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Determination of Coronavirus 229E Antibody by an Immune-Adherence Hemagglutination Method

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An immune-adherence hemagglutination (IAHA) method for coronavirus 229E antibody determination has been developed both for diagnosis of recent infections and for detection of long-past infections. Results have been compared with those obtained by complement fixation (CF), neutralization (Nt), and indirect hemagglutination (IHA) tests. The IAHA method has been shown to be as sensitive as the CF, Nt, and IHA tests in detecting cases of acute 229E infection. However, in a seroepidemiological survey of 343 healthy people of all ages, IAHA detected 229E antibody in 254 individuals (74.0%), Nt in 166 (48.3%), IHA in 89 (25.9%), and CF in 30 (8.7%). A study of the prevalence of coronavirus 229E IAHA antibody in the different age groups has shown that during the second decade of life nearly 100% of the population acquire this type of antibody, whereas only 50% are positive at the end of the first decade. In the older age groups, the high frequency of CF antibody ("marker" of recent infection) indirectly confirms the high rate of 229E reinfections and the nonprotective nature of IAHA antibody. CF titer $\geq$ 1:8 in 90% of cases corresponded to IAHA titers $\geq$ 1:64. However, sera with IAHA titers of $\geq$ 1:128 were often CF-negative. Recent 229E infections (or reinfections), as determined by the presence of CF antibody, were more frequent in April-May than in October-November. Three cases of acute infection showing 229E seroconversion (two adults and one child) were observed during the winter-spring season. IAHA appears to be the test of choice for seroepidemiological surveys.

Key words: coronavirus 229E, immune-adherence hemagglutination, viral respiratory infections

INTRODUCTION

Epidemiological surveys carried out in the United States [1–3], England [4], and Brazil [5] indicate that coronavirus 229E infections are worldwide. Studies of prevalence of coronavirus 229E antibody have been performed using complement fixation (CF) [6], neutralization (Nt) [1], and indirect hemagglutination tests (IHA) [7]. CF antibody

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tends to be transitory and is detectable only within some weeks or a few months after infection (or reinfection). Nt and IHA antibodies remain elevated somewhat longer but do not seem to be long-lasting [7, 8].

The present report describes the development and use of an immune-adherence hemagglutination test (IAHA) for a seroepidemiological survey of the prevalence of coronavirus 229E antibody in different age groups and for the diagnosis of 229E infections. In this test the underlying mechanism is that when antigen and antibody combine, the third-complement component (C′3) is activated, causing agglutination of human type O red blood cells, due to their C′3 receptor [9]. Results are compared with those obtained by CF, IHA, and Nt tests. When compared with the other tests, the IAHA method appears to be superior in detecting remote 229E infections and appears to be the method of choice for seroepidemiological surveys.

MATERIALS AND METHODS

Cell Cultures and Virus Propagation

A coronavirus 229E reference strain was kindly supplied by Dr H.S. Kaye (Virology Respiratory Unit, Center for Disease Control, Atlanta, Georgia) and grown in the RU-1 strain of diploid human fetal lung fibroblasts. Viral infectivity was assayed in RU-1 as well as WI-38 cells, and titers were calculated by the method of Reed and Muench [10].

Sera and Patients

Three groups of sera were examined: a) 343 sera from healthy individuals to study the prevalence of antibody to coronavirus 229E in different age groups; b) two groups of sera from adults (20–60 years) of which the first was taken in October-November 1976 and the second in April-May 1977, to investigate the incidence of coronavirus 229E infections (or reinfections) in two different periods of the year (of these, 31 were paired sera); c) 30 paired sera from infants (6–18 months), admitted to the hospital with acute respiratory infection, to determine the etiologic role of coronavirus 229E with respect to the pathogenesis of severe respiratory tract infections during infancy.

A reference guinea pig immune serum for serological methods was kindly supplied by Dr H.S. Kaye.

