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Effect of Urea on the Hemagglutinating and Complement-Fixing Antigens of Type 2 Dengue Virus

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Sephadex G-200 filtration was used for fractionating dengue type 2 (DEN-2)-infected suckling mouse brain (SMB) supernatant fluids. The high-molecular-weight fraction, which was eluted in the void volume, showed no increase in type specificity when tested against immune ascitic fluid to the four prototype dengue viruses. A void-volume fraction obtained after the infected SMB supernatant fluids were treated with urea displayed significant increases in complement fixation (CF) type specificity. Immune ascitic fluid prepared against the more type-specific DEN-2 antigen demonstrated neutralizing ability and greater CF type specificity. When DEN-2 sucrose-acetone-extracted hemagglutinating (HA) antigens were treated with 6 M urea at 37 C for various time intervals, all HA antigen was destroyed in 15 min. Urea treatment of infected SMB supernatant fluids indicated that the CF antigens were more stable to the effect of urea than were the HA antigens. After urea treatment of the SMB supernatant fluids, the CF type specificity increased as the hemagglutination inhibition titer decreased.

Antigenic similarities among the dengue (DEN) subgroup of viruses and several other group-B arboviruses have led to extensive serologic cross-reactions (9, 10). As a result, it is frequently difficult to differentiate DEN virus infection by serology in patients who have even a primary infection, and still more difficult if they had one or more previous group-B arbovirus infections.

Cell culture of suckling mouse brain (SMB)-derived DEN type 2 (DEN-2) virus results in the production of three physically distinct antigens. These antigens can be separated by rate-zonal centrifugation (20) and result in an infectious, rapidly sedimenting hemagglutinin (RHA); a noninfectious, slowly sedimenting HA (SHA); and a soluble, complement-fixing (CF) antigen (SCF).

Russell and co-workers (17) examined the SCF antigen from all four DEN prototypes by CF and immunodiffusion (ID). By CF there were various degrees of increased specificity when the SCF antigens were compared to crude SMB-derived antigens. By ID, however, it was concluded that the SCF antigens were multideterminant in that they contained both type-specific and common sites. Members of this same team (6) demonstrated that SCF antigen was not related to the antigens present on either the complete virion (RHA) or the incomplete virion (SHA).

Brandt et al. (4) demonstrated that an increase in homotypic CF specificity of sucrose-acetone-extracted (7) DEN type-1 virus (DEN-1) and DEN-2 antigens could be affected if treated with either sodium lauryl sulfate (SLS) and 2-mercaptoethanol (2-ME) or with 8 M urea. They postulated that the SLS and 2-ME reduced the CF titer of all virion-related antigens to undetectable levels and that the apparent increase in CF type specificity was due to reaction of antibody with the SLS-stable native SCF antigen.

The purpose of the present investigation (taken in part from a D.Sc. thesis [R. A. C.], University of Pittsburgh, Pittsburgh, Pa., 1972) was to examine the interrelationship between the rate of antigen inactivation by urea and the increase in homotypic CF specificity. In addition, we will present results of comparative studies by CF of several DEN-2 antigens other than the SCF antigen.

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MATERIALS AND METHODS

Antigens. The four prototype strains of DEN viruses were used in this study, all at mouse passage no. 24 (13) without intermediate passage in other systems to minimize possible antigenic variants (12). The brains were infected by intracerebral inoculation of suckling mice, and when the animals were moribund the brains were harvested under aseptic conditions and homogenized in *p*H 9.0 borate-saline (BS9) (2) at a 20% (w/v) concentration. Homogenization was performed in a chilled Lourdes blendor run at full speed for three 1-min cycles. The homogenates were clarified at 4 C for 30 min at 13,200 × *g*. The supernatant fluids were stored at −60 C until needed.

The sucrose-acetone-extracted DEN-2 antigens were prepared by the method of Clarke and Casals (7).

