JOURNAL OF VIROLOGY, Nov. 1984, p. 575–582
0022-538X/84/110575-08$02.00/0

Vol. 52, No. 2

# Identification of Distinct Antigenic Determinants on Semliki Forest Virus by Using Monoclonal Antibodies with Different Antiviral Activities

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Received 16 April 1984/Accepted 30 July 1984

**Fifteen monoclonal antibodies (MAs) directed against either the $E_1$ or $E_2$ glycoprotein of Semliki Forest virus (SFV) were characterized by immunoglobulin subclass, pI traject, hemagglutination inhibition, neutralization of infectious virus, and protection against virulent infection in mice. All MAs except UM8.4 (immunoglobulin M [IgM]) belonged to various subclasses of IgG and predominantly to IgG2a, but all were unique as indicated by their banding patterns in isoelectric focusing. Competitive binding assays with these MAs revealed the presence of at least six distinct antigenic determinants (epitopes) on the $E_1$ glycoprotein and five epitopes on the $E_2$ glycoprotein. Two of the epitopes on $E_1$, as defined by the properties of the MAs, were associated with hemagglutination inhibition ($E_1^c$ and $E_1^d$), three were associated with neutralization ($E_1^a$, $E_1^b$, and $E_1^f$), and five were associated in various degrees with protection ($E_1^a$, $E_1^b$, $E_1^c$, $E_1^e$, and $E_1^f$) of mice against virulent SFV infection. With the MAs against $E_2$, the epitopes on $E_2$ were similarly defined. Epitopes $E_2^b$ and $E_2^e$ were associated with hemagglutination inhibition, $E_2^c$ and $E_2^d$ were associated with neutralization, and three epitopes were associated with in vivo protection ($E_2^a$, $E_2^c$, and $E_2^d$). Furthermore, for each MA the relative avidity to purified SFV was determined with an enzyme-linked immunosorbent assay. The binding of some MAs to purified SFV was enhanced by a second MA. The relative avidities of individual MAs did not correlate with their neutralizing capacities. From the results, we suggest that the amino acid sequence which makes up determinant $E_2^d$ and is recognized by the highly protective MA UM5.1 is an excellent candidate for the production of a synthetic vaccine.**

Injection of mice with Semliki Forest virus (SFV) results in polyclonal formation of antibodies against the various antigenic components of the virus. Sera containing these antibodies cause hemagglutination inhibition (HAI), neutralize infectious virus, and protect recipient mice against lethal challenge (13, 20). In a previous paper (1), we described two monoclonal antibodies (MAs) which have specificity for the $E_2$ glycoprotein and which also protect mice against virulent SFV. One of these MAs (UM5.1) neutralizes SFV in a plaque reduction test; the other (UM4.2) does not. Similar results have been obtained by other workers with MAs against two closely related Togaviridae, Sindbis and Venezuelan equine encephalomyelitis viruses (18, 22).

In the present study, we extended our earlier passive protection experiments with 13 selected MAs directed against either the $E_1$ or $E_2$ glycoprotein of SFV. Furthermore, SFV reciprocal competitive binding assays (CBAs), using peroxidase-labeled MAs, allowed us to define on both the $E_1$ and $E_2$ glycoproteins distinct antigenic determinants with different activities regarding HAI, neutralization, and protection.

## MATERIALS AND METHODS

**Virus strains.** The avirulent SFV strain MRS MP 192/7 (10), obtained from K. G. Oei, Royal Tropical Institute of Amsterdam, The Netherlands, was used as an antigen. Infectious virus was used for primary immunization, and purified UV-inactivated SFV was used as a booster. The latter antigen was also used in enzyme immunoassays. The virulent SFV strain SF/LS 10Cl/A (2) was received from C. J. Bradish, The Porton Down Microbiological Research Establishment, Salisbury, United Kingdom. The 50% lethal dose ($LD_{50}$) for male BALB/c mice was 1 to 2 PFU intraperitoneally. The preparation of purified, inactivated SFV (MRS MP 192/7) and virulent SFV and the general virological methods (plaque titration and hemagglutination) have been described previously (14).

**Animals and immunization.** BALB/c mice were bred and maintained in our own animal house. For hybridoma production, female BALB/c mice of about 12 weeks of age were injected intraperitoneally with 0.5 ml of diluted avirulent SFV ($3 \times 10^3$ PFU) and boosted 4 weeks later intravenously with 0.5 ml of inactivated SFV ($10^4$ hemagglutinating units) 4 days before fusion. Hyperimmune mouse serum was collected from the retroorbital plexus 7 days after booster injection, pooled, and kept in small portions at −20°C.

