Identification of Respiratory Syncytial Virus Antigens by Agar Gel Diffusion and Immunoelectrophoresis* (34519)

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Respiratory syncytial (RS) virus has been demonstrated to be an important cause of severe lower respiratory illness in infants and young children (1). For this reason, the development of a safe and effective vaccine to prevent infection by this agent would be of great practical public health importance. Recently, the administration to infants of an experimental inactivated concentrated vaccine was shown to result in more extensive respiratory disease following natural RS infections in the immunized infants than in a comparable nonvaccinated control group (2–5). On the basis of both this paradoxical vaccine effect and the epidemiologic setting of natural RS virus disease, the immunological response to RS virus and its antigens has been postulated to be a critical determinant to host response to RS virus infection (2). For this reason, a systematic study of RS virus has been undertaken in an attempt to define these critical immunological reactions and their relationship to RS virus infection and immunization.

RS virus is an RNA containing virus which has been classified as a member of the myxovirus family on the basis of its nucleic acid composition, ether sensitivity, size, and ultrastructure (6). Previous studies have identified two soluble complement-fixing (CF) antigens of RS virus, which have been designated RS antigen A and RS antigen B (7). These antigens were separated by column chromatography and possessed different biological and physical properties. The present study was undertaken to characterize further the antigens of RS virus by agar gel diffusion and immunoelectrophoresis, with the expectation that this approach would provide additional information about the antigenic structure of the virus and aid in elucidating the immunological reactions to RS virus infection and immunization.

Materials and Methods. Virus. The Long strain of RS virus, which was first isolated from the throat of a patient with bronchopneumonia and since passed six times in tissue culture of KB cells, twice in Chang liver epithelium, and six or seven times in HEp-2 cells, was used in all experiments (7).

Tissue culture. HEp-2 cells were grown in 16-oz disposable Brockway bottles in Eagle's basal media with Hank's balanced salt solution containing 10% inactivated fetal bovine serum (56° for 30 min). Prior to inoculation, the cells were washed three times with Hanks' balanced salt solution. Eagle's basal media containing 5% inactivated agamma chicken serum (Hyland Laboratories, Los Angeles, California) was used to maintain the tissue cultures.

All media contained 0.1 ml of L-glutamine, 100 units of penicillin, 100 μg of streptomycin, and double the usual concentration of amino acids and vitamins. All tissue cultures were incubated at 33° to 34° in a stationary position.

Preparation of concentrated antigen pools. HEp-2 bottles were infected with approximately 10^2 to 10^3 $TCID_{50}$ of a standard preparation of the Long strain of RS virus. Tissue culture fluid was harvested when 75 to 100% of the tissue culture cells showed cytopathic effects. The fluid was then centri-

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fuged at 4° for 20 min to 2000 rpm and the pellet removed.

Tissue culture fluids were then concentrated 20 to 100 times by ultrafiltration. These concentrated antigen pools were used without further treatment in some of the gel diffusion and immunoelectrophoresis experiments. In addition, purified RS antigens A and B were prepared from these concentrated antigen pools by column chromatography on Sephadex G-200, by methods previously described (7). RS antigens A and B were further purified before use by reconcentration and rechromatography on Sephadex G-200 by methods previously described (7).

Control antigens. Uninfected HEp-2 was grown and maintained as indicated above. The cell sheet was frozen and thawed three times. This crude material was then processed in the same manner as the infected tissue culture fluids None of the sera used reacted with the control antigens in the CF test, agar gel diffusion, or immunoelectrophoresis.

Test sera. Guinea pig sera were prepared, as previously described, from rechromatographed bovine embryo kidney grown RS virus (7). Guinea pig serum A had a CF antibody titer of 1:16 when tested with four units of RS antigen A by methods previously described (7). It had a CF antibody titer of less than 1:2 when tested with four units of RS CF antigen B. Guinea pig serum B had a CF titer of less than 1:2 when tested with four units of RS antigen A and a titer of 1:8 when tested with four units of RS antigen B.

A human postinfection serum with a CF titer of 1:64 when tested with four units of crude RS virus antigen and a CF titer of 1:16 when tested with four units of either RS antigen A or B was also used in these experiments. This serum had a neutralized antibody titer of 1:128 when tested with the Long strain of RS virus as previously described (8).

