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Elimination of Nonspecific Cytomegalovirus Immunoglobulin M Activities in the Enzyme-Linked Immunosorbent Assay by Using Anti-Human Immunoglobulin G

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Direct enzyme-linked immunosorbent assay methods offer several advantages in assessing past or recent exposure to cytomegalovirus (CMV) infection, but there persist many pitfalls in the use of these methods for determining specific immunoglobulin M (IgM). The efficiency of absorption of sera by IgG-coated latex beads, aggregated human IgG, or *Staphylococcus aureus*, i.e., for removing nonspecific CMV IgM activities, was evaluated in comparison with the effect of an anti-human IgG hyperimmune serum. Large routine series comprising serum samples from patients of various clinical groups and healthy individuals were examined. The CMV IgM-positive samples were at first treated with latex or aggregated IgG, but these absorptions left too many CMV IgM-positive individuals. *S. aureus* increased the nonspecific activity of some sera and, in other cases, removed or impaired specific IgM activities. The anti-IgG treatment caused the disappearance of nonspecific CMV IgM activities that had resisted the other treatments, whereas specific activities remained intact. Utilizing this method, only 1.03% of the routine series patients remained CMV IgM positive by the enzyme-linked immunosorbent assay, a figure in good agreement with a mean probability of CMV antibody acquisition of 0.33% for the population living in Belgium. On the other hand, in a series of patients who were investigated for serological response to several viruses, eight individuals displayed multiple IgM activities after anti-IgG treatment. In these cases, most IgM activities were found in patients who had IgG toward the related antigen for a long time before transient IgM was detected. This result implies that to assess a diagnosis of primary infection, it is necessary to examine serial specimens for IgG acquisition accompanying specific IgM.

Well recognized as an important human pathogen, cytomegalovirus (CMV) produces congenital infections associated with a poor prognosis, especially regarding psychomotor development (22). CMV is capable of causing serious illness in immune-compromised patients, and it is regarded as a cause of mortality in recipients of bone marrow (30), kidney (17), or cardiac (20) transplant and in markedly immunodepressed patients with hematologic or other malignancies (14, 20). Now it has become clear that primary infection rather than recurrent infection or reinfection is involved in serious or fatal illness (3).

Recognition of specific immunoglobulin M (IgM) is the method of choice for detection of primary infections. Unfortunately, IgM monitoring does not usually discriminate between specific and nonspecific IgM activities, and in the case of CMV infection, an aberrant ratio of specimens displaying some CMV IgM activity may be observed with serum samples containing IgG-binding IgM, such as rheumatoid factor of the IgM class (5) or anti-IgG IgM associated with a wide range of infectious diseases and autoimmune conditions (10). As a matter of fact, recognition of genuine anti-CMV IgM may be difficult in any individual with anti-CMV IgG either actively or passively acquired, such as infants and gamma globulin prophylaxis recipients (22, 31).

Direct enzyme-linked immunosorbent assay (ELISA) methods (33) do permit detection and evaluation of specific IgG or of IgM activity. These methods offer several advantages over complement fixation in assessing past exposure to CMV infection (21), but there persist many pitfalls in their use for determining specific IgM. To eliminate nonspecific IgM activities, several methods for removing anti-IgG IgM from sera have been proposed. These methods include binding of IgG by protein A (9), serum fractionation (23), and absorption of rheumatoid factor with IgG-coated latex beads or with heat-aggregated human IgG (26). In this work, we have evaluated the efficiency of these methods in removing nonspecific CMV IgM activities in comparison with the effect of an anti-human IgG hyperimmune serum.

MATERIALS AND METHODS

Serum samples. Serum samples were taken from routine specimens submitted to the Medical Virology Laboratory of the University of Liège from January 1982 to May 1985. The series included specimens from the following clinical groups: patients with liver disorders, unexplained temperature episodes, or long-lasting fatigue; patients suspected of CMV infection; patients from the dialysis unit of the university; and kidney or bone marrow recipients. Individuals from the last three groups provided 5 to 25 serum samples. Moreover, our investigations covered a large series of healthy subjects comprising pregnant women, mothers at delivery and their babies, and donors of bone marrow, kidney, or blood who were screened for the absence of CMV infection. Every serum sample was given the patient's number, followed by a letter from A to Y according to the serial number of the patient's samples. All the specimens were stored at −25°C. Altogether, 2,862 serum specimens provided by 1,191 individuals were examined.

