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The role of complement in monoclonal antibody-mediated protection against virulent Semliki Forest virus

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SUMMARY

Monoclonal antibodies (MAs), specific for either the E_1 or E_2 glycoproteins of Semliki Forest virus (SFV), and belonging to various immunoglobulin subclasses (IgM, IgG2a, IgG2b and IgG3), effected lysis of SFV-infected L cells in co-operation with guinea-pig complement. In this antibody-dependent complement-mediated cytolysis (ADCMC) test, IgG1 MAs were not effective although these antibodies recognize the viral antigens on the surface of SFV-infected L cells. The latter was shown with horseradish peroxidase (HRPO)-labelled MAs in a direct enzyme immunoassay. The binding reactivities of HRPO-labelled MAs to infected L cells at selected time-intervals after infection correlated well with the amount of cytolysis in a parallel ADCMC test. Cytolysis was dependent on the duration of incubation with antibodies: more cytolysis was measured after a 4-hr incubation period with MA, starting at 4 hr after infection, compared to a 1-hr incubation period starting after 7 hr of infection. However, in the latter case (1-hr period) the amount of cytolysis measured correlated better to neutralization and/or protection by MAs than after the extended period (4 hr) of incubation. Complement (C3) depletion by cobra venom factor treatment led to a higher mortality and viraemia of mice prophylactically injected with critically protective doses of either the neutralizing MA UM 8·4 (IgM) or the non-neutralizing MA UM 4·2 (IgG2a). The results suggest a co-operative role of MA with complement in mediating protection against SFV. Passive immunization by administration of low amounts (0·1 μg/mouse) of neutralizing MA UM 5·1 resulted in protection of normal mice against a lethal infection with SFV. Mice immunosuppressed by cyclophosphamide were not protected by these doses. If the doses were increased however, these mice were protected both prophylactically and therapeutically. These results indicate that, using critical doses of MAs, an intact immune system ensures survival in normal mice after infection with virulent SFV.

INTRODUCTION

Since the development of the monoclonal antibody (MA) technique (Köhler & Milstein, 1975), many investigators have reported on the protective capacities of MAs against various viruses (Balachandran, Bacchetti & Rawls, 1982; Boere *et al.*, 1983, 1984, 1985; Lefrancois, 1984; Letchworth & Appleton, 1983; Mathews & Roehrig, 1982, 1984; Schmaljohn *et al.*, 1982; Schmaljohn, Kokubun & Cole, 1983; Walsh, Schlesinger & Brandriss, 1984). The protective properties of MAs were generally established by passive transfer of these antibodies to animals before or after infection with the virus under investigation. The mechanisms by which MAs mediate *in vivo* protection might depend on immunoglobulin subclass and recognized antigenic determinants, and might be correlated with neutralization titre or effectiveness in the antibody-dependent complement-mediated cytolysis (ADCMC) assay. Neutralization of infectious virus by MA may occur by different virus–antibody interactions, including hindrance of virus adsorption to the host cell, or reduction of infectious units by mere aggregation of virus particles by MA (Dimmock, 1984; Mandel, 1979). MA may enhance clearance *in vivo* by the promotion of virus uptake in Fc receptor-bearing non-permissive host cells, e.g. granulocytes and (with many viruses) macrophages (Boere *et al.*, 1983, 1985). Neutralization of some viruses by antibodies could be enhanced by the complement system as first shown for infectious bronchitis virus (Berry & Almeida, 1968). Furthermore, the production of membrane-budding viruses could be reduced by destruction of the host cells by antibody and complement (Sissons & Oldstone, 1980).

Little is known about the role of complement in pathogenesis of and immunity against Semliki Forest virus (SFV), but many data are available on the interaction of complement, both in the presence and absence of antibodies, with the closely related Sindbis virus and other alpha viruses (Aaskov, Hadding & Bitter-Suermann, 1985; Hirsch, Griffin & Winkelstein, 1978; Hirsch, 1982; King, Wust & Brown, 1977; Stollar, 1975).

