

Difference in Antibody Reactivity Between Complement Fixation and Immune Adherence Hemagglutination Tests with Virus Antigens

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Complement-fixing (CF) immunoglobulin M antibody to infantile gastroenteritis virus (a rotavirus) did not show the reactivity of immune adherence hemagglutination (IAHA). Early immunoglobulin G CF antibody produced both in patients and in guinea pigs experimentally infected with Japanese encephalitis virus (a flavivirus) had weak reactivity in IAHA tests. However, late antibody showed higher titers by IAHA than by CF. These results suggested that early antibodies with lower affinity are inefficient in the IAHA reaction. The implications of this study are: (i) a low ratio of IAHA/CF antibody titers in a serum suggests a recent rotavirus infection; (ii) the IAHA reaction is more type specific than the CF reaction for identifying the serotype of antigenically cross-reacting viruses with hyperimmune sera.

The immune adherence hemagglutination (IAHA) test (18) is similar to the complement fixation (CF) test in that the two tests employ complement (C) for the detection of antigens (Ags) and antibodies (Abs). After Mayumi et al. (24) introduced dithiothreitol for the stabilization of the hemagglutination pattern, the IAHA test has become widely used for the titration of viral Ags (23, 27, 30) and Abs (14, 20, 21, 26) because of its greater sensitivity.

We found, however, that, in contrast to late Abs, early Abs have weak IAHA reactivity. We studied two systems of virus Ag-Ab reactions: the rotavirus (large-size virus) system, in which immunoglobulin M (IgM) fixes C (1); and the flavivirus (small-size virus) system, in which it does not (2). This paper deals with the reactivity of early Abs by IAHA and discusses the mechanism of the IAHA reaction.

MATERIALS AND METHODS

IAHA and CF tests. The IAHA test was carried out according to Mayumi et al. (24), with slight modifications. We used Veronal-buffered saline with Ca^{2+} and Mg^{2+} containing 0.1% bovine serum albumin, 0.002% gelatin, and 0.02% NaN_3 as the diluent throughout the test. Ag and Ab, in 25- μl volumes each, were mixed in wells of U-bottom polystyrene microplates and incubated at room temperature for 1 h. Then, 25 μl of fresh guinea pig serum diluted 1:100 was added as a source of C. The plates were sealed with tape and incubated at 37°C for 40 min. Then, 25 μl of dithiothreitol (3 mg/ml) and 50 μl of 0.4% human type O

erythrocytes were added. The hemagglutination pattern was read after overnight incubation of the plates at room temperature.

The 0.4% erythrocyte suspension for the IAHA test was prepared in the following way. Four milliliters of blood was taken by venipuncture, mixed with 20 ml of Alsever solution to give an approximate 8% suspension, and stored at 4°C for at least 3 weeks. A 0.5-ml sample from this suspension was mixed with 24.5 ml of the cyanmethemoglobin reagent, and then the optical density at 540 nm was read and the dilution factor for 0.4% suspension was calculated according to Hierholzer and Suggs (16). In each test, only the required volume of this suspension, calculated by the dilution factor, was taken, and the erythrocytes were washed with physiological saline by three cycles of low-speed centrifugation and finally suspended in the IAHA diluent to the predetermined volume for 0.4% suspension. In this way, about 100 microplates could be examined with 4 ml of blood without wasting erythrocytes. We always used erythrocytes from the same donors.

The CF test was done in microplates with 5 50% hemolytic complement units of C. When IgM Ab to rotavirus was detected, the plates were incubated for 1 h at 37°C before the addition of sensitized sheep erythrocytes to increase the reactivity of IgM Ab (1).

Particulate rotavirus Ags were prepared from patients' feces by pelleting through 45% sucrose by the method of Bishop et al. (3). Japanese encephalitis virus (JEV) Ags derived from infected mouse brains by ether-acetone treatment were purchased from Takeda Pharmaceutical Co. (Osaka, Japan).

The Ag dose used for line titrations of Ab was 4 U, which was determined by checkerboard titrations using hyperimmune sera. Since Ag titers by IAHA were higher than by CF, dilutions of an Ag preparation for

use in IAHA Ab titrations were higher than those for CF Ab titrations.

