INFECTION AND IMMUNITY, Nov. 1980, p. 500-505
0019-9567/80/11-0500/06$02.00/0

Vol. 30, No. 2

Human Antibody to Influenza C Virus: Its Age-Related Distribution and Distinction from Receptor Analogs

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Sera from persons of four age groups (1 to 2 years, 2 to 5 years, 20 to 30 years, and 65 to 85 years) were analyzed for hemagglutination inhibition (HI) activity for influenza C virus. Significant HI activity was found in 66% of the 237 sera tested, and titers ranged from 8 to 512. In the young adult group, 96% had antibody and the highest mean titer (74.7) of any age group. Positive sera were far less common in young children (36 to 47%), and relatively low titers (18.3) were common among adults over 65. The high percentage of sera with antibody to influenza C virus suggests that infections with this virus occur at a rate greater than previously recognized. The high percentage of young adults with elevated levels of HI antibody suggested either that an immune response to influenza C infections is common or that the observed HI activity might be attributable, in part at least, to nonspecific inhibitors in the sera. We showed both directly and indirectly that most if not all of the inhibitory activity in the human sera we examined was due to specific antibody, mostly immunoglobulin G. This conclusion is based on the finding that the single serum protein fraction with HI activity was found to have a molecular weight equivalent to that of 7S antibody (150,000) and that the HI activity was removed by absorption to staphylococcal protein A. Moreover, immunoglobulin from only HI-positive sera bound specifically to cells infected with influenza C virus, as shown by inhibition of hemadsorption and immunofluorescence. These findings were supported by similar results obtained with chicken antisera to C virus.

Influenza C virus has been the cause of infrequent outbreaks of respiratory illness among institutionalized children (4, 15). It has also been discovered in association with other viruses during the course of epidemics of acute respiratory infection among institutionalized elderly persons (10). The incidence of influenza C infection, as measured by serum hemagglutination inhibition (HI) activity, appears to be much higher than is suggested by the limited reports of confirmed viral isolation. About 50% of sera collected from the general adult population in Jamaica (11), Germany (5), and Egypt (3) inhibited influenza C virus to significant titers. Such activity was absent in the sera of children less than 1 year of age but was found with increasing prevalence among children aged 1 to 8 years (5). Reported prevalence of HI-positive sera in persons over 65 years of age has varied widely. Gerth et al. (5) found that the percentage of HI-positive sera among persons over 65 was only half that of younger adults (25% versus 50%). The age-related difference was attributed either to changes in the molecular composition of the virus (antigenic drift) or to decreased elaboration of serum HI activity as a result of aging. Jennings (11), on the other hand, found that the percentage of HI-positive sera increased with age, even beyond age 65, and attributed the persistence of HI activity to periodic reinfections, a suggestion that is not in accord with the infection rate of less than 1% in adults reported by Mogabgab (16).

Epidemiological studies based on the HI titration of sera may not be a true measure of influenza C infections. They could be misleading because influenza viruses are inhibited not only by virus-specific antibody, but also by nonspecific inhibitors present in the serum. For influenza A and B viruses, nonspecific inhibitory substances have been shown to include glycoproteins containing *N*-acetylneuraminic acid (NANA) (19, 20). NANA-containing inhibitors can be circumvented for antibody titration by treating serum with receptor-destroying enzyme (RDE), specifically neuraminidase (10, 19, 20). Treatment of serum with RDE is recommended for inactivation of suspected nonspecific inhibitors of influenza C virus (10, 20); however, published data have not shown reductions in inhib-

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itor activity after RDE treatment (10). Gerth et al. (5) reported that RDE treatment failed to destroy a human serum inhibitor for one strain of influenza C virus. Furthermore, the benefits of RDE treatment are not conceptually in accord with the recent findings that receptors for influenza C virus do not contain NANA (9, 12, 20) and that the virus lacks neuraminidase activity (12, 18).

These considerations led to the present evaluation of the relationship between the HI activity of human sera and the presence of antiviral antibody. Included in this evaluation is a comparison of human sera to animal serum containing well-characterized antiviral antibody (18) and to sera containing a potent nonspecific inhibitor of influenza C virus (6, 7).