ELISA: general procedure. ELISAs were carried out in

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disposable polyvinyl microplates (no. 1-220-24; Dynatech) by a technique derived from that of Voller et al. (29). CMV antigen (complement fixation reagent; Calbiochem-Behring) was diluted in 0.05 M sodium carbonate-bicarbonate buffer at pH 9.6 containing 0.02% (wt/vol) NaN_3 (coating buffer). Samples (0.05 ml) of the CMV antigen diluted 1:100 (optimal concentration; see below, under Standardization of ELISA) were distributed into the wells. After one night at 4°C, the plates were washed five times with phosphate-buffered saline (PBS) at pH 7.5 plus 0.05% (vol/vol) Tween 20 (washing buffer) and drip dried. Twofold dilutions of the patients' sera in contact buffer, i.e., PBS plus 0.05% (vol/vol) Tween 20 and 2% (vol/vol) fetal calf serum, were distributed (0.05 ml; 1:100 to 1:12,800 for IgG and 1:40 to 1:5,120 for IgM detection). After 2 h at 25°C, the plates were washed five times and dried, and 0.05-ml samples of alkaline phosphatase-coupled anti-human IgG or IgM (produced in swine; Sigma Chemical Co.) diluted 1:3,200 in contact buffer (optimal concentration; see below, under Standardization of ELISA) were distributed. The plates were kept again for 2 h at 25°C and then washed and dried. Samples (0.05 ml) of a fresh solution of 4-nitrophenylphosphate (1 mg/ml in diethanolamine buffer) were added, and the plates were kept for 1 h at 37°C. Finally, the enzyme reaction was stopped by adding 0.15 ml of 0.4 M NaOH. The specific antibody activity was monitored at 405 nm with a multichannel spectrophotometer (Titertek Multiskan; Flow Laboratories, Inc.), allowing, at each serum dilution, for the difference between the absorbance of the test wells and that of the control well, i.e., the well containing extracts from uninfected cell cultures. The specific activity of a serum was defined as the maximum dilution at which that difference was ≥ 0.1 (maximum active dilution). Specific activities of sera toward different viral antigens were measured by the same procedure when the related antigens and control antigens were used.

ELISA for detection of rheumatoid factor (anti-IgG antibodies of the IgM class). Serum specimens were tested for anti-IgM antibodies by the procedure described above, but the wells were coated with human native IgG purified from a pool of sera from healthy subjects (Cohn fraction II; Sigma) diluted in coating buffer at a concentration of 0.001 mg/ml (32). The control wells were treated in parallel with buffer only. The serum samples were diluted from 1:40 to 1:5,120, and anti-IgG IgM activities were measured and expressed by the general procedure.

Standardization of ELISA. Paired sera showing seroconversion by complement fixation toward CMV were diluted from 1:100 to 1:12,800 and were tested with multiple chessboards with a twofold dilution of CMV antigen and conjugate. Optimal dilutions of the reagents were defined as those giving, respectively, <0.1 and >0.3 absorbance differences with the negative and the positive serum at the starting dilution. Optimal dilutions were 1:100 for the antigen and 1:1,600 to 1:3,200 for the conjugate, depending on the batch. The same procedure was applied for the detection of anti-IgG IgM activity. Latex-positive adult sera and serum samples from individuals without evidence of anti-IgG IgM activity were used to define the optimal dilution of the anti-IgM conjugate. In all tests, the results obtained were routinely checked with negative and positive control sera for reproducibility, specificity, and sensitivity. In the routine series, serum samples were initially checked for their anti-CMV IgG and IgM activities without preliminary treatment (see below) at 1:100 and 1:200 or at 1:40 and 1:80, respectively (preliminary screen test). Thereafter, the sera found to be positive were titrated by the maximum active dilution system.