Hemagglutination inhibition tests with JEV Ag. Hemagglutination inhibition Ab titers were determined by the method of Clarke and Casals (6) in a microtiter system.

Sucrose density gradient centrifugation of sera. Serum was diluted threefold and inactivated at 56°C for 30 min; 0.5-ml volumes were then centrifuged in sucrose gradients as previously described (1).

Infection of guinea pigs with JEV. Female guinea pigs were intraperitoneally infected with 10,000 plaque-forming units of JEV. One year later, the same dose of virus was injected as a booster.

RESULTS

Reactivity of rotavirus Ab by IAHA and CF. Serum samples collected from patients with infantile gastroenteritis were titrated for rotavirus Ab by CF and IAHA. The same Ag preparation was diluted 50-fold for CF tests and 800-fold for IAHA titrations. Table 1 shows that within 2 weeks after the onset of disease, IAHA Ab activity was either undetectable or very weak, whereas CF Ab was readily detected. After week 3, however, IAHA Ab titers were higher than CF titers. These results show that a low ratio of IAHA to CF titers is characteristic of a recent infection.

To examine the IAHA reactivity of both IgM and IgG Abs, the sera were centrifuged in sucrose gradients to separate these Ab classes. Figure 1 shows that 19S IgM Ab had no IAHA Ab reactivity. Furthermore, in early sera (Fig. 1a, e, and f) 7S IgG CF Ab possessed no IAHA Ab titers. In other early sera (Fig. 1c and g) IgG Ab gave a weak hemagglutination pattern. In late sera (Fig. 1b, h, and i), however, IAHA titers of IgG Ab were higher than CF titers. These results show that early IgM and IgG Abs are inefficient in IAHA reactions.

The whole serum of patient no. 2, taken 12 days after the onset of disease, showed a weak hemagglutination pattern in the IAHA test (Table 1), whereas the isolated 7S Ab gave a distinct pattern (Fig. 1d). Furthermore, the whole sera of patient no. 2, taken at day 9, and of patient no. 9, taken at day 11, gave no IAHA Ab titers (Table 1), although their separated IgG Abs showed weak reactivities in IAHA tests (Fig. 1c and g). These results suggest that, in some sera, IgM Ab inhibits the reactivity of IgG Ab when both Abs are present in the same serum.

A neonate patient (no. 23) had higher Ab titers in her early sera by IAHA than by CF (Table 1). Figure 1j shows that she had IgM CF Ab of low titer but IgG Ab with high IAHA reactivity. The former might have been produced by herself, whereas the latter was derived

from her mother.

Reactivity of anti-JEV Ab. Serum samples were sequentially collected from JEV-infected guinea pigs, and IAHA, CF, and HI Ab titers were determined. The same Ag preparation was diluted 20-fold for CF and 200-fold for IAHA tests. Figure 2 shows that 2 weeks postinfection guinea pigs no. 17 and 18 had CF, but no IAHA, Ab titers. At week 3, the sera gave weak hemagglutination patterns in IAHA tests. However, at later times the hemagglutination patterns became distinct, and IAHA Ab titers were 3 dilutions higher than CF Ab titers. These results again show that early Abs are inefficient for IAHA reactions. Sucrose gradient centrifugation of these sera revealed that both CF and IAHA Ab activities resided only in 7S fractions, but hemagglutination inhibition Ab activity occurred in 19S as well as 7S fractions (data not shown).

For a more detailed examination of early and late sera by IAHA and CF, checkerboard titrations were carried out. Serum taken 2 weeks postinfection from guinea pig no. 17 was used as early serum, and postbooster serum from the same animal was used as late serum. Figure 3 shows that a great difference in IAHA reactivity existed between the early and hyperimmune sera. The early serum gave low IAHA Ag titers with a weak hemagglutination pattern. However, the postbooster serum gave Ag titers 5 dilutions higher. This indicates that early sera may give no IAHA Ab titers if the Ag used for Ab line titrations is 4 U, as determined by checkerboard titrations with hyperimmune antisera. In contrast to the IAHA titers, CF Ag titers of the early serum were the same as those of the hyperimmune serum (Fig. 3).

