La Crosse Virus Soluble Cell Culture Antigen

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A virus-free soluble antigen, obtained by ammonium sulfate precipitation of the supernatant fluids of La Crosse virus-infected BHK-21 cell cultures, was more reactive and more specific than infected suckling mouse brain antigen when compared by immunodiffusion and counterelectrophoresis tests. By complement fixation tests, the antigen was cross-reactive with heterologous California group arbovirus hyperimmune mouse ascitic fluids, but to a lesser degree than was the standard sucrose-acetone-extracted infected suckling mouse brain antigen. The major virion nucleocapsid protein of La Crosse virus was found by polyacrylamide gel electrophoresis to be the soluble antigen protein responsible for precipitation in immunodiffusion and counterelectrophoresis tests.

La Crosse virus, a California group arbovirus of the Bunyaviridae family (17), was originally isolated from human brain tissue in 1964 (19). Although 11 other types or subtypes of California group virus exist in the United States (7, 16, 18), as shown by isolations from arthropods and by serological evidence in humans and animals, only La Crosse virus, thus far, is known to cause serious human disease. Symptoms range from a mild, undifferentiated febrile illness to transient aseptic meningitis or severe encephalitis (10). The increasing numbers of human La Crosse virus infections reported since 1964 emphasize the importance of including California group antigens in routine diagnostic tests for arboviral infections involving the central nervous system.

Serological methods used for the diagnosis of infections, the identification of isolates, and the study of antigenic relationships between California viruses included hemaggultination inhibition, complement fixation (CF), immunodiffusion (ID), counterelectrophoresis (CEP), and neutralization tests (G. D. Brooks, Ph.D. thesis, University of North Carolina, Chapel Hill, 1973; 2, 3, 8, 12, 20, 21). All the antigens used in these tests, except a cell culture hemaggultinin antigen for hemaggultination inhibition (5), were derived from infected suckling mouse brain (ISMB). The standard sucrose-acetone-extracted mouse brain antigen has been the most satisfactory for CF tests. However, simple homogenates of ISMB were the most suitable antigens for ID and CEP tests. Murphy and Coleman (12) investigated several virus preparations for use in ID tests and concluded that an undiluted suspension of ISMB was superior to either a crude tissue culture suspension or sucrose-acetone-extracted ISMB.

To meet the need for a less-complicated yet sensitive antigen for early diagnosis of California virus infections and for differentiation among these viruses, we prepared a virus-free soluble cell culture antigen (ccAg) from BHK-21 tissue culture fluids. This antigen was very reactive and was more specific than ISMB antigen when the two were compared in ID, CEP, and CF tests. The soluble antigen, extracted from immune precipitates in agar, was found to be indistinguishable from the major virion nucleocapsid protein (13).

MATERIALS AND METHODS

Chemicals and reagents. U-14C- or U-3H-labeled L-amino acid mixtures were obtained from New England Nuclear Corp., Boston, Mass. Specially purified reagents for preparing polyacrylamide gels were purchased from Bio-Rad Laboratories, Richmond, Calif. Other reagents used included: absolute-grade ammonium sulfate from Research Plus Laboratories, Inc., Denville, N.J.; sodium dodecyl sulfate (SDS), glycerol, sucrose, and potassium tartrate from Gallard-Schlesinger Chemical Corp., Long Island, N.Y.; and purified agarose from L'Industrie Biologique Française, Gennevilliers, France.

Preparation of La Crosse soluble and virion antigens. Confluent roller bottle monolayer cultures of BHK-21 cells, grown in reinforced Eagle medium with 10% (vol/vol) fetal calf serum, were infected with La Crosse virus at an input multiplicity of 0.001 plaque-forming units per cell. The infected cultures were maintained with medium 199 at 33°C. The culture fluids were harvested after 48 to 56 h, when extensive cytopathic changes were evident in all the cells. Cellular debris was removed from the infectious cell culture fluids by centrifugation at 10,000 x g for 20 min at 4°C. Virus particles were removed from the
cell-free fluid by centrifuging at 30,000 rpm for 2 h at 4°C in a Beckman type 30 rotor. The pellet from this centrifugation, which contained almost all of the viral infectivity of the culture, was suspended in TSE buffer [tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 7.8), 0.15 M NaCl, 0.002 M ethylenediaminetetraacetic acid], and a portion was stored at -70°C as a crude La Crosse virion antigen for subsequent serological studies. The remainder of the virus was purified by successive cycles of sucrose and potassium tartrate-glycerol equilibrium density gradient centrifugation, as described earlier (15). The purified virus was concentrated into a pellet by centrifugation, suspended by sonic treatment in TSE buffer, and stored at -70°C. This material was used in serological tests as a purified La Crosse virion antigen.

