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Group B Arbovirus Structural and Nonstructural Antigens

III. Serological Specificity of Solubilized Intracellular Viral Proteins

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Solubilized nonstructural antigen III from Saint Louis encephalitis (SLE)-Japanese B encephalitis (JBE)-West Nile (WN) and dengue-2 arbovirus-infected pig kidney cells was purified by employing Brij-58 solubilization, organic solvent extraction, and column chromatography. Diethylaminoethyl-cellulose column peak C eluate contained only intracellular viral envelope protein designated antigen I. Antigen I of SLE virus absorbed homologous neutralizing antibody; however, the intracellular nonstructural protein designated antigen III did not. The antigenic relationships of solubilized antigens I and III prepared from SLE, JBE, and WN virus-infected cells were determined by complement-fixation (CF) and immunodiffusion analyses. Solubilized antigen I of each virus cross-reacted broadly with both homologous and heterologous antibody at high antigen concentrations in the CF test. Antigen III of each virus reacted with only homologous antibody by both CF and immunoprecipitation. This study demonstrates that during SLE, JBE, WN, and dengue-2 infections, virus-specific proteins containing both type-specific and group-reactive determinants are synthesized. Antigen III, a nonstructural protein, is serologically virus type specific and may be useful as a type-specific diagnostic reagent.

The broad serological cross-reactivity among group B arbovirus antigen preparations often complicates the specific etiological diagnosis of these infections (26). This problem becomes even more complex when attempting to distinguish between different viruses within antigenically closely related subgroups. Serological studies indicate that, during group B arbovirus infections, antibodies are formed which cross-react with antigens produced by other viruses within the complex (7, 11, 15, 17, 30). Sera from animals and humans infected with viruses belonging to the dengue subgroup contain antibodies which neutralize Saint Louis encephalitis (SLE), Japanese B encephalitis (JBE), and West Nile (WN) viruses (2, 3, 12, 14, 23). Similarly, sera from the patients and experimental animals infected with WN virus contain antibodies which neutralize JBE, SLE, Murray Valley, and dengue viruses (10, 11, 17, 18). Heterologous antibody absorption experiments with suckling mouse brain suspensions of viruses in the JBE-SLE-WN and dengue subgroups indicate that these virus preparations contain viral antigens which share at least one group-reactive determinant in addition to type-specific components (8, 16). The dengue virus-soluble complement-fixing antigen (SCF), non-infectious hemagglutinin (SHA), and intact virion contain both type-specific and group-reactive determinants (5, 22). Recent studies have shown that cells infected by group B arboviruses contain at least seven viral-specific proteins: two structural and five which are nonstructural (25, 27). The serological specificity of these viral antigens is not yet known.

In the preceding paper (21) we described the isolation and purification of two solubilized intracellular SLE virus proteins designated as antigen I and III. The data in this report (a portion of which will be submitted by A.A.Q. in partial fulfillment of the requirement for the Ph.D. degree from The University of Texas Medical School at San Antonio) show that the major intracellular viral envelope protein of the antigenically related SLE, JBE, and WN arboviruses cross-react in both complement-fixation (CF) and immunodiffusion tests and appear to contain both group-reactive and type-specific determinants. The soluble viral non-

structural protein designated as antigen III reacts only with homologous antibody and is therefore serologically type specific.

MATERIALS AND METHODS

Viruses and cell cultures. The Tampa Bay human isolate of SLE virus (TBH-28), Nakayama strain of JBE, and Ar-248 strain of WN viruses were obtained from Center for Disease Control, Atlanta, Ga. The KB cell-adapted New Guinea B strain of dengue-2 virus was obtained from Victor Stollar of the Rutgers Medical School. Procedures used for the propagation and assay of these viruses in pig kidney (PS) cell monolayers have been previously described (27, 29).

Preparation of solubilized viral antigens and serology. Methods for preparation of solubilized viral intracellular antigens, hyperimmune ascitic fluids, and immunodiffusion analyses have been described before (20). Complement-fixing activity of the antigens was assayed by the Laboratory Branch Complement-Fixation modification of the microtechnique by using hyperimmune mouse ascitic fluids (28). Antigen was assayed in antibody excess and the titers were expressed as the reciprocal of the dilution giving 30% hemolysis in the test.

