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Immune Response in Rabbits to Virion and Nonvirion Antigens of the Flavivirus Kunjin

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The nature of the antibodies formed in rabbits in response to the following Kunjin virus antigens was examined: infectious suckling mouse brain (SMB), purified virion or rapidly sedimenting hemagglutinin (RHA), slowly sedimenting hemagglutinin (SHA), and envelope fragments prepared from RHA disrupted by 0.1 or 0.2% sodium deoxycholate (DOC). The hemagglutination-inhibiting (HI) and neutralizing antibody responses to SMB, RHA, and large envelope fragments (0.1% DOC) were remarkably uniform, antibodies appearing at the same time, attaining similar HI titers (lowest to envelope), and being of similar avidity early and late in the response. The 19S (immunoglobulin M) antibodies to all antigens were always relatively type-specific, whereas the 7S (immunoglobulin G) antibodies were always broadly cross-reactive in HI tests. These results confirm that the envelope antigen is the principal antigen involved in the stimulation of protective neutralizing antibodies and contains both type- and group-specific antigenic determinants. The results also establish that there is no significant advantage in using purified RHA or SHA either for immunization or as hemagglutinin antigens in attempts to obtain greater specificity in the HI test. No differences were detected in the antibody responses to infective Kunjin virus, within the range 1,400 to 10^9 plaque-forming units (PFU). Below 1,400 PFU, there was no detectable response. Inactivated virus (10^6 PFU) also stimulated the normal antibody response. In contrast, small envelope fragments (derived with 0.2% DOC) and a detergent-solubilized extract of infected cells were unable to stimulate a detectable antibody response and the small envelope fragments may have induced low dose tolerance in one of two rabbits.

The flaviviruses are a serologically related group of viruses in the family *Togaviridae*, many of which are human and/or animal pathogens (e.g., Murray Valley encephalitis, Japanese encephalitis, St. Louis encephalitis, yellow fever, dengue, Wesselsbron, louping ill viruses). Diagnosis of infection by use of the hemagglutination-inhibition (HI) test is difficult because of the cross-reactivity of antibodies to other members of the group (7, 11, 15, 16). Westaway (16) showed that the 7S (immunoglobulin G [IgG]) antibodies are cross-reactive, whereas the 19S (IgM) antibodies are relatively specific for the infecting virus in HI tests. Subsequently, it was demonstrated that, upon reinfection with another flavivirus, a specific 19S antibody response to this second virus can occur (7, 11, 18) which may be of diagnostic use.

Another approach to the problem of cross-reactivity has been the attempt to produce more specific antigens for serological tests. Flavivirus-infected suckling mouse brain (SMB) or tissue culture fluid contains the virion and other viral antigens separable by rate-zonal sedimentation in sucrose density gradients (5, 12, 13). There are two peaks of hemagglutinin (HA): the rapidly sedimenting hemagglutinin (RHA) or virion which contains three proteins, V1, V2, and V3, and the slowly sedimenting hemagglutinin (SHA), a nonvirion antigen made up of two of the three virion proteins (V1 and V3) and a virus-specified cytoplasmic protein (NV-2) (12, 20). A third antigen found at the top of the gradient is the soluble complement-fixing (SCF) antigen (5, 13). All these antigens were shown to possess both group- and type-specific antigenic determinants (2, 20). Other antigens studied include the virion envelope protein V3 (6) and solubilized virus-specified cytoplasmic proteins (10), of which one (NV-5) appeared to possess only a type-specific antigenic determinant.

Most studies on the immune response to flavivirus antigens have employed preparations of

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infected SMB (10, 13, 15, 16, 18) which, in addition to RHA, SHA, and SCF antigens, probably contain all the virus-specified cytoplasmic proteins (17). Comparisons of their antibody responses indicate that RHA and SHA can stimulate the production of both cross-reactive and type-specific HI antibodies (20) and that RHA can stimulate the production of neutralizing antibodies (2). However, no purified antigens have been used to study the nature of the immune response in further detail.

In this paper we report on the nature of the immune response in rabbits to various antigens from the flavivirus Kunjin, including infected SMB, RHA, SHA, and envelope preparations of the RHA.

MATERIALS AND METHODS

Viruses. The virus strain used for immunizations was Kunjin strain MRM61C. Murray Valley encephalitis strain MRM66, West Nile strain Sarafend, Japanese encephalitis strain Nakayama, and Kokobera strain MRM32 viruses (15, 16) were used as antigens also in HI tests.