**MA production and characterization.** Spleen cells from immunized mice were fused with the myeloma line P3-X63-AG8.653 (P3) (12) as described by Fazekas de St. Groth and Scheidegger (8). After 2 weeks, the cultures were screened for antibody production by a virus plaque reduction test and enzyme-linked immunosorbent assay (ELISA) as described earlier (1). SFV-positive clones were used for ascitic fluid production by injecting $10^6$ to $5 \times 10^6$ subcloned cells into pristane-primed BALB/c mice. After 1 to 2 weeks, ascitic fluid was collected, tested for the presence of anti-SFV antibody, and used for purification of anti-SFV immunoglobulins by the protein A-Sepharose method (7). The purity and integrity of the MAs were tested by isoelectric focusing

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(IEF) and polyacrylamide gel electrophoresis (PAGE) in a 4 to 30% gradient gel without sodium dodecyl sulfate and under nonreducing conditions with Tris-borate buffer (0.09 M Tris, 0.08 M boric acid, 2.5 mM EDTA [pH 8.4]) for 18 h at 50 mA. After PAGE, proteins were stained with Coomassie brilliant blue R250. The protein content of MA preparations was determined by the method described by Lowry et al. (17). Glycoprotein specificity was assayed by immunoblotting of SFV glycoproteins (1). The isoelectric point (pI) was determined by IEF in 1% agarose gels (Pharmacia Fine Chemicals).

**Immunoglobulin subclass determination.** MA subclasses were ascertained by ELISA. Briefly, microtiter plates were coated with 50 μl of rabbit anti-mouse immunoglobulin G class 1 (IgG1), IgG2a, IgG2b, IgG3, IgM, κ, or λ antiserum (Miles Research Products), diluted 1:500 in phosphate-buffered saline (pH 7.2) overnight at 4°C, and washed with phosphate-buffered saline containing 0.05% Tween 20. The plates were incubated with horseradish peroxidase (HRPO)-conjugated MA for 1 h at 37°C. The HRPO conjugates were prepared by the periodate method (19). After washing, the amount of bound HRPO was visualized by incubating the wells with 100 μl of a solution of 3'3'5'5'-tetramethylbenzidine (60 mg dissolved in 10 ml of dimethyl sulfoxide) and 100 μl of urea peroxide (1 tablet of UP dissolved in 7.5 ml of distilled water) in 0.11 M sodium acetate adjusted to pH 5.5 with saturated citric acid. After 10 min, the enzyme reaction was terminated by adding 100 μl of 2 M $H_2SO_4$ per well, and peroxidase activity was quantified by measuring the optical density at 450 nm with a Titertek Multiskan instrument (Flow Laboratories).

**CBA.** Competition among MAs for viral antigenic determinants was assayed by a modified ELISA. To test relative avidity, MAs were titrated against purified SFV in an indirect ELISA by coating microtiter plates with a previously determined virus concentration and using goat anti-mouse IgG and IgM with HRPO-labeled conjugates. Relative avidity was defined as the amount of MA protein needed to reach an ELISA absorbance value of 0.5 (see above). The CBA was performed at antibody concentrations where linear binding occurred (3).

The CBA was performed by coating with SFV diluted to 0.2 to 0.5 μg per well in carbonate buffer (pH 9.6) overnight at 4°C and incubating with 50 μl of purified competitor MA (1 to $10^5$ ng of protein) per well diluted in 0.5% Tween 20 and 5% heat-inactivated calf serum. After incubation for 1.5 h at 37°C, the HRPO-labeled MA (19) was added, and the incubation was continued for a further 1.5 h at 37°C. The plates were washed extensively, and finally the substrate was added as described above for the subclass determination. The percentage of competition between two MAs was calculated by the method of Lefrancois and Lyles (15). If inhibition was at least 50% at a competitor concentration of $\leq 10^4$ ng per well and at a virus coat concentration of 0.1 μg per well, two MAs were presumed to bind to the same or to two topographically related antigenic determinants (epitopes).

**HAI.** HAI experiments were performed with 4 hemagglutinating units of SFV ($\pm 10^6$ PFU) in microtiter plates as described by Clarke and Casals (5), using goose erythrocytes at pH 5.8 and twofold dilutions of purified MA. The virus antigen used in HAI was avirulent SFV purified by means of cesium chloride ultracentrifugation and column chromatography.

**Plaque reduction test.** Virus neutralization titers in vitro were determined by a plaque reduction test in 24-well plastic palettes. Briefly, 25 μl of 10-fold dilutions of MAs in culture

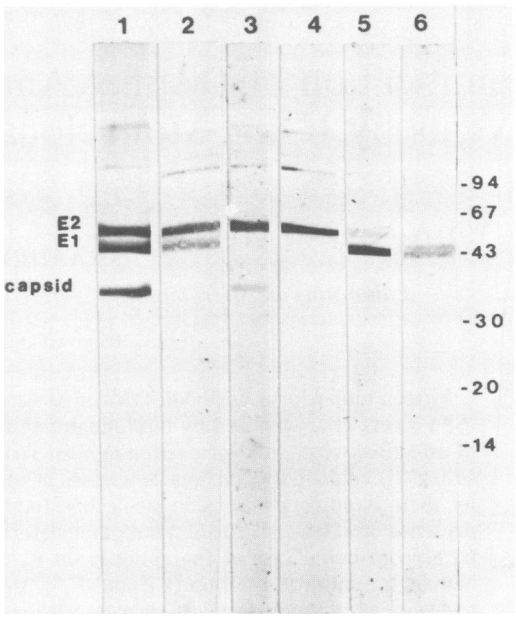


FIG. 1. Immunoblotting of SFV probed with MAs. Viral glycoproteins were solubilized in sample buffer with 1% sodium dodecyl sulfate without 2-mercaptoethanol, boiled, and separated by sodium dodecyl sulfate-PAGE. The nitrocellulose blots were stained with Coomassie brilliant blue (lane 1) or incubated with mouse hyperimmune serum (lane 2), UM4.2 (lane 3), UM5.1 (lane 4), UM.115 (lane 5), or UM8.64 (lane 6) followed by goat anti-mouse HRPO conjugate and HRPO staining. Molecular weights are in thousands.