La Crosse virus soluble BHK-21 ccAg was recovered from the virus-free cell culture supernatant fluid at 4°C by the dropwise addition of 1.5 volumes of saturated ammonium sulfate solution. Precipitation was allowed to continue at 4°C for an additional 2 h after the ammonium sulfate was added. The precipitate was collected by centrifugation at 14,000 × g for 45 min at 4°C and was washed twice with 50% saturated ammonium sulfate solution by resuspension and further centrifugation. The final pellet was dissolved in TSE buffer, and residual ammonium sulfate was removed by dialysis at 4°C against five 1,000-volume changes of TSE buffer.

La Crosse soluble ccAg that was to be tested for CF activity was prepared from virus-free supernatant fluid containing the culture fluid against Veronal buffer (4) for 3 days at 4°C and then concentrated the antigen 50-fold by evaporation. ISMB antigens used for comparative CF controls were prepared by a standard sucrose-acetone extraction method (6).

La Crosse ISMB antigens used for ID and CEP studies were recovered from infected mouse brain suspensions as described by Wellings et al. (20).

Radiolabeled La Crosse virus antigens. For the production of labeled virus or labeled soluble ccAg, we included in the Eagle virus growth minimal essential medium either a mixture of L-14C-amino acids (0.2 μCi/ml) or L-3H-amino acids (2 μCi/ml) and only 20% of the standard level of amino acids. After 48 h of growth at 33°C, purified virus particles and soluble ccAg were prepared from the cell culture fluids as described.

Serological tests. CF tests were performed by the standardized LBCF method (4). ID tests were done by first allowing 10 ml of a 0.9% agarose solution in TSE buffer to solidify on the surface of a glass slide (5 by 8 cm). After the gel was allowed to stand overnight at 4°C, wells to contain the antigen were cut out in a hexagonal pattern around a single center well for the test antisera. The wells were 3 mm in diameter and had a center-to-center distance of 7 mm between reactant wells (Fig. 1). Reactants were added to the wells with a capillary pipette, and, after incubation for 24 h at 4°C, the plates were examined for immunoprecipitates. The results on each slide were recorded by dark-field illumination photography.

The immune fluids used throughout this study were hyperimmune mouse ascites fluids (AF). These were prepared by multiple subcutaneous injections of adult mice with a vaccine derived from a 1% suspension of ISMB (8, 11). The hyperimmune AF collected were lyophilized and stored at -20°C until used. In addition, single-injection hamster antisera obtained from the Vector-Borne Diseases Division, Center for Disease Control, Fort Collins, Colo., were used to evaluate specificity of our La Crosse soluble antigen.

CEP. CEP was performed on glass slides (8 by 10 cm) in 0.7% agarose as described by Hatch (9) with the following modifications. The Veronal buffer used in this study had a pH of 7.7 and contained 0.1 M NaCl and 0.002 M ethylenediaminetetraacetic acid. Two parallel rows of 10 holes each (5 mm in diameter) with a center-to-center distance of 10 mm were cut into the agarose layer with a stainless-steel cutter. Immune AF were always added to the wells on the anodic side (right side), whereas the antigens to be tested filled the cathodic wells (left side). Electrophoresis was performed at room temperature for 1.5 h at a constant current of 35 mA per slide. Immediately after electrophoresis, each slide was examined under an indirect light and then photographed. This time schedule was necessary to avoid nonspecific reactions that sometimes developed if the slides were allowed to sit for more than 2 to 3 h after electrophoresis.

Polyacrylamide gel electrophoresis. The protein(s) present in the immune complexes formed either by ID or CEP tests was analyzed by SDS-polyacryl- amide gel electrophoresis. To do this, we cut several immunoprecipitate lines from the agarose gel and dialyzed the material four times against 100 volumes of TSE buffer at 4°C. This procedure was satisfactory for removing extraneous proteins that did not compose either the antigen or antibody of the immunoprecipitate line. After dialysis, the immune complexes were suspended in 0.06 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 6.8) and were solubilized by boiling for 3 min with a solution of 2.5% (wt/vol) SDS, 5% (vol/vol) 2-mercaptoethanol, and 8 M urea. The proteins were resolved by a discontinuous SDS-polyacrylamide gel electrophoresis procedure as described previously (13, 15). When radioactively labeled proteins were separated, their locations were determined by cutting frozen gels into 1-mm portions and determining the radioactivity of each slice after incubating them at 37°C for 18 h in a toluene-based scintillation cocktail containing 4% (vol/vol) NCS (Amersham/Searle, Arlington Heights, Ill.). In some experiments, the location of the proteins was detected by staining the gels with Coomassie brilliant blue, destaining in an acetic acid-methanol mixture, and scanning the gels at 620 nm in a Gilford recording spectrophotometer (13).