Preparation of antigens. Vero cells were infected at a multiplicity of infection of 1 to 10 plaque-forming units per cell with infected SMB pools, and the cells were maintained in Eagle minimal essential medium containing 0.1% bovine serum albumin. Infectious culture fluids were harvested when the cells showed a 3+ to 4+ cytopathic effect, the pooled fluids were clarified by centrifugation, and the antigens were concentrated by precipitation with 8% polyethylene glycol 6,000 (5). This material was used as the HA antigen in HI tests. Kunjin virus RHA and SHA, both labeled with [^{3}H]leucine during growth in Vero cells, were separated by sedimentation through sucrose density gradients (5, 19). Small or large fragments of the viral envelope were prepared by treatment of the RHA with 0.2 or 0.1%, respectively, sodium deoxycholate (DOC; Koch-Light Ltd., Colnbrook, Bucks, England) at 4°C and were immediately separated by sedimentation through sucrose density gradients. Infected SMB antigens were the 10% clarified homogenates normally used as infectious Kunjin virus pools.

Preparation of antibodies. Adult rabbits were immunized by intravenous inoculation. Reimmunization was usually at 6- to 8-week intervals. Blood was collected from the marginal ear vein; the serum was separated and clarified, heat-inactivated (56°C for 30 min), and stored in a number of small portions at −20°C. Serum was acetone-extracted (4) to remove nonspecific inhibitors before testing for HI antibodies. Separation of 19*S* and 7*S* antibodies was done by sedimentation in 10 to 40% sucrose density gradients in an SW39 rotor at 35,000 rpm for 18 h (16). After salt precipitation of the immunoglobulins at room temperature with 18% (wt/vol) sodium sulfate (15), they were fractionated on a column (25 by 120 mm) of Whatman diethylaminoethyl (DEAE)-cellulose, DE52 (Whatman Ltd., Springfield Mill, Maidstone, Kent, England). Stepwise elution was carried out by use of 0.02, 0.04, 0.08, 0.15, and 0.3 M phosphate buffers at pH 8.0 at flow rates of 30 to 45 ml/h. A small amount of residual protein was eluted with 0.7 M phosphate buffer at pH 5.0. The proteins in the various peaks were analyzed by immunoelectrophoresis as described by Westaway (15).

HI test. A microtiter modification of the HI test of Clarke and Casals (4) was used (5, 15) in which antibody dilutions were incubated with 4 units of HA for 1 h at room temperature before addition of goose red blood cells in appropriate buffer.

Neutralization test. Plaque reduction assays of the neutralizing activity of antibodies were carried out by use of methods described by Westaway (15).

Electron microscopy. Specimens were placed on thin carbon films supported on 400-mesh copper grids and were stained with 1% aqueous uranyl acetate. The negatively stained specimens were examined in a Philips EM300 electron microscope at various magnifications and at an acceleration voltage of 80,000 kV.

RESULTS

Characterization of the antigens. The virion (RHA) and SHA antigens were prepared by banding in sucrose density gradients (Fig. 1a). Electron microscopic examination of the RHA (Fig. 2a) showed it to consist of spherical-shaped virions approximately 53 nm in diameter. The SHA peak was found to contain "doughnut"-shaped particles approximately 14 nm in diameter (Fig. 2b), similar to those of dengue type 2 virus SHA described by Smith et al. (13), and was contaminated with a small quantity of infectious virus (4.0×10^3 plaque-forming units [PFU]/ml). The major peak of infectious virus was the RHA (1.8×10^7 PFU/ml). Purified virions were treated with DOC to produce small (0.2% DOC) or large (0.1% DOC) fragments of the viral envelope which were purified by rate-zonal sedimentation (Fig. 1b and 1c, respectively). Neither of the envelope preparations could be shown to possess infectious virus, and only the large envelope fragments (Fig. 1c, sedimentation coefficients in the range of 100*S* to 120*S*) retained some ability to hemagglutinate red blood cells. Envelope preparations obtained by DOC treatment contain only the viral glycoprotein V-3 (19). The Kunjin SMB antigens were supernatants of homogenates clarified at low speed and hence contain RHA, SHA, the SCF antigen, and probably the virus-specified cytoplasmic proteins (5, 13, 17).