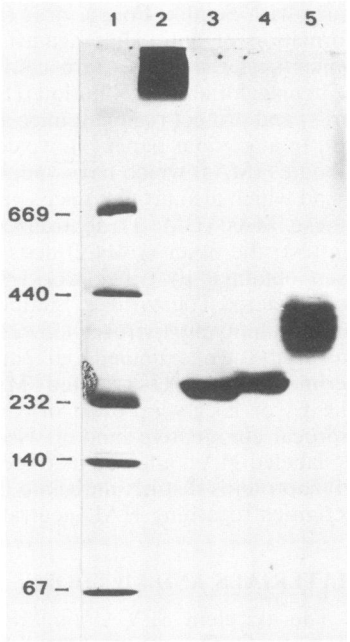


FIG. 2. PAGE MAs in a polyacrylamide gel without sodium dodecyl sulfate. MAs were solubilized under nonreducing conditions and in the absence of sodium dodecyl sulfate in Tris-borate electrophoresis buffer at pH 8.4. Proteins were stained with Coomassie brilliant blue R250. The proteins separated were: lane 1, high-molecular-weight references; lane 2, UM8.4; lane 3, UM8.20; lane 4, UM8.22; lane 5, UM5.1. Molecular weights are in thousands.

TABLE 1. Characteristics of MAs directed against either the $E_1$ or $E_2$ glycoprotein of SFV

| Glycoprotein specificity | MA | Immunoglobulin | pI traject (range) | Relative avidity (μg/ml) | Antigenic determinant | HAI[a] | Neutralization[b] | | In vivo protection[c] |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | V | A | |
| $E_1$ | UM8.4 | IgM | 6.3–6.7 | 10 | $E_1^a$ | − | +++ | +++ | + |
| | UM8.20 | IgG2a | 6.0–6.3 | 0.4 | $E_1^b$ | − | + | + | + |
| | UM8.47 | IgG2a | 6.5–7.3 | 10 | $E_1^c$ | ++ | − | − | + |
| | UM8.64 | IgG1 | 5.9–6.4 | 0.05 | $E_1^d$ | + | − | − | − |
| | UM8.115 | IgG2a | 7.0–7.5 | 5 | $E_1^e$ | − | − | − | + |
| | UM8.139 | IgG2a | 7.0–7.6 | 5 | $E_1^f$ | − | + | + | + |
| $E_2$ | UM4.2 | IgG2a | 7.5–8.0 | 0.2 | $E_2^a$ | − | − | − | + |
| | UM8.22 | IgG2a | 5.9–6.2 | 0.04 | $E_2^a$ | − | − | − | + |
| | UM8.77 | IgG2b | 5.2–5.6 | 10 | $E_2^a$ | − | − | − | + |
| | UM8.130 | IgG2a | 7.1–7.4 | 0.2 | $E_2^a$ | − | − | − | + |
| | UM8.73 | IgG2a | 7.2–8.3 | 0.05 | $E_2^b$ | + | − | − | − |
| | UM8.48 | IgG2a | 7.8–8.2 | 0.02 | $E_2^c$ | − | ++ | + | + |
| | UM8.55 | IgG2a | 7.0–7.7 | 0.02 | $E_2^c$ | − | ++ | + | + |
| | UM5.1 | IgG2a | 6.7–7.2 | 1 | $E_2^d$ | − | +++ | ++ | + |
| | UM8.107 | IgG1 | 5.3–5.8 | 0.4 | $E_2^e$ | + | − | − | − |

[a] HAI tests were performed with the avirulent strain of SFV. −, No HAI; +, HAI $\log_2$ titer of 6 to 8; ++, HAI $\log_2$ titer of 9 to 10.
[b] Plaque reduction tests were performed with both the virulent (V) and avirulent (A) strains of SFV. −, No neutralization; +, 50% plaque reduction with a $\log_{10}$ titer of 0.5 to 1.5; ++, $\log_{10}$ titer of 1.5 to 2.5; +++, $\log_{10}$ titer of 2.5 to 6.
[c] +, Full protection after intravenous injection of 0.1 ml of ascitic fluid against intraperitoneal challenge with 10 $LD_{50}$ units of virulent SFV; −, no full protection.

medium were made in 96-well palettes, mixed with an equal volume of either the virulent or avirulent SFV strain, and incubated for 1 h at 37°C. Subsequently, 200 μl of medium was added, and virus-MA mixtures were tested in quadruplicate (50 μl each) on monolayers of L cells (about $5 \times 10^5$ cells per well). After 40 min of adsorption at room temperature, 0.3 ml of overlay medium was added. After 24 h (avirulent strain of SFV) or 36 to 48 h (virulent SFV strain) of incubation at 37°C, plaques were developed with 50 μl of 0.02% neutral red. Controls contained about 100 plaques per 16-mm well. The reciprocal of the highest dilution of an MA or serum causing 50% plaque reduction was called the plaque reduction titer.

