Group B Arbovirus Structural and Nonstructural Antigens

II. Purification of Saint Louis Encephalitis Virus Intracellular Antigens

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Three serologically distinct antigens were identified in homogenates of Saint Louis encephalitis (SLE) virus-infected cells after Brij-58 solubilization and diethylaminoethyl (DEAE)-cellulose chromatography. These antigens were designated as antigen I, II, and III. Immunodiffusion analyses showed that all antigen peaks which eluted from the DEAE-cellulose column contained the intracellular major envelope protein, antigen I. This protein was the only viral antigen which eluted at 0.125 M KCl in DEAE-cellulose column peak C. Optical density peak D, which eluted at 0.2 M KCl, contained both antigens I and III. Antigen III was purified from this column fraction approximately 200-fold by organic solvent extraction and chromatography on hydroxylapatite and Sephrose 6B. Purified antigen III had a molecular weight of 80,000 to 85,000 and contained only one antigenic determinant as judged by immunodiffusion analysis.

Various cell-associated antigens have been solubilized and purified after detergent or enzyme treatments. The murine tumor cell H-2 alloantigen and HL-A antigen from hematopoietic cell lines were isolated by using nonionic detergents, proteolytic enzyme digestion, and ion exchange chromatography (13, 18). Spira et al. (21) isolated the simian virus 40-induced tumor (T) antigen from transformed cells by hydroxylapatite column chromatography after freezing and thawing and differential ammonium sulfate precipitation. The adenovirus-induced tumor antigen was isolated and purified by using a multistep procedure including detergents, organic solvent extraction, and chromatography (11). A virus-specific soluble antigen which stimulated the production of neutralizing antibodies was isolated from herpes simplex virus-infected cells by using sonication and calcium phosphate chromatography (9).

Ion exchange and gel filtration chromatography have also been used to separate arbovirus antigens from infected mouse brain suspensions (1, 5, 7, 11, 19). Calcium phosphate chromatography of group A and B arbovirus preparations revealed two hemagglutinating (HA) antigens, one which was the complete virion and the second, a less-dense HA antigen, which was thought to be a component of the viral envelope (19). Suspensions of group B arbovirus-infected mouse brain contain two particulate complement-fixing (CF) antigens which have been separated by ion exchange chromatography (19). These CF antigens have now been identified as the complete virion or rapidly sedimenting HA (RHA), the slowly sedimenting HA (SHA) antigen, and the nonsedimenting CF (SCF) antigen (5, 19, 20). The dengue SCF antigen is a nonstructural protein which has been partially purified by ultracentrifugation and Sephadex gel filtration (5). After sonication of group C arbovirus-infected mouse liver homogenates, CF antigens could be separated from HA antigens by high-speed centrifugation (1). Calcium phosphate chromatography of the separated HA antigens removed contaminating cellular proteins and yielded an antigen preparation with higher titers (1).

We have reported isolation of Saint Louis encephalitis (SLE) virus-solubilized structural and nonstructural antigens from infected cells, using sonication, detergent solubilization, and ion exchange chromatography on diethylaminoethyl (DEAE)-cellulose columns (15). 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) describes the techniques which have been developed for purification of SLE virus nonstructural protein designated antigen III.

**MATERIALS AND METHODS**

**Virus and cell cultures.** Procedures employed for the propagation of radioactively labeled solubilized intracellular viral antigens have been previously reported (15). Briefly, infected pig kidney (PS) cells were harvested 36 h postinfection, washed with 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.8, and frozen. After thawing, the infected cells were sonically treated and solubilized with Brij-58 (0.5%), cellular debris was removed by centrifugation, and the supernatant was applied to a DEAE-cellulose column equilibrated in Tris buffer, pH 7.8. Viral antigens were eluted stepwise by using 0.05, 0.075, 0.10, 0.125, 0.15, and 0.2 M KCl in Tris buffer, pH 7.8. Each optical density peak was concentrated 50-fold by passing the solution through a membrane ultrafilter (Diaflo XM-50).

**Hyperimmune ascitic fluids.** Group B arbovirus hyperimmune ascitic fluids were prepared in adult mice as described before (15).

**Complement-fixation and immunodiffusion tests.** Complement-fixing activity of the antigens was determined by the Laboratory Branch Complement-Fixation modification of the microtechnique by using hyperimmune mouse ascitic fluids. Antigen samples were assayed in antibody excess, and the titers were expressed as the reciprocal of the antigen dilution giving 30% hemolysis in the test (23). Immunodiffusion analyses of the antigen preparations were performed as previously reported (15).