HI antibody response to antigen preparations. Comparisons of the HI antibody responses in rabbits at 7 days after immunization with various preparations of Kunjin virus revealed a remarkably uniform response which is apparently independent of whether purified virions (RHA) or SMB pools were used. With

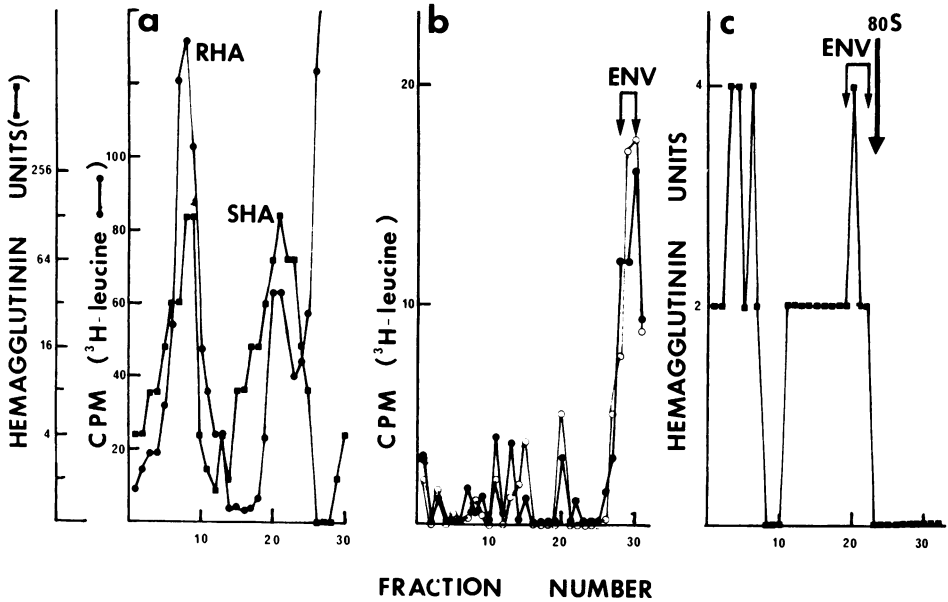

FIG. 1. *Preparation of Kunjin virus-derived antigens by sucrose density gradient ultracentrifugation in an* SW25.1 rotor at 25,000 rpm. (a) Purification of [³*H*]*leucine-labeled Kunjin rapidly sedimenting HA (RHA, the virion) and slowly sedimenting HA (SHA), by sedimentation of concentrated antigens through a 5 to 25% (wt/vol) gradient for 3 h. (b) Preparation of small envelope fragments by treatment of RHA from (a) with 0.2% DOC at 4°C immediately prior to layering onto 5 to 25% gradients and sedimenting for 3.5 h. The results of two identical preparations in parallel gradients are shown. There was no detectable HA activity in the gradients. (c) Preparation of large envelope fragments by treatment of purified unlabeled RHA (32 HA units) with 0.1% DOC at 4°C and sedimentation through a 5 to 20% gradient for 3 h. 80S Vero cell ribosome markers were sedimented in a parallel gradient. Sedimentation in all gradients is from right to left.*

RHA virus doses of 3.2×10^6 to 1.8×10^7 PFU/ml, titers from 1:160 to 1:1,280 were obtained (four rabbits). With SMB virus doses of 10^8 to 10^9 PFU/ml, titers of 1:320 to 1:640 were obtained (seven rabbits). A comparison of the HI antibodies found in whole serum from rabbits immunized with SMB, RHA, SHA, and large envelope fragments (Fig. 3) showed little difference in responses to SMB, RHA, and SHA on primary immunization. The large envelope fragments appeared to have a slightly slower response which was of a lower magnitude. On revaccination, SMB, RHA, and envelope induced an anamnestic response, whereas the SHA induced little, if any, response (Fig. 3).

19S and 7S HI antibody responses to RHA and to large envelope fragments. The 19S and 7S antibody responses to the intact RHA (virion) and large envelope fragments in rabbits are shown in Fig. 4. Antibody responses of both immunoglobulins to the envelope fragments were slower and lower in magnitude than those to the virion. The 19S antibody response to the envelope fragments was as high after revaccination as after the primary vaccination, whereas the 19S antibody response to RHA gradually decreased on subsequent vaccinations. The 7S antibody responses for both antigens were similar except that titers were lower when the rabbits were injected with envelope fragments. In comparisons of the specifities of the HI antibodies (Fig. 5), the early 7S antibodies were broadly cross-reactive whereas the 19S antibodies were relatively specific, the homologous reactions being at least eightfold greater than the cross-reactions; the 19S antibodies to the envelope fragments tended to be even more specific than those to the RHA. The 19S antibodies, whether found early (7 to 14 days after infection) or late (21 days after primary infection or after reinfection) in the response, were always relatively specific.