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Complement (C3) depletion of Sindbis-infected mice by treatment with cobra venom factor (CoF) resulted in enhanced virus titres in the main target organ, the brain, and rather surprisingly in delayed mean time of death. Overall, mortality was similar in CoF-treated and untreated mice. MAs against both Sindbis virus and SFV cause lysis of infected cells *in vitro* by combined action with guinea-pig complement (Schmaljohn *et al.*, 1982). Information is scarce, however, about the individual role of MAs in ADCMC, especially related to the corresponding antigenic determinants recognized on the virus particle or the infected cell. In the present study, we compare in the ADCMC test neutralizing and non-neutralizing SFV-specific MAs of various immunoglobulin (sub)classes and recognizing different determinants. The way by which complement interacts with virus-infected cells *in vitro* may be of relevance in the mediation of protection *in vivo*. Therefore, the role of complement in MA-mediated protection against SFV-induced encephalitis was analysed by depleting mice of C3 by CoF treatment.

MATERIALS AND METHODS

Virus

The virulent SFV strain SF/LS 10C1A was received from C. J. Bradish (Bradish, Allner & Maber, 1971) and passed three times through BALB/c mice. Stock SFV used in earlier studies (Boere *et al.*, 1983, 1984, 1985; Henderson *et al.*, 1970) yielded 6×10^5 PFU/ml. The 50% lethal dose (LD_{50}) for male BALB/c mice was 1–2 PFU per mouse. The general virological methods have been described previously (Boere *et al.*, 1984, 1985; Kraaijeveld, Harmsen & Khader Boutahar-Trouw, 1979).

Cells and media

L cells, a continuous line of mouse fibroblasts, grown in Dulbecco's minimal essential medium (DMEM) with 0·01 M *N*-2-hydroxyethyl-piperazine N′-2-ethane sulphonic acid supplemented with 5% calf serum and antibiotics were used for plaque titrations.

Animals

Inbred BALB/c mice were bred and maintained in our own animal house. Male mice of about 12 weeks of age were used for CoF treatment and protection experiments.

Monoclonal antibodies

The production, purification and biological characterization of MAs against SFV have been described in other papers (Boere *et al.*, 1983, 1984).

Cobra venom factor treatment

Mice were depleted of the third component of complement (C3) by treatment with purified CoF (Ballow & Cochrane, 1969). Mice were injected intravenously with 1 unit (5 μg) of purified CoF 22 hr before MA treatment and 24 hr before challenge with SFV. The serum of the CoF-treated mice was analysed for the presence of C3 by a colorimetric microassay (Klerx *et al.*, 1983). Mice treated with CoF had no detectable C3 1–3 days after the injection of CoF.

Passive protection by monoclonal antibodies

Protection experiments provided by MAs in BALB/c mice were performed as described previously (Boere *et al.*, 1985).

Virus titrations

Blood was collected from ether-anaesthized mice by retro-orbital puncture. Virus titres in sera were determined by plaque titration as described earlier (Boere *et al.*, 1984).

Antibody-dependent complement-mediated cytolysis (ADCMC)

Lysis of $Na_2{}^{51}CrO_4$-labelled SFV-infected cells by the mutual action of MAs and guinea-pig complement was measured as described previously (Boere *et al.*, 1985).

ELISA

Viral antigens were detected on L cells infected with SFV as described elsewhere (Van Tiel *et al.*, 1984).

Immunosuppression

Mice were immunosuppressed with cyclophosphamide (Bradish, Allner & Fitzgeorge, 1975; Gates, Brown & Wust, 1984). This drug was injected intraperitoneally (i.p.) in 0·5 ml phosphate-buffered saline (PBS) of pH 7·2 in a dose of 200 mg/kg (5 mg/mouse).

RESULTS

Biological characterization of SFV-specific MAs

Ten purified MAs, with specificities for either the E1 or E2 glycoprotein of SFV and belonging to various immunoglobulin Ig (sub)classes, were tested for their capacities to neutralize SFV, to protect mice against virulent infection, to mediate lysis of SFV-infected cells in conjunction with complement, and to react with SFV-infected L cells as enzyme conjugates. The results of four separate experiments are summarized in Table 1. Both neutralizing and non-neutralizing MAs protect mice against lethal infection except the two MAs of IgG1 subclass. In combination with complement, the latter MAs were unable to lyse SFV-infected L cells, although they bound to infected cells as indicated by the positive reactions in the direct enzyme immunoassay.

