JOURNAL OF VIROLOGY, Oct. 1970, p. 500-506
Vol. 6, No. 4

Dengue Virions and Antigens in Brain and Serum of Infected Mice

WALTER E. BRANDT, ROBERT D. CARDIFF, AND PHILIP K. RUSSELL
Department of Virus Diseases, Walter Reed Army Institute of Research, Washington, D.C. 20012

Received for publication 5 May 1970

The temporal relationships of the production of infectious dengue-2 virus and its antigens were investigated in intracerebrally infected suckling mice. Infectious virus, a slowly sedimenting noninfectious hemagglutinin (SHA), and a noninfectious soluble complement-fixing antigen (SCF) were found in the brain. Serum contained high concentrations of SCF antigen relative to infectivity when compared to SCF to infectivity ratios in the brain. Degradation of virions by Tween-80 and ether produced two antigens with sedimentation characteristics similar to the noninfectious antigens occurring naturally in infected tissues. However, the virion-derived SHA differed from native SHA when examined by electron microscopy and by equilibrium centrifugation in cesium chloride. Virion-derived SCF (as well as the virions and both SHA antigens) was denatured by sodium lauryl sulfate (SLS) and 2-mercaptoethanol (2-ME), whereas native SCF retained its complement-fixing activity. SLS and 2-ME treatment of dengue-1 and dengue-2 sucrose-acetone antigens increased their serotypic specificity. The hemagglutinin present in sucrose-acetone antigens was predominantly native SHA.

Dengue-2 virus infection of cell cultures or suckling mice results in the production of at least three physically distinct antigens (5, 6). These antigens are readily separated by rate zonal centrifugation into a rapidly sedimenting infectious hemagglutinin (RHA), a slowly sedimenting noninfectious hemagglutinin (SHA), and a nonhemagglutinating soluble complement-fixing antigen (SCF). RHA has been demonstrated to be the complete dengue-2 virion, but whether SHA and SCF are structural components of RHA or nonstructural antigens has not been determined. To examine the temporal and biophysical relationships between the virions (RHA) and other dengue antigens (SHA and SCF), two experimental approaches were used: examination of the sequential appearance of each antigen in the brain and blood of infected mice and comparison of noninfectious antigens found in brain tissue with those antigens derived from the virion by chemical degradation.

The recognition of several physically distinct antigens raised the question of which antigens are present in standard diagnostic antigen preparations. Sucrose-acetone antigens were analyzed by physical methods to determine the nature of their antigenically active components.

MATERIALS AND METHODS

Preparation of partially purified HA antigens. Suckling mice (1- to 3-day ICR strain) were inoculated intracerebrally with 10^4 plaque-forming units (PFU) of New Guinea "C" dengue-2 virus in the 33rd mouse passage. Brains were harvested from moribund mice 5 days later and homogenized (20%, w/v) in 0.02 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.2). These suspensions were clarified by the addition of 3 mg of protamine sulfate per ml and centrifugation at 9,000 × *g* for 1 hr at 4 C. Antigens were sedimented from the supernatant fluid at 105,000 × *g* for 2.5 hr and resuspended in a volume of buffer effecting a 100-fold concentration.

Density gradient centrifugation. Rate zonal separation of dengue virus antigens was carried out in 27-ml gradients preformed with 5 to 25% (w/v) sucrose in Tris buffer. Samples (2 ml) were layered on top of each gradient and centrifuged in a Beckman 25.1 rotor at 63,000 × *g* for 3 hr at 3 C. Approximately 30 fractions (1 ml) were collected dropwise through the bottom of the centrifuge tube. Under these conditions as previously described (5), RHA (virion) is found in fractions 4 to 8 near the bottom of the tube, SHA is found in fractions 18 to 22, and SCF is found in fractions 27 to 29.

Equilibrium centrifugation in CsCl was accomplished by adjusting samples to approximately 1.23 g/cm^3 with a saturated solution of CsCl in Tris buffer. After centrifugation of a 5-ml sample at 104,000 × *g* for 40 hr at 3 C (Beckman SW-39 rotor), fractions were collected as above. Refractive indexes of the CsCl fractions were obtained in a Bauch & Lomb refractometer and converted to density by the method of Ifft et al. (3).

