Sensitivity and Specificity of Viral Immunoglobulin M Determination by Indirect Enzyme-Linked Immunosorbent Assay

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Four sources of error associated with virus-specific immunoglobulin M (IgM) determination by indirect enzyme-linked immunosorbent assay were recognized and analyzed. First, competitive inhibition due to specific IgG was demonstrated by experiments involving addition and subtraction of rubella-specific IgG. Second, the interference due to rheumatoid factors (RFs) of the IgM class (IgM-RFs) was studied thoroughly, and it appeared that the level of false positivity was more dependent on specific IgG titers than on IgM-RF titers. Third, it was found that some IgM-RFs, differing from conventional IgM-RFs in that they reacted only with isologous IgG, were responsible for further cases of false positivity. Fourth, the interference of an IgM reacting with some virus-unmasked cellular antigens was demonstrated for some infected individuals. All four interfering factors could be readily eliminated by simple premixing serum samples with a sheep anti-human gamma-chain serum. This single pretreatment was shown to eliminate false-negatives as well as false-positives in a further 2,004 sera tested for six viruses. These results also emphasize the frequency of RFs and their heterogeneity.

Demonstration of virus-specific antibodies of the immunoglobulin M (IgM) class appears to be a precious and reliable indicator of current or recent infection. The small amount of antibodies of this class relative to specific IgG calls for assays of high sensitivity, such as enzyme-linked immunosorbent assays (ELISAs). Indirect ELISA, in which the solid phase is coated with the viral antigen, has been used for its ease in demonstrating specific IgM as well as IgG and IgA. However, IgM determination is frequently hampered by the attachment of rheumatoid factor (RF) of the IgM class (IgM-RF) to specific IgG, leading to a positive reaction in the absence of specific IgM.

Various techniques for elimination of RF-related nonspecific positivities have been tried. They only partially eliminate RF: aggregated IgG (16), IgG-coated latex particles (20), and Staphylococcus aureus protein A (7) or protein A-Sepharose saturated with IgG (11, 17). IgG immunoprecipitation assays have been recommended for immunofluorescence assays by Gispel et al. (9) for rubella and by Hekker et al. (10) for cytomegalovirus (CMV) and for ELISAs by Schmitz et al. (15) and Ziegelmayr et al. (19) for CMV, herpes simplex virus (HSV), and rubella and by Cerny et al. (2) for syphilis.

IgM class capture assays also have limitations, as emphasized in the Third Workshop on New and Useful Methods in Rapid Viral Diagnosis (13). IgM-RF can compete with viral IgM for binding sites on the solid phase; IgM-RF can react directly with the labeled viral antibody; it can also complex with virus-specific IgG, which in turn binds the virus antigen. Moreover, Naot et al. (12) showed that false-positive results do occur with sera containing IgM-RF with antinuclear activities. This type of assay requires virus-specific methods or reagents to evidence the captured antigen, but some assays with labeled viral antigen proved insensitive to IgM-RF (1). Also, this method does not allow parallel determination of specific IgG or IgA, a drawback for routine purposes.

We studied here the causes of false positivity and false negativity in indirect ELISA. We show that a simple and rapid pretreatment of sera with a sheep anti-human gamma-chain serum allowed sensitive and specific detection of viral IgM in 2,004 serum samples.

MATERIALS AND METHODS

Sera. Serum samples tested for the presence of viral IgM were either from healthy individuals, for systematic serology, or from hospitalized patients, sent to the virology laboratory, Centre Hospitalier Universitaire Bicêtre. Reference sera with known specific IgG and IgM virus titers were from a commercial source (Behringwerke, Marburg, Federal Republic of Germany); for rubella IgG, a positive control serum of 256 IU was used (Technique Biologique Française, Paris, France); for IgM-RF, a calibrator of 256 IU was used (Boehringer GmbH, Mannheim, Federal Republic of Germany).