Agar gel diffusion. Ouchterlony agar gel diffusion was performed using commercially prepared 1- × 3-in. slides containing 4 ml of agar gel (Hyland Laboratories). The agar gel had the following composition: Special Noble Agar (Difco) 2%; glycine 7.5%; sodium chloride 1%; and sodium azide 0.1%. The agar had a final pH of 7.2. The plates had six 2-mm wells, with a distance of 5 mm between the central and peripheral wells.

The wells were filled with the appropriate antigens and sera and incubated in a moist chamber at room temperature for 24 to 48 hr. The slides were then examined by incident light microscopy, washed for 2 to 3 days in normal saline, and rinsed for 1 day in distilled water. Finally, the slides were dried, stained with amido black, destained, and photographed.

Immunoelectrophoresis. Immunoelectrophoresis was carried out using a modification of the method of Scheidegger (9) 1- × 3-in. agar slides, containing the agar described above, were prepared. A 2-mm center well, 5 mm from a 58- × 2-mm trough, was used. A barbital buffer solution, with a pH of 8.2 and an ionic strength of 0.1, was placed in the trays. The center well was filled with the antigen to be treated. The slides were run at 5 ma per slide, with a total voltage between 70 and 90 volts for 2 to 4 hr. Following the completion of the electrophoresis, the appropriate antiserum was pipetted into the troughs, and the slides were incubated for 24 to 48 hr in a moist chamber. The slides were then washed thoroughly in a normal saline for 2 to 3 days, followed by a 1-day rinse in distilled water. The slides were dried, stained with amido black, destained, and photographed.

Results. Agar gel diffusion. Figure 1 illustrates the results of an agar gel diffusion experiment designed to determine the number of precipitating antigens present in the concentrated RS virus-infected tissue culture fluid (well C), and to compare these precipitin lines with those obtained using chromatographically purified RS antigens A (well A) and RS antigens B (well B). The center well (E) contained a human postinfection serum, and the HEp-2 control antigen was placed in well D. With this serum, two precipitating antigens were regularly and reproducibly demonstrated in the concentrated infected tissue culture fluids. In contrast, each of the

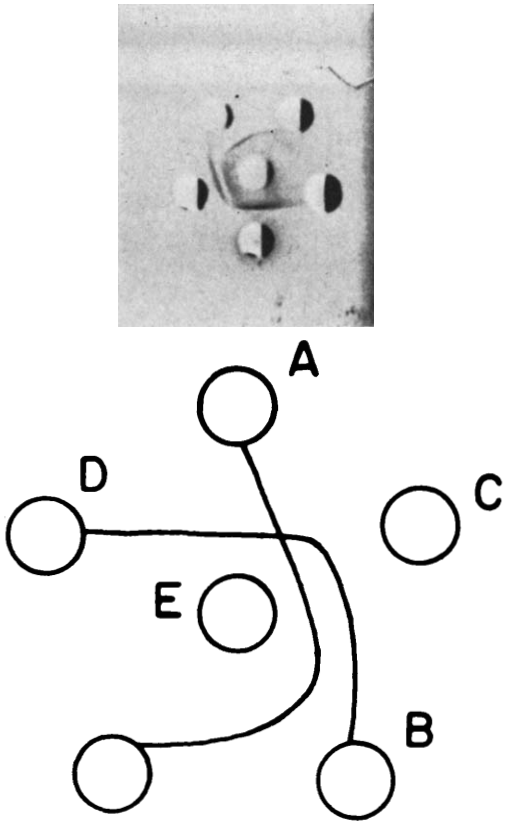

FIG. 1. Precipitating antigens of RS virus demonstrated by agar gel diffusion: well A = RS antigen A; well B = RS antigen B; well C= concentrated virus-infected tissue culture fluid; well D = tissue culture control antigen; well E = human post-RS virus-infection serum.

two chromatographically purified antigens, RS antigens A and B, contained only one precipitating antigen. The precipitating antigens of chromatographic RS antigens A and B appeared to be immunologically distinct from each other, and immunologically identical with different precipitating antigens present in the concentrated infected tissue culture fluids. There was no reaction between the control HEp-2 antigen and the test serum. Although the presence of three precipitating antigens in RS virus preparation has been reported previously (10, 11), a third precipitin line could not be regularly and reproducibly demonstrated in the present test system.