Antigen assay. Hemagglutinating (HA) antigen determinations were carried out in microtiter plates

(Linbro Chemical Co., New Haven, Conn.) by standard methods (2) with goose erythrocytes at *p*H 6.2. Complement fixation tests were carried out by a microtiter modification of the method described by Kent and Fife (4) utilizing hyperimmune mouse ascitic fluid as a source of excess specific antibody (1). Infectivity titrations were carried out by plaque assay in LLC-MK_2 cell cultures.

Electron microscopy. Single-drop samples of appropriate sucrose gradient fractions were placed directly onto grids covered with carbon-coated collodion membranes prewashed in chloroform. Specimens were allowed to settle for several minutes and the excess was removed by pipette. The grids were inverted on water to remove sucrose, dried, and stained with 1% aqueous uranyl acetate. Micrographs were taken at 58,000 magnification in a Hitachi 11B electron microscope.

RESULTS

Sequence of appearance of infectious virus and antigens. Approximately 100 suckling mice were sacrificed at daily intervals for 6 days after intracerebral inoculation of 10^4 PFU, and the antigen content of pooled brains was compared to that of serum. Crude 20% brain suspensions were clarified at 9,000 × *g*, and samples were stored at −70 C for analysis of SCF antigen and infectivity. HA antigens were recovered from the remainder as described above, except for a 16-fold rather than 100-fold concentration after sedimentation in the ultracentrifuge. Infectivity titers of brain suspensions and serum are shown in Fig. 1 in comparison with HA and SCF antigen assays. An increase of infectious virus in brain was detected 2 days after inoculation, and a peak titer of 5.5 × 10^6 PFU was attained on day 5. In contrast, no infectious virus was detectable in serum on day 2, and only low titers (2.1 to 3.0 × 10^2 PFU) were present on days 3 through 6. Brain HA antigen was detectable at low levels (1:8) on day 2 and reached a maximum titer of 1:8,192 on day 6. HA antigen was not found in serum until day 5 and only then in low titer (1:10); serum samples required acetone extraction (2) before antigen analysis to remove nonspecific inhibitors of HA.

In contrast to the marked differences observed

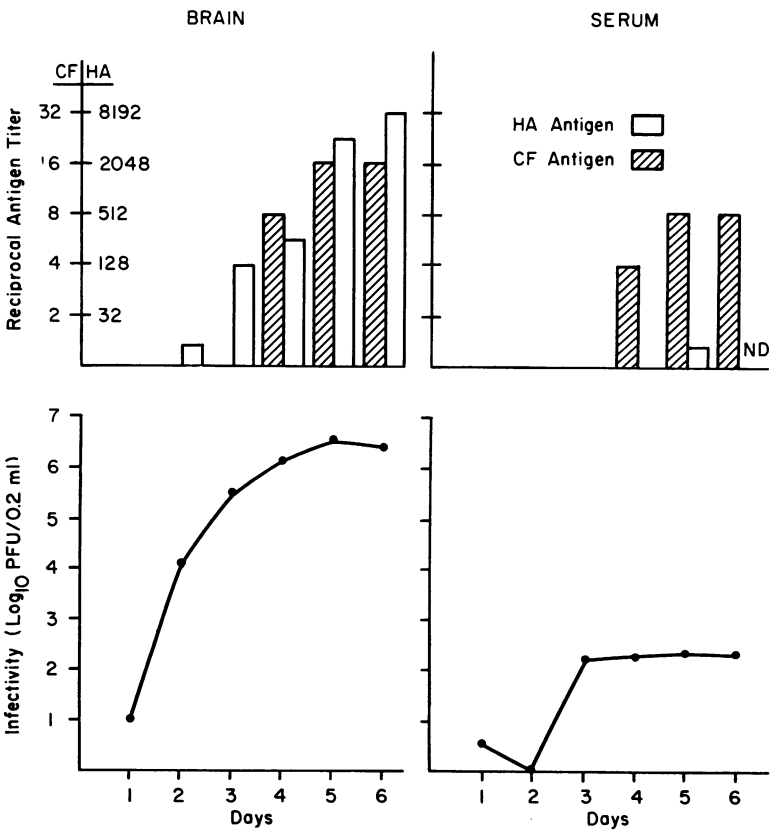

FIG. 1. *Upper quadrants depict the complement fixation (CF) and hemagglutinin (HA) titers of infected mouse brain (left) and serum (right) at daily intervals after intracerebral inoculation of 10^4 plaque-forming units (PFU). Lower quadrants show the amount of infectious virus in the same samples.*