Antibody response to purified RHA and to UV-inactivated Kunjin virus. A single immunizing dose of 1.4×10^5 or 1.4×10^3 PFU of purified RHA preparations produced normal 19S and 7S HI antibody responses in IgM and in IgG (similar to those in Fig. 4). A further 100-fold reduction in dose produced no response. Because amplification of the higher doses may have occurred as a result of virus multiplication, the response to ultraviolet (UV)-inacti-

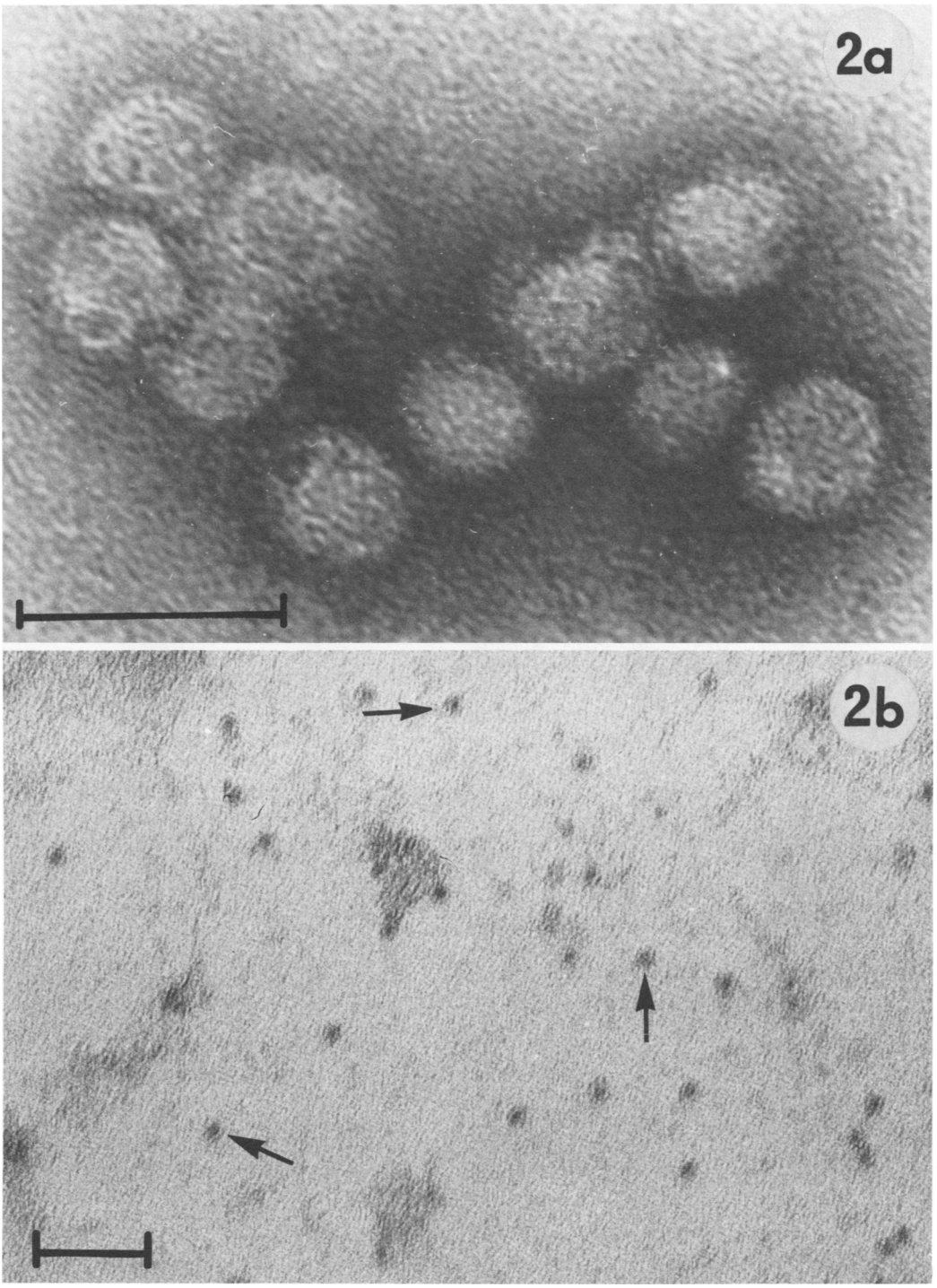

FIG. 2. *Electron micrographs of Kunjin virus antigens after negative staining with 1% aqueous uranyl acetate. (a) Purified Kunjin RHA (virons). (b) Slowly sedimenting HA (SHA) from Kunjin virus purification gradient (Fig. 1a). Arrowed are some of the approximately 14-nm diameter "doughnut"-shaped particles. The bars represent 100 nm.*