**Virus purification.** SLE virus released into the supernatant fluids of infected PS cells was purified by the methodology described before (15).

**Polyacrylamide gel electrophoresis.** Co-electrophoresis of concentrated purified 4H-leucine-labeled antigens with 4C-leucine-labeled infected cell cytoplasmic extract was performed on 10% polyacrylamide sodium dodecyl sulfate (SDS) gels as described previously (15).

**Chemical determinations.** The protein content of each sample was determined by the method of Lowry by using bovine serum albumin as standard (12). Quantitative phosphate determinations were done by the method of Bartlett (2).

**RESULTS**

**Procedure for purification of intracellular antigen III.** The techniques developed in the purification procedure are summarized in Fig. 1; a detailed description follows.

Solubilized intracellular antigens were eluted stepwise from a DEAE-cellulose column with KCl in Tris buffer, pH 7.8. Four regions of optical density and complement-fixing activity, designated as A, B, C, and D, were identified in the column eluate (Fig. 2). Immunodiffusion analyses of the antigen eluting from the DEAE-cellulose column revealed three serologically distinguishable antigens designated as antigen I, II, and III. Antigen I was found in all DEAE-column fractions which contained CF activity. Peak A1 also contained antigen II, whereas regions B and C contained only antigen I. Region D contained antigen III in addition to antigen I.

Antigens in column region D were extracted with an equal volume of cold 1,1,2-trichlorotrifluoroethane (Genetron) and vigorously mixed by hand for 5 min at 4 C, and the aqueous supernatant was collected. The aqueous phase was then extracted twice with an equal volume of a mixture containing equal proportions of chloroform, ether, and carbon tetrachloride (CEC) by vigorous hand shaking for 5 min at 4 C followed by centrifugation at 2,800 x g for 15 min to separate the organic solvent and aqueous phases. The antigen-containing aqueous phase was collected and concentrated by ultrafiltration (XM-50).

Immunodiffusion analyses revealed that both antigens I and III were contained in DEAE-cellulose column region D and that antigen I was removed by organic solvent extraction (Fig. 3). Genetron extraction did not remove all of antigen I, since precipitin lines formed by both antigens I and III were detected after this treatment. Extraction with CEC effectively removed phospholipids from the antigen preparation. (Table 1) and eliminated immunological demonstrable antigen I from DEAE-cellulose...
PURIFICATION OF SLE VIRUS ANTIGEN III

Fig. 2. DEAE-cellulose chromatography of SLE virus-solubilized intracellular antigens. Infected cells were harvested 36 h postinfection, solubilized with Brij-58, and applied to a DEAE-cellulose column which was eluted stepwise with increasing molarities of KCl in Tris buffer, pH 7.8. Optical density at 280 nm (O). Identity of the complement-fixing antigen in each DEAE-column peaks is indicated by the bar diagram at the bottom.

Fig. 3. Immunodiffusion analysis of organic solvent-extracted solubilized antigen in DEAE-cellulose peak D. Center well contains hyperimmune anti-SLE ascitic fluid. Outer wells contain concentrated antigen before and after extraction. C, Carbon tetrachloride-ether-chloroform-extracted antigen; D, DEAE-cellulose peak D eluate; G, Genetron-extracted antigen.

Table 1. Summary of the results of SLE virus intracellular antigen III purification

<table>
<thead>
<tr>
<th>Sample</th>
<th>CF units/mg of protein</th>
<th>^H-DPM/mg of protein</th>
<th>^32P-DPM/μg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original homogenate</td>
<td>77</td>
<td>72</td>
<td>164</td>
</tr>
<tr>
<td>DEAE-cellulose peak D</td>
<td>167</td>
<td>235</td>
<td>7</td>
</tr>
<tr>
<td>Genetron</td>
<td>261</td>
<td>393</td>
<td>8</td>
</tr>
<tr>
<td>Chloroform-ether-carbon tetrachloride</td>
<td>298</td>
<td>485</td>
<td>429</td>
</tr>
<tr>
<td>Hydroxylapatite chromatography</td>
<td>853</td>
<td>667</td>
<td>a</td>
</tr>
<tr>
<td>Sepharose 6B chromatography</td>
<td>421</td>
<td>934</td>
<td>a</td>
</tr>
</tbody>
</table>

a Chemically undetectable phosphorus.

After CEC extraction a single antigen III precipitin band was observed.