Figure 2 illustrates the results of an experiment designed to determine the number of precipitin lines produced by sera obtained by the immunization of guinea pigs with bovine kidney–grown RS antigen A (well F) and RS antigen B (well G) when they were tested with concentrated RS virus-infected HEp-2 tissue culture fluid (well H). This latter antigen produced two precipitin lines when tested with the human postinfection serum in the experiment described previously. Each serum produced only one precipitin line, and these precipitin lines were immunologically distinct. There was no reaction between these sera and the HEp-2 control antigens contained in wells I.

Immunoelectrophoresis. Following the demonstration of two reproducible precipitin

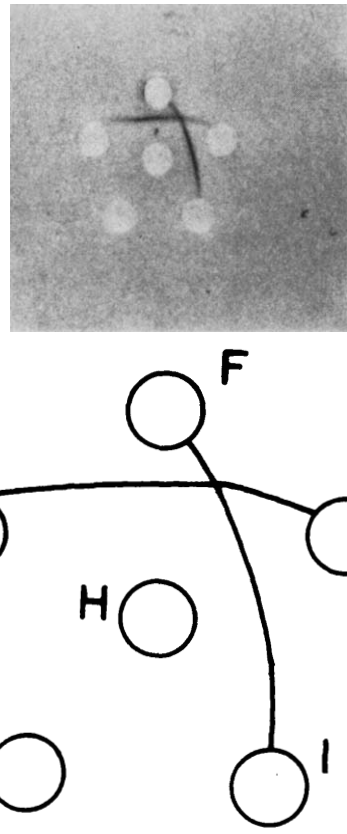

FIG. 2. Specificity of sera obtained by the immunization of guinea pigs with purified RS antigen: well F = guinea pig anti-RS antigen A; well G= guinea pig anti-RS antigen B; well H= concentrated RS virus-infected tissue culture fluid; well I = tissue culture control antigen.

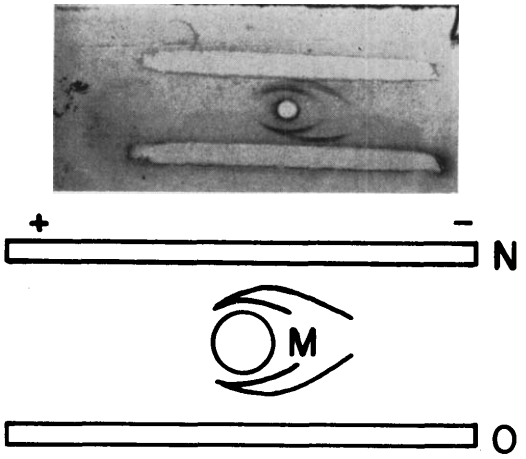

FIG. 3. Immunoelectrophoretic separation of RS virus antigens: well M = concentrated RS virus-infected tissue culture fluid; wells N and O = human post-RS virus-infection serum.

lines in concentrated RS virus-infected tissue culture fluids, and the identification of these precipitin lines with the previously described chromatographic RS antigens A and B, an attempt was made to separate and identify these antigens by immunoelectrophoresis. This method was used to determine the characteristic mobility of RS antigens A and B, and to detect any additional precipitating antigens.

Figures 3 and 4 illustrate the results of the electrophoretic separation of concentrated RS virus-infected tissue culture fluids. In each experiment, the concentrated tissue culture fluid was placed in the center well (M or J) and then subjected to electrophoresis, as described above. In Fig. 3, the results of an experiment in which the human postinfection serum had been placed in troughs N and O following electrophoresis are illustrated. The two precipitin bands previously noted in the agar diffusion experiment are present, and the two antigens appear to have different electrophoretic mobility. There is no evidence of a third precipitating antigen in this preparation. In Fig. 4, the results of a similar experiment, in which the previously described guinea pig serum A (trough L) and guinea pig serum B (trough K) were placed in the troughs following electrophoresis, are given. As previously noted, each serum produced only one precipitin line and these precipitating antigens had different electrophoretic mobilities. Under the conditions of these experiments, RS antigen A precipitated as a band moving toward the cathode, whereas RS antigen B remained close to the origin.

