Role of Rheumatoid Factor in Complement Fixation and Indirect Hemagglutination Tests for Immunoglobulin M Antibody to Cytomegalovirus

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Absorption of immunoglobulin M (IgM)-rheumatoid factor (RF) from serum samples by reaction with insolubilized gamma globulin reduced the complement-fixing (CF) antibody titer to cytomegalovirus (CMV) antigen to less than 1:2 in the IgM fraction of some, but not all, sera. Thus, IgM-CF activity in some sera appeared to be due to specific IgM anti-CMV antibody and in other sera to complexes of IgM-RF with antiviral IgG antibody. Prozones were present in the CF test on IgM fractions. Increasing the concentration of antigen from 2 to 4 U reduced the prozone titer by one or two doubling dilutions. This observation suggested that a competition for antigen may be operating at low dilutions of IgM antibody fractions. Removal of RF had little or no effect on the reaction of the IgM fraction of sera with CMV by the indirect hemagglutination test.

In cytomegalovirus (CMV) infection aberrant immunological reactions may occur (3, 17, 23, 29, 34). One such reaction is the formation of rheumatoid factor (RF), an antibody reactive with immunoglobulin G (IgG) which is usually of the IgM class but may also be of the IgG or IgA class (1, 15). Complexing of IgM-RF with viral antibody of the IgG class has led to false positive reactions for IgM viral antibody in the indirect fluorescent-antibody test (10, 29).

Analysis of antibody production in human CMV infection in a previous study indicated that IgM reactivity with CMV antigen detected by indirect hemagglutination (IHA) usually arose earlier than did that detected by complement fixation (CF) (8a). The difference in temporal appearance of these two reactions suggested that different populations of IgM molecules were involved. The present report, as an extension of our previous study (8a), investigates the contribution of RF to the IHA and CF reactivity seen between IgM fractions of serum and CMV antigen.

MATERIALS AND METHODS

Serum samples. Eight sera in which CMV antibody was found in the isolated IgM fractions by both IHA and CF were selected for study.

Antibody assays. IHA tests were done according to the method of Bernstein and Stewart (5) with slight modification. Antigens extracted by freezing and thawing infected cells and noninfected cells (9) were used to coat sheep erythrocytes treated with tannic acid.

The concentration of antigen for use was determined by block titration with a positive reference serum that contained IgM anti-CMV antibody. Complete or almost complete agglutination by antibody of cells coated with the optimal dilution of antigen was taken as the end point. A positive serum of known titer and a negative serum were included in each run. CF tests were performed as previously described (9), using antigen extracted with 0.1 M glycine (pH 9.5 buffer) from human fetal diploid lung cells infected with CMV strain AD169. Two units of antigen, as determined by block titration, were used in the test. A similar preparation was made from noninfected cells for use as a control antigen.

RF. Sera and fractions were checked for RF with Hyland Laboratories' latex globulin reagent in the slide test.

Absorption of RF. Gamma globulin (immune serum globulin, Armour Pharmaceutical Co., Chicago, Ill., or Pentex human gamma globulin, fraction II, Miles Laboratories, Inc., Elkhart, Ind.) was insolubilized with glutaraldehyde as described by Avrameas (2). After homogenization and washing, the insolubilized globulin was further reacted with 0.3 M glycine to block any unreacted glutaraldehyde bonds (23). After additional washing, the insolubilized material was made to a final 50% concentration (vol/vol) in phosphate-buffered saline (0.01 M phosphate buffer, pH 7.2).

To determine the amount of insolubilized gamma globulin necessary for removal of RF from the test sera, 0.05-ml samples (i.e., 0.2 ml of a 1:4 dilution) of a reference serum strongly reactive for RF were absorbed for 1 h at 37°C with increasing amounts of the 50% slurry, i.e., with the pelleted material from 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 ml. The amount found satisfactory for removal of RF in the reference serum was

then used for absorption of all sera. After absorption, the sera were retested for RF and for IgM, IgG, and IgA by radial immunodiffusion, using plates specific for human heavy chains (Oxford Laboratories, Foster City, Calif., or Kallestad Laboratories, Chaska, Minn.).