DISCUSSION

An interesting finding in this study was that IgM Ab to rotavirus did not show IAHA reactivity, and, furthermore, inhibited the reactivity of IgG Ab in some sera. Therefore, a comparison of IAHA with CF Ab titers of single sera may be useful for the serodiagnosis of infantile gastroenteritis caused by rotavirus; a low ratio of IAHA/CF Ab titers is suggestive of a recent primary infection which may have taken place within 2 weeks previously (Table 1). It should be noted, in this connection, that the virion Ag free of soluble components must be used in these tests (1, 4) for the comparison to be valid.

Early IgG Abs produced after rotavirus and JEV infections were found to be inefficient for the IAHA reaction, whereas they showed efficient CF reactivity. This finding is in accord with that on hepatitis A infection (20, 22, 26);

TABLE 1. Comparison of serum Ab titers by CF and IAHA

Patient no.	Sex	Age	Detection of rotavirus in feces ^a	Days after onset of disease	Ab titer	
					CF	IAHA
1	M	11 mo	+	6	128	<8
				12	256	<8
				34	128	512
2	F	1 yr, 3 mo	NT ^b	6	256	<8
				9	256	<8
				12	256	64* ^c
3	M	1 yr, 2 mo	+	3	<8	<8
				22	256	1,024
4	M	6 yr	+	7	64	<8
				11	64	<8
5	M	1 yr, 3 mo	+	7	128	<8
				10	128	<8
6	M	7 mo	+	6	64	<8
				10	128	64*
7	M	1 yr, 10 mo	NT	7	256	<8
				9	256	<8
8	F	8 mo	+	4	<8	<8
				9	256	<8
9	F	1 yr, 1 mo	+	6	64	<8
				11	256	<8
10	F	1 yr, 11 mo	+	8	256	<8
				10	256	128*
11	M	1 yr	+	7	128	<8
				10	256	512*
12	M	1 yr, 4 mo	NT	4	8	<8
				6	32	<8
13	M	11 mo	NT	4	8	<8
				10	256	32*
14	F	1 yr	+	6	32	<8
15	M	2 yr	NT	8	256	<8
16	M	1 yr	NT	6	256	<8
17	M	4 yr	NT	7	64	<8
18	M	2 yr	NT	9	256	<8
19	F	10 mo	+	9	64	<8
20	M	1 yr, 3 mo	+	6	256	<8
21	F	1 yr	+	54	128	512
22	M	1 yr, 6 mo	+	95	128	1,024
23	F	Neonate	+	9	16	256
				14	32	256
				21	32	256

^a Detection by electron microscopy or the IAHA test (23).^b NT, Not tested.^c *, Incomplete hemagglutination patterns were observed in several dilutions of serum.