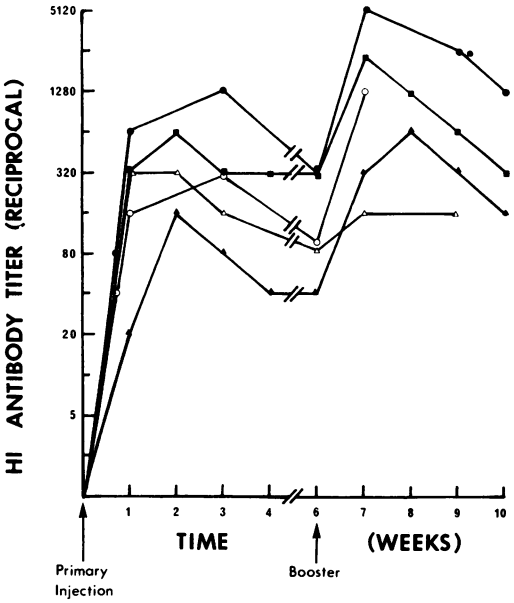

FIG. 3. *HI antibody response in rabbits immunized with various Kunjin antigens. Microtiter assays of antibodies in acetone-extracted serum were performed with 4 units of Kunjin virus HA. Symbols: ■, anti-SMB; ●, anti-RHA; ○, anti-RHA; ▲, anti-large (0.1% DOC) envelope fragments; △, anti-SHA.*

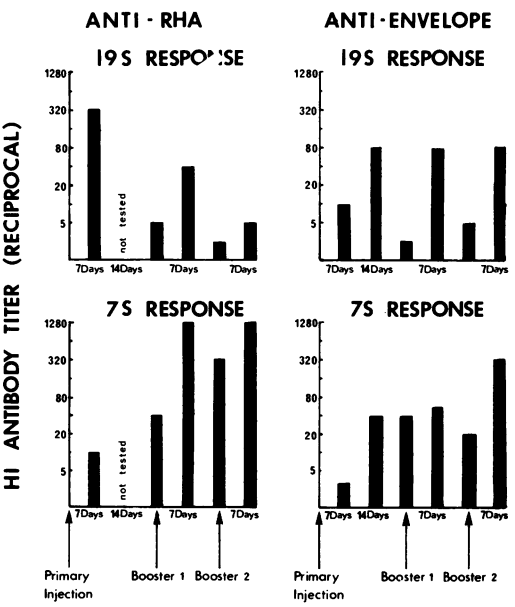

FIG. 4. *7S and 19S HI antibody response in rabbits when immunized with purified Kunjin RHA (virions) and large envelope fragments (prepared by treating RHA with 0.1% DOC). The sera were fractionated by sucrose density gradient ultracentrifugation, and the 7S and 19S fractions were then titrated against 4 units of Kunjin virus HA. There was a 6- to 8-week interval between immunizations.*

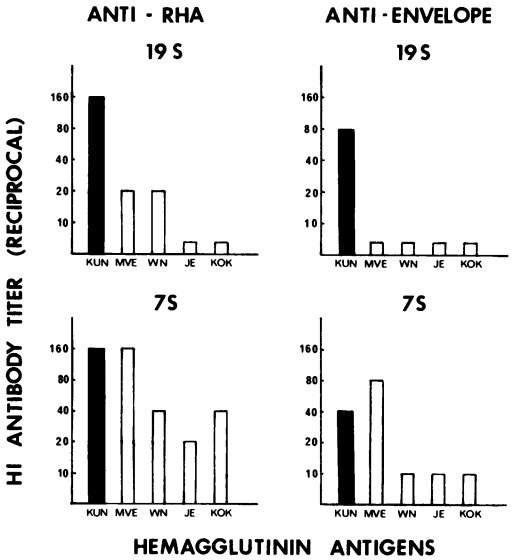

FIG. 5. *Specificity to related flavivirus HAs of the HI 7S and 19S antibodies formed in rabbits against purified Kunjin RHA virions or large envelope fragments (0.1% DOC) at 7 or 14 days, respectively, after immunization. The sera were fractionated by sucrose density gradient ultracentrifugation. Kunjin (KUN), Murray Valley encephalitis (MVE), West Nile (WN), and Japanese encephalitis (JE) viruses are members of the same serological subgroup (18). Kokobera (KOK) is outside this subgroup. Four units of viral HA were used in these titrations.*

vated virus was studied. Kunjin virus in 0.012 M tris(hydroxymethyl)aminomethane (Tris)-0.12 M NaCl buffer (pH 7.4) containing 0.2% bovine serum albumin and of 0.5 mm depth was exposed to irradiation by a UV germicidal lamp (Oliphant, South Australia) at a distance of 240 mm. Samples taken at 30-s intervals were titrated by plaque assay. A linear logarithmic decrease in residual infectivity was observed, the linear regression line equation being: log surviving fraction = $0.1 - 2.4 \times$ time (in minutes). After 3 min, less than 10^{-7} of infectious virus survived. A preparation of RHA containing 3.2×10^6 PFU/ml was divided into two fractions. One fraction was UV-irradiated for 6 min under conditions identical to those described above, so as to destroy all infectivity. The other fraction was used for the infectious virus controls. Pairs of nonimmune rabbits were inoculated with 1 ml of each preparation by the intravenous route. They were bled 7 and 10 days later. The 10-day HI antibody titers were similar to those in Fig. 3 for both sets of rabbits; however, the initial (7-day) responses to the inactivated virus were lower (titers of 1:40 and less than 1:20) than to the live virus (titers of 1:160 and 1:1,280). Clearly, the anti-