Determination of antibody class. Serum samples, diluted 1:4 with phosphate-buffered saline, were centrifuged before and after absorption in a 10 to 40% linear sucrose gradient as previously described (9). The total volume consisted of 5 ml comprised of a 4.5-ml gradient and a 0.5-ml sample. Fractions (0.5 ml) were collected from the bottom of the gradient. Each fraction was tested by the Ouchterlony method for immunoglobulin class, using antisera to μ , γ , and α chains (Behring Diagnostics, Somerville, N.J.) and for IHA and CF antibody to CMV. Fractions from the unabsorbed sera were also checked for RF. Sera and fractions from any one patient were evaluated in the same run to avoid differences between runs.

RESULTS

Absorption of RF. Absorption of the reference serum with 0.2 ml of insolubilized gamma globulin per 0.05 ml of serum removed the reactivity for RF at a 1:20 dilution of the serum, the recommended dilution for testing RF in the Hyland Laboratories latex test. There was also a reduction in the concentration of all of the immunoglobulins, with the greatest reduction in IgM (Fig. 1). Absorption of the test sera at this ratio of insolubilized gamma globulin to serum similarly removed RF activity and reduced the concentration of the immunoglobulins (Table 1). The greatest reduction of IgM occurred with serum C, the serum with the greatest RF activity.

A weak reaction for RF was observed at a 1:5 dilution in fraction 3 (the IgM fraction) of the unabsorbed sera from patients B and C. This dilution represents a 1:20 dilution of the original

FIG. 1. Reduction in classes of immunoglobulins in a reference serum containing RF after absorption with insolubilized gamma globulin.

TABLE 1. Percent reduction in immunoglobulins after absorption of serum with insolubilized gamma globulin

Patient	% Reduction			RF
	IgG	IgM	IgA	
A	28	45	27	1+ ^a
B ^b	35	55	37	2+
B ^c	48	69	39	3+
C	53	81	41	4+
D	52	47	37	2+
E	46	42	39	1+
F	48	45	35	Negative
G	38	53	34	2+

^a Reaction of serum at a 1:20 dilution before absorption. After absorption all sera were negative at a 1:20 dilution.

^{b, c} Acute-phase (b) and convalescent-phase (c) sera from the same patient.

serum sample. RF activity was not detected in any of the fractions of the unabsorbed sera from the other patients. Apparently the concentration of RF in these specimens was too low for detection after serum fractionation.

CF and IHA antibody titers in serum. Table 2 shows the antibody titers of the whole serum before and after absorption for RF and gives the RF reaction of the sera prior to absorption. The usual reduction in CF and IHA antibody titers after serum absorption was two- to fourfold.

Antibody titers of fractions from sucrose density gradient centrifugation. Table 3 gives the CF and IHA titers of the IgM and IgG fractions showing the greatest reaction with CMV antigen, i.e., fraction 3 and fraction 5 or 6, respectively. Depending upon the patient's antibody response, the time of sampling of the serum, the nature of the antigen under study, and the type of assay procedure, both IgM and IgG classes of serum antibodies may be seen, or either one or the other. Thus, in the acute-phase serum of patient B, both IgM and IgG antibodies were detected by CF (Fig. 2), but only IgM antibodies were found by IHA (Fig. 3). Good separation of the immunoglobulin classes of antibodies was achieved, as shown by the bimodal distribution of the CF antibodies in Fig. 2.

CF antibody. With the exception of the CF antibody in sera from patients B (Fig. 2) and E, the CMV antibody titer in the IgM fractions was reduced to less than 1:2 by absorption of the sera with insolubilized gamma globulin. Of interest was the presence of a prozone in the IgM fraction of seven of the eight unabsorbed sera and in the absorbed sera from patients B and E that varied from 1:2 to 1:16 (Table 3). No prozones were seen in any of the CF tests on the

TABLE 2. Serum antibody titers to CMV before and after absorption with insolubilized gamma globulin

Patient	Time (days) of serum collection from onset of disease	Antibody titer				RF
		CF		IHA		
		Not absorbed	Absorbed	Not absorbed	Absorbed	
A	26	2,048 ^a	512	4,096	4,096	1+ ^b
B	6	4,096	2,048	2,048	1,024	2+
B	29	512	256	4,096	2,048	3+
C	17	256	128	8,192	2,048	4+
D	16	512	128	4,096	2,048	2+
E	19	2,048	1,024	16,384	4,096	1+
F	22	256	64	1,024	512	Negative
G	33	512	128	16,384	8,192	2+

^a Titer = reciprocal of the dilution.^b Reaction before absorption at a 1:20 dilution of serum. After absorption all sera were negative at a 1:20 dilution.