The 20% DEN-2-infected SMB supernatant fluids were treated with crystalline urea, and added quickly to a final 6 M concentration while the supernatant fluid was stirred on a Vortex mixer. After incubation for 1 hr at 37 C, the urea-treated antigen was either placed on a descending Sephadex G-200 column or dialyzed against 100 volumes of cold phosphate-buffered saline (PBS). The controls consisted of the same untreated supernatant fluid, the volume of which was adjusted with PBS to correspond to the increase in volume resulting from the urea treatment. In addition, the control antigen was heated at 37 C for 1 hr and was either fractionated or dialyzed to match the urea-treated portion.

Gel filtration of antigens. In the gel filtration study, Sephadex G-200 (Pharmacia Fine Chemicals, Inc.) was used. The columns had a diameter of 2.5 cm and a bed height of 45 cm. After being equilibrated at 4 C with BS9, a flow rate of approximately 20 ml/hr was maintained. To calibrate the cclumns, the method of Ke and Ho (15) was used. Fractions of 6 ml were collected, and the elution patterns of optically active materials were monitored on an ISCO model UA-2 analyzer (Instrumentation Specialties Co., Inc.) at 280 nm.

Sucrose gradient centrifugation of antigens. Linear gradients of 4 to 18% sucrose in bovine albumin-borate-saline (11) were prepared. Gradients of 29 ml were overlaid with 1.0 ml of sample and centrifuged at 63,000 × *g* for 3 hr at 4 C. Fractions of 2.5 ml were collected dropwise from a pinhole in the bottom of the tube.

Concentration of fractions. Concentration of the gel filtration fractions was achieved by dialysis (16) against Carbowax 20M (Union Carbide Chemical Co.).

Antisera and immune ascitic fluids. Mouse hyperimmune antisera and ascitic fluids against the prototype strains of DEN viruses were perpared against infected 20% SMB supernatant fluids, as reported by Brandt et al. (3).

The immune ascitic fluid against a concentrated, urea-treated DEN-2 Sephadex G-200 void-volume CF antigen was prepared in female mice as described by Russell et al. (17).

CF tests. A microtiter CF test as described essentially by Hammon and Sather (11) was used.

RESULTS

Sephadex G-200 CF elution profiles of DEN-2-infected SMB supernatant fluids before and after urea treatment. To determine quantitatively which CF fraction(s) was affected by urea treatment, a 20% DEN-2-infected SMB supernatant fluid was divided into two portions. A 5.0-ml fraction of the supernatant fluid was adjusted volumetrically with PBS to account for the increase in volume which would have resulted if crystalline urea were added to a final concentration of 6 M. The sample (heat control) was incubated in a 37 C water bath for 1 hr, after which it was applied to a descending Sephadex G-200 column. Fractions of 6 ml were collected, and the CF antigen elution profile was determined.

Two CF fractions were obtained, one in the void volume (Vo-200) and the other which is the SCF antigen (Fig. 1, upper panel). Computations indicated that the large Vo-200 antigen represented approximately 90% of the total CF antigenic activity.

To ascertain the effect of urea on the DEN-2 supernatant fluid, a 5.0-ml sample was made 6 M in urea, incubated at 37 C for 1 hr, and then placed on the same Sephadex G-200 column. As with the "heat control," fractions of 6 ml were collected, and the CF antigen elution profile was determined.

Two CF fractions were obtained from the fractionated urea-treated sample (Fig. 1, bottom panel). Based on the heat control fractionation data, it was noted that the SCF antigen was not affected by 6 M urea, whereas the large void

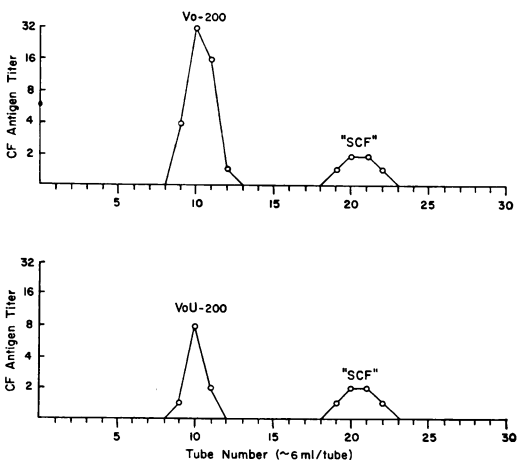

FIG. 1. *CF elution profiles of a 5.0-ml 20% DEN-2-infected SMB supernatant fraction from a Sephadex G-200 column (top) after incubation at 37 C for 60 min and (bottom) after treatment with 6 M urea at 37 C for 60 min.*

volume fraction (VoU-200) retained only 20% of its original activity.