Antigen Preparation

Human embryo fibroblast cell cultures (RU-1 and WI-38) were grown in 150-cm^2 flasks with Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum. When cell monolayers were complete, the cells were inoculated with coronavirus 229E at a multiplicity of infection (MOI) of 0.01 to 0.1. Following incubation at 34°C for 60 minutes, cell cultures were fed with Eagle's MEM containing 2% fetal calf serum, and incubation at 34°C was continued. When cytopathic effect was 1+ to 2+, cells were washed three times with Hanks' balanced salt solution (BSS). Finally, 2–3 ml of Hanks' BSS were added to each flask, and the cells were frozen at −80°C. The thawed contents of the flasks were pooled and then centrifuged at 1,500 rpm for 10 minutes. The super-

natant containing the antigen was collected and stored at −80°C. This antigen was used in the IHA and IAHA tests. The 229E CF antigen was prepared from the same cell cultures by the method of Hamre and Procknow [6].

Immune-Adherence Hemagglutination Test

The 229E IAHA test was performed as follows. Serial twofold dilutions from 1:4 to 1:512 of serum were made in triplicate using gelatin veronal buffer (GVB) as diluent and tissue culture grade microtiter V plates (Sterilin, Teddington, Middlesex, UK) (0.025 ml/well). The 229E antigen was added to the first, control antigen to the second, and GVB to the third set of serum dilutions (0.025 ml). Following incubation at 37°C for one hour, the optimal dilution of guinea pig complement (C′) in GVB (0.025 ml) was added to all wells and the plates were incubated at 37°C for 40 minutes. Dithiothreitol (DTT) at a concentration of 3 mg/ml in 0.04 M EDTA-GVB (two parts 0.1 M EDTA (pH 7.5) added to three parts GVB) was then added (0.025 ml). Finally, a 0.4% suspension of human type O red blood cells (RBC) in GVB (0.025 ml) was added to each well. The microplates were then left at room temperature for 45–60 minutes before hemagglutination patterns were read. Observed patterns ranged from reactions of nonagglutinated cells (negative reactions) to reactions of partially or completely agglutinated cells (positive reactions).

GVB was prepared according to the Laboratory Branch Complement Fixation (LBCF) procedure [11]. C′ (Microbiological Assoc., Bethesda, Maryland) was titered according to the same procedure and diluted for use in GVB. Optimal dilutions of C′ for the CF and IAHA tests were identical. Human type O RBC were obtained from suitable donors. Whole blood was collected in twice the volume of Alsever's solution and stored at 4°C for up to 2–3 weeks. Prior to use, RBC were washed with GVB and adjusted to a 0.4% suspension in GVB.

As a control for specificity, serum containing specific 229E antibody was absorbed with 229E antigen and control antigen for our hour at 4°C and then over night at 37°C. After centrifugation at 5,000g for 15 minutes, the supernatants were tested for IAHA reactivity. Specific antibody titer was completely abolished.

CF Test

The 229E CF antigen was prepared in RU-1 cells by the method of Hamre and Procknow [6]. The CF test was performed according to the LBCF procedure [11].

Neutralization Test

Neutralizing antibody to 229E was measured in microplates of WI-38 cell monolayers. Six sets of twofold dilutions (1:5–1:640) of heat-inactivated serum (56°C for 30 minutes) were made in TC-199 medium supplemented with 10% fetal calf serum (0.025 ml/well) and mixed with an equal volume of virus diluted to give 40–200 50% tissue culture infective doses ($TCID_{50}$) per 0.025 ml. After incubation at 34°C for one hour, a WI-38 cell suspension containing 2×10^5 cells per milliliter was added (0.05 ml) to each

well and microplates were incubated at 34°C for 3–4 days. When, in the virus controls, the amount of virus detected by microscopic observation was included in the range of 40–200 $TCID_{50}$, microplate cell monolayers were fixed and stained with crystal violet. Appropriate serum (1:5–1:10 dilutions) and cell controls were included in each test. Serum titers were calculated by the method of Reed and Muench [10].

