Detection of Coronavirus 229E Antibody by Indirect Hemagglutination

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Tannic-acid treated sheep erythrocytes (fresh or glutaraldehyde preserved) were sensitized with 229E antigens from human embryonic lung (RU-1) cell cultures. Indirect hemagglutination (IHA) antigen titers in 229E-infected cell cultures paralleled virus infectivity and complement fixation (CF) antigen titers. The identity of the IHA antigen was confirmed by testing extracts from inoculated and control cell cultures for ability to inhibit IHA. Also, significant increases in IHA antibody were demonstrated with acute and convalescent serum pairs from patients with proven 229E infections. A comparison of IHA, neutralization and CF titers for 229E antibodies was made on human sera drawn from different populations. The IHA and neutralization results were in agreement on 93% of the 129 sera found to be positive by at least one of three tests. The number of antibody titers detected by the CF test was insufficient to permit comparison. Hyperimmune sera from animals immunized with OC 43 did not react with 229E by IHA. Also no increase in IHA antibody was demonstrated with acute and convalescent serum pairs from patients with seroconversions to OC 43. These findings suggest that the IHA test provides (i) a rapid and sensitive method for serodiagnosis of 229E infections and (ii) a simple and inexpensive method for seroepidemiological studies.

Over the past 6 years viruses belonging to the newly identified coronavirus group (5) have been shown to cause common cold-like illnesses in man. At least three prototype strains designated B814, 229E, and NIH "OC" 43 (or 38) have been implicated (6, 12, 17). The major obstacle in studying these viruses is their fastidious nature which has hindered primary isolation and further adaptation to growth in animal or cell culture systems.

In the absence of virus isolations, emphasis has been placed on serodiagnostic and seroepidemiological studies. The adaptation of strains OC 38/OC 43 to growth in the suckling mouse brain has made available complement fixation (CF) and hemagglutination inhibition (HI) tests (10, 12). The growth of 229E in diploid fibroblasts provides antigens for the CF test as well as a system for performing neutralization (NT) tests (6). Although useful, the two tests available for 229E are not ideal for seroepidemiological studies: CF antibody does not persist long after infection (4), and testing large numbers of sera by NT is beyond the means of most laboratories. An HI test is not available since 229E does not agglutinate erythrocytes (6). In this study we describe the development and use of an indirect hemagglutination (IHA) test for detection of 229E antibody. The IHA test is specific, simple, and has many features which make it promising for coronavirus serological studies.

MATERIALS AND METHODS

Viruses. Coronavirus 229E (6) was grown in the RU-1 strain of diploid human fetal lung fibroblasts. Infectivity was assayed in RU-1 cells and titers were calculated by the method of Reed and Muench (15). Coronavirus OC 43, adapted to growth in mouse brain (11), was further passaged in our laboratory in 3-day-old Swiss white mice (ICR) and then adapted to African green monkey kidney heteroploid cells (BSC-1). Herpes simplex type 2 (MS strain) was also grown in RU-1 cell cultures.

Sera. Three hundred and forty-five sera from adults with and without respiratory illness were selected from studies previously undertaken by the Center for Disease Control (CDC). Two hundred and
thirteen sera were from adult men participating in a chronic bronchitis study (1969-70). Eighty-eight acute and convalescent sera were from adults with upper respiratory illness (1965). Forty-four sera were from adults participating in an influenza vaccine study (1971-72). One hundred and four acute and convalescent serum pairs with diagnostic fourfold or greater rises in antibody titers (seroconversion) to 229E or OC 43 were from children included in a longitudinal survey (1960-68) of respiratory illness (10).

Acute and convalescent sera from patients w2, w3, upper respiratory illness from whom 229E-like viruses were isolated were kindly supplied by A. Z. Kapikian, National Institutes of Health.

Various animal immune and hyperimmune sera to OC 43-purified antigens were prepared using techniques and schedules described elsewhere (6a).

Antigen preparation. RU-1 cells were grown in 32-oz (ca. 0.9 liter) prescription bottles with Eagle minimum essential medium (MEM) containing 10% fetal calf serum and aureomycin (25 μg/ml). Care was taken to eliminate calf serum proteins from antigens to be used in the IHA test. When cell monolayers were complete, the cells were washed with serum-free Eagle MEM and reincubated with serum-free Eagle MEM overnight at 35 C. The wash cycle was repeated and the cells were inoculated with 10 ml of 229E at 10⁴ tissue culture infective dose (TCID₅₀)/ml. The plates were placed in a 35 C incubator for 30 to 60 min. Twenty ml of serum-free Eagle MEM was added and incubation at 35 C was continued. When the cytopathic effect (CPE) was 1 to 2%, all but 2 to 3 ml of media was decanted, and the cells were frozen at -70 C and thawed. Any adhering cells were scraped off the glass by shaking the bottle or using a rubber policeman. The contents of the bottles were frozen and thawed three times in this manner, pooled, and then centrifuged at 1,500 rev/min for 10 min. The supernatant containing the antigen was collected and stored at -70 C.