NAB. The neutralizing antibody blocking (NAB) activity of the antigen was determined by the method of Appleyard et al. (1). Serial twofold dilutions of antigens I and III were prepared in medium 199 containing 3% calf serum and incubated with that dilution of anti-SLE hyperimmune ascitic fluid which, during 1 h at 37 C, would neutralize 95% of 800 plaque-forming units (PFU) of SLE virus. After the antigen-antiserum incubation, 800 PFU of SLE virus were added and the antigen-antibody-virus mixture was incubated for 1 h at 37 C. The residual virus infectivity in the mixture was assayed on PS cells (27, 29).

RESULTS

Complement-fixation analysis of antigens in infected mouse brain and cell culture suspensions. Hyperimmune mouse antiviral ascitic fluids used in these studies had homologous CF titers of 256 against SLE, 128 against JBE, 64 against WN, and 256 against dengue-2 virus-infected crude mouse brain antigens. No CF activity was detected when these ascitic fluids were tested against a suspension of normal mouse brain. A significant cross-reaction was observed when each mouse ascitic fluid was tested against both homologous and heterologous infected mouse brain suspensions (Fig. 1). Anti-SLE hyperimmune ascitic fluid which had a homologous titer of 256 reacted with the heterologous crude mouse brain antigens of JBE at 8, WN at 16, and dengue-2 at 64. Anti-JBE antibody reacted with homologous antigen at a CF titer of 128 and reacted with SLE, WN, and dengue-2 antigens at approximately the same titers. Anti-WN antibody had a homologous titer of 64 and reacted with heterologous crude antigens of dengue-2 at 64, SLE at 16, and JBE at 2. Anti-dengue-2 ascitic fluid reacted with the heterologous SLE antigen at 16 and WN at 8, but did not cross-react with the JBE antigen.

SLE, JBE, WN, and dengue-2 mouse brain antigens cross-reacted with antibodies in ascitic fluid prepared against heterologous viruses at almost the same titer as they reacted with antibody in homologous ascitic fluids (Fig. 1). The crude mouse brain JBE antigen, however, did not cross-react with anti-dengue-2 antibody.

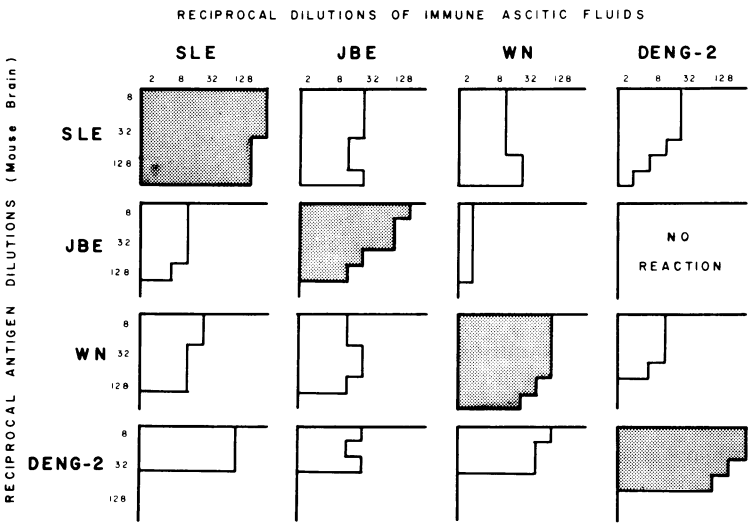

FIG. 1. *Complement-fixation analysis of infected crude mouse brain antigens and immune ascitic fluids. Homologous reactions are shaded in this and other CF grids.*

A significant cross-reaction was observed when each mouse ascitic fluid was tested against both homologous and heterologous virus-infected PS cell culture homogenates (Fig. 2). Anti-SLE hyperimmune ascitic fluid which had a homologous CF titer of 256 reacted with JBE, WN, and dengue-2 cell antigens at titers of 16, 2, and 16, respectively. Anti-JBE and WN ascitic fluid cross-reacted with heterologous SLE, WN, JBE, and dengue-2 antigens at low titers. Anti-dengue-2 antibody did not react with the WN antigen and reacted only at low dilutions with other heterologous antigens.