Indirect Hemagglutination

The IHA test was performed according to the method reported by Kaye, Ong, and Dowdle [7], using either fresh or glutaraldehyde-fixed sheep erythrocytes [12]. According to a procedure described for IHA tests applied to human cytomegalovirus and human herpesvirus antibody determination [13], sheep cell agglutinins were eliminated by absorption of the sera with tanned sheep erythrocytes in a water bath at 37°C for 45 minutes. Specificity of the IHA test was determined by indirect hemagglutination inhibition [7].

RESULTS

Specificity of the IAHA Test for Determination of Antibodies to Coronavirus 229E

The specificity of the IAHA method has been tested 1) using reference guinea pig immune serum and preimmune serum; 2) using negative (from three infants 6–8 months old) and positive (from one child and two adults) human sera from recent 229E infections (these sera were well characterized by CF, IHA, and Nt); 3) using a blocking test in which 229E antigen blocked the IAHA reaction of positive sera, whereas control antigen did not.

Comparative Sensitivity of the 229E IAHA Test for the Determination of Coronavirus 229E Antibody

A comparison of IAHA, IHA, Nt, and CF tests for detection of 229E antibody was made on human sera drawn from healthy people of different ages (Fig 1 and Table I). Of the 343 sera tested, 254 (74%) were positive by the IAHA test. Of these IAHA-positive sera, thirty (8.7%) showed a CF titer ≥ 1:4, 89 (25.9%) an IHA titer ≥ 1:16, and 166 (48.3%) an Nt titer ≥ 1:5. No titer was detected in 89 sera (25.9%) by any test. As shown in Figure 1, antibody would not have been detected in as many as 80 sera (23.3%), if it had been tested only by Nt and IHA. These two tests agreed in only 81 (23.6%) positive sera. That is, the IHA added only eight positive results to those already detected by Nt.

CF antibody was always detected also by IAHA, Nt, and IHA tests.

Prevalence of 229E Antibody in Different Age Groups

As reported in Table I, no antibody was detected in infants of 4–6 months by any test. Antibody started appearing in the group of 7 months to 5 years and increased its frequency in the 6- to 12-year-old group. Prevalence of 229E antibody, as determined by all four tests, did not show large variations among the older age groups. Titers were consistently found in less than 20% of the cases by the CF test and in more than 90% by IAHA. IHA and Nt tests showed intermediate values.

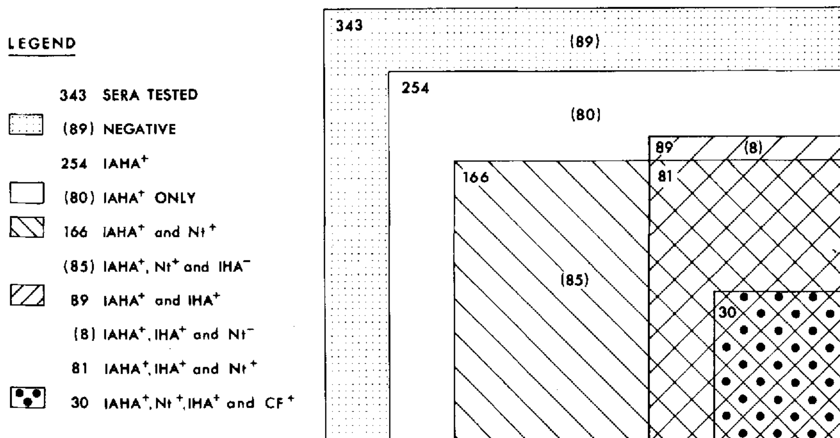

Fig 1. Schematic comparison of coronavirus 229E antibody titers obtained by the immune-adherence hemagglutination (IAHA), neutralization (Nt), indirect hemagglutination (IHA), and complement fixation (CF) tests (each rectangle, representing the number of sera in its upper left corner, is a subdivision of all larger rectangles in which it is included).