The following DEN-2 antigens were compared for CF type specificity: (i) a sucrose-acetone-extracted preparation, (ii) the 20% infected SMB supernatant fluid which was heated at 37 C for 1 hr and then dialyzed against cold PBS (20% SMB heat), (iii) the 20% infected SMB supernatant fluid which was treated with 6 M urea for 1 hr at 37 C and then dialyzed against cold PBS (20% SMB urea), (iv) the concentrated DEN-2 heat control, Sephadex G-200 Vo fraction (Vo-200), (v) the concentrated DEN-2, urea-treated Sephadex G-200 void volume fraction (VoU-200).

The CF results (Table 1) indicate that significant CF cross-reactions occurred with the sucrose-acetone-extracted, 20% heated SMB, and Vo-200 antigens. On the other hand, both the 20% SMB urea and VoU-200 antigens, which were treated with urea, gave increased CF type specificity.

Sucrose gradient CF sedimentation profiles of a supernatant fluid and the Sephadex G-200 void volume fraction of DEN-2 before and after urea treatment. To partly characterize the urea-treated DEN-2 VoU-200 fraction, rate-zonal centrifugation in a linear 4 to 18% sucrose gradient was performed. For comparison, a 20% DEN-2-infected SMB supernatant fraction and a DEN-2 Vo-200 fraction that was heated at 37 C for 1 hr prior to gel filtration were included. Fractions of 2.5 ml were collected, dialyzed, and tested for CF antigen titers.

The 20% DEN-2-infected SMB supernatant fraction (Fig. 2, upper panel) yielded three CF fractions which probably correspond to RHA, SHA, and SCF antigens (20). Neither the Vo-200 (middle panel) nor the VoU-200 (lower panel) fractions contained the quickly sedimenting or the light CF fractions, but instead contained a single CF peak which sedimented to approximately 13% sucrose.

Comparative serological results with immune anti-DEN-2 VoU ascitic fluid against various DEN virus antigens. After a series of fractionations of urea-treated, DEN-2-infected SMB supernatant fluids on Sephadex G-200, a sufficient quantity of concentrated VoU-200 antigen became available to immunize mice. The immunogen had a CF antigen titer of 1:64 when tested against an excess of immune ascitic fluid prepared against crude SMB antigen and two exact units of complement.

The anti-DEN-2 VoU immune ascitic fluid (Table 2) was tested for CF type specificity against 20% SMB supernatant fluids of the four prototype strains of DEN viruses and the SCF antigen from DEN-2.

TABLE 1. *Reciprocal of CF titers of homologous and heterologous DEN-infected mouse hyperimmune ascitic fluids obtained against various DEN-2 antigens*

DEN-2 antigen[a]	Reciprocal of CF titer			
	DEN-1	DEN-2	DEN-3	DEN-4
Sucrose-acetone..	<8	*128*	64	512
20% SMB heated..	<4	*64*	32	16
20% SMB urea....	<4	*32*	4	4
Vo-200.....	<4	*256*	128	128
VoU-200.....	<4	*64*	8	<4

[a] The 20% SMB heated antigen resulted from incubating a supernatant fraction at 37 C for 1 hr followed by overnight dialysis. The 20% SMB urea antigen was treated with 6 M urea at 37 C for 1 hr prior to dialysis. Vo-200 was a concentrated void volume from a Sephadex G-200-fractionated 20% SMB heated antigen. VoU-200 was a concentrated void volume from a Sephadex G-200-fractionated 20% SMB urea antigen.