The influence of incubation time and MA dose on the specific lysis in ADCMC

In order to assess the relative effectiveness of purified MAs to induce lysis of SFV-infected L cells, incubation time and MA dose were varied in the ADCMC assay. L cells, labelled with $Na_2{}^{51}CrO_4$, were infected with virulent SFV at a multiplicity of infection (MOI) of 8, and subsequently seeded as monolayers in wells of 96-well plates. After either 4 or 7 hr of infection, five-fold dilutions of purified MAs (80, 16 or 3·2 μg per well) and a 1:30 dilution of guinea-pig complement were added. At 8 hr post-infection (after incubation times of 4 and 1 hr, respectively) samples of supernatant fluids were collected and analysed for ^{51}Cr release from infected cells. The amount of specific lysis in the ADCMC depended on the period of incubation with MA and the amount of MA used (Fig. 1). A rapid lysis with relative low doses of MA occurred with the highly protective MAs UM 8·4 (IgM) and UM 5·1 (IgG2a). An optimal dose of 16 μg MA per well resulted in approximately 30% specific lysis of infected cells during 1 hr incubation with these MAs. When the infection period was kept constant for 8 hr, but the MAs were added 4 hr post-infection, the specific lysis increased to 45%.

Table 1. Properties of Semliki Forest virus-specific monoclonal antibodies

MA	Antigenic determinant*	Class of antibody	Reciprocal of neutralization titre	*In vivo* minimal protective dose (μg)	% lysis of virulent SFV-infected L cells in ADCMC† with:		Reactivity‡ (absorbance at 450 nm) against:	
					Inactivated complement	Active complement	Non-infected L cells	SFV-infected L cells
UM 8·4	$E1^a$	IgM	10^5	1	2	45	NT§	NT
UM 8·64	$E1^d$	IgG1	< 1	Non-protective	0·2	2	0·163	0·761
UM 8·115	$E1^e$	IgG2a	< 1	100	0·6	13	NT	NT
UM 8·139	$E1^f$	IgG2a	10	100	0·3	23	0·110	0·834
UM 4·2	$E2^a$	IgG2a	< 1	10	0·9	18	0·141	0·609
UM 8·77	$E2^a$	IgG2b	< 1	10	−0·2	9	NT	NT
UM 8·107	$E2^c$	IgG1	< 1	Non-protective	−0·2	3	0·049	0·216
UM 5·1	$E2^d$	IgG2a	10^6	0·1	0·7	53	0·002	0·735
UM 8·1	E2	IgG3	10^6	1	2	13	0·040	0·801
UM 8·79	E2	IgG2a	10^4	1	0	7	NT	NT

* Results are taken from previous experiments (Boere *et al.*, 1984).

† L cells were infected with virulent SFV at a MOI of 8. Addition of 16 μg purified MAs and guinea-pig complement was performed 4 hr or 5 hr after virus infection. Supernatants were harvested 8 hr after infection

‡ L cells were infected with virulent SFV at a MOI of 15. After 6 hr infected and control cells were fixed with 0·05% glutaraldehyde and incubated with HRPO-labelled MAs.

§ NT, not tested.