Antiserum to human gamma chain. One anti-human gamma-chain serum was selected among sera produced either in rabbits (Dako, Copenhagen, Denmark; Organon, Turnhout, Belgium), in goats (Hyland, Nivelles, Belgium), or in sheep (Behring; Organon). Selection was performed by taking into account (i) the highest neutralizing activity on sera with known IgG rubella titers, (ii) total IgG immunoprecipitation activity as measured by radial immunodiffusion, and (iii) the lowest background activity in wells coated with control antigen. The sheep antiserum from Behring was retained and used undiluted, i.e., four times the amount necessary to totally eliminate IgG from serum.

Pretreatment of sera. Immunoprecipitation of serum IgG was performed as follows. Anti-gamma-chain antiserum (200 µl) and 200 µl of a 1:50 dilution of test serum in phosphate-buffered saline–Tween–albumin buffer (described below) were mixed and agitated on a Vortex mixer. After incubation

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overnight at 4°C and vigorous reshaking, the mixture was processed for specific IgM determination and control of IgG elimination.

ELISA for IgM-RF and antibodies to viruses. Dilution of all sera was performed in 0.01 M phosphate-buffered saline (pH 7.4) containing 4% Tween 20 and 2% bovine serum albumin. Microplates were washed in the same buffer lacking albumin in a Tittertek automatic washer (Flow Laboratories, Inc.). Sodium carbonate-bicarbonate, 0.05 M, pH 9.6, was used as a coating buffer. Phosphatase-labeled rabbit antiserum to human gamma and mu chains (whole antibody) (Behring) were used in all ELISAs, except in an experiment shown in Fig. 4, for which an F(ab′)2 goat anti-human mu chain was necessary (Tago, Burlingame, Calif.). Microplates, U-shaped, were coated with either rubella, mumps, CMV, measles, varicella-zoster virus (VZV), or HSV antigens or with control antigen, i.e., uninfected cells prepared in the same way as viral antigens: BHK-21 cells for rubella, Vero cells for mumps, HeLa cells for HSV, and human embryonic fibroblasts for VZV, CMV, and measles (Enzygnost; Behring). For IgM-RF determination, U-shaped microplates (Nunc immunoplate II; Roskilde, Denmark) were coated with human IgG (Nordic, Tilburg, The Netherlands), 0.1 μg/ml, and saturated with a 4% bovine serum albumin solution. Sera were tested in duplicate; no control antigen could be used. Determination of autologous IgM-RF was performed similarly but on plates coated with a rabbit anti-human gamma-chain serum (Dako), 1:250.

Enzyme immunoassays were performed as described previously (3–5) and as follows: 100 μl of untreated or pretreated serum was added to virus- and control antigen-coated wells and incubated for 60 min at 37°C; after the washing, anti-gamma- or anti-mu-chain conjugates were distributed in corresponding wells and incubated similarly. After the washing, para-nitrophenyl phosphate substrate was distributed and incubated for 45 min; absorbancy was measured at 405 nm in a Multiskan photometer (Flow Laboratories).

For specific immunoglobulin quantitation, a Commodore computer, running homemade software, was directly connected to the photometer. For viral assays, but not necessary for IgM-RF determinations, the absorbancy of sera on control antigen was subtracted from the absorbancy on viral antigen (net absorbancy). The net absorbancy of each test serum was compared with that of control sera for determination of titer. For calibration, positive control sera were serially diluted twofold, starting at 1:100, and all dilutions were tested; a standard dose-response curve, plotting the absorbancy values against the dilutions, was then calculated by the regression analysis method. An A0.05 cutoff value of 0.200 was necessary to assess positivity, and negative sera were regularly under an A0.05 of 0.070; for rubella IgG, the threshold corresponded to 10 IU; for IgM-RF, to 4 IU; for viral IgM, to 1 U (arbitrary unit); and for other virus-specific IgGs, to a titer of 100 (dilution of test sera, 1:100). The titer of each test serum was deduced by comparison of its net absorbancy with that of the serially diluted control serum.

RESULTS

Effect of rubella-specific IgG on the sensitivity of determination of rubella IgM. The influence of the simultaneous presence of specific IgG on specific IgM titers was studied in a rubella ELISA. Levels of rubella-specific IgG and IgM were determined in 10 mixtures containing a constant concentration of specific IgM and increasing amounts of rubella-specific IgG from 0 to 2,560 IU. With rubella-specific IgM at 1 and 2 U, it was observed that the measured apparent IgM titer could be much lower than the expected titer or even negative when high quantities of specific IgG were simultaneously present (Fig. 1A and B).