TABLE I. Detection by CF, IHA, Nt, and IAHA of Antibody to Coronavirus 229E

Age	No. tested	No. positive by							
		CF		IHA		Nt		IAHA	
4–6 months	18	0		0		0		0	
7 months–5 years	68	3	(4.5%)	2	(2.9%)	4	(5.9%)	12	(17.6%)
6–12 years	20	2	(10.0%)	3	(15.0%)	5	(25.0%)	10	(50.0%)
13–20 years	46	0		11	(23.9%)	26	(56.5%)	43	(93.5%)
21–30 years	49	4	(8.2%)	15	(30.6%)	42	(85.7%)	49	(100.0%)
31–40 years	65	9	(13.8%)	20	(30.7%)	39	(60.0%)	65	(100.0%)
41–50 years	45	8	(17.8%)	23	(51.1%)	29	(64.4%)	43	(95.5%)
>50 years	32	4	(12.5%)	15	(46.8%)	21	(65.6%)	32	(100.0%)
Total	343	30	(8.7%)	89	(25.9%)	166	(48.3%)	254	(74.0%)

CF, complement fixation; IHA, indirect hemagglutination; Nt, neutralization test; IAHA, immune-adherence hemagglutination.

Seasonal Distribution

Since it is well known that the highest frequency of 229E infections takes place during the winter-spring season, we have compared a group of sera drawn from healthy adults in October-November 1976 with a group of sera taken in April-May 1977. Of these, 31 were paired sera. Results reported in Table II show that the percentage of sera showing CF antibody in the October-November group is 16.8%, whereas in the April-May group it is 26.7%. The same sera showed presence of 229E antibody with a frequency close to 100% by IAHA. Furthermore, in the October-November group 13 of 18 (72.2%) CF-positive sera had a CF titer of 1:4, whereas in the April-May group only 21 of 38 CF-positive sera (55.3%) had a titer of 1:4. CF titers ⩾ 1:8 in 20 of 22 cases of both groups

TABLE II. Detection by CF and IAHA of Antibody in Two Groups of Sera From Healthy Adults Tested in Two Different Periods of the Year

Sera drawn in	No. sera tested	No. sera positive by CF	No. sera positive by IAHA
October-November 1976	107	18 (16.8%)	105 (98.1%)
April-May 1977	142	38 (26.7%)	137 (96.5%)
Total	249	56 (22.5%)	242 (97.2%)

(90%) corresponded to IAHA titers ⩾ 1:64 (Fig 2). However, sera with IAHA titers ⩾ 1:128 were CF-negative in 64 cases (25.7%).

Of 31 paired sera, two showed seroconversion by all four tests (Table III).

Acute Pediatric Infections

In 30 cases of acute upper and lower respiratory tract infections in children of 6–18 months, 229E antibody was determined by all four methods in acute and convalescent sera. One case showed seroconversion, as reported in Table III. Clinical symptoms were fever, cough, and otitis. Other possible etiologic agents (viral and bacterial) were ruled out.

DISCUSSION

The IAHA test described in the present report appears to be the most reliable serological test for determination of coronavirus 229E antibody in seroepidemiological surveys.

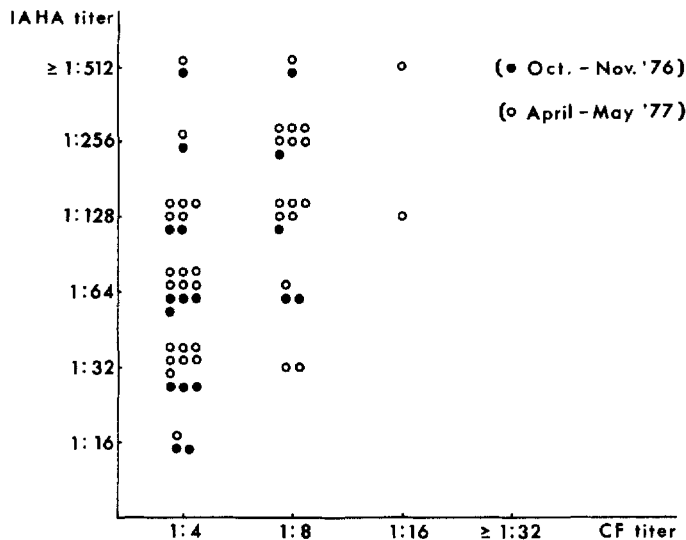

Fig 2. Comparison of coronavirus 229E CF and IAHA antibody titers in two groups of CF-positive sera drawn in October-November 1976 and in April-May 1977.