Serum absorption with polystyrol IgG-coated beads (latex reagent). Serum absorption with polystyrol IgG-coated beads was performed by the method described by Ziola et al. (35). After dilution of the serum sample to 1:8 in contact buffer, 0.05 ml of the solution was mixed with 0.2 ml of a homogenized suspension of latex-rheumatoid factor reagent (Calbiochem-Behring) in a small centrifuge tube. The mixture was allowed to stand for 30 min at room temperature, and after centrifugation for 15 min at 200 × *g*, the supernatant was collected for further IgM investigation by ELISA.

Serum absorption with *Staphylococcus aureus* suspension. According to Jankowski et al. (9), lyophilized *S. aureus* walls (Ig-sorb, Enzyme Center) were rehydrated and washed twice with PBS. To 0.2 ml of sediment, 0.2 ml of a 1:20 dilution of the test serum in PBS was added. The mixture was gently mixed five or six times within 45 min at room temperature. After centrifugation at 2,000 × *g*, the supernatant was collected and mixed with 0.05% (vol/vol) Tween 20 plus 2% (vol/vol) fetal calf serum for further IgM investigation by ELISA. Under these conditions, more than 92% of the total IgG serum content would be absorbed (2).

Serum absorption with aggregated IgG. Human IgG (Cohn fraction II; Sigma) was first aggregated by heating and then stored at −20°C in PBS by the method of Kurtz and Malic (13). For use, a 1:2 dilution of the aggregated IgG in contact buffer was mixed with an equal volume of serum diluted 1:20 in the same buffer. The mixture was shaken for 1 h at 37°C and then held at 4°C overnight. After centrifugation at 2,000 × *g*, the supernatant was ready for testing.

Serum treatment with anti-human IgG hyperimmune serum. Patient serum diluted 1:20 in contact buffer was added to the same volume of hyperimmune anti-human IgG serum from sheep (RF-absorbent; Calbiochem-Behring). This reagent was utilized at a dilution providing a binding capacity of 15 mg of IgG per ml as defined by the manufacturer (34). After careful mixing and 15 min of contact at room temperature or overnight at 4°C, the suspension was mixed again and directly used for IgM determination.

RESULTS

Aberrant frequency of CMV IgM activity in ELISA. A series of 527 specimens including 365 routine specimens collected from January to March 1983, 87 samples taken from mothers at delivery between February 1979 and February 1980, and 75 cord blood samples collected at the same time were initially tested for CMV IgM activity at dilution 1:40 (ELISA IgM preliminary screen test). Displaying IgM activity were 92 specimens in the first group, 49 in the second group, and none in the third group. IgM-positive specimens from the first group were tested for rheumatoid factor. Of these, 24 were positive, of which 9 became CMV IgM negative after latex absorption. Therefore, only 10% of the CMV IgM-positive individuals of this group displayed a nonspecific activity due to IgM antibodies capable of binding an IgG-coated solid phase, leaving 83 (22.7%) apparently IgM positive. However, 22 serum samples from 21 individuals with CMV IgM activities but no apparent IgM activity in the anti-IgG IgM ELISA tests (rheumatoid factor-negative sera) were examined for CMV IgM after latex absorption. Of these specimens, 13 remained CMV IgM positive, and the others became negative, presumably through removal of anti-IgG IgM that was undetectable by direct ELISA.

Assays for the elimination of nonspecific CMV IgM activi-

TABLE 1. Effect in ELISA of absorption with latex, aggregated IgG, *S. aureus* suspension, or anti-IgG serum on CMV IgM activity in a series of serum samples

Serum code	CMV IgM activity in ELISA				
	Untreated samples	After treatment with:			
		Latex	Aggregated IgG	*S. aureus*	Anti-IgG serum
20120	160	<40	<40	<40	<40
21213	640	640	640	80	320
19720	320	160	160	<40	<40
21272	320	<40	<40	<40	<40
19871	160	80	160	<40	320
21273B	1,280	640	160	160	<40
19962	40	<40	<40	80	<40
21399	320	160	80	<40	<40

ties. Eighteen sera CMV IgM positive in ELISA were absorbed with either latex or aggregated human IgG. In this series, all the activities found after treatment with latex remained also after aggregated-IgG absorption. However, two sera were fourfold less active after treatment with aggregated IgG than after latex absorption.

