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Passive Protection across Subgroups of Alphaviruses by Hyperimmune Non-Cross-Neutralizing Anti-Sindbis Serum (42446)

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Abstract. Extended hyperimmunization of rabbits with Sindbis (SIN) or Semliki Forest (SF) viruses causes the production of antisera that are cross-reactive with virus-infected cells in antibody-dependent, complement-mediated cytotoxicity assays but that do not cross-neutralize viruses *in vitro*. C3H/HeJ mice given γ globulin fractionated from the extended hyperimmune antiserum against SIN, but not control sera, were protected from challenge by 100 LD_{50} of SF, a virus which is in a different subgroup than SIN. All mice survived if the γ globulin was given 24 hr before challenge virus and partial protection occurred if the globulin was given 24 hr after the virus. Cobra venom factor treatment of normal C3H mice challenged with SF did not reduce the protection, suggesting that complement was not involved. Methyl palmitate (40 mg/mouse) given before γ globulin and virus challenge suppressed macrophage activity and reduced the level of protection 23% in females and 70% in males. Silica treatment (3 mg/mouse) reduced the protection equally in both males and females by 92%. *In vitro* experiments were done to test if it were possible that cross-antibody-dependent cellular cytotoxicity (ADCC) could accout for the passive cross-protection observed in this system. Cross-ADCC could be demonstrated *in vitro* at high dilutions of antiserum (1:25,600). On the basis of the *in vitro* and *in vivo* results presented, we suggest that cross-ADCC against SF-infected target cells is one of the likely mechanisms to explain the passive cross-protection observed.

We previously reported that two immunizations with infectious Sindbis virus (SIN) results in cross-protection against challenge by a virus from a different alphavirus subgroup, namely Semliki Forest (SF) virus (1–3). This was observed in the absence of cross-neutralizing antibody (the basis for division of alphaviruses into subgroups) (4) and the proposed mechanism to account for the cross-protection was cross-cell-mediated immunity (CMI) mediated by cytotoxic T cells (1–3, 5). Other experiments in our laboratory related to the humoral response showed that cross-antibody-dependent, complement-mediated cytotoxicity (ADCMC) could be demonstrated *in vitro* in both directions against infected cells using antisera obtained only after extended hyperimmunization as opposed to normal hyperimmunization (6–8). Both types of hyperimmune antisera possessed very high titers of homologous neutralizing antibody ($>10^5$) but no detectable cross-neutralizing activity (6–8). The virally encoded antigens involved in cross cytolysis, demonstrated in cross-ADCMC or cross-CMI, appear to be present only on the surface of infected cells and not on virions (8–10). These target antigens include a unique conformation of the E1 glycoprotein (8, 9) and the capsid protein that is exposed on the infected cell membrane late in infection (10).

The above findings, taken together, led us to test whether passive immunization with our high titered cross-ADCMC antisera (1:1600), but undetectable cross-neutralizing activity, could cross-protect mice from a virus of a different subgroup (anti-SIN serum in SF-challenged mice). Others have shown that non-neutralizing antibodies to alphaviruses could protect against homologous virus, or virus within a single subgroup (11–17). It is important to note, however, that no tests were reported for possible cross-protection against viruses belonging to different subgroups, where cross-protection after immunization is more difficult to demonstrate and/or quantitatively lower.

The present study was undertaken to test the cross-protective efficacy of extended hyperimmune anti-SIN against SF challenge. Such cross-protection is reported here for the first time; the mechanism(s) could not be explained by cross-ADCMC occurring *in vivo*.

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Instead, we suggest on the basis of our *in vivo* and cross-ADCC *in vitro* experiments that cross-antibody-dependent cellular cytotoxicity (cross-ADCC) against SF-infected cells *in vivo* is one likely mechanism that could explain the cross-protection we observed.

Materials and Methods. *Cells.* Mouse L929 fibroblasts were cultured in McCoy's 5A medium (GIBCO, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS, Sterile Systems, Logan, UT). Primary chicken embryo fibroblasts (CEF) were prepared from 10- to 11-day-old embryonated eggs after decapitation and evisceration. They were cultured in Medium 199 (GIBCO) supplemented with 5% FBS.