RESULTS

Production and evaluation of La Crosse soluble ccAg by ID and CEP tests. Separate La Crosse virus-infected BHK-21 monolayers were harvested at either 16, 24, 30, or 48 h postinfection, and the soluble ccAg was recovered by precipitation with ammonium sulfate. Although 2+ to 3+ cytopathic effects were observed in cultures, precipitating ccAg was not
detected at 24 h by ID (Fig. 1). At 30 h postinfection, some of the cultures harvested gave inconsistent precipitin reactions. However, definite reproducible ID patterns were always observed for ccAg collected by 48 h, when the cytopathic effect was 4+. In contrast to the multiple lines seen when ISMB antigen was tested, our preparation of ccAg gave a single distinct line by ID. On one occasion, the ccAg preparation did give, unexplainably, a second very faint ID line that became more evident when photographed (Fig. 1). Appropriate controls of uninfected BHK-21 cultures, uninfected suckling mouse brain suspension, and TSE buffer were included and emphasize the specificity of the reactions observed.

CEP was not only a more rapid test (1.5 h) than ID, but was also more sensitive, since we were able to detect ccAg in cultures harvested as early as 24 h postinfection (Fig. 2). This procedure also gave a more concise immunoprecipitate line with ccAg than with La Crosse ISMB antigen. An unexpected single reaction line was evident when either crude or purified virion antigen was tested by CEP. A similar line did not appear when these same two antigens were tested by simple ID. Perhaps the combination of the increased sensitivity of the CEP and some damage to the virus particles during the standard purification procedure allowed us to detect one of the virion antigens with this test procedure. Information that relates to the latter suggestion was obtained by concentrating the virus particles by polyethylene glycol 6000 precipitation before density gradient purification. When La Crosse crude and purified virion antigens prepared by this method were tested by CEP, no reaction with the test hyperimmune AF was detected (unpublished data).

Cross-reactivity of La Crosse soluble ccAg. Group specificity was observed by ID and CEP procedures when La Crosse ccAg was tested against heterologous mouse AF of the California group viruses (Fig. 3 and 4). Strong immunoprecipitin lines (4+) were evident against California encephalitis and snowshoe hare viruses. Jamestown Canyon, San Angelo, and Trivittatus viruses showed moderate immunoprecipitation (2+), whereas Melao, Keystone, and Tahyna viruses reacted to a lesser extent (1+). There was no reaction when either eastern equine encephalomyelitis virus, an alphavirus, or St. Louis encephalitis, a flavivirus, or normal mouse AF was tested. Similarly, the same cross-reactivity observed by the ID tests was also noticed when these same reagents were tested by the CEP procedure (Fig. 4).

By the CEP procedure, the extent of cross-
reactivity of our La Crosse ccAg with heterologous hyperimmune AF was measured by testing varying dilutions of antigen against varying dilutions of antisera (Table 1). The results of this one-way cross-reaction show La Crosse virus to be more closely related to snowshoe hare, California encephalitis, and Jamestown Canyon viruses, less related to Trivittatus and San Angelo viruses, and even more distantly related to Key- stone, Melao, and Tahyna viruses. Our observations concerning the serological relationship of La Crosse virus to the other California group viruses agree with the findings of other studies in which different test antigens were used (Brooks, Ph.D. thesis; 2, 12, 16, 20).

When ISMB antigen was tested by ID and CEP tests, we found the same degree of reactions among the group members (Fig. 4 and 5). However, the immunoprecipitate lines varied from single arcs to double and triple arcs, which sometimes obfuscated the interpretation of the results. Problems of reproducing our results with the ISMB antigen preparation were evident, particularly in the CEP test system (Fig. 4). Often extraneous mouse brain tissue diffused in the anodic direction during electrophoresis and made subsequent examination of the CEP slides difficult. Since single, distinct precipitation was observed in both test systems, it was apparent that the La Crosse ccAg clarified the ID and CEP reactions. In addition to clarifying ID and CEP reactions, the sensitivity and specificity of the La Crosse ccAg was demonstrated using single-injection hamster sera collected 10 days postinoculation (Table 1). Even though high homologous CF and neutralization titers were evident against all the California group viruses, cross-reactions were not detected by CEP with undiluted antisera. Antibody to La Crosse virus produced in rabbits was detected as early as 7 days postinoculation by this antigen (data not shown).