Conversely, progressive IgG immunoprecipitation in a serum containing rubella-specific IgM demonstrated a rise in specific IgM titers. Serum from a patient with rubella, containing 640 IU of rubella-specific IgG and 64 U of rubella-specific IgM but devoid of IgM-RF, was pretreated with serial twofold dilutions of anti-gamma-chain serum. Results of a typical experiment are reported in Fig. 2. They clearly show that the IgM titer was minimal, 8 U, when all IgGs were present and dramatically increased up to 64 U as soon as specific IgGs were immunoprecipitated.

Effect of rubella-specific IgG and IgM-RF on the specificity of the determination of rubella IgM. It was demonstrated, by varying the amount of rubella-specific IgG and IgM-RF (Fig. 3), that reactivity in the IgM ELISA could be observed although all the mixtures were devoid of rubella-specific IgM. The degree of this false positivity in IgM was proportional to the concentrations of both rubella-specific IgG and IgM-RF. It appears clearly that in this built-up experiment a proper combination of both components is necessary to obtain false positivity. Thus, neither the combination of 20 IU of rubella-specific IgG and 128 IU of IgM-RF nor that of 40 IU of rubella-specific IgG and 32 IU of IgM-RF led to false positivity in IgM. Conversely, positivity in IgM could be observed with a rubella-specific IgG titer as low as 20 IU if the IgM-RF titer was 256 IU; similarly, when the IgM-RF titer was as low as 8 IU, RF was combined with 160 IU of rubella-specific IgG. It was also demonstrated that with more than 40 IU of rubella-specific IgG and 64 IU of IgM-RF false positivity was regularly observed. Moreover, the false positivity increased more rapidly when the concentration of
rubella-specific IgG was doubled than when the concentration of IgM-RF was doubled. To eventually obtain the maximum level of false positivity of 32 U, the concentration of IgM-RF had to be increased sixfold and that of specific IgM to be increased only fourfold. Thus, calculation of the corresponding mean slopes, tan \( \gamma \), gave 1.2 for the IgG false-positivity dose-response curves and 0.8 for the RF false-positivity dose-response curves.

Conversely, elimination of RF-induced false positivity was investigated by pretreating samples with anti-gamma-chain serum. Several sera containing no rubella-specific IgM, rubella-specific IgG, or IgM-RF and giving rise to false positivity in the rubella IgM ELISA were pretreated with various amounts of anti-gamma-chain serum. It appeared (Fig. 4A shows a typical experiment) that at a working dilution of antiserum of 1:4, no rubella-specific IgG or IgM or RF reactivity was detected. From the working dilutions of 1:8 up to 1:024, rubella-specific IgG reappeared first, IgM-RF reappeared second, and false positivity in rubella-specific IgM reappeared third.

For the highest working concentrations of antiserum, 1:1 and 1:2, unexpected positive responses were observed for IgM-RF. A similar phenomenon was demonstrated in control experiments with either a serum devoid of RF (Fig. 4B) or buffer in the place of serum (not shown). The phenomenon could be completely eliminated by the use of an F(ab')

\[ ^2 \text{anti-mu-chain conjugate (not shown). Although total neutralization of rubella-specific IgG was already obtained at a dilution of 1:4, antiserum was routinely used at a dilution of 1:1.} \]

**Effect of treatment with anti-gamma-chain serum.** To test the efficacy of the method in medical practice, titers of specific IgM and IgG to rubella virus, CMV, HSV, VZV, mumps virus, and measles virus were determined in ELISA before and after treatment. The absence of residual specific IgG after treatment was confirmed for each of 2,004 sera by the negativity of the IgG ELISA. Results of this study (Table 1) show that serum pretreatment led to 73 (38%) of the 190 initially positive IgM determinations becoming negative, i.e., leaving 117 positive, and to a further 8 determinations initially negative becoming positive. On the other hand, clinical and virological evidence of infection had been observed in 125 cases. The presence of specific IgM correlated with all 125 infections (117 + 8) and only in those, and thus there was no false positivity or false negativity.