Crude cell culture antigens reacted broadly with heterologous hyperimmune ascitic fluids (Fig. 2). The SLE crude cell culture antigen reacted more broadly with the anti-JBE and anti-WN viral ascitic fluids than with dengue-2. The JBE cell culture antigen reacted with anti-SLE and anti-WN ascitic fluid at a higher titer (64) than it did with the anti-dengue-2 antibody. The WN antigen reacted with the heterologous anti-SLE and anti-JBE antibody at the same CF titer; however, it did not react with the anti-dengue-2 antibody at all. The dengue-2 cell culture antigen reacted with anti-SLE, anti-JBE, and anti-WN ascitic fluids at the same titer as homologous dengue-2 ascitic fluid.

A comparison of the CF analyses of infected mouse brain and infected cell culture antigens indicated that mouse brain antigens exhibit a broader serological cross-reaction than do the infected cell culture antigens. These results indicate that antigens prepared from mouse brain infected with viruses of the SLE-JBE-WN complex and dengue-2 virus contain broadly cross-reactive group-antigenic determinants in addition to viral-specific components.

Complement-fixation analyses of solubilized antigens I and III. The serological specificity of soluble antigen I was evaluated by heterologous and homologous CF tests. As shown in Fig. 3, antibodies in SLE hyperimmune ascitic fluid reacted with homologous antigen I and also JBE antigen I. Antibodies in dengue-2 ascitic fluid reacted at high titer with homologous antigen I and also with SLE antigen I at high antigen concentration. Antibodies in JBE ascitic fluid reacted at much higher titers with WN antigen I than they did with SLE antigen I and did not react with dengue-2 antigen I. Similarly, anti-WN immunoglobulins reacted at higher titers with JBE antigen I than they did with SLE antigen I and very weakly with dengue-2 antigen I.

The SLE antigen I reacted at approximately the same CF titer with either anti-JBE or anti-WN antibodies in ascitic fluid and with significantly lower titers with antibodies in anti-dengue-2 ascitic fluids. JBE antigen I reacted with immunoglobulins in both anti-SLE and anti-WN ascitic fluids at the same CF titer, whereas anti-dengue-2 antibodies did not react with this antigen. WN antigen I cross-reacted with antibodies in anti-JBE ascitic fluid in a reaction pattern almost identical to that observed with homologous antibodies in anti-WN ascitic fluids. Dengue-2 antigen I cross-reacted with antibodies in the anti-WN ascitic fluids. These

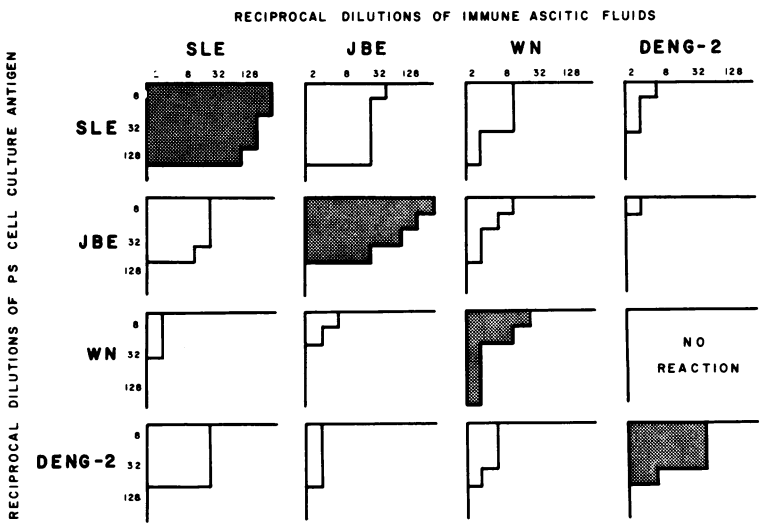

FIG. 2. *Complement-fixation analysis of infected unfractionated cell culture homogenates and immune ascitic fluids.*