CF reactivity of La Crosse ccAg. The CF reactivity of the virus-free infected cell culture fluids was low; optimal dilutions were usually 1:4 and 1:8. However, after concentration by pervaporation, the optimal dilution for the antigen increased to about 256. When the concentrated CF La Crosse ccAg was tested for reactivity with California group virus hyperimmune AF in comparison with the standard sucrose-acetone-extracted suckling mouse brain antigen, greater specificity of the ccAg was observed (Table 2). Whereas the mouse brain antigen exhibited only 2-fold to 4-fold differences between homologous and heterologous AF, the ccAg gave 8-fold to 16-fold differences.

Identification of the proteins in La Crosse soluble ccAg. To identify the soluble antigen protein responsible for precipitation by ID and CEP, we cut immune complexes from ID and CEP agar gels and examined their protein con-
tent by SDS-polyacrylamide gel electrophoresis. Stained gel electropherograms revealed three distinct bands (Fig. 6A) and indicated that the soluble antigen was probably the major virion nucleocapsid protein N (molecular weight, 25,000) (13). The two additional proteins observed in the stained gel tracing are the heavy and light chains of the dissociated immunoglobulin. The association of the N-protein with the immunoprecipitates was confirmed by subjecting purified preparations of $^{3}C$-amino acid-labeled La Crosse virus to electrophoresis with $^{3}H$-amino acid-labeled soluble antigen recovered from CEP immune complexes (Fig. 6B).

**DISCUSSION**

In previous serological studies of California group arboviruses with ISMB antigen in ID tests, several antigenic subgroups among these viruses were identified by unique lines of immunoprecipitation (12). Many of the ID patterns obtained were difficult to interpret, however, because of multiple arc formation. Wellings et al. (20) adopted a more successful approach for distinguishing these viruses by using ID tests with rabbit antisera that was collected after a single injection of ISMB antigen. These investigators also observed that, if an antigen booster was given to the rabbits, the immune sera collected were less specific in the ID reactions. In the study reported here, we were successful in isolating virus-free soluble ccAg from La Crosse virus-infected BHK-21 cells. When compared with ISMB antigen by ID and CEP tests, the ccAg was superior in that it gave single, precise, clear, and reproducible precipitate lines of greater specificity. Production of a soluble ccAg for the remaining less-significant California group viruses was neither intended nor feasible, making two-way comparisons impossible. However, cross-reactivity of La Crosse ccAg in ID and CEP analyses against hyperimmune mouse AF of eight other California group viruses showed equally strong precipitation against California encephalitis, snowshoe hare, and Jamestown Canyon viruses; a lesser reaction for San Angelo and Trivittatus viruses; and little or no reaction to Melao, Keystone, and Tahyna viruses (Fig. 3 and 4). Specific reactions were demonstrated when single-injection hamster antisera were used rather than hyperimmune AF. Although heterologous rabbit antisera were not available to us, others have found that rabbit antisera show more homotypic reactivity than do hyperimmune mouse AF (1).

La Crosse ccAg exhibited some cross-reactivity when tested by CF with heterologous California group virus immune AF. However, the degree of cross-reactivity was considerably less than that found with standard sucrose-acetone-extracted ISMB antigen, emphasizing the purity of the ccAg (Table 2).

The CEP test with La Crosse ccAg was a very rapid (test read in 1.5 h) and sensitive procedure and consistently gave a single distinct precipitation line (Fig. 2). Balfour and associates have also used the CEP technique with an ISMB antigen, and they were able to detect La Crosse antibodies in about 40% of patients during acute illness (1, 2). Although not reported here, we
FIG. 4. CEP of La Crosse ccAg (cathode wells, −; pattern A) or La Crosse ISMB antigen (cathode wells, −; pattern B) with control reagents and virus-specific immune mouse AF (anode wells, +; both patterns). Reagents in the anode wells: (1) TSE buffer; (2) La Crosse virus; (3) eastern equine encephalomyelitis virus; (4) California encephalitis virus; (5) St. Louis encephalitis virus; (6) snowshoe hare virus; (7) normal mouse AF; (8) Jamestown Canyon virus; (9) TSE buffer; (10) San Angelo virus; (11) TSE buffer; (12) Trivittatus virus; (13) TSE buffer; (14) Melao virus; (15) normal mouse AF; (16) Keystone virus; (17) TSE buffer; (18) Tahyna virus.
TABLE 1. Cross-reactivity of La Crosse ccAg with California group heterologous antisera by CEP