Moreover, it appeared that IgM-RF was a significant occurrence in patients with or without viral infection directed for serological determinations: IgM-RF titers of >4 IU were found in 28% of sera with IgM to viruses versus 22% of others; titers of >32 IU were found in 5.6% versus 3.4%, respectively (not significant). The proportions of false positivity were 12% among samples with IgM-RF titers of >4 and 23% among samples with IgM-RF titers of >32.

### Table 1. Result of 2,004 determinations by indirect ELISA of IgM antibodies to six viruses by the method of anti-gamma-chain pretreatment

<table>
<thead>
<tr>
<th>Virus (no. of samples)</th>
<th>No. of sera with high RF titer/total no.*</th>
<th>Became negative</th>
<th>Unchanged, negative</th>
<th>Unchanged, positive</th>
<th>Became positive</th>
<th>Evidence of virus infection( ^\text{a} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMV (715)</td>
<td>12/19</td>
<td>143/671</td>
<td>6/22</td>
<td>6/22</td>
<td>3/3</td>
<td>25</td>
</tr>
<tr>
<td>Measles (102)</td>
<td>3/3</td>
<td>13/94</td>
<td>1/5</td>
<td>1/5</td>
<td>0/0</td>
<td>5</td>
</tr>
<tr>
<td>Mumps (173)</td>
<td>6/11</td>
<td>22/134</td>
<td>2/25</td>
<td>2/25</td>
<td>0/3</td>
<td>28</td>
</tr>
<tr>
<td>HSV (390)</td>
<td>9/10</td>
<td>77/373</td>
<td>3/7</td>
<td>3/7</td>
<td>0/0</td>
<td>7</td>
</tr>
<tr>
<td>VZV (145)</td>
<td>0/1</td>
<td>19/132</td>
<td>5/12</td>
<td>5/12</td>
<td>0/0</td>
<td>12</td>
</tr>
<tr>
<td>Rubella (479)</td>
<td>18/29</td>
<td>65/402</td>
<td>14/46</td>
<td>14/46</td>
<td>0/2</td>
<td>48</td>
</tr>
<tr>
<td>Total (2,004)</td>
<td>48/73</td>
<td>339/1,739</td>
<td>31/117</td>
<td>31/117</td>
<td>3/8</td>
<td>125</td>
</tr>
</tbody>
</table>

* RF titer of >4 IU. Categories reflect results after pretreatment.

\( ^\text{a} \) Based on positivity of virus culture and clinical evidence of infection, for each virus (top to bottom): congenital or mononucleosis; rash or encephalitis; parotitis or meningitis; vesicles; vesicular rash; congenital, postvaccinal, or rash.
The 25 sera that became negative and did not contain a significant amount of IgM-RF, i.e., titers of <4 IU, were analyzed further. A determination of the patient’s IgM directed to his own IgG revealed such autoantibodies in 21 samples. These autoantibodies were thus different from IgM-RF, defined as reacting with a pool of human IgG, such as that used in the standard IgM-RF ELISA as well as in the Singer-Plotz latex agglutination test. The four remaining sera gave rise to IgM false positives with CMV and HSV antigens, grown in human embryonic fibroblasts and Vero cells, respectively.

Negativity was obtained as well by pretreatment with sheep serum devoid of anti-IgG activity. These four sera were found to agglutinate sheep erythrocytes but not Formalin-treated horse erythrocytes and were negative for antinuclear IgG, IgA, and IgM antibodies.

**DISCUSSION**

These findings clearly indicate that four causes of error may interfere with virus-specific IgM assays by indirect ELISA: (i) virus-specific IgG-IgM competition, (ii and iii) complexes of virus-specific IgG with IgM-RF (ii) or with autologous IgM-RF (iii), and (iv) IgM to intracellular components unmasked by viruses. It was found that pretreatment of sera with a sheep anti-human gamma-chain serum eliminated all of them through either its anti-IgG activity or its specificity.