Viruses. Sindbis AR339 and Semliki Forest viruses were grown in primary chicken embryo fibroblasts at a multiplicity of infection (moi) of 0.01. Stock virus suspension of SF was diluted to 5×10^6 plaque-forming units (PFU)/ml in brain heart infusion broth and 0.1 ml containing 5×10^5 PFU (100 LD_{50}) was injected ip as the challenge dose. Virus used for the immunization of rabbits was purified as described previously (8, 18) by low speed clarification (5000*g*), polyethelene glycol precipitation (10% in 0.5 *M* NaCl), and isopycnic density centrifugation for 16.5 hr at 25,000 rpm (SW 27 rotor) in a 20 to 60% linear sucrose gradient in TNE medium (0.01 *M* Tris, 0.05 *M* NaCl, 5 m*M* EDTA, pH 7.4). The virus band was collected, pelleted at 25,000 rpm in an SW27 rotor for 2.5 hr, and suspended in TNE for storage at −70°C.

Production of extended and normal hyperimmune antisera. Purified SIN or purified SF (250μg, 10^{10} PFU) were emulsified in complete Freund's adjuvant and injected into the gastrocnemius muscle of rabbits. Secondary immunization with 250 μg of purified virus was given ip in incomplete adjuvant 1 week later. Additional immunizations over a period of 2 to 4 months (normal hyperimmune antisera) or 10 to 12 months (extended hyperimmune antisera) were given iv, each with 250 μg purified virus alone. Antisera from animals on each immunization schedule contained homologous virus neutralizing titers $> 10^5$ and nondetectable heterologous virus neutralizing titers (<1:5) when assayed by plaque reduction on CEF monolayers. Extended hyperimmune antisera, but not normal hyperimmune antisera, obtained from every rabbit immunized thus far (eight rabbits) showed cross-reactivity by ADCMC of virus-infected L929 and CEF cells using assays previously described (8, 18).

Gamma globulin was precipitated from preimmune and immune antisera with 33% $(NH_4)_2SO_4$, centrifuged at 2000*g* for 30 min, dissolved in phosphate-buffered saline (PBS, pH 7.4), and dialyzed against PBS. The γ globulin solution was diluted to the original serum volume before use. The globulin was injected ip at a dose of 0.5 ml per mouse.

Animals. C3H/HeJ mice were obtained from the breeding colony of the University of Tennessee Memorial Research Center and used at 5 to 8 weeks of age.

Cobra venom factor (CoVF). CoVF (Cordis Laboratory, Miami, FL) was reconstituted in water to 100 units/ml and given at 0.5 units/g body wt in each of three injections (two given ip, one iv) for a total of 30 units per mouse. The first injection was given at 30 hr before virus challenge; the second injection was given at 6 hr after virus; the third injection was given at 30 hr after virus.

RES blockage. Methyl palmitate (palmitic acid methyl ester; Sigma Chemical Co., St. Louis, MO) was dissolved in 0.1% Tween 20–saline. The solution was sonicated 5 min until an emulsion resulted and then kept at 37°C. Each mouse was injected with 40 mg in a total of 0.3 ml given in several doses iv, 30 min apart. Using this procedure, which is reported to act selectively on macrophage function, maximum suppression of the reticuloendothelial system (RES) occurs at 48–72 hr post injection and the spleen then is small and amorphous; recovery requires up to 2 weeks (19, 20).

RES blockage was tested by injecting colloidal carbon iv into treated mice and comparing the clearance with that in untreated mice. After injection, heparinized blood samples from treated or untreated mice were diluted in 0.1% Na_2CO_3 solution. The mixtures were then read photometrically at 610 nm. Mice not treated with palmitate cleared 50% of injected carbon in about 6.5 min. No clearance was observed in treated animals over a 15-min time period. There was no apparent difference between males and females.

Another method to block the RES was to inject mice iv with 3 mg of silica particles

(Whittaker, Clark and Daniels, South Plainfield, NJ; No. 216 Silica Min-U-Sil; average particle size, 1.5 μ) in 0.3 ml of PBS (pH 7.4) 24 hr before the injection of hyperimmune γ globulin and 48 hr before SF challenge (21, 22). The silica was suspended in PBS, autoclaved, and sonicated for 10 sec before use.

