsible for the protection in vivo. The fact that in this experiment 100% protection was not observed (survival of five of seven mice) may imply that some cellular protection mechanism is also triggered by the LMN block. However, this quantitative difference in the extent of protection in the two types of experiments (passive versus active immunization) may also be explained by technical differences in the two protocols (e.g., differences in distribution of antibody or age of challenged animals). There are several reports demonstrating that virus neutralization or lack of neutralization activity in vitro may not be a criterion for protection in vivo. A group of nonneutralizing monoclonal antibodies directed against the E_1 protein of another alphavirus, Sindbis, was found to protect mice against virus challenge through complement-dependent antibody-mediated cytolysis of Sindbis virus-infected cells (18). Similarly, nonneutralizing monoclonal antibodies against SFV E_2 protein of the IgG2a and IgG2b subclasses were found to protect mice in vivo, again through complement-mediated lysis (3, 4). Others (5) identified nonneutralizing IgG monoclonal antibodies to the envelope protein of the 17D vaccine strain of yellow fever virus that conferred passive protection when administered at high doses. An additional example of an in vitro nonneutralizing monoclonal antibody with protective effect in vivo was reported for West Nile virus (Y. Ol-

shevsky, S. Lustig, B.-E. Lachmi, Y. Akov, and N. Epstein, VII Int. Congr. Virol, p. 107, 1987). It should be noted, however, that in all of these three cases, antibodies with neutralizing activity were 2 orders of magnitude more potent in protection than were the protective nonneutralizing antibodies.

In conclusion, we have delineated three regions on the SFV E_2 protein that contain blocks of two to three linear cassettes that can trigger a protective immune response in mice against a lethal dose of virulent virus. At least for one of the linear cassettes (LMN), protection could be linked to a humoral response with no in vitro neutralization activity. The type of humoral activity induced by the LMN block remains to be determined. It is possible that anti-LMN antibodies trigger complement-mediated lysis of infected cells or are involved in an antibody-dependent cell cytotoxicity-type mechanism. In addition, we are now dissecting the two most potent blocks, LMN and HIK, into their individual cassettes to test our assumption that individual cassettes, characterized by a virus-specific hydrophilic sequence flanked by conserved amino acids, are sufficient for eliciting protective antibodies in the alphavirus family.

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