**Passive protection by MAs.** Protection provided by MAs in BALB/c mice against lethal encephalitis caused by 10 $LD_{50}$ units of SFV (16 PFU) was determined after intravenous injection of serial dilutions in phosphate-buffered saline of either 0.1 ml of ascitic fluid or 0.1 to 100 μg of protein A-purified MA. After 2 h, groups of four to six mice received 16 PFU of virulent SFV intraperitoneally. To quantitate protection, mice were observed for 21 days. Control mice that received phosphate-buffered saline instead of MA had after challenge with virus a mean survival time of 5 to 6 days. Only 1 out of 42 control mice survived the challenge.

## RESULTS

**Production and characterization of SFV-specific MAs.** Spleen cells of SFV-immunized mice were fused with P3 myeloma cells and grown as hybridomas in cell cultures. Screening of the hybridoma supernatants with ELISA and plaque neutralization yielded 130 clones positive either in the ELISA or in both tests. Fifteen clones were selected for further study mainly because of their rapid growth in cell cultures and their neutralizing and nonneutralizing properties. The $E_1$ or $E_2$ glycoprotein specificity was determined by immunoblotting (Fig. 1). Of the four clones shown, two

TABLE 2. Antigenic determinants on $E_1$ and $E_2$ glycoproteins of SFV detected by CBA

| Unlabeled MA | HRPO-labeled MA blocking[a] by unlabeled MA | | | | | | | | | Antigenic determinant |
|---|---|---|---|---|---|---|---|---|---|---|
| Anti-$E_1$ | 8.4 | 8.20 | 8.47 | 8.64 | 8.115 | 8.139 | | | | |
| UM8.4 | + | − | − | − | − | − | | | | $E_1^a$ |
| UM8.20 | − | + | − | − | − | − | | | | $E_1^b$ |
| UM8.47 | − | − | + | − | − ↑ | − ↑ | | | | $E_1^c$ |
| UM8.64 | − | − | − | + | − | − | | | | $E_1^d$ |
| UM8.115 | − | − | − ↑ | − | + | − | | | | $E_1^e$ |
| UM8.139 | − | − | − ↑ | − | − | + | | | | $E_1^f$ |
| Anti-$E_2$ | 4.2 | 8.22 | 8.77 | 8.130 | 8.73 | 8.48 | 8.55 | 5.1 | 8.107 | |
| UM4.2 | + | + | + | + | − | − | − | − | − | $E_2^a$ |
| UM8.22 | + | + | + | + | − | − | − | − | − | $E_2^a$ |
| UM8.77 | + | + | + | + | + | − | − | − | − | $E_2^a$ |
| UM8.130 | + | + | + | + | + | − | − | − | − | $E_2^a$ |
| UM8.73 | − | − | + | + | + | − | − | − | − | $E_2^b$ |
| UM8.48 | − | − | − | − | − | + | + | − | − | $E_2^c$ |
| UM8.55 | − | − | − | − | − | + | + | − | − | $E_2^c$ |
| UM5.1 | − | − | − | − | − | − | − | + | − | $E_2^d$ |
| UM8.107 | − | − | − | − | − | − | − | − | + | $E_2^e$ |

[a] +, Competition of ≥50% at an unlabeled MA concentration of $\leq 10^4$ ng per well; −, no competition; ↑, enhancement of second MA binding.