Contaminating proteins were removed from partially purified antigen III preparation by chromatography on hydroxylapatite (Fig. 4). The organic solvent-extracted aqueous phase was dialyzed overnight against 0.001 M phosphate buffer, pH 7.8, and applied to a hydroxylapatite column (1.5 by 6.5 cm) equilibrated in the same buffer. The column was washed with 0.001 M phosphate buffer, and the antigen was eluted stepwise with 0.02, 0.06, and 0.10 M phosphate buffer, pH 7.8. Antigen III eluted from the column with 0.1 M phosphate buffer as evidenced by the CF activity in the 0.1 M phosphate peak. Other contaminating proteins
were eluted both at 0.001 and 0.06 M phosphate (Fig. 4). The antigen which eluted with 0.10 M phosphate was concentrated 50-fold by ultrafiltration (XM-50) and mixed with 1 M NaCl in Tris buffer, pH 7.8. The antigen III concentrate was mixed with purified φX-174 phage, dextran blue, hemoglobin, and phenol red, and applied to a Sepharose 6B column (4 by 90 cm) equilibrated with 1 M NaCl in Tris buffer, pH 7.8. The column was developed with the same buffer, and the eluate was collected in 2-ml fractions which were analyzed for optical density (280 nm), radioactivity, and CF activity. Sepharose 6B column chromatography of the concentrated antigen III preparation revealed one peak of radioactivity and CF activity (Fig. 5). The antigen eluted at an estimated molecular weight of approximately 80,000 in relation to the standard markers of known molecular weight.

The results of a representative purification are presented in Table 1. Antigens in the DEAE-cellulose region D eluate had a specific activity of 235, as compared to 72 for antigen in the original infected cell homogenate (Table 1). After cold Genetron extraction of antigens in the DEAE-column region D eluate, the preparation had a specific activity of 393. After CEC organic solvent extraction, protein in the aqueous phase had a specific activity of 485, which represented a 12-fold purification as compared to the original homogenate. Hydroxylapatite chromatography increased the specific activity to 667, an approximately 55-fold purification. Sepharose 6B chromatography of the antigen increased the specific activity to 934, which represents an approximately 200-fold purification of antigen III.

The antigen after each purification step was analyzed for residual phosphates by chemical and by 32P radioactivity determinations. Data from such analyses are presented in Table 1. The original homogenate had a 32P-DPM to chemical phosphorus ratio of 164. DEAE-cellulose chromatography removed relatively less chemically detectable phosphorus than the 32P radioactivity, which is indicated by low 32P-DPM to phosphorus ratio. Genetron extraction removed approximately similar amounts of chemically detectable phosphorus and the 32P counts resulting in the 32P-DPM to phosphorus ratio to 8, whereas CEC organic solvent mixture removed both chemically detectable phosphorus and 32P radioactivity to very low levels. However, the

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**Fig. 4. Purification of antigen III on a hydroxylapatite column.** Organic solvent-extracted antigen was applied to a hydroxylapatite column and eluted stepwise with increasing phosphate concentrations. Symbols: optical density at 280 nm (O); radioactivity (●); complement-fixation titer (■).
amount of chemically detectable phosphorus removed at this step was more than the $^{32}$P counts, which is evident by the high $^{32}$P-DPM to phosphorus ratio of 429. After the CEC extraction, the amount of chemically detectable phosphorus and $^{32}$P radioactivity was negligible. This indicates that more than 99% of the total phosphorus present, either as cellular lipids or as nucleic acid, was removed by organic solvent extraction.

The serological activity of the antigen during purification was evaluated at each step with respect to the residual antigen present. The results of a typical experiment are summarized in Table 1. The infected original homogenate had a CF unit (CFU) to protein ratio (serological specific activity) of 77. The antigen in DEAE-cellulose column region D had an increased CFU to protein ratio of 167, which was approximately twice that of the original. Organic solvent extraction resulted in a fourfold increase in the serological specific activity. Hydroxylapatite column chromatography of the partially purified antigen preparation increased the serological specific activity almost threefold as compared to the antigen after organic solvent extraction. Sepharose 6B column chromatography reduced the serological specific activity to nearly half of that observed after hydroxylapatite chromatography. The reduction in serological specific activity during Sepharose chromatography probably resulted from antigen deterioration during the long time interval required.

In summary, approximately 2.5% of the antigen in the infected cell homogenate was obtained as purified antigen III. The purified preparation represented only 0.5% of the total protein in the crude cell homogenate and was purified approximately 200-fold. The specific activity of this antigen was increased about 13-fold during purification, whereas the serological specific activity of the purified antigen increased 5-fold.