TABLE 3. Antibody titer of isolated immunoglobulins to CMV before and after absorption of serum with insolubilized gamma globulin

Patient	CF					IHA			
	IgM		IgG		Prozone (IgM)	IgM		IgG	
	Before	After	Before	After		Before	After	Before	After
A	64 ^a z	<2	128	32	4	1,024	256	16	4
B ^b	256 z	256 z	128	128 ^c	8	512	256	2	<2
B ^c	64	16 z	32	8	4	1,024	256	64	32
C	32 z	<2	8	2	8	512	512	64	16
D	128	<2	32	8	0	1,024	512	32	16
E	256 z	64	64	32	16	2,048	1,024	16	<4
F	32 z	<2	8	4	2	128	64	32	32
G	64 z	<2	16	8	4	2,048	2,048	32	16

^a Titer = reciprocal of the dilution of the gradient fraction. A 1:4 dilution of the serum samples was placed on the gradient. z, Prozone.^{b, c} Acute-phase (b) and convalescent-phase (c) sera from the same patient.

whole sera. The CF tests on the IgM fractions were repeated with 2 and 4 U of antigen. Increasing the concentration of antigen to 4 U caused a two- to fourfold reduction in the titer of the prozone. A concentration higher than 4 U of antigen could not be used because at greater concentrations the antigen became anticomplementary.

CF reactivity in the IgG fractions of the absorbed sera was either not reduced or was reduced two- to fourfold from that of the unabsorbed sera.

IHA antibody. With the exception of two sera which showed a fourfold reduction in IHA titer in the IgM fraction after serum absorption, none of the other absorbed sera showed more than a twofold reduction in IgM titer (Table 3). With regard to IgG antibody, there was either no reduction in titer (serum from patient F) or a two- to fourfold reduction after serum absorption.

DISCUSSION

Absorption of serum with particulate material

usually results in some nonspecific as well as specific loss of serum proteins, depending upon the nature of the absorbing material. Thus, *Staphylococcus aureus* protein A reduces IgG concentration specifically, but there is also some nonspecific loss of IgM and IgA (7, 14, 20, 24). Similarly, in the present study, insolubilized IgG specifically removed RF through binding to the antibody-combining site on the RF molecule, but it also caused some nonspecific loss of IgG, IgA, and possibly also of non-RF-IgM.

Absorption with insolubilized gamma globulin reduced the IgM-CF antibody titer of five of the eight sera (Table 3) to less than 1:2 (a 32- to 128-fold reduction). However, there was no change in IgM-CF antibody titer of the first serum of patient B (Fig. 2) and only a partial reduction (fourfold) in that of the second serum from patient B and in that of the serum from patient E.

The antibody titers remaining after absorption are considered to be specific. The absorbed sera were negative for RF, and the nonspecific controls in the CF test were negative.

FIG. 2. Biphasic distribution of CMV antibodies. Sucrose density gradient centrifugation pattern of CF antibodies in the acute-phase serum of patient B. Symbols: (●) not absorbed; (○) absorbed; (■) prozone, not absorbed; (□) prozone, absorbed.

There are reports of serum specimens which do not react in the RF test and yet contain the factor (21, 29). The serum from patient F appears to be such a seemingly negative specimen, since absorption completely removed the IgM-CF activity. The reduction in serum titer was 32-fold greater than would be anticipated for nonspecific absorption, and particularly in relation to the loss in CF-IgG antibody titers. CF-IgG titers were reduced by absorption two- to fourfold. Since a specific loss of IgG would not be expected unless RF was present in the insolubilized gamma globulin, this level of titer reduction appears to be due to nonspecific absorption. (Our gamma globulin preparation did not react in the RF test.) Twofold reductions are also considered within the error of any doubling dilution test.