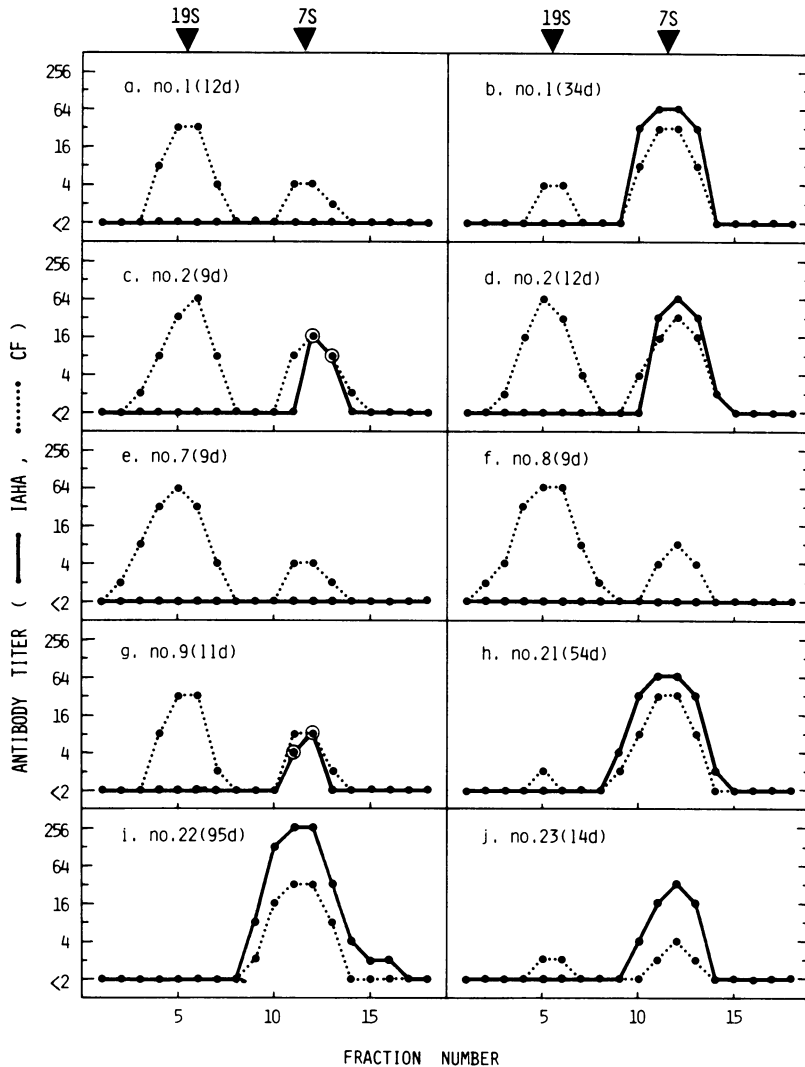


FIG. 1. Sedimentation profile of CF and IAHA Ab activity in the serum of patients with infantile gastroenteritis. Serum samples were centrifuged in sucrose density gradients after heat inactivation. The patient number corresponds to that shown in Table 1. *d*, Days after the onset of disease. O, Incomplete hemagglutination pattern.

the delayed appearance of IAHA Ab activity after this infection has also been reported by other investigators (7, 10, 11, 15, 28, 29). Late IgG Abs, however, had higher titers by IAHA than by CF. Thus, the IAHA test should be used rather than the CF test for the seroepidemiological studies of rotavirus infection. We found that the IAHA test using a cultivatable human rotavirus, "WA," as antigen is useful for such studies (manuscript in preparation).

It is known that Ab produced shortly after antigenic stimulation has low affinity, and late Ab has higher affinity to Ag (12). Thus, Ab with

low affinity is considered to have weak IAHA reactivity. We previously reported that, in the serotyping of dengue viruses with hyperimmune antisera, IAHA reactions were more type specific than CF reactions: dengue type 3 Ag reacted with the homologous antiserum more specifically by IAHA than by CF (17). The reason could be explained in the following way. When a virus Ag is reacted with a heterotypic antiserum, the Abs are considered to behave as low-affinity ones because the reactions are heterologous. Thus, the IAHA reaction in the heterologous Ag-Ab system is expected to be a weak

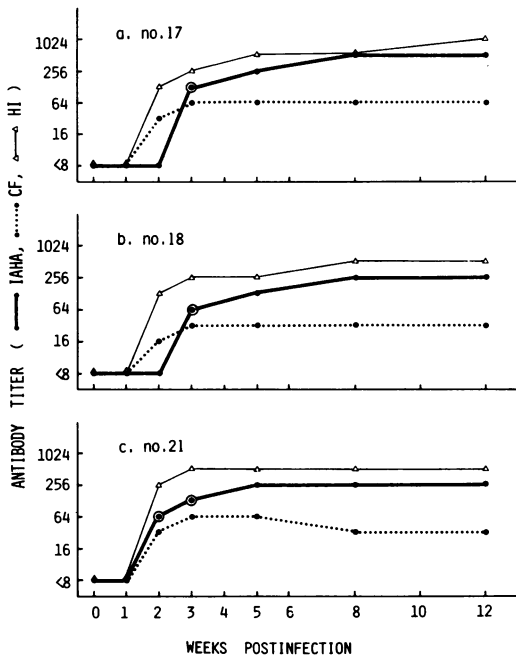


FIG. 2. Ab responses after experimental infection of guinea pigs with JEV. Three different tests were used for determination of Ab titers. The time course of the appearance of IAHA Ab is not parallel to that of CF Ab. \odot , Incomplete hemagglutination pattern.