MATERIALS AND METHODS

Virus. The propagation of influenza C virus (JJ) in eggs and in Madin-Darby canine kidney (MDCK) cells has been described previously (18). The virus used in this study had been passed amniotically in eggs 302 to 314 times since primary isolation.

HA and HI. Hemagglutination (HA) assays of influenza C virus (100 μl) were done with phosphate-buffered saline (PBS; 0.01 M phosphate–0.146 M NaCl, pH 7.2) as diluent, using microtiter plates, and an equal volume of chicken erythrocytes (0.5%). Settling patterns were allowed to develop at 2 to 4°C.

For HI assays, serial twofold dilutions of sera in 50 μl of PBS were mixed with an equal volume of virus containing 4 HA units. One hundred microliters of 0.5% chicken erythrocytes was added, and the reactions were stored at 2 to 4°C until patterns had settled (60 to 90 min).

RDE (neuraminidase treatment). The enzymatic activity of neuraminidase (purchased from Sigma Chemical Co., St. Louis, Mo.) was titrated by measuring the destruction of receptors on chicken erythrocytes for influenza A (PR8) virus (19). For inhibitor destruction, neuraminidase (0.75 ml of 100 U/0.25 ml) was added to serum (0.25 ml) and incubated overnight at 37°C. Sodium citrate (0.75 ml of 2.5%, wt/vol) was added, and the sera were heated at 56°C for 30 min. Sera were then titrated for remaining HI activity. For controls, equal portions of each serum were processed in an identical manner except that the neuraminidase was omitted.

Molecular sieve chromatography. Serum (2 to 4 ml) or solutions of known proteins (8 mg in 2 ml) were pumped (18.6 ml/h) at 4°C into an ascending column (2.5 by 120 cm) packed with Sephadex G-200. Fractions (4.3 ml) were analyzed for HI activity, and optical density was read at 280 nm. Rat serum, before chromatography, was overlaid with 10 ml of water and centrifuged at 105,000 × *g* for 16 h at 4°C. The lipid-rich water layer and the solid pellet, both lacking HI activity, were discarded. The serum infranatant used for chromatography contained over 95% of the original HI activity. Protein standards used for molecular weight determination included bovine serum albumin (67,000), aldolase (158,000), catalase (240,000), phosphorylase A (360,000), and ferritin (540,000). All were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) except phosphorylase, which was purchased from Worthington Biochemicals Corp. (Freehold, N.J.).

Spectrophotometric assays. Protein concentrations were determined, using bovine serum albumin as a standard, according to the method of Lowry et al. (14).

Staphylococcal protein A. According to the method of Edwards and Larson (2), the Cowan I strain of *Staphylococcus aureus* was grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), washed (four times) in PBS, and suspended in PBS containing 0.5% Formalin. After a 3-h incubation at 23°C, the bacteria were washed three times in PBS and heated at 80°C for 1 h. Bacteria were washed in PBS twice more and stored at 4°C for use. For immunoglobulin extraction, 10^9 *S. aureus* cells were centrifuged (10,000 × *g* for 10 min), and the supernatant was removed. Serum (0.2 ml) was added to the pellet, and the bacteria were suspended to maximize immunoglobulin binding. After incubation at 23°C for 15 min, bacteria were centrifuged (12,000 × *g* for 20 min) and the serum sample was removed. The efficiency with which immunoglobulin could be removed by protein A was confirmed by determining the agglutination titer of rabbit antiserum to sheep erythrocytes before and after absorption to staphylococci. A single absorption reduced the agglutination titer for sheep cells by 94%. We assumed that antibody to influenza C virus in human sera would be similarly absorbed.

Sera. This study included two pediatric groups (1 to 2 years old and 2 to 5 years old), young adults (20 to 30 years old), and older adults (65 years and older). Human sera samples were collected from pediatric and geriatric patients admitted to a general hospital for reasons other than respiratory illness. Half of the young adult sera came from pregnant women, and the remainder came from medical students before clinical service. Rat serum was purchased from Microbiological Associates (Rockville, Md.). Several lots of pooled rat serum had identical HI titers and were therefore used interchangeably.