between brain tissue and serum in HA antigen and infectious virus content, SCF antigen titers in brain and serum were similar (Fig. 1). SCF antigen was first detected on day 4 in both brain and serum. Brain SCF antigen titered 1:16 and serum SCF antigen titered 1:8 on days 5 and 6; on those same days, there was more than a 10,000-fold difference in brain and serum infectivity titers. SCF antigen titers represent peak titers of fractions in the SCF region at the top of a sucrose gradient similar to those in Fig. 2 (shaded area).

HA antigen in brain harvested on days 3 through 6 was of sufficient concentration for sedimentation studies on sucrose gradients. As shown in Fig. 2, HA activity on day 3 consisted predominantly of RHA (virions), SHA being barely detectable. Subsequently, the SHA component gradually increased until peak titers were the same or higher than RHA.

Sedimentation analysis of Tween-80 and ether-treated RHA. To determine if SHA and SCF could be derived by chemical degradation of whole virus, a purified preparation of virions was obtained in the following manner. Partially purified and concentrated HA antigens (described above) were centrifuged in a sucrose gradient (Fig. 3a); a pool of fractions 3 to 7 contained virions when examined by electron microscopy (Fig. 4a). One sample of the pool was dialyzed in Tris buffer and placed on another sucrose gradient (Fig. 3b). Another sample was treated with Tween-80 (5 mg/ml) and was vigorously agitated (Vortex mixer) for 5 min. This treatment was followed by addition of ether (USP) to a final concentration of 10% and mixed for another 5 min. Ether was removed by evaporation under a stream of nitrogen, and the sample was dialyzed in Tris buffer for 18 to 40 hr before analysis on sucrose gradients (Fig. 3c). The Tween-80-ether treatment converted the virions to a slowly sedimenting HA antigen (derived SHA) that

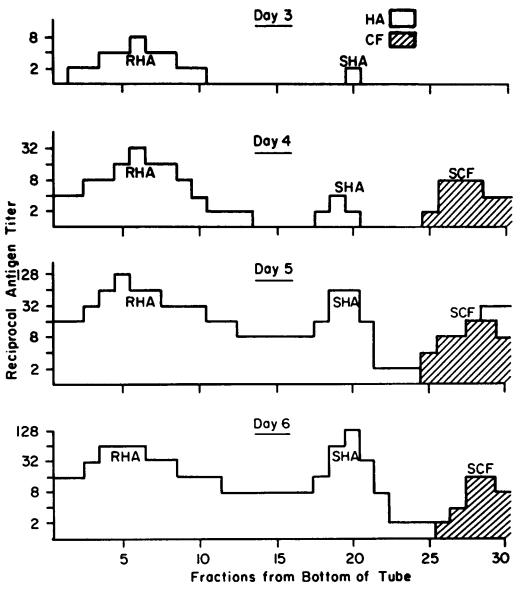

FIG. 2. *Rate zonal centrifugation of infected mouse brain suspensions taken at daily intervals after intracerebral inoculation. The sucrose gradients (5 to 25%) were centrifuged at 63,000 × g for 3 hr at 4 C. RHA, rapidly sedimenting hemagglutinin; SHA, slowly sedimenting hemagglutinin; SCF, soluble complement-fixing antigen.*