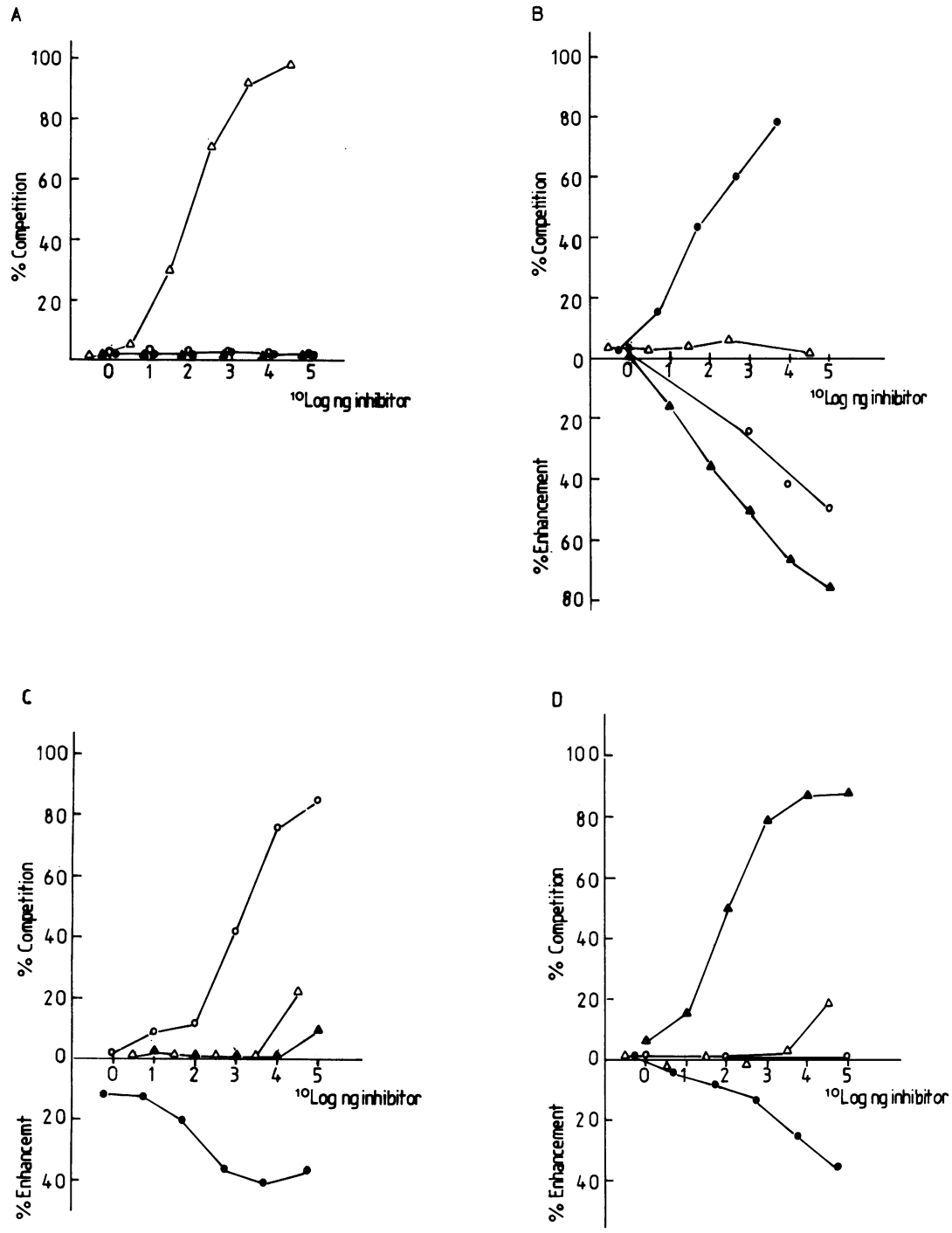


FIG. 3. CBAs with anti-$E_1$ MAs. Assays were performed as described in the text, using HRPO-labeled UM8.64 (A), UM8.47 (B), UM8.115 (C), and UM8.139 (D). The percentages of competition and enhancement were estimated from the optical density at 450 nm in the presence of competitor compared with that in the absence of competitor as described in the text. Symbols: △, UM8.64; ●, UM8.47; ○, UM8.115; ▲, UM8.139.

were specific for $E_2$, whereas the other two were specific for $E_1$. The results of the immunoglobulin subclass determination of the 15 MAs are shown in Table 1. MAs of subclass IgG2a predominated, although one IgM, two IgG1, and one IgG2b subclass that produced hybridomas were generated. All MAs shared the kappa light chain. The subclass of each MA was confirmed by its specific elution pattern during the purification procedure with protein A-Sepharose affinity chromatography (7). Analysis of the purified MAs by PAGE (Fig. 2) and IEF (1; data not shown) revealed that all MAs were free of contaminating proteins. The high molecular weight (>900 × $10^3$) of MA UM8.4 confirmed that this MA

was of the IgM isotype. The other MAs were predominantly of subclass IgG2a (Table 2). By using the IEF technique, it could be proved that each MA was unique as indicated by distinct banding patterns at specific pH trajects (Table 1).

**Antigenic determinants on the $E_1$ and $E_2$ glycoproteins of SFV.** A CBA was used to identify separate antigenic determinants on SFV. Fifteen protein A-purified MAs were labeled with HRPO and cross-matched with unlabeled MAs. First, the relative avidities of these MAs for purified SFV were measured (Table 1). Two of the anti-$E_1$ MAs showed high relative avidities (UM8.20 and UM8.64) (low value), whereas the others were in the low-relative-avidity range. The binding patterns found in relative avidity measurements were comparable when indirect ELISA was performed with goat anti-mouse IgG or IgM conjugates or direct ELISA with HRPO-labeled MAs (see Fig. 5). All MAs with specificity for $E_2$ displayed high relative avidities for $E_2$, except for clones UM8.77 and UM5.1.