A series of eight rheumatoid factor-positive and CMV IgM-positive specimens provided by eight patients suspected of CMV primary infection were treated with either latex, aggregated IgG, *S. aureus*, or an anti-human IgG serum (Table 1). In this series, five sera remained positive after both latex or aggregated-IgG absorption. *S. aureus* absorption left only three CMV IgM positives. Either latex, aggregated IgG, or *S. aureus* treatment abolished the IgM activity of two sera (no. 20120 and 21272). The IgM activity of one serum (no. 19962) was unaffected by *S. aureus* treatment, but disappeared after latex or aggregated IgG absorption. In three cases (no. 19720, 19871, and 21399), *S. aureus* treatment abolished IgM activity persisting after latex or aggregated IgG absorption. Finally, *S. aureus* abolished or caused an eightfold decrease of the IgM activity of two specimens (no. 21213 and 19871), both of which revealed subsequent acquisition of anti-CMV IgG, confirming true primary infection. We observed a similar effect while investigating other specimens for IgM directed against rubella or other viruses.

The activities of six sera were abolished after treatment with anti-IgG serum (Table 1). In one case (no. 21213), CMV IgM activity persisted after treatment with anti-IgG serum, corroborating the results obtained with the other three methods. In another case (no. 19871), CMV IgM activity that was later on revealed to be specific remained after treatment with anti-IgG, latex, or aggregated IgG, but disappeared after *S. aureus* absorption (see above).

Efficiency of anti-human IgG serum in eliminating nonspecific IgM activities. To further evaluate the efficiency of the anti-human IgG serum, 40 serum samples displaying CMV IgM activity at a dilution of 1:40 (40 of 711 serum samples provided by 483 patients) were tested both with and without anti-human IgG. Only 5 serum samples remained positive after that control procedure (Table 2, group I). Moreover, the anti-IgG treatment was applied to samples from individuals in five groups (Table 2, groups II, III, IV, V, and VI). Among kidney recipients, only three of the five CMV IgM-positives presented serological evidence of primary infection. Of the three dialyzed patients, one remained IgG negative and the other two were known as CMV IgG positive before acquisition of IgM. The bone marrow recipient was CMV IgG positive before grafting. She became negative after the graft, as was the bone marrow donor. Sometime later, she was found to be IgM positive, and later on, she was found to be CMV IgG positive.

Altogether (Table 2), 9 of 14 CMV IgM-positives acquired CMV IgG. CMV IgG acquisition was not observed in any individual detected as CMV IgM negative after the control test. These results indicate that anti-IgG serum is the only tested reagent that allows detection of CMV IgM-positive specimens in keeping with other serological evidences of primary infection.

Instability of some nonspecific IgM. In routine series, and more strikingly in group III (Table 2), up to 100% of the individuals found to be CMV IgM positive in the preliminary screen test became negative in a second analysis performed after storage for 2 weeks or more at −25°C and at least two rounds of freezing and thawing. This phenomenon occurred with a high frequency in mothers at delivery who were known as CMV IgG positive. In these cases, the IgM activities first detected were low, ranging from 1:40 to 1:80. On the other hand, all their babies had CMV IgG, and none had CMV IgM. In any case, in sera from mothers, nonspecific activities were low and were associated with low optical densities, suggesting that these activities might be related to

TABLE 2. Distribution of the individuals CMV IgM positive in ELISA among patients from routine series and from various clinical groups before and after treatment with anti-IgG serum

Clinical group[a]	n[b]	No. of sera	Preliminary screen test[c]		Maximum active dilution system[d]		Maximum active dilution after treatment with anti-IgG	
			n	%	*n*	%[e]	*n*	%[f]
I	483	711	40	8.3	20	4.15	5	1.03
II	126	1,020	ND		38	30.16	3	2.38
III	87	87	49	56.32	0	0		
IV	74	75	0	0				
V	11	128	3	27.27	3	27.27	1	9.09
VI	45	476	ND		11	24.44	5	11.11