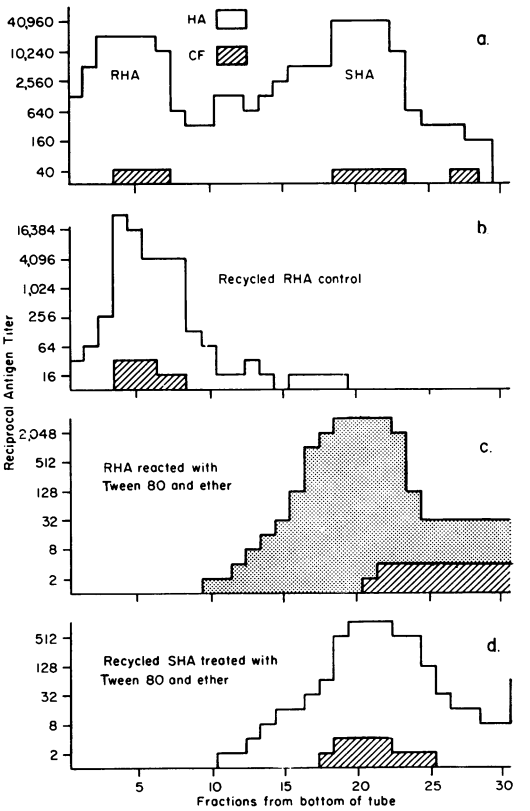

FIG. 3. *(a) Rate zonal centrifugation of concentrated dengue-2 antigens on 5 to 25% sucrose gradients at 63,000 × g for 3 hr at 4 C. RHA, rapidly sedimenting hemagglutinin; SHA, slowly sedimenting hemagglutinin. (b) Recentrifugation of a sample of RNA after dialysis in Tris buffer. (c) Recentrifugation of a second sample of RHA after treatment with Tween-80 and ether and dialysis in Tris buffer. Stipled area indicates a "derived" HA antigen to distinguish it from naturally occurring or "native" SHA shown in panels (a) and (d). (d) Recentrifugation of native SHA after treatment with Tween-80 and ether and dialysis in Tris buffer.*

sedimented in a similar manner as native SHA (Fig. 3d). However, electron micrographs of derived SHA revealed ill-defined aggregates of 6 to 7-nm particles (Fig. 4c) in contrast to 14-nm ring structures in native SHA (Fig. 4b). Virion-derived SHA and native SHA similarly treated with Tween-80-ether were compared by equilibrium centrifugation in CsCl (Fig. 5). Native SHA banded at 1.23 g/cm^3, whereas derived SHA appeared to peak at 1.19 g/cm^3 but extended in a smear from 1.22 to 1.17 g/cm^3.

A slowly sedimenting complement-fixing antigen was also derived from RHA by treatment with Tween-80 and ether (Fig. 3c). The titer of this heterogeneous virion-derived CF antigen was reduced to undetectable levels by 0.1% sodium lauryl sulfate (SLS) and 2-mercaptoethanol (2-ME) at 37 C for 30 min, whereas the titer of native SCF antigen from either infected mouse brain or serum was not affected. The CF activity of intact virions and native SHA obtained from sucrose gradients was also reduced to undetectable levels by the combination of SLS and 2-ME followed by dialysis in Tris-saline buffer for 18 hr, whereas native SCF was unaffected. Since mouse brain contaminants remain near the surface of sucrose gradients where native SCF is found, normal mouse brain was added as a control to all dengue antigens purified on sucrose gradients before addition of SLS and 2-ME. Thus,