<table>
<thead>
<tr>
<th>Serological test system</th>
<th>LAC</th>
<th>CEV</th>
<th>SSH</th>
<th>JC</th>
<th>TVT</th>
<th>SA</th>
<th>MEL</th>
<th>KEY</th>
<th>TAH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperimmune AF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Homologous CF titer(^{b})</td>
<td>256</td>
<td>64</td>
<td>256</td>
<td>128</td>
<td>256</td>
<td>64</td>
<td>32</td>
<td>64</td>
<td>16</td>
</tr>
<tr>
<td>CEP titer(^{c})</td>
<td>32</td>
<td>8</td>
<td>16</td>
<td>8</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Single-injection hamster sera</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homologous CF titer</td>
<td>64</td>
<td>256</td>
<td>256</td>
<td>64</td>
<td>512</td>
<td>256</td>
<td>32</td>
<td>NT(^{d})</td>
<td>NA (^{e})</td>
</tr>
<tr>
<td>Homologous neutralization titer</td>
<td>250</td>
<td>1,050</td>
<td>1,050</td>
<td>250</td>
<td>1,800</td>
<td>2,000</td>
<td>250</td>
<td>160</td>
<td></td>
</tr>
<tr>
<td>CEP titer</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^{a}\) Viruses: LAC, La Crosse; CEV, California encephalitis; SSH, snowshoe hare; JC, Jamestown canyon; TVT, Trivittatus; SA, San Angelo; MEL, Melao; KEY, Keystone; TAH, Tahyna.

\(^{b}\) Each California subtype CF antigen was a sucrose-acetone-extracted ISMB tissue preparation.

\(^{c}\) CEP titer is expressed as the reciprocal of the highest dilution giving a 1+ precipitation reaction against an optimal dilution of La Crosse ccAg. Undiluted antisera giving a 1+ reaction were given a titer of 1.

\(^{d}\) Negative reaction.

\(^{e}\) NT, Not tested.

\(^{f}\) NA, Not available.

FIG. 5. Gel diffusion reactivities of La Crosse ISMB antigen (center wells) with hyperimmune mouse AF (outer wells) against the same reagents as those used for Fig. 3.

TABLE 2. Complement fixation reactivity of La Crosse ccAg and La Crosse ISMB antigen

<table>
<thead>
<tr>
<th>Antigen</th>
<th>LAC</th>
<th>CEV</th>
<th>SSH</th>
<th>JC</th>
<th>TVT</th>
<th>SA</th>
<th>MEL</th>
<th>KEY</th>
<th>TAH</th>
<th>EEE/SLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAC (ccAg)</td>
<td>256</td>
<td>16</td>
<td>32</td>
<td>16</td>
<td>16</td>
<td>8</td>
<td>&lt;8</td>
<td>&lt;8</td>
<td>&lt;8</td>
<td>&lt;8/&lt;8</td>
</tr>
<tr>
<td>LAC (ISMB)</td>
<td>256</td>
<td>64</td>
<td>128</td>
<td>64</td>
<td>32</td>
<td>64</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>&lt;8/&lt;8</td>
</tr>
<tr>
<td>Homologous (ISMB)</td>
<td>64</td>
<td>256</td>
<td>128</td>
<td>256</td>
<td>64</td>
<td>32</td>
<td>64</td>
<td>16</td>
<td>128/64</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Hyperimmune AF CF antibody titers were determined by block titration, i.e., varying dilutions of antigen versus varying dilutions of antisera. The heterologous cross-reaction antibody titer is the serum titer against the optimal antigen dilution of the homologous system. EEE, Eastern equine encephalomyelitis virus; SLE, St. Louis encephalitis virus. For other abbreviations, see Table 1, footnote \(^{a}\). Underlines indicate the homologous system.
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have also used La Crosse ccAg to detect antibodies in a limited number of human sera collected from patients infected with La Crosse virus. Our preliminary findings indicate positive results with patients' immune sera that also were positive by CF tests. Our results were negative, however, with sera that had no detectable CF antibodies but were positive by hemagglutination inhibition and neutralization tests. Apparently CF and precipitating antibodies are analogous, and these findings agree with similar ideas expressed earlier by others (3, 20).

Polyacrylamide gel electrophoresis analysis indicated that the soluble antigen responsible for precipitation by ID and CEP tests was the major virion nucleocapsid protein N (molecular weight, 25,000) (13). At present, we do not know whether the antigen expelled into the extracellular fluids during infection is free N-protein or whether the antigen is bound to the virus ribonucleic acid, as it is found in mature virions (14).

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LITERATURE CITED


