Production in Guinea Pigs of Antibodies to Cross-Reactive Antigenic Determinants of Human Enteroviruses

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We studied the type specificity of the antisera which were produced in guinea pigs by immunization with heat-disrupted virions of human enteroviruses (echovirus types 7 and 11 and coxsackievirus type B5). When guinea pigs were immunized with heated virions (H particles), the antibodies produced reacted type specifically by complement fixation with the homologous H particles. By contrast, when guinea pigs were immunized with disrupted virions prepared by heating in the presence of sodium dodecyl sulfate, antibodies were produced which reacted broadly. Heterotypic complement-fixing antibody responses of humans to enterovirus infections are discussed.

Complement fixation (CF) tests are widely used for serodiagnosis of many viral infections. In the case of enterovirus infection, however, the sera of patients often react by CF even with unheated antigens of many different serotypes; thus, CF antibody assays are not useful for determining the serotype of the infecting enterovirus (2, 4, 7, 13, 16, 23). On the other hand, hyperimmune sera to native virions produced in guinea pigs are type specific in CF tests (8, 11, 26).

In this study, we prepared antisera in guinea pigs, using two kinds of immunogens: (i) heated virions (H particles), and (ii) virions disrupted by heating in the presence of sodium dodecyl sulfate (H + SDS fragments). We found that the two kinds of antisera had different reactivities: antisera to H + SDS immunogens were cross-reactive by CF with H particles of heterologous viruses, whereas antisera to H particles reacted type specifically only with homologous viruses. This paper deals with the CF reactivity of such antisera and the sedimentation character as well as the viral protein composition of the immunogens. It also discusses the reasons for heterotypic CF antibody responses in humans infected with enteroviruses.

MATERIALS AND METHODS

Cells and viruses. LLCMK2 cells were cultivated in roller bottles (110 by 260 mm), using Eagle minimum essential medium with 3% calf serum for virus propagations. Echovirus type 7 (E7) (Wallace) and type 11 (E11) (Gregory) and coxsackievirus type B5 (CB5) (Faulkner) were used.

Propagation and purification of viruses. Cells were infected with virus at a multiplicity of infection of 30 to 50 and then incubated at 37°C for 6 h. Cells were then frozen and thawed three times. After the cell debris was removed by low-speed centrifugation, virus was precipitated with 8% polyethylene glycol 6000 in the presence of 0.5 M NaCl. The pellet was suspended in phosphate-buffered saline (pH 7.2), and CsCl was added to a final density of 1.33 g/cm³. The virus was banded by isopycnic centrifugation in a Spinco SW 50.1 rotor at 100,000 × g for 18 h at 4°C. The virion peak (1.33 g/cm³) was collected and dialyzed against phosphate-buffered saline. For the preparation of radioactive E7, [35S]methionine (5 μCi/ml) was added to the virus-infected cells 2.5 h postinfection, and virions were purified as described above.

Preparation of immunogens and antisera. Purified E7, E11, and CB5 virions were treated in the following way for use as immunogens: (i) heated at 56°C for 30 min (H particles) or (ii) heated at 56°C for 30 min in the presence of 0.1% SDS (H + SDS fragments). These preparations were mixed with equal volumes of Freund complete adjuvant. Guinea pigs weighing 400 to 600 g received footpad injections of the immunogens (0.4 ml) and, 4 weeks later, intraperitoneal injections of 1.0 ml of the immunogen without adjuvant. One week later, sera were collected and stored at −20°C until used.

Velocity sedimentation of heated virions. Heated virions were centrifuged in 15 to 30% (vol/wt) sucrose gradients in a Spinco SW41 rotor for 4 h at 100,000 × g at 4°C.

SDS-polyacrylamide gel electrophoresis. Electrophoresis of radioactive polypeptides was performed in 13% acrylamide gels (18) with the discontinuous buffer system described by Laemmli (17) at room temperature for about 4 h at a constant voltage of 100 V.

CF test. CF tests were carried out in microplates, using five 50% hemolytic units of complement. The antigens used were: (i) native virions (N antigenicity) (12) and (ii) H particles (H antigenicity) (12).
RESULTS

Characterization of immunogens. To study the character of the two kinds of immunogens, we subjected [35S]methionine-labeled E7 virions to the same treatments as used for unlabeled virions: (i) heating at 56°C for 30 min (H) or (ii) heating in the presence of 0.1% SDS at 56°C for 30 min (H + SDS). They were then centrifuged in sucrose gradients as described above. Heating virions in the absence of SDS converted 160S virions to 80S empty capsids (H particles) (Fig. 1b); similar results have been reported by others (10, 21). In contrast, heating virions in the presence of SDS disrupted the virion into smaller fragments which remained at the top of the gradients (Fig. 1c). For examination of the polypeptide composition, each peak of radioactivity (I, II, and III) was collected, dialyzed against phosphate-buffered saline, and then subjected to SDS-polyacrylamide gel electrophoresis. Both H particles (II) and H + SDS fragments (III) contained VP1, 2, and 3 (Fig. 1, II and III).