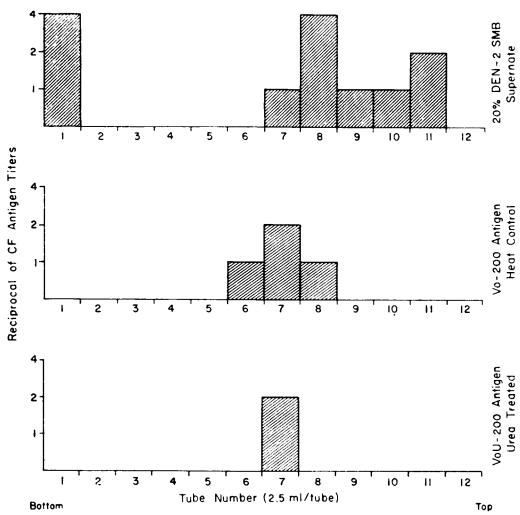

FIG. 2. *CF sedimentation profiles of a 20% DEN-2-infected SMB supernatant fraction, and the void volume CF fraction from a Sephadex G-200 column of both a heat-treated supernatant fraction (Vo-200) and a urea-treated supernatant fraction (VoU-200) by rate-zonal centrifugation on a linear 4 to 18% sucrose gradient at 63,000 × g for 3 hr.*

The ascitic fluid had a CF titer of 1:8 against the DEN-1 antigen, but failed to react with the DEN-3 and DEN-4 antigens, as well as with the DEN-2 "SCF" antigen. A CF titer of 1:32 resulted when the ascitic fluid was tested against the DEN-2 antigen.

In addition (not shown), the anti-DEN-2 VoU immune ascitic fluid contained 2.0 $\log_{10}$ of neutralizing antibody to DEN-2.

TABLE 2. *Reciprocal of CF antibody titers of anti-DEN-2 VoU against various DEN virus antigens*

Antigen[a]	Reciprocal of CF titer
20% DEN-1-infected SMB supernatant fluid	8
20% DEN-2-infected SMB supernatant fluid	32
20% DEN-3-infected SMB supernatant fluid	<4
20% DEN-4-infected SMB supernatant fluid	<4
DEN-2 SCF	<4

[a] The 20% SMB antigens were clarified by centrifugation prior to testing. The DEN-2 SCF antigen was kindly supplied by W. F. Brandt. The VoU-200 fraction was the immunogen. It was the concentrated void volume from a Sephadex G-200 fractionation of 20% DEN-2 supernatant fluids that were treated with 6 M urea at 37 C for 1 hr prior to gel filtration.

HA titers of DEN-2 sucrose-acetone-extracted antigens as a function of time after urea treatment. Since it was shown that hemagglutinin (HA) activity could not be detected in the void volume fraction of Sephadex G-200-fractionated DEN-2-infected SMB supernatant fractions after treatment with 6 M urea at 37 C for 1 hr, it was decided to examine the rate of HA inactivation by urea.

A sucrose-acetone HA preparation of DEN-2 was divided into two equal portions. These portions were equilibrated in a 37 C water bath. One portion was made 6 M with crystalline urea, and the volume in the other portion (heat control) was quickly adjusted with *p*H 9.0 BS9 which was prewarmed to 37 C. Both portions were shaken vigorously and then reincubated. At 0 (~15 seconds), 1-, 2-, 4-, 6-, 8-, 10-, 12-, 14-, 15-, 20-, and 25-min intervals, 1.0-ml fractions were removed from both portions and were dialyzed immediately under pressure against 200 volumes of cold BS9 for 16 hr, with dialysate changes at 2 and 6 hr. The samples were then tested for HA activity.

The HA titer in the heat control portion remained constant for the duration of the experiment (Fig. 3). In the urea-treated sample, the HA titer dropped quickly, and by 4 min HA activity could be demonstrated only in the undiluted sample. A urea-resistant fraction seemed to persist since HA activity could be detected in the undiluted fractions for up to 14 min.

CF antigen titers and CF specificities of DEN-2-infected SMB supernatant fluids as a function of time after urea treatment. From our data, it became apparent that there was an increase in CF type specificity when either the urea-treated DEN-2-infected SMB supernatant fraction or the large void volume antigen from Sephadex G-200 gel filtration of such supernatant fractions was used as the antigen. There was no need for fractionation after urea treatment to demonstrate this increase in CF specificity since the two antigens remaining after urea treatment of crude SMB, the VoU-200 and the SCF, both displayed increased CF specificity when compared to either the crude SMB supernatant fluid or the sucrose-acetone-extracted antigens.