Chicken antisera to influenza C virus and control sera were prepared, collected, and analyzed as described previously (18).

Hemadsorption inhibition assays. Monolayers of MDCK cells growing on cover slips in petri dishes were infected with influenza C virus as previously described (18). After 24 to 48 h of incubation at 32°C, infected and mock-infected monolayers were tested for hemadsorption by means of chicken erythrocytes. For inhibition assays, infected and mock-infected monolayers were incubated with 0.2 ml of serum or PBS at 4°C for 15 min, washed in cold PBS (four times), and tested for hemadsorption.

Fluorescent-antibody test for immunoglobulin. Monolayers of control and influenza C virus-infected cells were incubated with tris(hydroxymethyl)aminomethane-hydrochloride-buffered saline (pH 7.2) or sera containing known amounts of HI activity for influenza C virus. Monolayers were washed in PBS

(four times), fixed with acetone, and stained with fluorescein-conjugated rabbit antibody to chicken or human immunoglobulin G (IgG) (Behring Diagnostics, Somerville, N.J.). Cover slips were washed with PBS (four times) and once in distilled water and were then mounted in buffered saline.

RESULTS

Sera from subjects in the four age groups were analyzed for HI activity against influenza C virus (Table 1). Sera from persons between 20 and 30 years of age had the highest percentage of HI activity and the highest mean titer, the latter only slightly higher than that of children 2 to 5 years old. In the 20- to 30-year-old group, the titers and percentage of positive sera showed no differences attributable to sex or economic status. Adults over 65 years of age had a high incidence of HI-positive sera (66.7%), but the lowest mean titer of all age groups.

To determine the contribution, if any, of RDE-sensitive inhibitors to the HI activity of human sera, equal portions of 44 human sera showing HI activity were incubated overnight with 100 U of RDE and then heated at 56°C for 30 min to inactivate the enzyme. As controls, equal portions of the same sera were incubated in buffer without RDE. Comparisons of controls with RDE-treated sera showed no loss in HI activity attributable to RDE activity. Both treated and untreated sera retained equal levels of HI activity after incubation and heating at 56°C. RDE treatment of rat and immune chicken sera caused no reduction in HI titer. The HI titer of rat serum inhibitor increased when the serum was heated at 56°C.

We tried to distinguish between antiviral antibody and nonspecific inhibitors on the basis of molecular size, as reported by Hana et al. (7). Ten human sera with HI activity (titers of 64 to 256) were subjected to chromatography on Sephadex G-200, and the HI activity of the fractions was determined (Fig. 1). Chicken antisera to influenza C virus and a variety of proteins with known molecular weights were also chromatographed as molecular weight standards. The inhibitory factor in the human sera eluted as a single peak having an R_f nearly identical to that of the 7S antibody of chicken antiserum (150,000 daltons).

Inhibitory activity in rat serum eluted from Sephadex G-200 has a major peak with a molecular weight of about 400,000 and a minor peak with a molecular weight of about 70,000 (Fig. 1).

To confirm the identity of HI-activity component of human serum as immunoglobulin, the latter was removed from whole serum by reaction with protein A. The high HI activity of each of 60 sera was virtually eliminated by incubation with the Cowan strain of staphylococcus, which contains protein A (Table 2). The HI activity of chicken immune serum was unaffected by absorption with staphylococcal protein A. Retention of HI activity in chicken serum absorbed with protein A was expected because protein A does not react with chicken immunoglobulin (13). The inhibitory activity of rat serum was unaffected by protein A exposure even though protein A binds rat immunoglobulin (13). The results of immunofluorescence analysis further confirmed the immunoglobulin nature of the HI activity in human sera. Only human sera with high HI activity when reacted with infected monolayers gave specific reactions by hemadsorption inhibition and with fluorescent antiglobulin (Table 3). Infected monolayers exposed to sera with little or no HI activity were unreactive, as was normal chicken serum.

TABLE 1. *HI activity of human sera for influenza C virus*

Age of serum donors (years)	No. of:		% Positive	Mean titer[b]
	Sera tested	Positive HI[a]		
1–2	50	18	36	33.1
2–5	40	19	47.2	65.1
20–30	75	72	96	74.7
65–85	72	48	66.7	18.3

[a] Defined as a titer of 8 or greater.