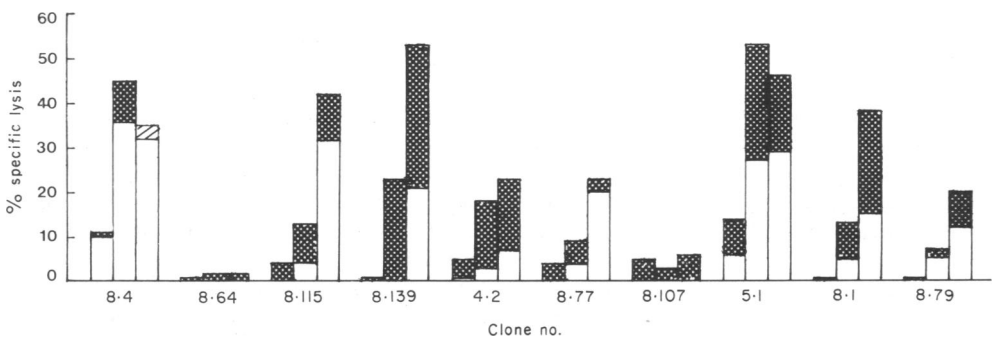

Figure 1. The effect of incubation time and MA dose on specific lysis in ADCMC. The assay was performed as described in the test. Doses of 3·2 (left column), 16 (middle column) and 80 μg (right column) per well of each MA clone were added at 4 hr (total column) or 7 hr (open column) post-infection and supernatants harvested at 8 hr after infection with SFV. The striped column indicates a diminished specific lysis if antibodies were added at 4 hr post-infection.

The two non-neutralizing MAs UM 4·2 (IgG2a) and UM 8·77 (IgG2b) were less effective, but 4 hr incubation with 80 μg of these MAs caused a considerable specific lysis of 23% of infected cells. The same phenomenon was observed with the two non-neutralizing anti-E1 MAs UM 8·115 and UM 8·139. The MAs UM 8·64 and UM 8·107 (both IgG1 subclass) showed no specific lysis when added 1 hr after infection. On the other hand, for the two protective MAs UM 8·1 and UM 8·79, at least 16 μg MA per well were required before specific lysis occurred after a 1-hr period. Comparison of these results with the properties of the MAs in mouse protection (Table 1) reveals that a short incubation period (especially at the 16 μg dose) correlates well with protection.

The appearance of viral glycoproteins on infected L cells as measured by direct enzyme immunoassay and ADCMC

During a one-step multiplication cycle, SFV glycoproteins are increasingly inserted in the cell membrane. After a latent phase the host cell becomes vulnerable to lysis due to the combined action of antibody and complement. The appearance of the E_2 glycoprotein on the surface of SFV-infected cells was studied with a direct enzyme immunoassay with two E_2-specific HRPO-conjugated MAs (UM 5·1 and UM 4·2). In the enzyme immunoassay, L cells were infected in suspension with virulent SFV at a MOI of 10 and seeded in 96-well plates to form monolayers. After selected time-intervals (3, 4, 5, 6 and 7 hr) the