Preparation of peritoneal exudate cells (PEC). Untreated C3H/HeJ female mice, 8 to 9 weeks old, were killed by cervical dislocation and then injected ip with 5 ml of ice-cold PBS. The peritoneum was gently massaged and the fluid was withdrawn by syringe. The fluid was centrifuged at 500*g* for 10 min, the supernatant fluid was discarded, and the cells were suspended to 4×10^6/ml in RPMI 1640 supplemented with 5% FBS (GIBCO). Typical harvests yielded 5×10^6 PEC per mouse.

Antibody-dependent cellular cytotoxicity assay. L929 cells were infected with SIN or SF, labeled with ^{51}Cr, and washed (18). They were then suspended to 2×10^5 cells/ml in RPMI 1640 with 2% FBS. At 6–7 hr post infection, a 20-to-1 mixture of PEC and target cells (100 μl of each suspension) was seeded into microtiter wells (96-well round-bottom plate; Corning) containing 50 μl of antiserum dilutions. The antisera were first heat-inactivated at 56°C for 30 min and fourfold serial dilutions were made in HBSS (GIBCO).

After the cells were added, the plates were centrifuged at 500*g* for 5 min and then incubated for 6 hr at 37°C in a 5% Co_2 humidified incubator. At 12–13 hr post infection, the plates were centrifuged at 500*g* for 5 min and 150-μl samples of the supernatant fluids of triplicate assays were analyzed for ^{51}Cr release in an LKB 1274 RiaGamma spectrometer. Controls consisted either of target cells with antibody alone or PEC alone, or of target cells treated with 3% Triton X-100 (total releasable counts). Percentage specific cytotoxicity was calculated as

$$\frac{\text{cpm released by target cells with PEC and antibody dilution} - \text{cpm released by target cells with PEC alone}}{\text{total releasable cpm} - \text{cpm released by target cells with PEC alone}} \times 100.$$

Results. Extended hyperimmune rabbit anti-SIN serum does not have cross-neutralizing activity for SF but it does have fairly high titers (1:1600) of cross-ADCMC for SF-infected as well as homologous SIN-infected L cells and CEF as previously described (7, 10). Owing to the cross-ADCMC activity, we thought that this extended hyperimmune serum (injections for >10 months) might cross-protect mice from SF challenge. To test this, mice were injected ip with γ globulin fractionated from control and test sera and challenged at various times with 100 LD_{50} of SF. Control virus-challenged mice received preimmune rabbit gamma globulin or globulin from sera of rabbits that had been injected several times over 2–4 months with SIN and considered to be normal hyperimmune. Such normal hyperimmune sera showed equally high titers of homologous neutralizing antibody activity ($>10^5$) similar to extended hyperimmune sera, but are negative (<1:5) in both cross-neutralization and cross-ADCMC assays (7, 8). Homologous ADCMC titers with normal hyperimmune antisera were about one-fourth of those found with extended hyperimmune antisera (e.g., 1:400 versus 1:1600).

The extent of protection of mice from SF challenge by the passively administered hyperimmune anti-SIN γ globulin is presented in Table I. Maximum protection was seen when the extended hyperimmune globulin was given 24 hr before challenge. In contrast, no protection was conferred by γ globulin prepared from either preimmune or normal hyperimmune sera given 24 hr after challenge and minimal protection when given 24 hr before challenge. This low level of protection was considered nonspecific. Repeated experiments gave essentially similar results. As an additional control for the specificity of the protection, i.e., the rabbit γ globulin, was not per se a significant factor, γ globulin prepared from extended hyperimmune rabbit anti-herpes simplex virus serum was injected 24 hr before SF challenge and a similar very low level of protection to SF challenge, as in other controls, was observed with this γ globulin.