[a] I, Routine specimens; II, patients from the dialysis unit; III, women just after delivery; IV, newborns (cord bloods); V, bone marrow recipients; VI, kidney recipients.
[b] *n*, Number of patients.
[c] See Materials and Methods: Standardization of ELISA. ND, Not determined.
[d] See Materials and Methods: ELISA: general procedure.
[e] Total percentage for 72 of 826 patients, 8.72.
[f] Total percentage for 14 of 826 patients, 1.69.

low-affinity anti-IgG IgM molecules or other serum components.

Multiple IgM activities. In the presence of anti-IgG serum, most specimens exhibited IgM activity toward one antigen only. In a large routine series, including patients who were investigated for serological response to several viruses, we found only eight sera retaining IgM activity toward up to five antigens. Incidentally, four of these eight specimens were obtained from four patients who acquired anti-CMV IgG after anti-CMV IgM and should be considered as undergoing true primary CMV infection. Most of the multiple IgM activities persisting in the presence of anti-IgG serum were found in patients who had IgG toward the related antigens long before transient IgM was detected.

DISCUSSION

Of the individuals we studied, 14.5% had anti-IgG IgM antibodies detectable by ELISA. Assuming that less than 4% of these individuals have anti-IgG IgM associated with rheumatoid arthritis (6), the remaining 10% could have IgM antibodies which were produced after various infectious episodes (1) or which could contribute to the regulation of IgG antibodies (7). To prevent nonspecific IgM activities due to anti-IgG IgM, the serum specimens were at first treated with latex reagent or aggregated IgG, but these absorptions obviously left too many anti-CMV IgM-positive individuals. Moreover, serum specimens initially found to be CMV IgM positive and rheumatoid factor negative became CMV IgM negative after treatment with latex, aggregated IgG, *S. aureus*, or anti-IgG. Therefore, even the sera devoid of anti-IgG IgM detectable by ELISA should be submitted to extensive controls. As noticed by Torfason and Diderholm (28) in herpes simplex virus IgM radioimmunoassay determination, we observed sometimes that *S. aureus* increased the ELISA IgM activity of sera investigated for IgM directed against CMV or other viral antigens. This result could depend on the impairment of a competition for the viral antigen between free IgG or IgG-IgM complexes and specific IgM. Conversely, some specific IgM activities were removed or impaired after *S. aureus* treatment, and we made the same observation in 3 of 21 sera with specific rubella IgM activity. According to Kangro et al. (12) and Jankowski et al. (9), protein A treatment of sera may reduce the specific IgM activity by about twofold as a consequence of the removal of 40 to 50% of the total IgM content. *S. aureus* treatment should be discarded, as it is a possible cause of some false-negative results, as well as possibly a failure in the removal of anti-IgG3–IgM complexes or IgG-IgM complexes with a steric blocking up of the IgG heavy chains.

In an attempt to avoid fastidious absorptions, we tested solid-phase techniques, in which the first step consists in binding IgM to the wells (27, 32). These methods that imply the use of conjugates specific for each type of antigen and require one more step than direct ELISA did not prove more specific than direct ELISA performed without anti-IgG serum. It is clear that a mere inversion of one step would not eliminate the indirect binding of unspecific IgM or of IgG-IgM complexes. Indeed, Kurtz and Malic (13) also considered that when indirect methods are applied, causes of nonspecific IgM activities must be eliminated first.