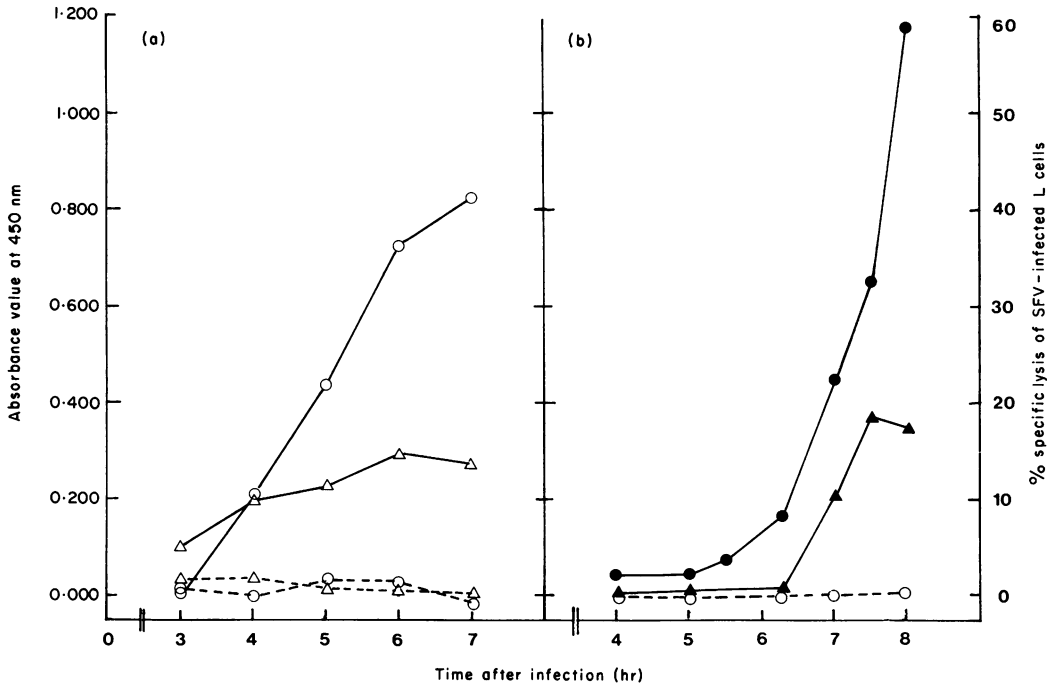

Figure 2. The appearance of viral glycoproteins on L cells infected with SFV tested by ELISA and ADCMC. (a) Absorbance values at 450 nm are presented for MA UM 5·1 (○) and UM 4·2 (△). Dotted lines are control values for uninfected L cells. (b) Percentage specific lysis in ADCMC is measured by taking supernatant at different times after infection and 1 hr after the addition of MA UM 5·1 (●) and UM 4·2 (▲). The dotted line indicates control ^{51}Cr-release values of cells if no antibodies are added.

infection was stopped by fixation with 0·05% glutaraldehyde. The monolayers were screened for the presence of viral antigen with the individual enzyme-conjugated MAs. The mean absorbance values at 450 nm of duplicate determinations are shown in Fig. 2a. With HRPO-conjugated MA UM 5·1 the absorbance values increased steadily from 4 to 7 hr post-infection (OD_{450}: 0·200–0·800). A minimal increase of absorbance was observed using HRPO-conjugated MA UM 4·2. In a parallel experiment the rise in specific lysis of SFV-infected cells was followed. Similarly, L-cell suspensions were infected at a MOI of 10 and seeded as monolayers in 96-well plates. After 2, 3, 4, 5, and 6 hr of infection, respectively, 80 μg of MA (UM 5·1 or UM 4·2) and guinea-pig complement were added to each well and incubated for 2 hr at 37°. Thereafter (4, 5, 6·25, 7, 7·5 and 8 hr after infection), the supernatant fluids were harvested and analysed for ^{51}Cr release. The percentages of specific lysis at the time-points of addition of MA and complement are given in Fig. 2b. Lysis of SFV-infected L cells by the neutralizing MA UM 5·1 and complement starts between 5 hr and 6 hr after infection. The lysis mediated by the non-neutralizing MA UM 4·2 starts later and reaches lower levels.

The effect of cobra venom factor treatment on monoclonal antibody-mediated protection of mice against virulent SFV

The capacity of both neutralizing and non-neutralizing MAs to induce, in co-operation with guinea-pig complement, lysis of SFV-infected cells suggests an accessory beneficient role of complement in the mediation of protection *in vivo* by monoclonal antibodies. This problem can be approached to some extent by comparison of viraemia and mortality in CoF-treated and control infected mice.

Two groups of 75 mice were injected i.v. with PBS or with one unit of the toxin CoF to deplete complement C3. Twenty-four hours later, groups of 25 control and C3-depleted mice received a critically protective dose of the neutralizing IgM MA UM 8·4 (3 μg) or the non-neutralizing MA UM 4·2 (30 μg). The minimal protective doses of these MAs were determined previously (Boere *et al.*, 1984). Control mice received PBS again.

Two hours later, the mice were infected i.p. with a normally lethal dose of virulent SFV. Blood was collected at several time-intervals (45 min, 24 hr and 48 hr after infection) to follow viraemia in these mice. Five mice of each group received 50 LD_{50} (80 PFU) in order to determine the number of mice with viraemia at 45 min after infection, while the others (groups of 25 mice) received 10 LD_{50}. No blood was taken from five mice of each group. To quantify survival, the mice were observed for 21 days. Furthermore, in each group the mean survival time of non-surviving mice was calculated. The results are presented in Table 2. In control mice receiving no MA, a viraemia was observed in 24 out of 25 mice. No infectious virus could be detected in serum at 45 min after infection in recipients of neutralizing MA UM 8·4. At that time the serum of recipients of the non-neutralizing MA UM 4·2 already contained infectious virus. The viraemia was not halted by these two MAs, for at 24 hr and at 48 hr after infection most mice had detectable virus in serum. Pretreatment of mice with CoF affected viraemia, virus titres and survival. Slightly more CoF-treated recipients of MAs were viraemic than untreated mice. Furthermore, CoF-treated recipients of MAs had consistently higher virus titres