Discussion. The present studies demonstrate the presence of two precipitating antigens in concentrated RS virus-infected tissue culture fluids. Each one of these two antigens appears to be immunologically distinct and is apparently immunologically identical with a different one of the two chromatographically separated RS virus antigens A and B. The two precipitating antigens have different electrophoretic mobilities and can be identified by immunoelectrophoresis, utilizing specific sera prepared by immunizing guinea pigs with chromatographically purified RS virus antigens A and B.

Although several reports have suggested the presence of three precipitating and CF antigens in RS virus preparations, the presence of a third RS virus precipitating antigen has not been a consistent or reproducible finding in the experiments reported in this paper (7, 10, 11). There are several possible explanations for the inability to demonstrate more than two precipitating antigens in our experiments. The agar gel diffusion and

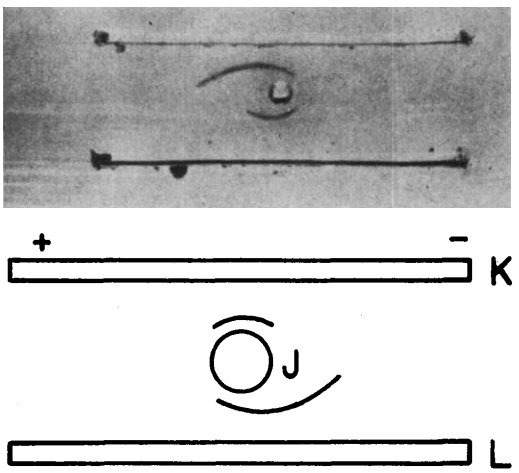

FIG. 4. Immunoelectrophoretic identification of RS virus antigen: well J = concentrated RS virus-infected tissue culture fluid; well L = guinea pig anti-RS antigen A; well K = guinea pig anti-RS antigen B.

immunoelectrophoretic methods are insensitive means of detecting RS virus antigens, and concentration of infected tissue culture fluids up to 100 times is necessary to produce any reaction. It is possible that other antigen(s) was present in insufficient quantity to be detected by this method, or that this additional antigen(s) was labile and thus denatured during the concentration of the infected tissue culture fluids. It is also possible that any additional antigen(s) was a cell-associated antigen and therefore not present in the material tested. Since previous studies have indicated that the animal and human sera differ in their immunological response patterns to the various RS virus antigens following natural infection or immunization, it is possible that the test sera used in these experiments lacked sufficient antibody to any additional antigen(s) (7). Further studies are currently in progress using other antigen preparations, antisera, and diffusion techniques, in order to elucidate this problem.

RS antigens A and B have been demonstrated previously to possess different biological properties. Guinea pigs immunized with chromatographically purified RS virus antigen A produced neutralizing antibody, whereas guinea pigs immunized with chromatographically purified RS antigen B failed to produce any neutralizing antibody (7). Both groups of animals produced homologous but not heterologous CF antibody. Assay of the protective antigen content of various tissue culture and vaccine preparations would be very important in the production of an efficacious inactivated RS virus vaccine. The identification and quantification of the various RS antigens in these preparations has been hampered because of the difficulty in producing chromatographically purified antigens and specific homologous antisera. The techniques described in this study should make it possible to identify RS antigens A and B by their distinctive electrophoretic mobilities in various RS virus tissue fluids or in potential vaccine preparations without using antigen-specific CF antisera. These methods should also be useful in characterizing human and animal serological responses following both natural RS virus infection and immunization with experimental live and inactivated RS virus vaccines, thus aiding materially in the development and evaluation of a safe and efficacious RS virus vaccine.

Summary. Two precipitating antigens of RS virus, which are immunologically distinct from each other, have been identified by agar gel diffusion. Each of the previously described chromatographic RS virus antigens A and B appear to be immunologically identical with a different RS virus-precipitating antigen and to be immunologically distinct from each other. RS virus antigens A and B also possess distinct electrophoretic mobilities which make possible their identification by immunoelectrophoresis.

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