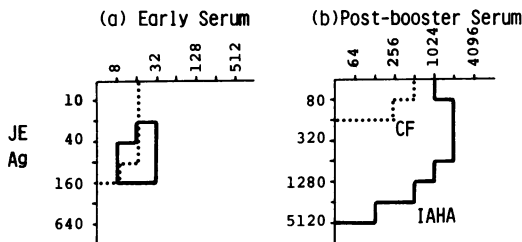


FIG. 3. Checkerboard titrations of early and hyperimmune sera by IAHA and CF. Guinea pig no. 17 was given a booster injection of JEV 1 year after the first infection. The early serum (a) was taken 2 weeks after the first infection, and the hyperimmune serum (b) was taken 2 weeks after the booster injection. Figures on abscissas and ordinates represent Ab and Ag dilutions, respectively. Note that the scales of dilution are different between (a) and (b).

one, whereas the homologous IAHA reaction should be strong. Therefore, the IAHA test is also useful for identifying the serotype of antigenically cross-reactive viruses (17, 30) because of its higher specificity and sensitivity.

The mechanism of the IAHA reaction of IgG Abs with particulate virus Ags is postulated to be as follows (see Fig. 4). (Soluble virus Ags do not give an efficient IAHA reaction [17]). At the

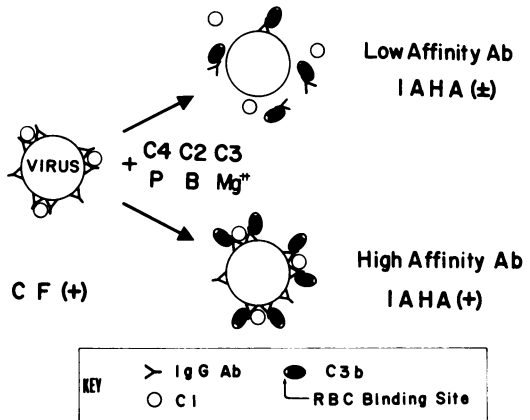


FIG. 4. Mechanism of the IAHA reaction (hypothesis). The classical pathway of C activation starts with binding of C1 to the Ab molecules on virus particles. For efficient generation of C3b, C components of the alternative pathway (factor B and properdin [P]) are also required (31). Nascent C3b attaches to nearby Ab molecules (5, 31). Virus particles carrying C3b via Ab can then agglutinate human erythrocytes. However, if the Ab has low affinity, it dissociates from the Ag by the effect of C3b attachment (9, 25), resulting in inefficient hemagglutination.

first step of C activation, Ab molecules on the surface of virus particles fix C component 1 (C1), which causes the C "cascade." Once C1 is activated and consumed, sensitized sheep erythrocytes, later added to the reaction mixture as indicators of CF activity in the original mixture, would not be lysed, which would give a CF positive result. When C3b molecules having a binding site for human erythrocytes are activated, they could then attach to nearby Ab molecules (5, 31). Then, virus particles coated with Abs carrying C3b could acquire hemagglutinating activity. However, if the affinity of Ab is low, the Ab molecules might dissociate from the Ag by the effect of C3b attachment (9, 25), resulting in inefficient hemagglutination. In contrast, the binding of C1 to Ab might take place irrespective of the Ab affinity, which would mean that the CF reaction might be less specific.

IgM Abs show CF reactivity with large-sized viruses such as rotavirus (1, 19) and herpesvirus (4, 8). However, they do not show IAHA reactivity with rotavirus, although they give a positive IAHA result with varicella-zoster virus (13). Possibly IgM Ab molecules carrying C3b dissociated from the rotavirus Ag, especially after the addition of dithiothreitol, which also converts IgM to its 7S subunits, to give negative hemagglutination in the IAHA test. On the other hand, herpesvirus has an envelope and is larger in size than rotavirus, and thus activated C3b may at-

tach directly to the envelope. In this case, Ab would have no effect on the dissociability of C3b from the Ag.

In summary, low-affinity Abs are generally inefficient for the IAHA reaction with virus particles.

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