Competition between IgG and IgM was demonstrated by progressive addition, as well as by progressive subtraction, of specific IgG relative to specific IgM. Low levels of specific IgM may even become no longer detectable by the assay when specific IgGs are concomitantly present, as in Fig. 2. In such a situation, it is difficult to distinguish between antigen limitation and the different accessibility, diffusibility, avidity, and affinity of the various classes of specific antibodies.

The role of IgM-RF in false positivity in specific IgM determination by ELISA was confirmed in this study (8, 14, 18). However, we observed that the level of specific IgG had more influence than the level of RF on the resulting false positivity. As measured by the slopes of the false-positive results (Fig. 3), the affinity of RF for complexed IgG was 1.5-fold weaker than that of IgG for the antigen. Thus, in the presence of low titers of specific IgG, the influence of RF was not perceptible. This explains why 339 of the 2,004 sera with an IgM-RF titer of >4 IU did not give rise to false positivity. Vejtorp (18) had determined that false-positive results were produced with IgM-RF titers of >3.5 IU/ml if rubella IgG titers were >30 IU/ml, but the cutoff was an A405 of 0.040; in our assay an IgM-RF titer of 32 IU was necessary for a cutoff of 0.200. The prevalence of IgM-RF above 4 IU in our series was not significantly different in virus-infected and noninfected patients; however, it was 5.6 versus 3.4%, respectively, for titers of >32 IU. Salonen et al. (14) observed a higher prevalence of IgM-RF in rubella and influenza patients than in controls or patients with six other viral infections.

A number of false-positive results could not be related to the presence of IgM-RF since RF titers, i.e., their reactivity on a pool of aggregated human IgG, were insignificant. However, we found in these sera some IgM with specificity restricted to autologous complexed IgG and thus different from the classic IgM-RF. Such cases of strictly autologous virus IgM-RF were infrequent, 21 among the 1,800 tested (ca. 1%).

Lastly, a few false-positive reactions, also occurring in individuals negative for viral isolation and for clinical symptoms, were attributable to some IgM which, although not virus specific, still reacted more strongly with virus-infected cells than with control antigen. Such IgMs were not antinuclear antibodies, and adsorption and agglutination studies showed that they were not of the Forssman or infectious mononucleosis heterophile type. Thus, only 4 of 1,800 individuals (0.2%) had such an autoantibody, a natural IgM directed against a ubiquitous antigen hidden in human and simian cultured cells, where it is unmasked when they are infected by a virus, and also unexpectedly present in sheep serum. It is relevant to notice that Cunliffe et al. (6) reported that some murine IgM reacted with isologous murine bromelain-treated erythrocytes.

Further work will be necessary to explain why sera, either with autologous IgM-RF or with IgM to virus-unmasked antigen, should become negative following pretreatment. However, it happened by chance that such antibodies recognized similar antigens in sheep serum (not necessarily on the IgG) and were thus removed following pretreatment.

Two precautions must be taken when selecting an anti-IgG
serum for the pretreatment described here. This serum must be devoid of antiviral activity lest it block antigenic sites on the solid phase and lower assay sensitivity. Second, the working dilution of anti-gamma-chain serum must be sufficient to neutralize physiological levels of IgG: here we used a fourfold excess. Consequently, the treated serum contains the immunoprecipitate and some anti-IgG in excess; neither was found to interfere in any way with the viral ELISA, and there is no theoretical reason why they should. In the IgM-RF assay, the unexpected positivity observed in Fig. 4, when the anti-IgG serum was used pure, may be explained by the excess of antisera which readily bound the plastic-adsorbed human IgG and also captured the anti-mu-chain rabbit conjugate, this probably because of a heterologous human-rabbit activity. Indeed, simply using an F(ab')2 rabbit conjugate made the reaction return to normal, i.e., negative. Anyway, pretreatment is pointless in IgM-RF assays.

More generally, this simple and efficient method could be used as an alternative to ultrafiltration or centrifugation for serum fractionation whenever a specific activity is to be demonstrated in the IgM or IgA class independently of IgG for neutralization, hemagglutination inhibition, passive hemagglutination, or complement fixation tests.

LITERATURE CITED