TABLE III. Coronavirus 229E CF, IHA, Nt, and IAHA Antibody Titers in Acute/Convalescent Sera From Patients With 229E Infections

Patient No.	Age	Coronavirus 229E antibody titer by CF	IHA	Nt	IAHA
607	57 years	<4/16	<16/512	<5/80	<4/512
655	55 years	<4/16	<16/128	<5/120	<4/128
3	7 months	<4/8	<16/256	<5/160	<4/64

This test has already been applied to determination of specific antibody following both hepatitis A [14] and B [15] as well as varicella-zoster virus infections [16]. We have modified the test for coronavirus 229E antibody determination by using a 0.4% suspension of RBC and incubating at room temperature for 45–60 minutes before the final reading. Technical problems such as nonspecific hemagglutination may sometimes arise with contamined sera or with frozen sera stored for a long time. However, it is usually easy to distinguish between the fine granular hemagglutination pattern due to the specific antigen-antibody reaction and the fuzzy pattern due to a nonspecific reaction.

So far, extensive epidemiological surveys have been carried out using CF, IHA, or Nt tests. When only the CF test was employed, relatively low levels of seropositivity were found in population samples of different countries [3, 5]. Levels of 229E seropositivity reached higher values [4] only when very potent antigens were prepared using special concentration procedures. IHA and Nt test have been reported to be in good agreement in more than 90% of sera tested for determination of 229E antibody [7]. Our results apparently show the Nt test to be more sensitive than IHA. The reason for this discrepancy may be the low initial dilution (1:5) and the more accurate test performance in microplates than with tubes. However, our results show that the IAHA test is far more sensitive than both IHA and Nt tests in detecting long-lasting 229E antibody.

Looking at the prevalence of 229E antibody detected by the IAHA test in the different age groups it appears that coronavirus 229E infections start occurring in the first five years of life, and more than 90% of adults show the presence of antibody to coronavirus 229E in their serum. In parallel, 229E CF antibody (a "marker" of recent infection) does not present remarkable variation in the frequency of its distribution after the second decade of life. These data indirectly confirm the high rate of coronavirus 229E reinfections reported previously [17]. In regard to this point in particular, neither IHA nor neutralizing antibody has been shown to be protective, and reinfections have been described in carriers of both types of antibody [17]. On the basis of our results, we conclude that even IAHA antibody is not protective. Whether immune status to coronavirus 229E depends on the presence of specific secretory IgA or on cell-mediated immunity is still to be determined.

As far as the seasonal distribution of coronavirus 229E infections is concerned, our results show that coronavirus 229E infections occur more frequently in Italy during the winter-spring period. This agrees with data previously reported for other countries [2, 3]. In our study, using as parameter of recent infection a titer of CF antibody ≥ 1:8, we found almost three times the frequency of 229E CF antibody in the spring as compared with the fall season. When a titer of 1:4 was used as a parameter, the frequency of 229E CF antibody during the spring dropped to less than double. Furthermore CF titers of 1:4

were often difficult to read because of incomplete complement fixation. So far, both epidemiological and technical data suggest that the performance of the CF test starting from a 1:4 dilution could be misleading in the diagnosis of a recent 229E infection. However, CF titers of 1:4 were always in agreement with IAHA titers, testifying to the specificity of the reaction. In our opinion, CF titers of 1:4 may be longer lasting than higher titers. This interpretation seems to agree with the results of Bradburne and Somerset [4], showing a very high prevalence of CF antibody when very potent antigens were employed in the CF tests.

In conclusion, when paired (acute and convalescent) sera are available, all four tests employed in this study can be used for diagnosis of a recent infection or reinfection. Using the CF test, a single serum sample can be sufficient for diagnosis of recent infection. However, for seroepidemiological surveys, the new IAHA test appears greatly superior to the other tests available (IHA and Nt).

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