Since extended hyperimmune anti-SIN sera gave cross-ADCMC against SF-infected L or CEF cells *in vitro,* a similar mechanism could be operating to mediate cross-protection *in vivo.* To assess a role for complement in the cross-protection, the effects of CoVF admin-

TABLE I. EFFECT OF γ GLOBULIN OF HYPERIMMUNE ANTI-SIN SERUM ON MORTALITY OF FEMALE C3H MICE GIVEN SF CHALLENGE

Globulin[a]	Time given (hr)	Challenge	Number dead/total	% Survival
Extended hyperimmune	−24	SF	0/22	100
Extended hyperimmune	+24	SF	14/23	39
Extended hyperimmune	0	None	0/22	100
Normal hyperimmune	−24	SF	15/20	25
Normal hyperimmune	+24	SF	21/21	0
Normal hyperimmune	0	None	0/22	100
Preimmune	−24	SF	17/20	15
Preimmune	+24	SF	19/22	14
None		SF	22/22	0
Hyperimmune anti-HSV	−24	SF	9/10	10

[a] 0.5 ml of γ globulin from designated serum given ip. Anti-HSV γ globulin was used as a specificity control.

istration on the passive cross-protection were measured (23). Such treatment did not reduce the protection afforded by hyperimmune globulin (Table II) and together with results showing the efficiency of CoVF treatment in reducing C levels, we concluded that the protection was not complement mediated. For example, undiluted serum from CoVF-treated mice did not cause the lysis of rabbit hemolysin-sensitized sheep erythrocytes whereas serum from nontreated mice at dilutions up to 1:100 did cause cytolysis. This test served to show that complement was inactivated below detectable levels by CoVF treatment *in vivo*.

Another possible mechanism for cross-protection *in vivo* is antibody-dependent cellular cytotoxicity against virus-infected cells. The primary effector cells are often assumed to be the killer lymphocyte (K cell) and the macrophage (24, 25). To test for the participation of macrophages, two inhibitors of macrophage function were used. Methyl palmitate was used first to block macrophage function (19, 20). The results in Table II indicate that the blockage minimally reduced the protection to SF

TABLE II. EFFECT OF TREATMENT WITH COBRA VENOM FACTOR (CoVF) OR METHYL PALMITATE ON CROSS-PROTECTION OF C3H MICE USING HYPERIMMUNE ANTI-SIN γ GLOBULIN GIVEN 24 hr BEFORE SF CHALLENGE

Globulin	Treatment	Challenge	Number dead/total	% Survival
Females				
Extended hyperimmune	CoVF	SF	1/10	90
Normal hyperimmune	CoVF	SF	11/12	8
Extended hyperimmune	Palmitate	SF	15/66	77
Normal hyperimmune	Palmitate	SF	21/24	12
Extended hyperimmune	None	SF	4/32	88
None	Palmitate[a]	None	0/14	100
None	None	SF	9/10	10
None	Palmitate	SF	10/10	0
Males				
Extended hyperimmune	Palmitate	SF	22/31	29
Normal hyperimmune	Palmitate	SF	10/10	0
Extended hyperimmune	None	SF	2/12	83
None	Palmitate[a]	None	0/10	100
None	Palmitate	SF	10/10	0
None	None	SF	10/10	0

[a] No obvious clinical signs of illness observed in mice treated with palmitate alone.

challenge provided by extended hyperimmune anti-SIN globulin in female mice but the protection was reduced significantly in male mice.

To further test for the putative role of macrophages in ADCC *in vivo,* silica was injected into mice, which were then given hyperimmune γ globulin and SF challenge. This treatment did markedly reduce the protection afforded by the γ globulin (Table III). In contrast to the previous palmitate experiment, no differences in the reduction of protection by silica were noted between males and females. Reasons for the differences in results between the male and female mice after palmitate vs silica treatments are not understood at this time. Included in this set of experiments were mice injected with silica and SIN to show that silica did not enhance the virulence of that virus. Further, silica did not affect homologous protection provided by the neutralizing antibody of anti-SF γ globulin against SF challenge.

As an *in vitro* correlate for ADCC, PEC were obtained from untreated C3H female mice and used in *in vitro* assays. The PEC were mixed with ^{51}Cr-labeled infected L cells at a ratio of 20:1 and varying dilutions of hyperimmune antisera were added to the mixtures in microtiter wells. Representative results are presented in Fig. 1 and show that resident, unstimulated PEC function in *in vitro* ADCC at serum dilutions up to 1:25,600 against heterologous SF-infected L929 cells and up to 1:4 $\times$ 10^5 against homologous SIN-infected cells. There is a prozone-like phenomenon at high concentrations of antiserum. These titers are, incidentally, considerably higher than ADCMC titers (26) since our titers for homologous ADCMC are about 1:25,000 and heterologous titers are about 1:1600. PEC are known to be stimulated by various substances. Thus, as one control, preimmune globulin- or immune globulin-stimulated PEC were used and showed from 13 to 20% greater cytotoxicity toward infected cells in the absence of added antiserum in controls than did the basic infected cell controls without PEC but with added antiserum at highest concentration. The former control values were subtracted from experimental results. Background cytotoxicity with preimmune serum was less than 5%.