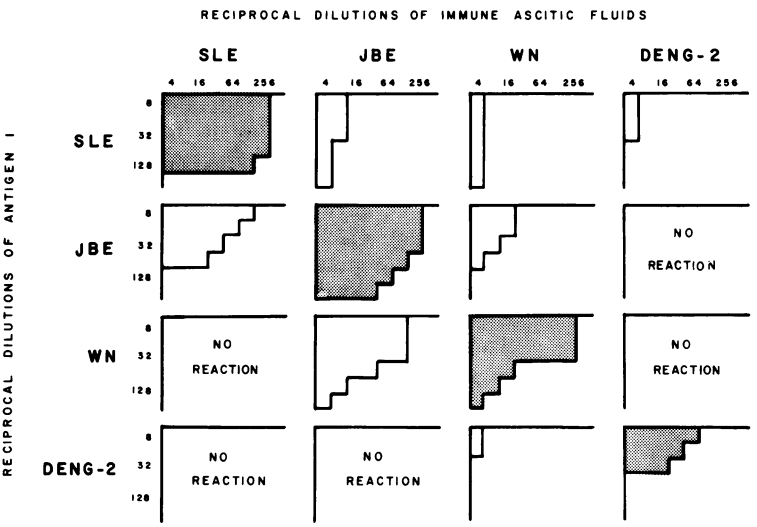

FIG. 3. *Complement-fixation analysis of group B arbovirus-solubilized antigen I and immune ascitic fluids.*

results indicated that intracellular envelope protein antigen I of SLE-JBE-WN group B viruses contain group-reactive determinants which stimulate the formation of group-reactive antibodies which are able to fix complement in the presence of very small amounts of homologous antigen.

Purified antigen III was analyzed by homologous and heterologous CF tests (Fig. 4). Antigen III preparations for SLE, JBE, WN, and dengue-2 viruses reacted only with antibodies in their homologous ascitic fluids. These results indicated that viral nonstructural protein antigen III of the SLE-JBE-WN complex and dengue-2 virus contains only virus type-specific antigenic determinants.

Immunodiffusion analyses of soluble antigens I and III. As shown in Fig. 5a, antigen I from SLE, JBE, and WN viruses formed one major precipitin line when reacted with antibodies in homologous ascitic fluid. The ends of the SLE, WN, and JBE antigen I precipitin lines fused, indicating that the envelope proteins of these viruses share common antigenic determinants. This observation is in contrast with that in Fig. 3, where WN antigen I did not fix complement in presence of anti-SLE mouse ascitic fluid. A second very faint precipitin line is detectable in SLE, JBE, and WN virus antigen I preparations. This second precipitin line may be due to the minor envelope protein or different immunoglobulins reacting with the group- and type-specific antigens present on the envelope protein molecule.

In Fig. 5b are presented the immunodiffusion patterns of antigen III specified by SLE, JBE, and WN viruses when reacted with both homologous and heterologous ascitic fluids. Antigen III of each virus was only precipitated by its homologous antibody. These results indicate that antigen III of these group B arboviruses is serologically type specific and does not share antigenic determinants which cross-react by CF or immunoprecipitation with antigen I or III of these antigenically related viruses.

NAB activity of antigen I and III. The ability of SLE virus-solubilized antigen I, the major virion envelope protein, and antigen III to absorb anti-SLE-neutralizing antibodies was determined. As shown in Fig. 6, SLE antigen I effectively absorbed neutralizing antibody at low antigen concentrations. Approximately 2.5 CF units of solubilized antigen I were required to absorb 50% of the neutralizing antibody activity of the serum in the reaction mixture. The neutralization inhibition curve was linear to a final antigen concentration of approximately 2 CF units. This indicated that the amount of neutralizing antibody absorbed by antigen I is directly proportional to the concentration of antigen in the reaction mixture. In contrast to solubilized antigen I, antigen III did not absorb a significant amount of anti-SLE-neutralizing antibodies even at high antigen concentrations.