To investigate the effects of urea on the CF antigen titers and CF specificity at various time intervals, a 20% SMB supernatant fraction of DEN-2 was divided into two equal portions. After equilibration to 37 C, one portion was made 6 M in urea, and the other portion was adjusted to an equal volume with PBS. At 0-, 15-, 30-, 45-, 60-, 90-, 105-, 120-, 240-, and 480-min intervals, 1.5-ml samples were withdrawn and pressure-dialyzed immediately against cold PBS. Each sample was tested for CF antigen titer against an excess of homologous antibody.

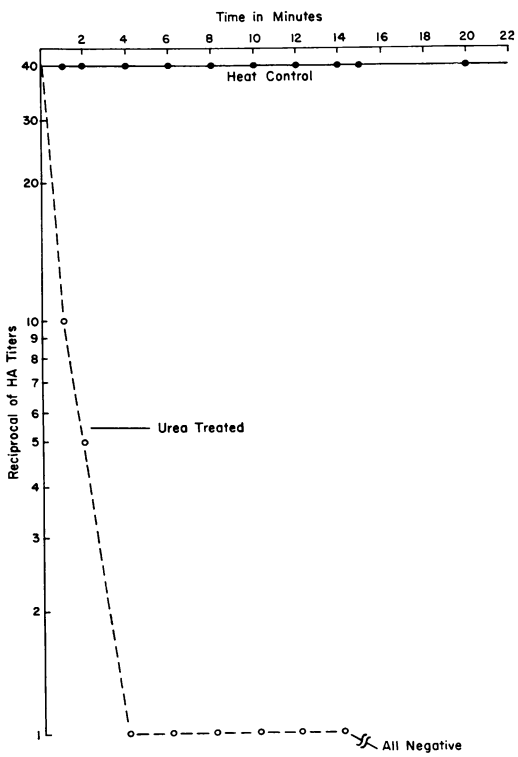

FIG. 3. *Reciprocal of HA titers of a DEN-2 sucrose-acetone preparation as a function of time when treated with 6 M urea at 37 C.*

The DEN-2-infected SMB supernatant fraction (Fig. 4), which was equilibrated at 37 C over the duration of the experiment (heat control), demonstrated a gradual decline in CF antigen titer. The urea-treated portion demonstrated a greater decrease in CF antigen titer that the heat control portion over the same time intervals. The remaining urea-stable CF antigen probably represents the stable SCF (4, 5) antigen.

Since the lowest antigen titer was 1:8, the above samples were adjusted by dilution to a final 1:8 CF antigen titer and then were tested for CF type specificity. The results (Fig. 5) demonstrated that

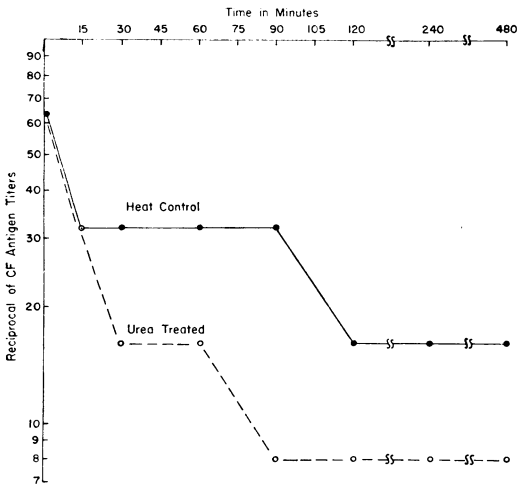

FIG. 4. *The CF antigen titers of 20% DEN-2-infected SMB supernatant fraction as a function of time after exposure to either 37 C or 6 M urea at 37 C.*