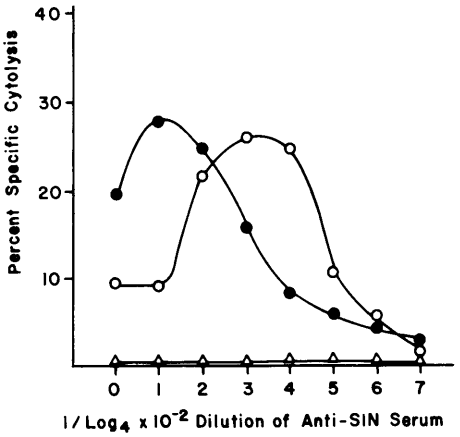

FIG. 1. ADCC assays of peritoneal exudate cells and virus-infected L929 cells (20:1) with varying dilutions of hyperimmune rabbit anti-SIN serum: SIN-infected (○), SF-infected (●), uninfected (△).

TABLE III. EFFECT OF SILICA TREATMENT ON THE CROSS-PROTECTION GIVEN BY EXTENDED HYPERIMMUNE ANTI-SIN γ GLOBULIN IN C3H MICE

Treatment[a]	Number dead/total	% Survival
Silica, anti-SIN globulin, SF	11/12	8
Anti-SIN globulin, SF	3/20	85
Silica[b]	0/12	100
SF	20/20	0
Silica, SIN (10^8 PFU)	0/11	100
SIN	0/11	100
Silica, anti-SF, SF	1/11	91
Anti-SF, SF	1/10	90

[a] 3 mg silica given iv at 24 hr before 0.5 ml γ globulin from extended hyperimmune anti-SIN serum, which was given ip 24 hr before 100 LD_{50} of SF (5 $\times$ 10^5 PFU) or SIN (10^8 PFU) given ip.

[b] No obvious clinical signs of illness observed in mice treated with silica alone.

Discussion. It is generally recognized that cross-protection across subgroups, as opposed to cross-protection within subgroups (4), is more difficult to demonstrate and/or that it is quantitatively lower. In our earlier reports on cross-serological reactions (6–8), we found that extended hyperimmunization with SIN caused the production of cross-reactive antibodies demonstrable in cross-ADCMC assays using SF-infected target cells. The presence of this cross-reactivity between different subgroups of alphaviruses provided us with a rationale to test such antisera for cross protection *in vivo* in the complete absence of any cross-neutralizing activity. We emphasize again that in

contrast to the reports of others (11–17, 27) who showed that nonneutralizing antibodies could protect mice from virulent virus challenge within a subgroup of alphaviruses, our experiments demonstrate passive cross-protection across subgroups.

The interpretation we give for our data is that extended hyperimmunization with an alphavirus of one subgroup (SIN) causes the production of antibodies directed against unique cross-reactive antigens on the surface of cells infected with an alphavirus of a second subgroup, namely, SF (9, 10). During normal, or short-term, hyperimmunization, antibodies showing cross-ADCMC were not detected (6–8) and the antisera would not passively immunize mice when SIN only was used as a vaccine to elicit cross-protection to SF (3). In our previous reports (1–3, 5), we proposed that cross-protection after two virus immunizations is established with cross-reactive CTL. However, we believe that the target antigens for cross-reactions observed for extended hyperimmune antibodies as seen, for example, in cross-ADCMC (9, 10) (and perhaps in cross-ADCC) and CTL (2, 3) may be similar. Since the target antigens are cell-bound, cytotoxicity of infected cells could be the basis of the mechanism involved in cross-protection of animals from lethal virus challenge. Further, these anti-SIN antibodies, which cross-react only with cell-associated SF-target antigens in ADCMC and passively cross-protect *in vivo,* provide a reagent that may be useful in the isolation and characterization of the antigens, or fragments therefrom, that might be employed in cross-subgroup vaccines.