Taking into account the different relative avidities of these MAs, we used them in CBAs in a region where linear binding occurred. The CBA data of four anti-$E_1$ HRPO-labeled MAs (UM8.47, UM8.64, UM8.115, and UM8.139) and their unlabeled counterparts for antigenic determinants on $E_1$ are shown in Fig. 3A to D. In tests with homologous MAs (e.g., UM8.64 versus UM8.64), complete inhibition was obtained; however, in assays with heterologous MAs, there was no competitive inhibition. Instead, enhanced binding of the enzyme-labeled MA was observed (Fig. 3B and Table 2). UM8.115 and UM8.139 enhanced the binding of clone UM8.47. In the reciprocal assays, enhanced binding was also observed (Fig. 3C and D). The CBA data indicated that each anti-$E_1$-specific MA recognized a separate ($E_1^a$ to $E_1^f$) antigenic determinant (six in total) (Table 2).

CBAs with anti-$E_2$-specific MAs revealed that a number of MAs recognized the same antigenic determinant. Two examples are shown in Fig. 4A (MA UM8.130 inhibited by itself, UM4.2, and UM8.22 but not by UM8.55) and B (UM8.55 inhibited by itself and UM8.48 but not by UM8.130 and UM5.1). In reversed assays, similar results were obtained. At least five antigenic determinants ($E_2^a$ to $E_2^e$) could be distinguished with nine MAs. Four MAs were specific for site $E_2^a$, and two were specific for $E_2^c$. MA UM8.73 showed competition with UM8.77 and UM8.130 but not with the other MAs specific for the $E_2^a$ epitope. Therefore, UM8.73 was directed to a separate antigenic determinant ($E_2^b$), which was probably adjacent to the $E_2^a$ epitope.

**Protective immunity afforded by MA.** In preliminary experiments, 12 of 15 clones induced ascitic fluids that were capable of protecting mice against lethal encephalitis caused by SFV (Table 1). The experiments were repeated with the purified MAs. BALB/c mice were injected intravenously with graded amounts (0.01 to 100 μg) of MA, followed 2 h later by an intraperitoneal challenge with 10 $LD_{50}$ units of virulent SFV (16 PFU). The patterns of protection related to different antigenic determinants are shown in Fig. 5. Three MAs (UM8.64, UM8.73, and UM8.107, corresponding to epitopes $E_1^d$, $E_2^b$, and $E_2^e$, respectively) were not fully protective at the highest tested dose of 100 μg per mouse. MA UM5.1 displayed the highest capacity of passive protection (0.1 μg per mouse). The four MAs specific for the $E_2^a$ epitope had the same protective properties, although different relative avidities of these MAs were found in ELISA (Table 2).

**Virus neutralization and HAI.** In vitro neutralizing activities of 15 MAs were assayed by plaque reduction tests against both the virulent and avirulent strains of SFV. Three out of six anti-$E_1$ MAs (UM8.4, UM8.20, and UM8.139) and three out of nine anti-$E_2$ MAs (UM8.48, UM8.55, and UM5.1) were positive for neutralization (Table 1), although with different efficiencies. Maximum neutralization was obtained with MA UM5.1. Clones which showed no neutralization of the avirulent strain of SFV also did not neutralize the virulent strain. Neutralization titers were in each case higher for the virulent strain than for the avirulent strain. The neutralization patterns were compared with in vivo protection (Fig. 5). In vivo protection was not concordant with in vitro neutralization (plaque reduction). For the $E_1^a$ determinant, there was agreement between both activities, but for the $E_1^b$ and $E_1^f$ epitopes, 100% plaque reduction was reached at lower MA concentrations than were necessary for in vivo protection. For two $E_1$ epitopes ($E_1^c$ and $E_1^e$) and one $E_2$ epitope ($E_2^a$), 100% protection was reached, whereas there was no plaque reduction at all.

HAI of goose erythrocytes infected with SFV occurred

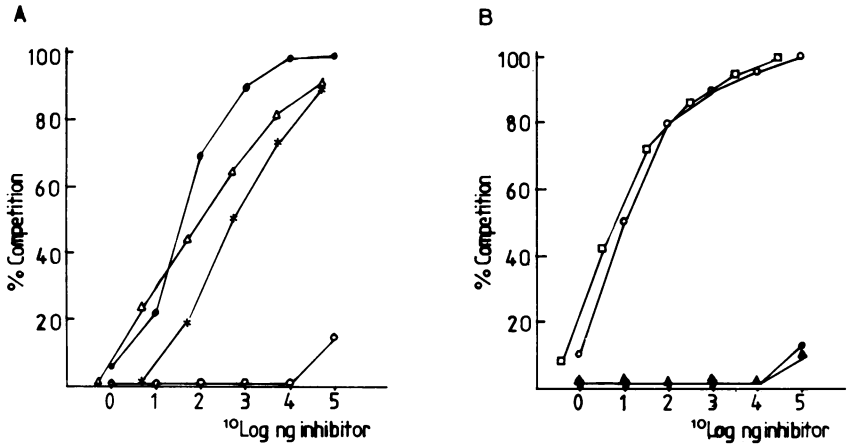


FIG. 4. CBAs (see legend to Fig. 3) with HRPO-labeled anti-$E_2$ MAs UM8.130 (A) and UM8.55 (B). Symbols: ●, UM8.130; △, UM4.2; *, UM8.22; ○, UM8.55.

with two of the anti-$E_1$ MAs and two anti-$E_2$ MA (Table 1). Moreover, MA UM8.47 was the most effective and was also able to give HAI of Sindbis virus, a closely related alphavirus (data not given).