genic mass represented by about 10^6 PFU of inactivated virus (possibly up to 100-fold more virus particles; 1) was sufficient to induce a slightly delayed but substantially normal antibody response.

Is there a lower limit to the size of the envelope antigen needed to elicit an immune response? RHA containing 128 HA units was treated with 0.2% DOC to prepare (Fig. 1b) small envelope fragments, which may be no more than monomers or dimers of the envelope glycoprotein V3 (19). Two rabbits were given two intravenous inoculations of this preparation with 1 month between the inoculations, and were bled each week for 2 months. No HI nor neutralizing antibodies could be detected in any of the sera. Each rabbit was then challenged with UV-inactivated Kunjin virus (3.2×10^6 PFU prior to inactivation) to determine whether either had been primed by the small envelope antigen. There was an HI and a neutralizing antibody response in only one rabbit, and its response could not be distinguished from the response in two control, previously unimmunized rabbits. The nonresponder was the first rabbit failing to respond in our experience with more than 30 rabbits (Westaway and Della-Porta, unpublished data).

We attempted also to produce HI antibody to envelope protein V3 in monomeric form by immunization with Kunjin virus-infected cells solubilized in 1% sodium dodecyl sulfate and 1% dithiothreitol (17). Rabbits immunized twice with such extracts (dialyzed to remove sodium dodecyl sulfate) were unable to respond.

Increase in charge distribution of IgG antibodies during the immune response. Westaway (15) demonstrated that very little electrophoretically slow IgG with antibody activity was formed early in the immune response in rabbits to SMB-derived flavivirus antigens, and that the main activity resided in the electrophoretically fast IgG molecules. As the response progressed, the amount of electrophoretically slow IgG antibodies with HI activity increased. Salt-precipitated globulins from sera collected from rabbits immunized with RHA or large envelope fragments were separated by ion-exchange chromatography on DEAE-cellulose (Table 1), and the immunoglobulin fractions so obtained were characterized by immunoelectrophoresis. Fractions 1 and 2 eluted with 0.02 and 0.04 M phosphate buffers, respectively, and contained only IgG, the second fraction being electrophoretically faster. Fraction 3 eluted with 0.08 M phosphate buffer contained IgG and four to five other serum proteins (but not IgM). Fractions 4 and 5 eluted with 0.15 and 0.3 M phosphate buffers contained mainly IgM, with a very small amount of IgG, and several other serum proteins. The IgM antibodies showed no variation in distribution of charge during the response, and their HI antibody titers and specificities were similar to those observed for the corresponding 19S antibodies in Fig. 4 and 5, and as reported by Westaway (16). In contrast, the IgG fractions showed a variation of charge distribution in relation to HI antibody titers (Table 1) during the immune response. Initially, the major activity resided in fraction 2; subsequently, the titers in fractions 1 and 2 slowly increased. Upon reimmunization, the HI antibody activity increased in all fractions, particularly in the electrophoreti-

TABLE 1. *Sequential development of HI antibody activity in immunoglobulin fractions of antisera produced by rabbits immunized with Kunjin virions or with large fragments of envelope*

Immunoglobulin fraction	Elution buffer (M)[a]	Antibody activity[b]					
		Antivirion (RHA)[c]			Antienvelope[d]		
		7 days[e]	21 days	7 days PB	14 days[e]	21 days	7 days PB
1	0.02	5	20	2,000	10	20	40
2	0.04	80	160	2,560	20	20	80
3	0.08	5	80	320	5	5	10
4	0.15	160	40	80	40	20	40
5	0.3	160	20	20	320	10	80

[a] Fractions of salt-precipitated globulins eluted from a DEAE-cellulose ion-exchange column by use of phosphate buffers, at pH 8.0, with increasing molarities. Each increase in buffer molarity eluted an IgG fraction of greater electrophoretic mobility. IgM was detected only in fractions 4 and 5, where it was the main immunoglobulin.

[b] Reciprocal of the HI antibody titer against 4 units of Kunjin virus HA. Titers of all fractions were adjusted in comparison with original serum volume.

[c] Anti-virion (RHA) serum produced using 1.8×10^7 PFU of purified virus. The second immunization was given 2 months after the primary immunization.