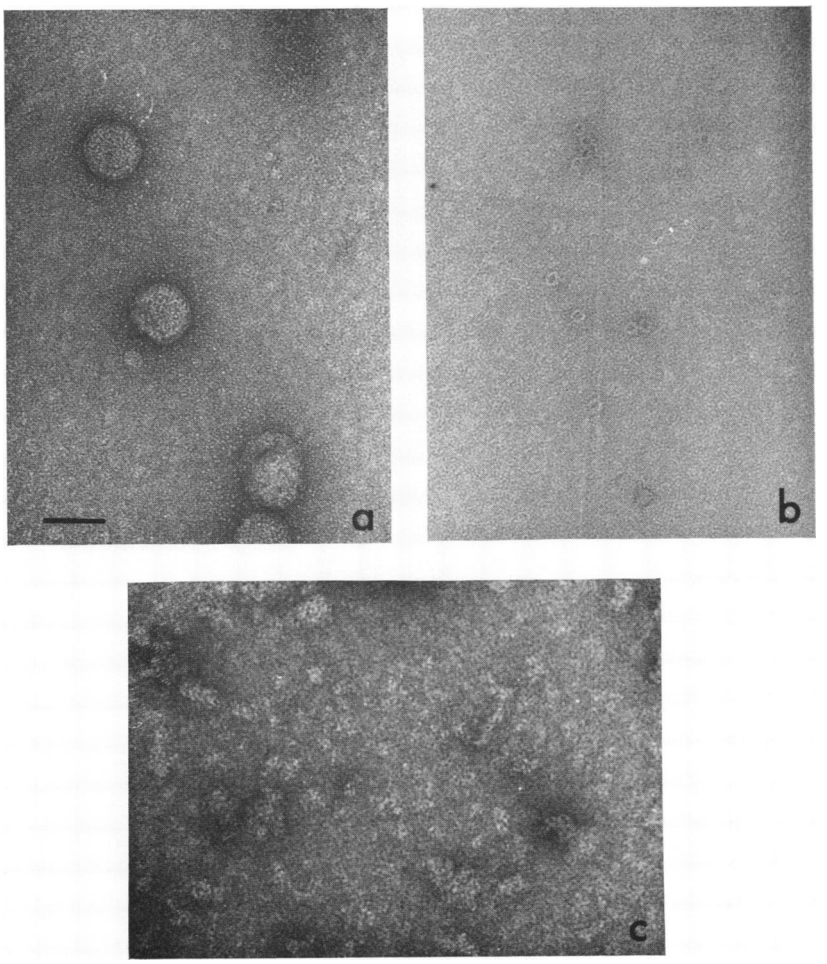

FIG. 4. *Electron micrographs of dengue HA antigens isolated in sucrose gradients (145,000 magnification); bar = 50 nm. (a) Rapidly sedimenting HA (RHA) contains complete virions. (b) Slowly sedimenting native HA (SHA) treated with Tween-80 and ether contains "ring" structures. (c) Slowly sedimenting HA derived from complete virions by Tween-80-ether treatment (derived SHA) contains aggregates of small doughnut particles each measuring 7 nm in diameter.*

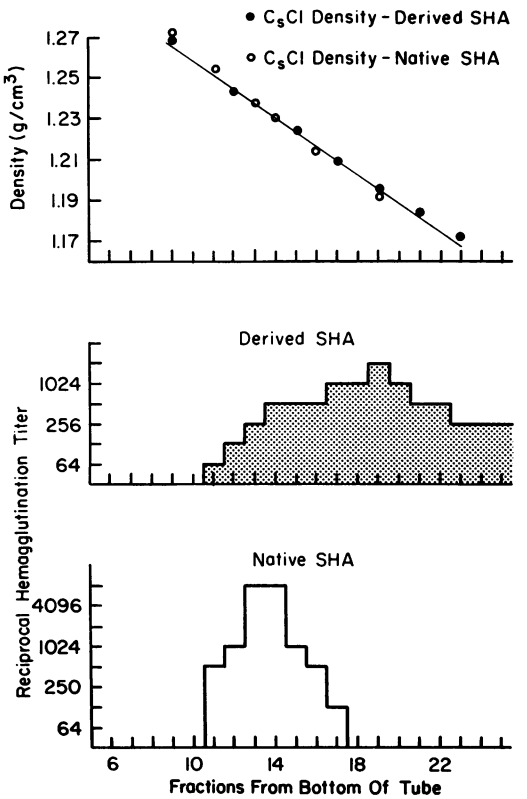

FIG. 5. *Equilibrium centrifugation in CsCl of virion-derived and naturally occurring (native) slowly sedimenting hemagglutinins (SHA). The gradients were centrifuged for 40 hr at 104,000 × g.*

the native SCF antigen found in infected tissue differed from all other detectable dengue antigens.

To determine the nature of antigen preparations usually employed for comparison of virus strains and for measuring serological responses to arbovirus infections, a sucrose-acetone HA antigen prepared by the standard method of Clarke and Casals (2) was analyzed on sucrose gradients (Fig. 6a). Compared to the unextracted brain suspension (Fig. 6c), this antigen preparation had relatively little HA activity due to RHA (virions) and contained primarily a slowly sedimenting HA antigen. Equilibrium centrifugation of the standard sucrose-acetone antigen in CsCl resulted in an HA peak at 1.22 to 1.23 g/cm³, very similar to equilibrium centrifugation of native SHA rather than derived SHA shown in Fig. 5.