Table 2. Effect of cobra venom factor treatment on viraemia and mortality in recipients of marginal protective doses of neutralizing IgM (UM 8·4) and non-neutralizing IgG2a (UM 4·2) monoclonal antibodies

Treatment			Parameters of infection									
			No. of mice with viraemia/no. injected at:			Log_{10} virus titres in serum at:						
MA	Dose of MA (μg)	Cobra venom factor	45 min	24 hr	48 hr	24 hr	*P*	48 hr	*P*	No. survivors/ no. injected	*P*	Mean survival time (days) of non-surviving mice
None (PBS)	0	–	4/5	10/10	10/10	3·95±0·84	0·046	3·51±0·52	0·08	1/30		5·6
None (PBS)	0	+	5/5	10/10	10/10	3·22±0·66		4·06±0·76		0/30		6·0
UM 8·4	3	–	0/5	6/10	7/10	1·13±0·58	0·02	1·57±1·08	0·11	13/30	<0·05	6·7
UM 8·4	3	+	0/5	9/10	9/10	1·94±0·80		2·60±1·60		4/30		6·0
UM 4·2	30	–	4/5	9/10	9/10	2·90±1·19	0·014	1·59±1·22	0·06	17/30	<0·02	7·5
UM 4·2	30	+	5/5	10/10	10/10	4·02±0·49		2·77±1·34		6/30		6·2

than the untreated recipients. In both recipients of MA UM 8·4 and MA UM 4·2, CoF treatment was associated with greater mortality reaching statistical significance. The mean survival time of non-surviving mice not treated with CoF was longer. No mice died before Day 4 of infection, indicating that blood withdrawal, in this particular experiment, had no immediate influence on the observed mortality.

Monoclonal antibody-mediated protection in the immuno-compromised host

In the foregoing experiment we showed that the majority of recipients of critical doses of protective MAs developed viraemia upon infection, suggesting that in surviving mice their own immune response may be responsible for the protection observed. This is also exemplified in the following experiments. Groups of mice were immunosuppressed by i.p. injection with the drug cyclophosphamide (200 mg/kg) and a control group received the diluent PBS. Twenty-four hours later, all mice were challenged i.p. with either 10 or 1000 LD_{50} of virulent SFV. Various groups received 2 hr earlier either PBS or neutralizing MA UM 5·1 (0·1 μg and 1 μg) i.v. A dose of 0·1 μg MA UM 5·1 is the minimal protective dose in normal mice (Boere *et al.*, 1983). The other groups of mice received MA UM 5·1 (10 μg and 100 μg) 24 hr after infection. The results, presented in Table 3, clearly indicate that high doses of neutralizing MA are prophy-

Table 3. Prophylaxis and therapy of lethal SFV infection in immuno-compromised hosts with neutralizing MA UM 5·1

Treatment			Mice infected with SFV†	
			10 LD_{50}	1000 LD_{50}
Dose of monoclonal antibody per mouse	Dose of cyclophosphamide* (mg/kg)	Interval between antibody treatment and infection (hr)	No. survivors/ no. injected	No. survivors/ no. injected
0 (PBS)	0	−2	0/6	0/6
0 (PBS)	200	−2	0/6	0/6
0·1	0	−2	6/6	5/6
0·1	200	−2	3/6	2/6
1	200	−2	6/6	6/6
0 (PBS)	0	+24	1/6	NT‡
0 (PBS)	200	+24	0/6	NT
10	0	+24	6/6	NT
10	200	+24	0/6	NT
100	200	+24	6/6	NT

* Cyclophosphamide was given intraperitoneally in a volume of 0·5 ml PBS 24 hr before infection. Graded doses of purified MA UM 5·1 were injected intravenously in 0·2 ml PBS at the indicated time-points.