[d] Antienvelope serum against large envelope fragments produced by treatment of RHA with 0.1% DOC. A 2-month interval occurred between injections.

[e] Days after immunization; 7PB, 7 days post-booster immunization.

cally slowest, fraction 1. Comparisons of HI antibody specificity (Table 2) showed that antibodies in fraction 3 were, in general, strongly cross-reactive at all times; fractions 1 and 2 tended to be more specific, but were inferior to IgM (Fig. 5) in specificity. These results show that the immune responses to purified infectious virus and to separated envelope antigen are similar to those observed previously with crude infectious pools, both in regard to the charge and to the antibody specificity of the immunoglobulin classes.

Neutralizing antibody response. A comparison of the antibody responses to infected SMB, RHA, and large envelope fragments shows (Fig. 6) that all these antigens stimulate production of neutralizing antibodies. Regression line analyses established that there was little, if any, difference in the magnitude of the response (represented by the intercept A) and in the avidity (represented by the slope B) of the antibodies formed at comparable times for all these antigens. The SHA neutralizing antibody response was not included in this comparison because the SHA antigen contained infectious virus (4×10^3 PFU/ml) which was above the minimal dose required to stimulate an antibody response.

TABLE 2. *Cross-reactivity of HI antibodies in IgG fractions of rabbit antisera to purified antigens of Kunjin virus*

Serum[a]	HA antigen in test[b]	Normalized value[c] of HI titer of antibodies in fraction[d]		
		1	2	3
7-day immune anti-RHA	KUN	100	100	100
	MVE	–[e]	3	100
	JE	–	<3	100
	KOK	–	3	400
21-day immune anti-RHA	KUN	100	100	100
	MVE	25	25	25
	JE	12	6	6
	KOK	<12	3	6
7-day post-booster anti-RHA	KUN	100	100	100
	MVE	25	50	200
	JE	25	12	50
	KOK	1	3	6
14-day immune anti-ENV	KUN	100	100	100
	MVE	100	25	50
	JE	<12	<12	<25
	KOK	<12	<12	<25
21-day immune anti-ENV	KUN	100	100	100
	MVE	200	50	100
	JE	<12	6	<6
	KOK	25	6	<6
7-day post-booster anti-ENV	KUN	100	100	100
	MVE	25	25	400
	JE	12	12	100
	KOK	<12	12	100

[a] Same sera as described in Table 1.

[b] Four units of HA in test. Kunjin (KUN), Murray Valley encephalitis (MVE), and Japanese encephalitis (JE) viruses are in the same serological subgroup; Kokobera (KOK) virus is outside this subgroup (18). HA antigens were prepared from infected cell culture fluids (5).

[c] Values of the HI antibody titers were normalized by taking the value of 100 for the HI antibody titer against homologous (Kunjin) HA and adjusting titers of heterologous reactions against this value.

[d] Fractions eluted from a DEAE-cellulose column with 0.02, 0.04, and 0.08 M phosphate buffers as described in Table 1.

[e] Heterologous HI antibody titer was less than the homologous titer, and the latter was at the limit of detection (1 in 5).

DISCUSSION

The SMB, RHA, and large envelope fragment antigens of Kunjin virus all stimulate an HI antibody response in rabbits in which the 19*S* antibodies are relatively type-specific and the 7*S* antibodies are clearly cross-reactive. This confirms that the previous use of crude SMB flavivirus antigens for immunization probably suffered little in regard to specificity in immunoglobulin fractions of HI antibodies produced against flaviviruses (15, 16, 18). There was little, if any, difference between the antibody reactions in HI tests with HA antigens, such as crude infected SMB, with tissue culture antigens concentrated by polyethylene glycol precipitation, or with purified RHA and SHA (20). Our present results establish that there is no significant advantage in using purified RHA or SHA either for immunization or as HA antigens in attempts to obtain greater specificity in the HI test.

The HI antibody responses to infectious virus (either SMB or RHA) in doses of 10^6 to 10^9 PFU were shown to be remarkably uniform, no rabbit failing to respond. The final magnitude of the response was independent of the dose of infectious virus until below 1,400 PFU, but there was a slight delay in the onset of the response as the virus dose was decreased. These results are similar to those obtained by Mason et al. (8) for the flavivirus yellow fever in monkeys, in which an immunizing end point of around 3,000 LD_{50} was observed and in which there was little dependence on dose, above this lower level. In contrast, the flavivirus Powassan produced in hamsters a better response (and greater viremia) with low doses (10 LD_{50}) than with high doses ($10^{5.0}$ LD_{50}) of virus (3). In this report we show that UV-inactivated Kunjin virus (3.2×10^6 PFU) stimulates an immune response in rabbits; the responses to inactivated virus, to separated envelope antigen (pre-