**Purification of intracellular antigen I.** Intracellular solubilized antigen I, the major virion envelope structural protein (15), is the only intracellular SLE viral antigen which elutes from the DEAE-column in region C. $^{3}H$-leucine-labeled SLE virus antigen in DEAE-column region C (soluble antigen I) was concentrated

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**Fig. 5.** Sepharose 6B gel filtration of solubilized antigen III. The radioactively labeled 0.10 M phosphate buffer eluate from the hydroxylapatite column was concentrated and made to 1 M NaCl in Tris buffer, pH 7.8. The antigen preparation was mixed with phase φX-174, dextran blue (DB), human hemoglobin (HH), and phenol red (PR) to serve as molecular weight markers and applied to a Sepharose 6B column which was developed with Tris-buffered 1 M NaCl, pH 7.8. Symbols: molecular weight markers (O); radioactivity (●); complement fixation (□).
50-fold by ultrafiltration (XM-50) and used for further study.

**Polyacrylamide gel electrophoresis of purified antigens.** To estimate the molecular weight of solubilized antigen III, radioactively labeled purified antigen was subjected to co-electrophoresis on 10% polyacrylamide SDS gels with protein standards of known molecular weights. Figure 6 shows that, in relation to the standard polypeptides, SLE virus intracellular solubilized antigen III had a molecular weight of approximately 85,000.

Purified and concentrated antigen I and III preparations labeled with 3H-leucine were subjected to co-electrophoresis with 14C-amino acid-labeled SLE virus-infected PS cell homogenate on 10% polyacrylamide SDS gels (Fig. 7). As seen in the top panel, solubilized antigen I contained only one radioactively labeled polypeptide which migrated in coincidence with the major virus envelope structural protein SP-1 (Fig. 7). As shown in the bottom panel, purified solubilized antigen III contained only one 3H-leucine-labeled polypeptide which migrated in coincidence with the viral nonstructural polypeptide NSP-1 (Fig. 7). These co-electrophoretic data confirm our previous observation that SLE virus-solubilized antigen I is the major virion envelope structural polypeptide, and solubilized antigen III is a nonstructural polypeptide (15, 21).
DISCUSSION

Antigen III isolated from cells infected with SLE virus was purified by freezing and thawing, sonication, detergent solubilization, organic solvent extraction, and column chromatography. This procedure yielded an antigen which had a final chemical specific activity of 934 and serological specific activity of 421. Approximately 99.5% of the protein and 99.9% of detectable phosphorus and $^{32}$P radioactivity in the crude antigen preparation were removed during purification. Antigen III was purified approximately 200-fold by this procedure and represented only 2.5% of the total antigenic activity based on CF titers in the crude infected cell homogenate. The relatively low serological specific activity obtained by this multistep procedure is undoubtedly due to antigen deterioration during purification.

Cellular lipoproteins are reported to be removed selectively from cell homogenates by Genetron and carbon tetrachloride-ether-chloroform extraction (10, 11). The selective removal of viral antigen I and 99.9% of chemically detectable phosphorus (Table 1) in the DEAE-column region D eluate by organic solvent extraction suggests that antigen I is a lipoprotein. This observation was expected, since antigen I, the virion envelope major structural protein, is a glycolipoprotein (unpublished data).

Calcium phosphate chromatography of tissues infected with arboviruses belonging to groups A, B, and C facilitated the separation of HA and CF antigens. Antigens separated in group B preparations were determined to include the virion, virion envelope component, and a CF antigen of then unknown nature (6, 19, 20). These antigens in dengue virus-infected mouse brain suspension have been identified as the complete virion (RHA), noninfected hemagglutinin (SHA), and a nonstructural soluble CF (SCF) antigen (6, 20).

Immunoprecipitation and CF tests have demonstrated that the dengue virus SCF antigen was also cross-reactive in heterologous tests (17). The molecular weight of SCF is reported as 39,000 (5, 7), whereas solubilized antigen III has a molecular weight of 85,000 by polyacrylamide SDS gel electrophoresis. These results indicate that solubilized antigen III is distinct from the nonstructural SCF antigen protein isolated from dengue virus-infected mouse brain.

Group B arbovirus antigens prepared from infected mouse brain cross-react with similar antigen preparations from viruses within this antigenic group (3, 4, 8, 14, 24). This problem has prompted a search for a virus type-specific antigen to be used as diagnostic reagent. From the data now available, it appears that the whole virion, the virus envelope, or viral structural subunits contain both group-reactive and type-specific determinants and therefore are not ideal as serologic reagents (8). The serological specificity of purified antigen III and solubilized antigen I is described in the accompanying paper (16).

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LITERATURE CITED