The 229E CF antigens were prepared in RU-1 cells by the method of Hamre and Pocknow (6). OC 43 antigens for the CF and HI tests were prepared by methods described previously (10, 12).

Sero logical methods. The IHA test was based on the method described by Stavitsky (16). Sheep erythrocytes were fixed with glutaraldehyde as described by Bing et al. (1). Erythrocytes were washed before and after glutaraldehyde treatment with phosphate-buffered saline (PBS), pH 7.2, diluted to 2.5%, and treated with a 1:20,000 dilution of tannic acid.

The optimal dilution of 229E antigen for sensitization of erythrocytes was determined by block titrations against immune sera. The lowest concentration of antigen which yielded the highest specific serum titer was taken as the optimal dilution.

Sensitized erythrocytes were prepared in graduated centrifuge tubes by adding equal volumes of tanned 2.5% erythrocytes and optimal dilutions of antigen suspended in PBS, pH 6.4. The mixture was incubated for 15 minutes at 37 C, and the erythrocytes were then washed twice in a 1:150 dilution of normal horse serum (NHS) in PBS, pH 7.2. After the second wash the packed erythrocytes were added to 1% by the addition of NHS diluent. Control tubes of unsensitized tanned erythrocytes were treated in the same manner.

The IHA test was performed in microtiter "U" plates. A 0.05-ml volume of NHS was used as the diluent for all tests. Each test serum was titrated in 0.05-ml volumes in twofold dilutions beginning with a 1:10 dilution. An equal volume of sensitized erythrocytes was added to each dilution. Controls consisted of (i) the first two dilutions of each serum plus tannic acid-treated unsensitized erythrocytes, (ii) sensitized erythrocytes plus diluent, and (iii) two known positive serum titrations. The plates were shaken and permitted to stand for 3 to 4 hr at room temperature before erythrocyte settling patterns were read.

Indirect hemagglutination inhibition (IHA-I) tests were performed with homologous and heterologous antigens to confirm the specificity of the IHA test. Each antigen to be tested was diluted in twofold dilutions in 0.025-ml volumes beginning with 1:2. Four antibody units from known specific sera were added in 0.025-ml volumes to each dilution of antigen. Plates were incubated at room temperature for 1 hr and 0.05 ml of sensitized erythrocytes was added. Erythrocyte patterns were read as described for the IHA test.

NT tests were performed with 30 to 300 TCID₅₀/0.1 ml of virus and serial twofold dilutions of serum starting at 1:10. Equal volumes of virus and serum dilutions were incubated at room temperature for 1 hr. A 0.2-ml volume of each mixture was inoculated into three RU-1 cell culture tubes without media and incubated at 35 C for 30 to 60 min. Eagle MEM plus 2% fetal calf serum was added, and the tubes were reincubated. Final readings were made on the 10th day.

CF (14) and HI (7) tests were performed using the microtiter technique. All sera were inactivated at 56 C for 30 min.

RESULTS

Relation of IHA and CF activity to 229E growth curve. Bottles of RU-1 cell culture monolayers were inoculated with 229E and incubated at 35 C. Supernatant fluid and cell pack were harvested at 12-hr intervals up to 96 hr from replicate cultures, at which time a 4+ CPE became apparent in the remaining cultures. Infectivity assays, IHA, and CF titrations were performed on the supernatant fluids and extracts of cell packs (Fig. 1).

Cell-pack virus reached maximum infectivity titer (10⁶.⁴ TCID₅₀) 60 hr after inoculation when only minimal CPE was present. Peak infectivity titers (10⁶ TCID₅₀) in supernatant fluid were reached 72 hr after inoculation when CPE was more extensive. Although IHA antigen was detected in the cell pack before CPE was detected, maximum titers were not reached until 72 hr, at which time CPE
was more evident and infectivity titers had passed their peak. Supernatant fluids had only minimal IHA activity well after CPE was judged to be complete (96 hr). CF antigens were detected in cell packs 36 hr before CPE but the highest titers were observed at the same time as peak infectivity. CF antigens in low titers were detected only in supernatant fluids from the 72 to 96 hr harvests.

**Specificity of 229E IHA test.** Various tissue culture components and infected cell extracts were examined for their ability to inhibit the IHA reaction. IHA was not inhibited by infected (herpesvirus type 2) or noninfected RU-1 cells, OC 43 (BSC-1) extracts, or fetal calf serum. Only RU-1 cell cultures infected with 229E specifically inhibited hemagglutination.

The specificity of the 229E IHA test was further demonstrated by fourfold or greater increases in titer in acute and convalescent serum pairs from patients from whom 229E-like viruses were isolated (Table 1).