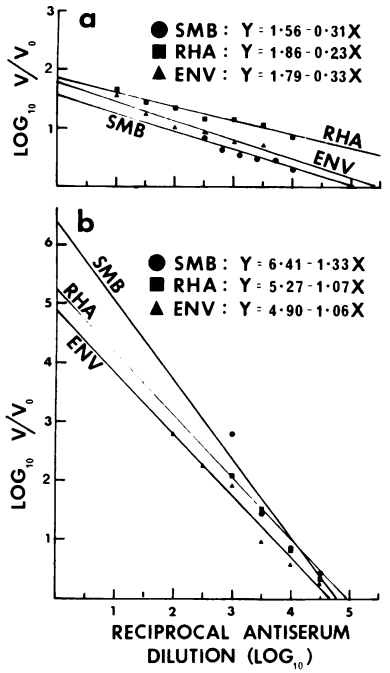

FIG. 6. *Dose-response relationships in plaque reduction neutralization assays using antisera against different Kunjin virus antigens: SMB (●) is the virus pool from infected suckling mouse brain, RHA (■) is rapidly sedimenting HA (purified virions), and ENV (▲) is derived envelope, prepared by treatment of RHA with 0.1% deoxycholate to produce large envelope fragments (Fig. 1c). The linear regression line equation for each dose-relationship is shown on the curve and was calculated as described by Westaway (15). The regression line equation is of the form Y = A + BX, where Y represents the $\log_{10}$ decrease in virus titer ($\log V/V_0$) and X is the $\log_{10}$ reciprocal of the antiserum dilution. (a) Sera collected 7 days after primary immunization. (b) Post-booster sera collected 7 and 14 days after second immunization of RHA and ENV antigens, respectively, and 7 days after a third immunization of SMB antigens.*

pared with 0.1% DOC), and to low doses of infectious purified virus are slower than to high doses of infectious virus, but similar antibody titers are eventually attained in all groups of animals.

The sequential development of IgM (19S) and IgG (7S) antibodies to the SMB, RHA, and large envelope fragments is similar to that described for other SMB-flavivirus antigens (15, 16, 18). The IgG antibodies, initially electrophoretically fast, later developed a large increase in the electrophoretically slow IgG fraction (Table 1; 15). Electrophoretically slow fractions of IgG (eluted with 0.02 and 0.04 M phosphate buffer) were more specific in HI tests (Table 2), and this specificity tended to increase during immunization, compared with that of the small amount of electrophoretically faster IgG (eluted with 0.08 M phosphate buffer). These results are in accord with our previous studies in rabbits on the sequential development and specificity in HI (and neutralization) tests of IgM and IgG fractions separated by ultracentrifugation or by anion-exchange chromatography (15, 16, 18), but using ion-exchange resins from different sources.

The envelope antigen, protein V3, is clearly of paramount importance (i) in the induction of neutralizing antibodies, necessary for protection (21), and (ii) as the major, possibly sole, antigen in the simplest serological test (HI) and in the most specific test (neutralization). In this report we show unequivocally that large envelope fragments from the virion (containing V3) stimulate both an HI and a neutralizing antibody response in rabbits. The production of cross-reactive 7S antibodies and of type-specific 19S antibodies confirms that protein V3 incorporates both group- and type-specific determinants, as proposed previously by independent but less direct methods (10, 18). The type specificity and magnitude of the immune response suggest that the larger envelope fragments could provide the basis for useful and specific subunit vaccines for selected flaviviruses. Despite the cross-reactivity of flavivirus antibodies, these antibodies confer protection only against homologous virus infection (7, 11). Envelope protein V3 in the form of monomers or small envelope fragments is less attractive as an immunogen, although both group- and type-specific determinants may be expressed in some serological tests (6, 10). We were unable to stimulate a detectable antibody response to envelope preparations apparently fully solubilized from the virion (RHA) by 0.2% DOC or from infected cells by 1% SDS; indeed, low dose tolerance (9) may have been induced in the rabbit failing to respond to challenge.

Recent reports indicate that the determinants of the envelope protein V3 in extracts from flavivirus-infected cells and in virions are not antigenically equivalent (6, 14). In the present study, there appears to be a difference in the antibody activities of antisera prepared against V3 in large envelope fragments and in RHA; although the neutralizing antibodies are equivalent, the HI antibody response to large envelope fragments is much reduced (compare results in Fig. 4 and 6). Further studies on the role of envelope protein V3 in immunization and in regard to specificity in diagnostic tests are required to define precisely this antigen of most interest and challenge in flavivirus infections.

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