The complement-fixing activity of the standard sucrose-acetone antigen was heterogeneous when analyzed on sucrose gradients (Fig. 6a), extending in a smear from the middle to the top of the gradient. The CF activity of all except the upper fractions (native SCF region) was reduced to undetectable levels when the antigen was exposed to SLS and 2-ME before gradient analysis (Fig. 6b). The effect of SLS and 2-ME on the CF activity of dengue type 1 (Hawaiian) and type 2 sucrose-acetone antigens was then tested with heterologous as well as homologous antibody. It can be seen in Table 1 that treatment of the antigens with SLS and 2-ME effected a 2-fold reduction of the homologous antibody titer but generally reduced the heterologous titers 4- to 16-fold. Essentially the same results were obtained when these sucrose-acetone antigens were treated with 8 M urea and dialyzed overnight in Tris-saline buffer before testing.

DISCUSSION

Present knowledge of the antigenic relationships among arboviruses is derived in large part from the results of serological tests utilizing sucrose-acetone-treated antigens. The greatest part of the HA activity of such antigens appears to be due to native SHA. Although not readily detected, virion-derived SHA in small quantity is most likely present in the standard antigen preparation because of disruption of the virions during the acetone extraction procedure.

The complement-fixing activity of sucrose-acetone reagent antigens appears to result from

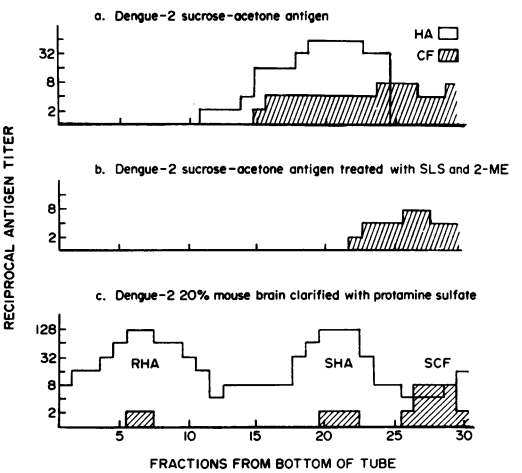

FIG. 6. *(a) Rate zonal centrifugation of a dengue-2 sucrose-acetone antigen on a 5 to 25% sucrose gradient for 3 hr at 63,000 × g. The predominant hemagglutinin is a slowly sedimenting form (SHA). (b) The same antigen preparation after treatment with 0.1% sodium lauryl sulfate (SLS) and 0.1% 2-mercaptoethanol (2-ME). (c) Protamine clarified mouse brain showing the location of rapidly and slowly sedimenting hemagglutinins (RHA and SHA) and the soluble complement-fixing antigen (SCF).*

TABLE 1. *Effect of 0.1% sodium lauryl sulfate (SLS) and 0.1% 2-mercaptoethanol (2-ME) on complement-fixing activity of sucrose-acetone antigens of dengue-1 and dengue-2*

Antigens	Units	Hyperimmune mouse ascitic fluids			
		Dengue-1	Dengue-2	Dengue-3	Dengue-4
Dengue-1 (untreated)	2	*1,024*[a]	64	128	32
	4	*1,024*	128	256	64
	8	*256*	128	256	64
Dengue-1 (SLS- and 2-ME-treated)	2	*512*	<8	32	<8
	4	*512*	32	32	<8
	8	*256*	32	32	<8
Dengue-2 (untreated)	2	256	*1,024*	256	64
	4	256	*1,024*	512	128
	8	256	*512*	256	128
Dengue-2 (SLS- and 2-ME-treated)	2	64	*512*	32	8
	4	128	*512*	32	8
	8	64	*256*	64	8

[a] Reciprocal antibody titer, homologous reaction in italics.

the combined activity of virion-derived SCF, native SHA, and native SCF. Antigens treated with SLS and 2-ME exhibited significantly fewer cross-reactions with heterologous dengue antibody preparations. Since SLS and 2-ME reduce the CF titer of the broadly reactive HA antigens and all virion-related antigens to undetectable levels, it is suspected that the apparent enhancement of specificity of the sucrose-acetone antigens may be due to the reaction of antibody primarily with the SLS-stable native SCF antigen. The demonstration of a type-specific antigenic determinant on the SCF molecule by immunoprecipitin analysis supports this interpretation (Russell et al., J. Immunol., *in press*).