## DISCUSSION

MAs reactive with either $E_1$ or $E_2$ glycoprotein of SFV were isolated and characterized by ELISA, virus neutralization, and HAI. The MAs were applied to detect the antigenic determinants (epitopes) on the glycoproteins of SFV by CBAs, and these results were compared with the different in vitro and in vivo properties of the associated MAs.

Before the CBA was performed, relative avidities of MAs for purified SFV were measured by ELISA. The relative avidities differed between the anti-$E_1$ and anti-$E_2$ MAs. They were generally higher for the anti-$E_2$ MAs and varied be-

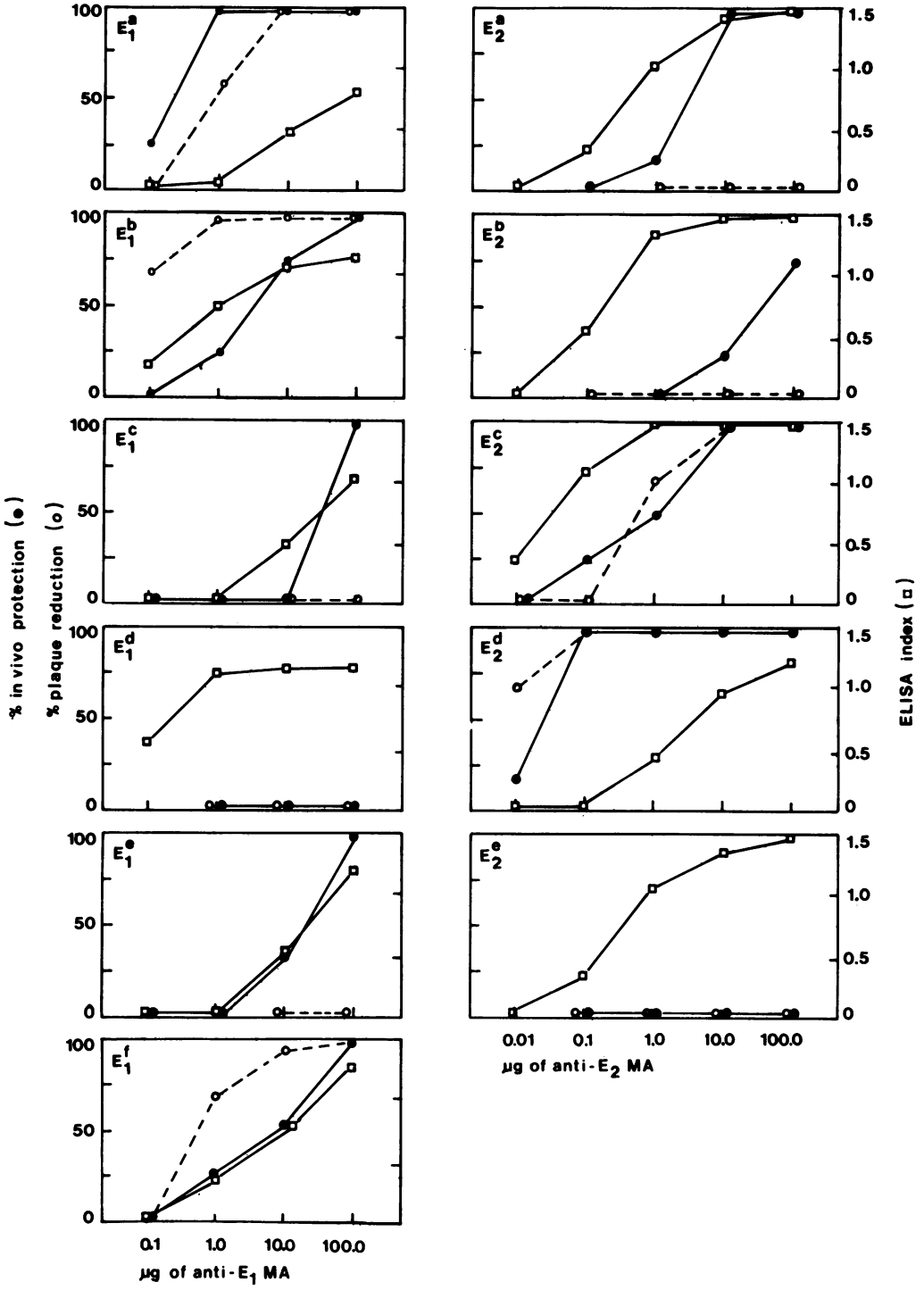


FIG. 5. Comparison of in vivo protection (●), plaque reduction of avirulent SFV (○), and optical density in ELISA (□) for $E_1$ and $E_2$ antigenic sites of SFV at 10-fold-increasing MA concentrations. The ELISA index is the optical density at 450 nm.

tween 0.02 to 10 μg of MA binding per ml. In CBAs, we observed enhanced binding of the second HRPO-labeled MA (Fig. 2). Such enhanced binding has been reported for MAs against vesicular stomatitis virus (15) and La Crosse virus (11) and might be related to an advantageous allosteric alteration of the antigenic glycoprotein after binding with the first MA, thereby resulting in increased binding of the second MA. Another explanation of this enhancement phenomenon might be the formation of a multicomponent complex (6).