† Mice received an i.p. injection of 0·5 ml PBS containing 16 PFU (10 LD_{50}) or 1600 PFU (1000 LD_{50}).

‡ NT, not tested.

lactically and therapeutically effective in the immunocompromised host, but critically low doses of MA (UM 5·1, 0·1 μg) are not effective. Therefore, besides the passive administration of low doses of MA to mice, the intact immune system of these mice also contributes to the protective immunity.

DISCUSSION

The present study was designed to investigate the co-operative role of complement and SFV-specific MAs. The ADCMC test was used *in vitro* to measure the relative effectiveness of MAs of various immunoglobulin subclasses (IgM, IgG1, IgG2a, IgG2b and IgG3) to cause complement-mediated lysis of SFV-infected L cells. MA-mediated protection experiments were performed in normal mice and mice depleted of complement and tested on the outcome of infection with virulent SFV.

All MAs tested, neutralizing and non-neutralizing SFV, induced lysis of SFV-infected L cells in the presence of guinea-pig complement except the two IgG1 MAs UM 8·64 and UM 8·107. The latter two MAs bind to SFV-infected L cells, which are shown by their reactivity as enzyme conjugates in an enzyme immunoassay, but are unable to fix complement, which might be related to a relative inflexibility of the Fc part of these molecules (Oi *et al.*, 1984). MAs UM 8·64 and UM 8·107 are also non-neutralizing antibodies and, moreover, they are unable to protect mice against lethal infection with SFV. In contrast, the non-neutralizing but complement-fixing MAs (UM 8·115, UM 4·2 and UM 8·77) of IgG2a and IgG2b subclasses were clearly protective. Earlier, we provided suggestive evidence to indicate that, besides cell lysis mediated by complement, the protective capacity of non-neutralizing IgG2a MAs might be explained by accessory mechanisms of protection such as growth inhibition and enhanced clearance of virus by non-permissive Fc receptor-bearing phagocytes (Boere *et al.*, 1985). Growth inhibition *in vitro* and enhanced clearance *in vivo* mediated by non-neutralizing MAs are the subjects of more detailed studies.

The profile of relative effectiveness of MAs in the ADCMC test is most pronounced when short incubation times and low doses of MAs are used. In our opinion, the selection of MAs as candidates for *in vivo* protection should occur during this time-period and not at conditions when maximum lysis is observed. As a parallel assay to the ADCMC test, the sensitive direct enzyme immunoassay in cell culture may be used to observe the appearance of viral determinants at the cell surface. The latter was performed with HRPO-labelled MAs as used in the ADCMC test.

MA-conferred protection against intraperitoneal infection with SFV is negatively affected by depleting mice of complement (Table 2). Intravenous injection of purified CoF results in a loss of functional C3 in the circulation for at least 6 days (C. J. Beukelman, personal communication). This period covers the first three critical days of infection before the appearance of specific immunity could have occurred. Co-operation of the mouse's own immune system with the passively transferred minimal protective doses of MAs is essential as indicated by the death of immunosuppressed recipient mice and survival of recipients with an intact system (Table 3). Therefore, the harmful effect of complement depletion is very pronounced by the excess mortality rate in mice receiving critically protective doses of either the neutralizing MA UM 8·4 (IgM) or the non-neutralizing MA UM 4·2 (IgG2a). The mortality in CoF-treated recipients of either MA was associated with an enhanced viraemia compared to control mice (Table 2). The observations mentioned above point to an *in vivo* mechanism similar to ADCMC as proposed for Sindbis virus (Schmaljohn *et al.*, 1982), although other mechanisms of protection may be involved. Firstly, complement might function as an opsonin for SFV that results in enhanced phagocytosis. Secondly, complement may have a synergistic effect on antibody-dependent cell-mediated immune mechanism(s), and thirdly, complement may play an important part in the induction of specific immune responses (Pepys, 1972). Although the problem of the role of complement in MA-mediated protection is not yet resolved, it is unequivocally clear that an intact complement system favours survival after infection with virulent SFV.

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