Two conclusions may be drawn from the above results. First, IgM-RF may lead to false positive reactions in the CF test for IgM viral antibody, and, second, some patients produce an IgM antibody capable of fixing complement. With regard to the first point, anti-CMV IgG antibody complexed with IgM-RF and therefore present in the IgM fraction would specifically react with the CMV antigen and fix complement. Such activity should then be obviated by removal of the IgM-RF, and such was the case. Whether CF would be enhanced in the unabsorbed whole serum because of the combined reaction of the CMV antibody with CMV anti-

FIG. 3. Monophasic distribution of CMV antibodies. Sucrose density gradient centrifugation pattern of IHA antibodies in the acute-phase serum of patient B.

gen and with IgM-RF is not known. IgM-RF in its reaction with IgG was considered at one time incapable of binding complement. More recent reports indicate the IgM-RF can lyse sheep erythrocytes sensitized with reduced and alkylated IgG antibody (31, 32). In the present study complexing IgM-RF with IgG in the absence of CMV antigen did not result in CF, since the reactions with the control antigen were negative.

With regard to the second point, IgM antibody in its reaction with viral antigen has usually been found incapable of fixing complement (4, 6, 8, 12, 13, 16, 18, 19, 25, 26, 28, 30). There are only a few studies to the contrary, and in these studies the presence of RF was not determined (11, 27, 33). Therefore, the contribution of RF to the results is not known. In the present study, CF activity was not removed by removal of RF from the sera of two individuals, suggesting that CF-IgM antibody can at least occasionally occur.

The occurrence of a prozone in the IgM fraction of all of the unabsorbed sera as well as the absorbed sera that retained CF-IgM activity suggests the additional presence of a non-complement-fixing IgM antibody to CMV in low titer. The non-complement-fixing antibody would have to be of high avidity to compete successfully for CMV antigen and thus block CF at low dilutions. This theory has some support since by increasing the concentration of antigen from 2 to 4 U the prozone was reduced, although it was

not eliminated in all cases. If a high concentration of such an antibody were the usual response in viral infections, it could completely mask CF-IgM activity. Competing antibody of this nature could explain the inability of most investigators to demonstrate CF-IgM antibody to viral antigens.

Other explanations are possible. Binding sites for complement become available on the Fc portion of the antibody molecule through spatial reorientation of the molecule in its reaction with antigen. Human IgM has ten binding sites for antigen (22); IgG has two binding sites. For effective binding of the first component of complement (C1q) by IgM antibody, antigen-binding sites present on the Fab portion of two adjacent subunits of the IgM molecule must be complexed with antigen. Therefore, there may be competition for antigen at the intramolecular level in the case of IgM antibody, necessitating concentrations of antigen greater than that required for IgG antibody. Usually in diagnostic virus laboratories, block titrations for determination of the antigen unit are done with convalescent-phase sera where IgG antibody is predominant.

Contrary to the CF results, IgM-RF appeared not to be significantly involved in the IHA reaction of IgM antibody. IgM titers with most of the sera after absorption were either unchanged or reduced by one doubling dilution. Only two of the eight sera showed a fourfold reduction in antibody titer. IHA-IgG antibody titers were similarly reduced. Since IgM-RF may cause false positive reactions for IgM by the indirect fluorescent-antibody method (10, 29), the same problem will no doubt occur with tests utilizing newer techniques for labeling antibody such as the enzyme-linked immunosorbent assay and radioimmunoassay. Although more time-consuming, IHA performed on isolated IgM fractions provides an alternate and sensitive method for determination of IgM antibody to CMV in which RF appears not to participate to any significant degree. IgM-RF-IgG antibody complexes may not be of optimal size or configuration for binding to antigen-coated erythrocytes. Also, the IHA test is reported to be more sensitive to IgM antibody than to IgG antibody (8), so that any IgG antibody in the IgM-CF complex may not be in a very competitive position for the viral antigen.

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