Sheep anti-human IgG treatment caused the disappearance of many nonspecific IgM activities that had resisted either absorption with latex reagent or aggregated IgG or treatment with *S. aureus* and did not impair activities which were specific. We excluded the possibility that pretreatment of the serum with the reagent has a negative influence on specific IgM by the fact that all known IgM-positive samples were found IgM positive in an IgM ELISA performed in the presence of anti-IgG serum. Moreover, in three cases of documented rubella, by using direct ELISA in the presence of anti-IgG, we were able to detect specific IgM that could not be recovered after centrifugation through sucrose density gradient. The reagent is standardized by the manufacturer so that it completely neutralizes the IgG of a serum containing 15 mg/ml (see Materials and Methods), a concentration much higher than normally occurs in human sera. The procedure is short and simple. Anti-IgG serum would allow (i) precipitation or blocking of free specific IgG that could compete for the antigen with specific IgM, (ii) fixation of various types of IgG-IgM complexes, and (iii) absorption of free anti-IgG IgM on solid IgG–anti-IgG aggregates. Taking as evidence of primary infection the presence of IgM (in anti-IgG-treated serum) followed by or accompanied with IgG acquisition, we found nine cases of CMV primary infection in our series of 826 patients. If it is assumed that the series encompassed a high proportion of people at risk, in particular three primary infections in kidney recipients, the observed percentage is in good keeping with the 0.33% expected from the seroepidemiological data available for the Belgian population (21).

Various control procedures were performed to assess the nature and the specificity of the molecules giving rise to IgM as well as to multiple IgM activities. These controls included successive absorptions with anti-IgG serum and centrifugation through sucrose density gradients followed by treatment with β-mercaptoethanol (4) or with an anti-IgM serum after absorption with anti-IgG serum. However, it was never possible to conclude that these activities were due to another factor than specific IgM molecules.

In particular groups such as mothers at delivery and patients from a dialysis unit, up to 100% of positives in the preliminary screen test became negative after at least two rounds of freezing and thawing and a storage period of 2 to 8 weeks at −25°C. The reproducibility of our ELISA being well established, this labile activity could depend on either low-avidity anti-IgG IgM or labile serum components causing the conjugate to stick to the antigen-coated wells. Such phenomenon was also observed by Shillitoe (25), who suggested adsorbing sera with kaolin to overcome this nonspecific binding. In our hands, this method was unsatisfactory, but in any case, these low nonspecific activities were abolished by RF-absorbent treatment.

Generally, multiple IgM activities disappeared after anti-IgG treatment, but some persisted. Epstein-Barr virus stimulation of B lymphocytes has been put forward to explain some nonspecific IgM accompanying infectious mononucleosis (16). In our series, there were eight patients with multiple IgM activities, including two who had transient Epstein-Barr virus IgM. Four patients who had anti-CMV IgM concurrently acquired CMV IgG and displayed elevated IgG and transient IgM toward a variety of viruses. This finding suggests that specific-IgM-producing lymphocytes were nonspecifically stimulated during the CMV primary immune response (18). Alternatively, as suggested by others (8, 24), the decrease in the relative concentration of thymus-derived lymphocytes that accompanies CMV infection could induce a secondary generation of IgM-producing cells. The last four patients and similar cases of IgM activity toward a variety of viruses accompanying varicella and primary herpes (unpublished results) lead us to suggest that problems

of multiple IgM activities could also be related to various infections or other immune stimulation.

In patients with CMV or infections with other herpes viruses, transient production of specific IgM could be observed in case of reactivation (or reinfection). Indeed, the development of an immune response to the whole variety of herpesvirus antigenic components might require several infectious episodes. Serological IgM responses toward CMV early and late antigens (15) and toward capsid, envelope, and nonstructural herpes simplex virus antigen (11) have been documented, as have responses to varicella-zoster virus antigens (19).

In conclusion, at present we consider that the safest method for investigation of sera for specific CMV IgM is first to screen IgM activities and then to examine serial dilutions of positive sera both without and after treatment with anti-IgG antibodies. To assess a diagnosis of primary CMV infection, it is necessary to examine several successive serum specimens and to note the disappearance of IgM together with a significant rise in IgG. Two or more serum samples from each patient found to be CMV IgM positive by this method should be investigated for both IgM and IgG activities toward several agents to detect IgG seroconversions or possible multiple IgM activities. This rule has been successfully applied to the serological diagnosis of CMV in our laboratory and has been revealed as reliable for recognizing other primary infections.

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