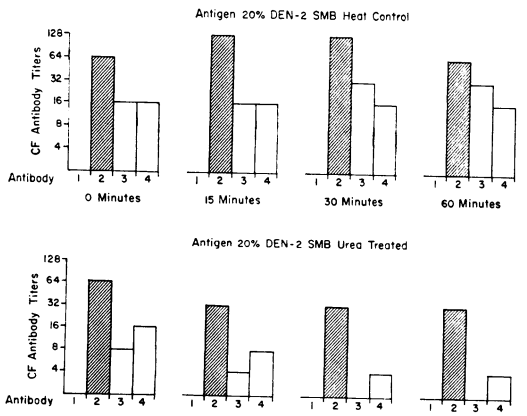

FIG. 5. *CF titers of homologous and heterologous antibody against a 20% DEN-2-infected SMB supernatant fraction that was either heated at 37 C or treated with 6 M urea at 37 C for 0-, 15-, 30-, and 60-min intervals, dialyzed, and adjusted to a final 1:8 antigen titer prior to testing.*

significant CF cross-reactions occurred in all samples of the heat control, whereas the urea-treated samples gradually demonstrated increased homotypic CF specificity over the first 30 min. After 30 min, there was no further change.

DISCUSSION

The hemagglutination inhibition (HI), CF, and neutralization (NT) tests are the conventional serological methods utilized for the serodiagnosis of DEN virus infections and serotyping of DEN virus isolates. Of these, the NT test is usually considered the most nearly type specific, but it is also the most laborious test. The easier HI and CF tests, utilizing the antigens presently in common use, frequently fail to differentiate the infecting DEN virus or the viral isolate, or both (9, 10).

To separate the nonvirion SCF antigen from the larger virion-associated antigens in DEN-2-infected SMB supernatant fractions, a Sephadex G-200 column was utilized. The large-molecular-sized antigen, which was eluted in the void volume before treatment with urea (Vo-200), failed to display increased CF type specificity over the sucrose-acetone-extracted antigen. After urea treatment of the SMB antigen, the void volume fraction (VoU-200) demonstrated increased homotypic CF specificity. Whereas the Vo-200 antigen had HA activity, the VoU-200 antigen did not. No difference between the Vo-200 and VoU-200 antigens could be demonstrated by comparing their CF sedimentation profiles by rate-zonal centrifugation.

The DEN viruses are classified as group B arboviruses by the HI test. It should follow, therefore, that one of the common group B antigens is an HA antigen. Other investigators, using a variety of ribonucleic acid viruses other than group B arboviruses, and serological tests other than HI, have suggested that group-specific serological reactions were attributable to nucleocapsid antigens (14, 18, 19), whereas the type-specific serological reactions were attributable to antigens on the viral surface (1, 14). If one assumes that the more nearly type-specific CF and NT DEN antigen is an envelope component, it is possible that urea treatment destroys not only a common nucleoprotein antigen but also the highly cross-reactive HA antigen, but leaves the highly specific NT determinant which fixes complement in the presence of antibody. Serological results with the antibody prepared against the fractionated urea-treated DEN-2 VoU-200 antigen (Table 2) are in agreement with this suggestion since it displayed homotypic CF specificity and reacted by NT.

These urea treatment experiments tend to confirm the work of Brandt et al. (4) who found an increase in CF type specificity of sucrose-acetone-extracted DEN-1 and DEN-2 antigens after treatment with 8 M urea. These investigators, however, attributed the enhancement of CF specificity to the SCF antigen which was stable to urea. In these fractionation studies, however, the SCF antigen was removed, and a large-molecular-weight antigen also displayed increased homotypic CF specificity.

Cooper (8) demonstrated that the protein shell of the poliovirus was left intact after treatment with urea but that the ribonucleic acid was released.

If the urea-treated, fractionated DEN antigens from the other prototypes can stimulate specific CF antibody as was possible with DEN-2, such sera may possibly have special value as serotyping reagents.

When the effect of 6 M urea on the CF antigen titers and CF specificities of DEN-2-infected SMB supernatant fluids were investigated as a function of time, the results indicated that in order to maintain CF antigen activity and to obtain type specificity the optimal time for urea treatment at 37 C was 30 min.

Under similar conditions, the HA activity of a sucrose-acetone-prepared DEN-2 antigen was destroyed totally in 15 min (Fig. 3), about the same time that CF specificity of a simple supernatant fraction began to increase (Fig. 5). These results support a hypothesis that urea treatment either destroyed or reduced to insignificant levels a group-reactive CF HA antigen leaving intact a more nearly type-specific CF, neutralization antigen.

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