**Sensitivity of 229E IHA test.** A comparison of IHA, NT, and CF tests for detection of 229E antibody was made with human sera drawn from different populations. Of the 345 sera, 129 were positive by at least one of the three tests (Table 2). The IHA and NT tests were in agreement on 93% of the positive sera. Antibody would not have been detected in 5% of the sera if it had been tested only by NT, or in 2% of the sera if tested only by IHA. The numbers of antibody titers detected by the CF test were insufficient to permit comparison, only 3 of 345 sera were positive.

**Absence of reactivity of 229E IHA with OC 43 antibodies.** Sera from guinea pigs, chickens, and mice hyperimmunized with purified OC 43 virus and with homologous HI titers $\geq 160$ (6a) did not react in the 229E IHA or CF tests. One thousand eight hundred and seventeen (1817) paired sera from children participating in a survey of respiratory illnesses were examined for fourfold or greater rises in titer for 229E and OC 43. Sixty-three seroconversions to 229E were detected by IHA. Only one of these had a simultaneous rise to OC 43 by HI. Forty-two seroconversions to OC 43 were detected by HI; none of these had rises to 229E (Table 3).

## DISCUSSION

Serological studies with known human coronaviruses have been hampered by difficulties in virus propagation and problems inherent in some of the available serological techniques. In

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**TABLE 2. Comparison of IHA and NT tests for detection of 229E antibody in human sera**

| Tests       | No. sera positive | Percent |
|-------------|-------------------|---------|
| IHA or NT   | 129               |         |
| IHA and NT  | 120               | 93      |
| IHA only    | 7                 | 5       |
| NT only     | 2                 |         |

* 345 Sera from adults with and without respiratory illnesses.

**TABLE 3. Heterotypic coronavirus antibody response among children with 229E IHA and OC 43 HI seroconversions**

| Strain | No. of seroconversions | GMT* |
|--------|------------------------|------|
| 229E   | 62                     | 7/136|
| OC 43  | 41                     | 8/8  |
| Both   | 1                      | 8/56 |

* Fourfold or greater antibody rises in paired sera.

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* Sera supplied by A. Z. Kapikian, NIH, from patients with 229E virus isolations.

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**FIG. 1. Replication of 229E virus in RU-1 cell cultures and titration of IHA and CF antigens.**
this paper we have described a known and widely used serological test which has been adapted for use with 229E virus. The IHA test for 229E antibodies is specific and sensitive, and the results closely parallel those obtained with the NT test.

Glutaraldehyde-fixed erythrocytes increase the usefulness of the IHA test. When stored at 4 C they can be used for at least 6 months; this saves time that would otherwise be spent in handling fresh erythrocytes and increases test reproducibility because the same cell lot is used. Further studies have revealed that fixed erythrocytes sensitized with optimal dilutions of 229E virus can be frozen at -70 C for an indefinite period of time. They may also be stored at 4 C for at least several months.

Previous studies suggested that serology may be of limited value for diagnosing the specific serotype responsible for human coronavirus infection. Simultaneous and, presumably, heterotypic antibody rises to more than one serotype have been reported to occur by CF, NT, and HI tests (3). Of particular concern have been possible heterotypic antibody rises resulting from 229E and OC 43 infections. Bradburne and Tyrrell (3) reported several fourfold NT antibody rises to LP and 229E viruses in paired sera from volunteers infected with OC strains. He also reported that only 5 of 14 volunteers receiving OC 43 virus challenge had fourfold or greater HI rises to OC 43 but that 2 of 14 developed neutralizing-antibody rises to LP or 229E virus (3). In addition, Bradburne also reported that, of 70 paired sera from volunteers given coronavirus other than OC 43, 14% had fourfold rises to OC 43 HI antibody. However, McIntosh et al. (13) reported that sera from patients yielding 229E-like virus did not neutralize OC 43. Using hemadsorption as an indicator system in NT tests with human sera, Kapikian et al. (8) also reported that two individuals with significant CF antibody rises to 229E virus did not develop significant OC 43-neutralizing antibody rises.

Bradburne, using several serological methods with hyperimmune animal sera, reported OC 43 and 229E antigens to be related (2). McIntosh et al. (13), on the other hand, found no relationship by NT tests between 229E and OC 38/43 when animal sera were used (13).

In our studies of antibodies to 229E by IHA and to OC 43 by HI, simultaneous serodiagnosis of infection did not occur among 103 of 104 paired sera with diagnostic antibody rises to either virus. Heterotypic rises were absent in spite of the fact that over a third of these pairs had preexisting antibody to the other virus.

A more complete understanding of antigenic relationships between 229E and OC 43 can only be obtained by studying both viruses by using the same serological tests. This is not possible at present since an HI test is not available for 229E and an IHA test has not yet been developed for OC 43. Further studies are also needed to elicit information regarding the incidence of heterotypic 229E antibody rises caused by infection with other human coronaviruses. Nevertheless, our findings clearly show that where a serodiagnosis of OC 43 infection has been made, the likelihood of a heterotypic rise in titer to 229E by the IHA test is remote.

We propose the IHA test to be a rapid and sensitive method for serodiagnosis of 229E infections and a simple and inexpensive method for seroepidemiological studies.

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