CF reactivity of antisera produced with H particles as immunogens. CF reactivity of the antisera to H particles was tested with H and N particles of homologous and heterologous types as antigens. Fig. 2 shows the results of CF checkerboard titrations with H antigens. Each antiserum was found to react type specifically only with the homologous antigens. When N virions were used as antigens, the antisera also showed type-specific reactions, although antigen and antibody titers were slightly lower than with H antigens (data not shown).

CF reactivity of antisera produced with H + SDS fragments as immunogens. Antisera against H + SDS fragments were also tested by CF checkerboard titrations with H and N antigens of homologous and heterologous types. These antisera cross-reacted with H particles of all three serotypes (Fig. 3). When N virions were used as antigens, the antisera reacted type specifically with antigens of homologous types with slightly lower titers than with H antigens (data not shown).

DISCUSSION

By immunizing guinea pigs with human enterovirus virions which were disrupted by heat and SDS (H + SDS immunogen), we have succeeded in producing antisera cross-reacting by CF with H particles of heterologous types. So
far, it has been reported that antisera from animals artificially immunized with human enteroviruses reacted only with homologous viruses, as studied by CF (3, 5, 15), immunodiffusion (11, 27), or immune electron microscopy (11). Mietens et al. (20) found recall of CF antibodies in guinea pigs after successive injections of different enteroviruses, but they did not detect heterotypic antibody responses to viruses not previously injected. Forsgren (6) found that poliovirus antisera prepared by repeated injections of virus with adjuvant did react with heterologous polioviruses by CF and immunodiffusion but that the heteroreactivity was limited, being found only within the poliovirus group. By using the enzyme immunoassay with ureda-disrupted virions as solid-phase antigens, Katze and Crowell (14) also detected heterotypic reactivity of rabbit antisera to group B coxsackieviruses with other members of the same group, but the heteroreactivity did not extend to other viruses beyond that group. In addition, we found that those antisera which were produced in guinea pigs immunized with H particles had type-specific reactivity with H antigens in CF tests (Fig. 2). In contrast to these results, if virions (E7, E11, and CB5) were disrupted by both heat and SDS and then used as immunogens, the antisera produced reacted by CF not only with H particles of homologous viruses but also with heterologous types belonging to different groups of human enterovirus (Fig. 3).

The data from this study indicate that H particles have both type-specific and cross-reactive antigenicity on their surfaces but do not possess cross-reactive immunogenicity in animals. After solubilization of virions by heat and SDS (Fig. 1c), cross-reactive antigenic determinants acquired immunogenicity. Antigenicity and immunogenicity do not necessarily coincide (1). In the case of enterovirus H particles, cross-reactive determinants on the surface, to which free antibody molecules could attach, might be located in a position so as not to stimulate antibody production; possibly, in a topographical state such that specific receptors on the immunocompetent B cells would not be accessible. Further, H particles probably have a rigid structure and may not be easily degraded within the body to expose such cross-reactive determinants.

![CF reactivity of antisera prepared with H particles as immunogens](image)

**FIG. 2.** CF reactivity of antisera prepared with H particles as immunogens. CF checkerboard titrations were carried out with H particles of homologous and heterologous types as antigens. Figures on abscissas and ordinates indicate reciprocals of antiserum and antigen dilutions, respectively. Shading shows reactions with homologous type antigens.
nents. Thus, a rather strong treatment, such as heat and SDS, may be needed to destroy the quaternary structure before immunization for production of cross-reactive antisera.

Contrary to animal antisera prepared by conventional immunization, human sera usually have heterotypic CF reactivity after enterovirus infection (2, 4, 7, 13, 16, 19, 23). They react with naturally occurring empty capsids present in crude enterovirus antigens (22). Schmidt et al. (24, 25) reported that monkeys orally infected with enteroviruses show type-specific CF antibody responses after the primary infection, whereas after the second infection, the antibody responses are heterotypic. We also observed that of 13 patients from whom enteroviruses were isolated and their serotypes identified, only 2 young infants responded with homotypic CF antibodies to the infecting serotypes; the rest showed heterotypic CF responses (9). Probably, when enterovirus multiplies in the body, viral subunits having cross-reactive immunogenicity are released from the infected cells. After repeated infections with different enteroviruses, the host responds to produce a large amount of antibodies to such cross-reactive determinants.

In the primary infection, however, antibody responses to those determinants would be weak; thus, the initial infection would produce mostly type-specific antibodies.

Finally, if the cross-reactive determinants detected by the above antisera are shared by all of the human enteroviruses, those sera could be used to determine whether a putative enterovirus isolate is really an enterovirus or not when it is difficult to identify with conventional antisera. Alternatively, if the cross-reactive determinants are not shared so broadly, antisera prepared with H + SDS immunogens of different serotypes could be used for the study of antigenic relatedness among human enteroviruses.

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LITERATURE CITED