None of the described anti-$E_1$ MAs (except MA UM8.4, a pentameric IgM molecule [Fig. 2], at very high inhibitor concentrations) showed competition with one of the other MAs in CBAs. Therefore, with the six MAs specific for $E_1$ (UM8.4, UM8.20, UM8.47, UM8.64, UM8.115, and UM8.139), six distinct antigenic determinants ($E_1^a$ to $E_1^f$) could be distinguished. Each of the anti-$E_1$ MAs was unique when tested for a number of biological parameters like plaque reduction, HAI, and in vivo protection (Table 1). Although MA UM8.4 had a low relative avidity for SFV, it was very effective in in vivo protection. This might be due to either in vivo complement activation by the IgM MA (24; unpublished data) or a higher relative avidity for viral antigens expressed on the surface of virus-infected cells (21).

Five distinct antigenic determinants on the $E_2$ glycoprotein were detected ($E_2^a$ to $E_2^e$) by using nine different MAs. To each epitope, a different biological function as mediated by the corresponding MA could be ascribed. Determinant $E_2^a$ was recognized by four different MAs which did not neutralize SFV in vitro, but all four afforded protective immunity. Analysis of biochemical parameters of the MAs (IgG isotype and PI) indicated that different clones were involved (Table 1). These MAs will be used in future research to elucidate the mechanism of nonneutralizing MA-mediated protection. MA UM8.73, which recognized the $E_2^b$ and partially the $E_2^a$ epitopes, was not completely protective in the dose range tested (Fig. 5) but showed HAI. None of our MAs exhibited both functions (neutralization and HAI), which is in contrast to the findings of Chanas et al. (4), who studied MAs (IgG2a subclass) directed to the closely related Sindbis virus. On the other hand, three MAs (UM8.48, UM8.55, and UM5.1) directed to $E_2$ epitopes $E_2^c$ and $E_2^d$ and all of the IgG2a subclass neutralized SFV both in vivo and in vitro. Although virus neutralization might be related to IgG isotypes (none of our IgG1 isotypes showed protective immunity) (24), the efficacy of virus neutralization largely depended on the epitope recognized by a specific MA. Our results (Fig. 4) indicated that determinant $E_2^d$, which was recognized by UM5.1, was superior to all other epitopes defined on the surface of SFV in providing protection. UM5.1 protected mice at a dose of 0.1 μg of MA protein, whereas 10 to 100 μg was required for most of the other MAs. The two MAs which were the most effective in in vivo protection (UM8.4 and UM5.1, corresponding to epitopes $E_1^a$ and $E_2^d$, respectively) did not have reactivity in ELISA at the MA concentration at which they could afford 100% protection (Fig. 5). The lack of reactivity in the ELISA might be caused by "deformation" of antigenic determinants during the purification procedure by the cesium chloride gradient, column chromatography, or by immunoadsorption of the virus to the solid phase at pH 9.6.

Schmaljohn et al. (23) identified six different antigenic determinants on another alphavirus, Sindbis virus. They found five distinct epitopes on $E_1$ and only one epitope on the $E_2$ glycoprotein of Sindbis virus. The $E_1$ glycoproteins of SFV and Sindbis virus have a great similarity in amino acid sequences (9). We found one more antigenic determinant on the $E_1$ glycoprotein of SFV. The number of epitopes on $E_1$ involved in neutralization of SFV exceeded by two the known number found on $E_1$ of Sindbis virus (23) and Venezuelan equine encephalomyelitis virus (18). That more epitopes on $E_1$ of SFV were found may be caused by the immunization procedure. Mice were immunized with live avirulent SFV without addition of an adjuvant and were boosted with highly purified, concentrated SFV. The influence of the immunization procedure on the expression of more antigenic determinants was also shown by Lerner (16) with purified hemagglutinin of an influenza virus strain. We showed in this study that MAs against $E_1$ of SFV were also involved in neutralization, albeit less than were anti-$E_2$ MAs. This is in agreement with other data for MAs against Sindbis virus (23) and Venezuelan equine encephalomyelitis virus (18). The MA UM8.47, which recognized $E_1^c$, cross-reacted with Sindbis virus both in ELISA and HAI tests and probably recognized a determinant with the same amino acid sequence for both alphaviruses.

In conclusion, we identified a major antigenic determinant on the $E_2$ glycoprotein of SFV ($E_2^d$) which is associated with the induction of a highly protective MA, and therefore, synthetic peptides with amino acid sequences identical to that of the $E_2^d$ determinant seem to be perfect candidates for further research on synthetic vaccines.

## ACKNOWLEDGMENTS

We thank R. van den Akker for introducing us to peroxidase coupling.

This study was supported by the Foundation for Medical Research project no. 13-27-67.

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