DISCUSSION

The previously reported antigenic relationships among group B togaviruses of the SLE-JBE-WN complex and dengue-2 viruses and the solubilized envelope protein reported here are in agreement with those described by other workers (11, 15, 19). Antigens in infected cell culture homogenates are not as broadly cross-reactive

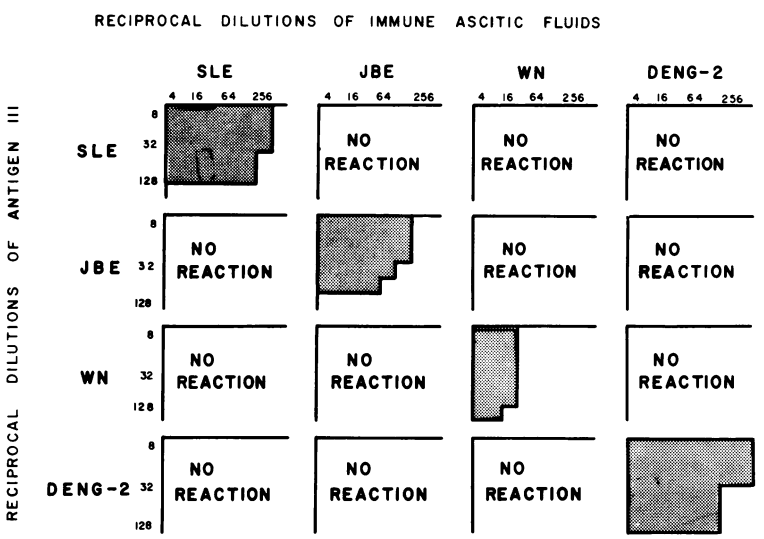

FIG. 4. *Complement-fixation analysis of group B arbovirus-solubilized antigen III and immune ascitic fluids.*

as those prepared from infected mouse brain (Fig. 1, 2). This suggests that infected mouse brain suspensions contain either higher concentrations or cross-reactive antigens and/or serologically different cross-reactive virus proteins than are found in antigen preparations from infected cell cultures. Comparison of the serological reactivity of antigens in crude homogenates of infected cell cultures and solubilized antigen I reveals that WN virus envelope protein contains more antigenic determinants common to JBE virus than it does with SLE virus. Dengue-2 virus antigens from infected cell cultures cross-reacted with SLE virus (Fig. 2); however, dengue-2 virus antigen I did not show any cross-reaction with antibodies in anti-SLE ascitic fluid. SLE virus envelope contains more antigenic determinants that are common with JBE virus envelope than it does with the envelope protein of either WN or dengue-2 viruses. The envelope of JBE virus contains antigenic determinants common to only SLE and WN viruses (Fig. 2). These observations may explain why infection by dengue-2 virus induces formation of antibodies which react with the surface antigens of JBE and SLE viruses in hemagglutination inhibition and neutralization tests (2, 3, 12, 14, 23).

Clarke (8) suggested that both group-reactive and type-specific determinants are specified by the group B arboviral genome. Group-reactive and type-specific determinants may both be located on the viral envelope or on two different molecules. Dalrymple et al. (9) described three types of antigens on the Sindbis virus envelope. On the basis of radioimmune precipitation-inhibition kinetics of Sindbis virus with anti-Western equine encephalitis virus serum, the authors suggested the presence of: (i) a type-specific determinant, (ii) a group-reactive determinant, and (iii) an intragroup complex specific antigen. Our data indicated the presence of a second minor antigenic determinant in immunodiffusion tests with antigen I when analyzed with homologous and heterologous antibody (Fig. 5a). Our data support the concept that the envelope glycoprotein of the group B arboviruses contains both group- and type-specific antigenic determinants on the same molecule, and each of these antigens reacts with immunoglobulins of different specificities. The second precipitin band may also be explained as the minor envelope protein, or as a second envelope glycoprotein which could not be resolved in polyacrylamide gel electrophoresis. Two glycoproteins are contained in the Sindbis virus envelope protein (24). The two glycoproteins were not detected when the virus was subjected to electrophoresis in continuous sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (24). The antigenic specificity and polypeptide composition of this minor envelope antigen are not known at this time.