[b] Mean titer $= \frac{\Sigma(n \times T^{-1})}{N}$, where n is the number of sera with a given titer T, T is the HI titer of each serum sample (n), and N is the number of HI positive sera in each age group.

DISCUSSION

Human antibody to influenza C virus has been measured for epidemiological studies by the HI titration of sera samples. In HI tests, the HI activity of each serum is assumed to be mediated by antiviral antibody; however, serum proteins analogous to receptors for the virus can contribute to the total HI activity of serum. Distinction between HI activity of antibody and of nonspecific viral inhibitors is made possible in many virus systems by destroying the nonspecific inhibitors with enzymes. Neuraminidase has been used effectively for this purpose in the assay of influenza A and B viruses and has been recommended in the HI titration of influenza C virus (8, 20). Demonstration of neuraminidase action on inhibitors for influenza C has not been reported, and evidence exists of its failure to destroy influenza C inhibitors present in both human (5) and rat (7) sera. The absence of NANA in influenza C virus receptors on erythrocytes (9) and the absence of neuraminidase in type C

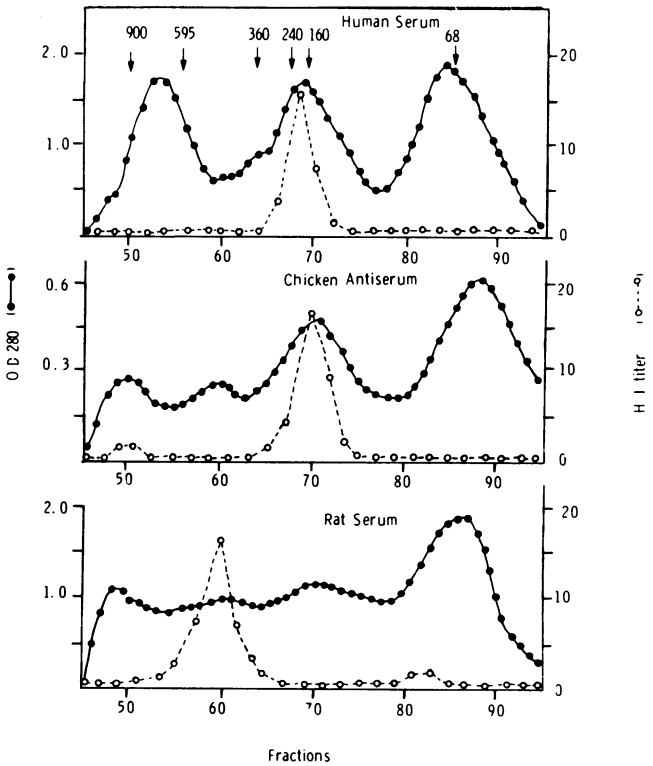

FIG. 1. *Molecular sieve chromatography of human, chicken, and rat sera. Portions (2 to 4 ml) of whole human serum, chicken serum, or a partially purified preparation of rat serum were applied to Sephadex G-200 columns and chromatographed as described in Materials and Methods. The ultraviolet absorption (optical density at 280 nm [OD 280];* ●*) and the HI activity* (○) *for influenza C virus of the fractions were measured. Elution positions and molecular weights* ($\times 10^{-3}$) *of the standards are indicated on the top graph.*

virions (12, 18) further suggest that neuraminidase action would not aid in the distinction between antibody and inhibitors.

The present study was an attempt to determine whether antibody does indeed account for the HI activity frequently present in human sera. The distinction between antibody and inhibitors was based not on the removal of inhibitor by enzyme action but rather on the direct demonstration of antibody as the active serum moiety. The HI-active component was shown to have the molecular weight, staphylococcal protein A-binding capacity, and antigenicity consistent with IgG antibody. Fortunately, binding to staphylococcus protein A was a rapid and economical method that distinguished antibody from nonspecific inhibitor.