Observations made in the analysis of ADCMC assays indicated that not unexpectedly, the more extended the immunization schedule, the higher the cross-ADCMC titers (unpublished findings); however, cross-neutralization activity was always undetectable. It appeared reasonable to test whether antisera with high cross-ADCMC titers could be used passively to demonstrate cross-protection. The motivation to do this was increased by reports from two different laboratories which showed that nonneutralizing monoclonal antibodies (MoAb) could protect mice from challenge by homologous virus or by virus within the same subgroup. One laboratory (11, 12) reported that nonneutralizing MoAb to SIN E1 and E2 glycoproteins protected mice against a neuroadapted variant *in vivo.* They demonstrated that the protective capacity of MoAb to either E1 or E2 occurred in mice decomplemented with CoVF but specific binding could be shown *in vitro* with enzyme-linked immunosorbent (ELISA) or ADCMC assays. Further, this group found that the majority of E1 epitopes present on SIN-infected cells appeared to become cryptic during SIN maturation because, except at low pH, they were undetectable on virions (17). Another laboratory (15) carried out homologous passive antibody experiments involving prevention and therapy with respect to wild-type and neuroadapted SIN virus, which extended in part the finding of others with SF virus (16). Monoclonal antibodies to a variety of epitopes could be differentiated into groups that were primarily effective in prevention and groups that were primarily effective in therapy after virus was found in the brain in substantial amounts. Additional reports indicate that nonneutralizing MoAb to glycoproteins of the alphavirus Venezuelan equine encephalitis (13, 14) and Western equine encephalitis (27) protected mice from homologous virus challenge. There was no correlation between passive protection and a role for complement, but there was a role suggested for the Fc portion of the antibody.

In a recent report, passive protection of mice against SF was ascribed to a number of factors, including mechanisms involving nonneutralizing MoAb that inhibited virus growth in cells and enhanced the uptake and clearance of virus by macrophages (16). The latter conclusion was based on *in vitro* effects of the nonneutralizing MoAb to retard virus growth in L cells and promote the uptake of virulent SF in Fc receptor-bearing WEHI-3 cells. The role of the Fc portion of the antibody in complement activation (ADCMC) and macrophage function was emphasized when it was noted that $F(ab')_2$ fragments of nonneutralizing MoAb gave no protection and $F(ab')_2$ fragments of neutralizing MoAb were 300 times less effective than intact MoAb in protecting mice.

In other virus systems, nonneutralizing MoAb to vesicular stomatitis virus serotypes Indiana and New Jersey protected mice from lethal challenge. The MoAb reacted with cell-

associated viral determinants and lysed virus-infected cells in the presence of complement (28). It was also found that nonneutralizing MoAb to Sendai virus protected mice from challenge (29).

As indicated earlier, our experiments with alphaviruses, unlike the reports of others on alphaviruses, emphasize cross-protection and cross-reactions among viruses from different subgroups; passive administration of γ globulin prepared from extended hyperimmune anti-SIN sera did protect C3H/HeJ mice against 100 LD_{50} of SF. However, even with high cross-ADCMC titers, it was found that cross-protection did not depend on complement, since CoVF given *in vivo* had no effect on cross-protection in mice. CoVF was shown to reduce complement markedly, in fact, to undetectable levels. On the other hand, macrophages appear to be required since methyl palmitate and silica substantially reduce the protection to SF challenge afforded by the γ globulin of extended hyperimmune anti-SIN serum. The inhibition of macrophage function by each macrophage-inhibiting agent did not induce untoward clinical signs in normal C3H/HeJ animals. In addition, our studies on cytolysis using ADCC assays *in vitro* show a far greater sensitivity when compared to the extent of cytolysis using ACDMC assays both in homologous and cross-reactions.

Thus, we believe we are the first to report that passive antibody-mediated protection across subgroups can be demonstrated with extended hyperimmune serum. In addition, on the basis of the *in vivo* and *in vitro* experiments together, one likely mechanism for the observed cross-protection is proposed to be ADCC, primarily involving macrophages.

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