Immunodiffusion and CF analyses of purified solubilized antigen III of SLE, JBE, WN, and dengue-2 viruses indicated an absence of common antigenic determinants on these viral-specific proteins (Fig. 4, 5b). Solubilized antigen III of dengue-2 virus does not cross-react by CF and immunodiffusion tests with heterologous antibody prepared against the related group B viruses. The nonstructural SCF antigen of dengue-2 virus cross-reacts with other dengue types in both immunodiffusion and CF tests

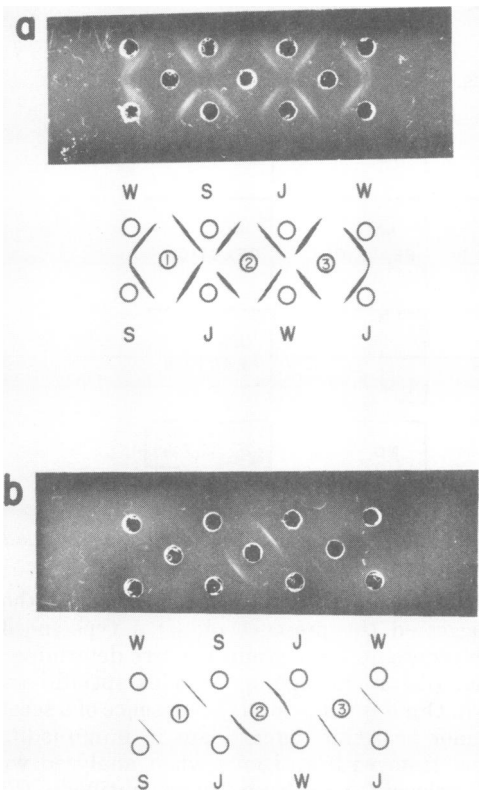

FIG. 5. *(a) Serological cross-reaction of solubilized antigens I (outer wells) and homologous and heterologous immune ascitic fluids (center wells). Note fusion of precipitin lines between heterologous antigen I. (b) Serological specificity of solubilized antigen III (outer wells) and homologous and heterologous immune ascitic fluids (center wells). Note precipitin lines only against the homologous antibody. Outer wells: W, WN antigen; S, SLE antigen; J, JBE antigen. Center wells: 1, anti-SLE ascitic fluid; 2, anti-JBE ascitic fluid; 3, anti-WN ascitic fluid.*

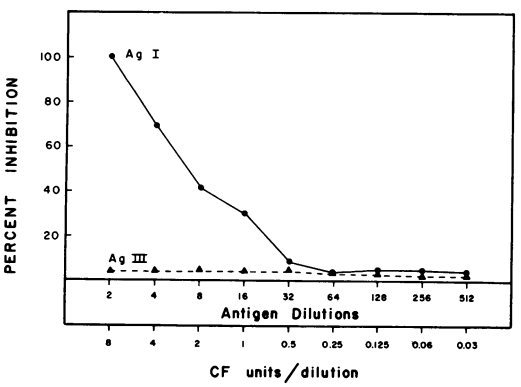

FIG. 6. *Neutralizing antibody blocking activity of solubilized SLE virus antigens I and III. Symbols: antigen I (○); antigen III (▲).*

(22). Two strains of dengue type 1, however, could be separated by subtle differences in cross CF tests (13). Dengue SCF antigens have molecular weights of approximately 39,000 (4, 6), as compared to solubilized antigen III which has a molecular weight of approximately 85,000 in polyacrylamide SDS gels (21). These data indicate that antigen III from group B arbovirus-infected cells and the dengue-2 virus SCF antigens isolated from infected mouse brains are serologically and biophysically distinct from each other.

Infection of mice with infected mouse brain suspensions induced the formation of antibodies which react with solubilized antigens I and III. If human infections with these viruses have a similar pathogenesis, it would be expected that viral nonstructural and structural proteins are released into the circulation during the infection. It is therefore anticipated that antibodies which develop against antigen III will react serologically in a type-specific manner. Under these conditions purified antigen III could be employed as a reference reagent for specific serological diagnosis of group B arbovirus infection. Studies with sera from humans naturally infected with group B arboviruses are needed to determine the antigens to which immunoglobulins are produced during human infection and the specificity of the human immunological response to structural and nonstructural proteins. Characterization of the appearance and persistence of antibodies to the structural and nonstructural antigens of the group B arboviruses will help resolve the complex immunological response often observed in man and animals after group B arbovirus infection. This knowledge will also provide a scientific basis for the preparation of type-specific diagnostic reagents and more effective immunogens.

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