Having established with reasonable certainty that antibody accounted for the HI activity of the sera studied, we can make a number of epidemiological observations on the basis of the HI data. Antibody to influenza C virus was found to be somewhat more prevalent than previously reported (3, 5, 11). That finding is especially true in the young adult population. Factors that might account for the higher prevalence of antibody found in our study population are: (i) higher incidence of influenza C infections and (ii) the limited age range of persons included in the basic adult group studied. This limitation was imposed to maximize differences that might be encountered between these young adults (aged 20 to 30 years) on the one hand and the other subjects, who were either in early childhood or over 65 years old. When the data were examined in this framework, the children were found to have a much lower incidence of infection (36 to 47%) than the young adults (96%). The incidence in much older adults dropped again to lower levels (18.3%). The mean titers of the several groups showed a similar pattern of being far lower in the extremely young and old than in young adults. These results support the epidemiological findings of Gerth et al. (5), who described childhood infection with influenza C virus as causing the appearance of antibody in high titer that persisted into much later life. These results contrast with the findings of Jen-

TABLE 2. *Properties of HI-active components of human, rat, and chicken sera*

Serum treatment	Serum tested		HI titer	
	Species	No.	Pretreatment	Posttreatment
RDE digestion[a]	Human	25	128-256	64-256
		19	64-128	32-128
	Rat	Pooled	256	512
	Chicken	3	512	512
Staphylococcal protein A extraction[b]	Human	60	64-256	0-2
	Rat	Pooled	256	256
	Chicken	3	512	512

[a] Sera were mixed with 4 volumes of calcium saline containing 100 U of RDE incubated at 37°C overnight, mixed with 3 volumes of sodium citrate (2.5%), and incubated at 56°C for 30 min before HI titration. For controls, sera were processed in the same manner except the calcium saline lacked RDE.

[b] Sera were twice extracted with 10^9 cells of *S. aureus* Cowan I strain for removal of immunoglobulin as described in Materials and Methods.

TABLE 3. *Interaction of sera with influenza C virus-infected cells*

Sera tested			Hemadsorption inhibition[a]	Surface[b] bound IgG[b]
Species	No.	HI titer		
Control (PBS)		0	−	−
Human	15	128-256	+	+
	10	64	+	+
	8	32	+	+
	3	0-4	−	−
Rat	Pooled	256	+	−
Chicken				
Preimmune	3	0	−	−
Immune	3	512	+	+

[a] MDCK cells infected with influenza C were incubated with 0.2 ml of sera being tested at 4°C for 15 min, washed four times with PBS, and tested for hemadsorption inhibition (+).

[b] Fluorescein-conjugated rabbit antiserum to human or chicken IgG was added to mock-infected or influenza C-infected MDCK cells that had been previously incubated with PBS (control) or a portion of the serum being tested. Monolayers were washed extensively and scored for specific fluorescein staining (+) relative to controls (−).

nings (11), who suggested that antibody titers increased throughout adulthood, presumably in response to multiple infections by influenza C virus.

The relationship between serum HI activity and protective immunity to influenza C has never been defined. A high degree of correlation, however, may exist between HI activity and protective immunity. Age groups in which low mean HI titers (i.e., those over 65) or a low percentage of positive sera (i.e., children under 5) account for nearly all confirmed influenza C infections (10, 15).

The C viruses have been shown to contain a segmented genome (1), making genetic reassortment theoretically possible; however, no evidence yet suggests that any major antigenic changes have occurred in the years since the C viruses were first recognized. This apparent, though not proved, relative antigenic stability would make this infection more akin epidemiologically to the other respiratory diseases (e.g., parainfluenza virus and respiratory syncytial virus) (17) than to the constantly changing patterns of epidemic group A or B influenza. This theory is not incompatible with the generally accepted concept of group C influenza as a predominantly inapparent or mild respiratory infection that is rarely specifically recognized. Much could be learned in this regard from a prospective effort to isolate C viruses from cases of undifferentiated respiratory infection, particularly in infants and children.

ACKNOWLEDGMENTS

We thank Gizelle Poche and Sue Wilson for their technical assistance. We also thank Calderon Howe for his advice in the preparation of this manuscript.

This work was supported by grants from the Louisiana Health Association through an award from Frank Delia, Jr., by the Edward G. Schlieder Educational Foundation, and by grant AI 10945 from the National Institutes of Health.

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