The sequential appearance of antigens in infected mouse brain does not indicate clearly whether the noninfectious antigens (SHA and SCF) are subunits synthesized in excess, subunits derived from disintegrating virions, or are nonstructural antigens. An unexpected finding was the appearance in serum of relatively high titers of complement-fixing antigen from the fourth day on in spite of low infectivity and HA titers. The SCF antigen found in serum is presumably produced in the brain since the primary site of virus replication in intracerebrally inoculated suckling mice is the brain (G. A. Cole, Ph.D. Thesis, Univ. of Maryland, 1966). The presence of a circulating noninfectious antigen would, however, add an additional factor which might affect the immunological response and the pathogenesis of diseases associated with group B arboviruses. The suckling mouse is a highly artificial host system, however, and the possibility that SCF antigens may circulate in the blood of natural hosts of group B arboviruses awaits further investigation.

The chemical degradation experiments showed that two antigens chemically derived from the dengue-2 virion have sedimentation characteristics similar to the two naturally occurring (native) noninfectious antigens found in infected mouse brains (SHA and SCF). However, the virion-derived antigens differed from the native antigens when compared by other parameters. Electron micrographs of native SHA revealed 14-nm "ring" structures, whereas electron micrographs of virion-derived SHA contained aggregates of 7-nm "doughnut" structures similar to those seen on the surface of dengue virions (5). Virion-derived SHA was also heterogeneous in CsCl density gradients, whereas native SHA banded at 1.23 g/cm^3. The virion-derived slowly sedimenting complement-fixing antigen and the native SCF antigen were markedly different in their stability to the combined action of SLS and 2-ME; virion-derived SCF was destroyed, whereas the activity of native SCF was unaffected. In addition, all antigens except native SCF lost their complement-fixing activity after exposure to SLS and 2-ME. This striking disparity between the virion-associated antigens and native SCF suggests that SCF is a nonstructural antigen distinct from the virion. Its relationship, if any, with one of the virus-specific but nonstructural proteins found in group B arbovirus-infected cell cultures (7; Trent et al., Bacteriol. Proc., p. 161, 1969) remains to be determined.

ACKNOWLEDGMENTS

We thank E. L. Buescher for reviewing the manuscript and E. St. Martin and J. M. McCown for valuable assistance.

LITERATURE CITED

1. Brandt, W. E., E. L. Buescher, and F. M. Hetrick. 1967. Production and characterization of arbovirus antibody in mouse ascitic fluid. Amer. J. Trop. Med. Hyg. 16:339–347.
2. Clarke, D. A., and J. Casals. 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Amer. J. Trop. Med. Hyg. 7:561–573.
3. Ifft, J. B., D. H. Voet, and J. Vinograd. 1961. The determination of density distributions and density gradients in binary solutions at equilibrium in the ultracentrifuge. J. Phys. Chem. 65:1138–1145.
4. Kent, J. F., and E. H. Fife, Jr. 1963. Precise standardization of reagents for complement fixation. Amer. J. Trop. Med. Hyg. 12:103–116.
5. Smith, T. J., W. E. Brandt, J. L. Swanson, J. M. McCown, and E. L. Buescher. 1970. Physical and biological properties of dengue-2 virus and associated antigens. J. Virol. 5:524–532.
6. Stollar, V., T. M. Stevens, and R. W. Schlesinger. 1966. Studies on the nature of dengue viruses. II. Characterization of viral RNA and effects of inhibitors of RNA synthesis. Virology 30:303–312.
7. Westaway, E. G., and B. M. Reedman. 1969. Proteins of the group B arbovirus Kunjin. J. Virol. 4:688–693.