JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1984, p. 972–976
0095-1137/84/110972-05$02.00/0

Vol. 20, No. 5

Comparison of Nonspecific Reactivity in Indirect and Reverse Immunoassays for Measles and Mumps Immunoglobulin M Antibodies

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Received 3 April 1984/Accepted 7 August 1984

Serum specimens collected from patients convalescing from acute measles or mumps infections, other viral infections, or rheumatoid arthritis and from blood donors were tested in indirect and reverse assays for measles and mumps immunoglobulin M (IgM) antibodies. All the samples from patients convalescing from acute mumps and measles infections gave positive IgM results in both tests. However, 6% of sera from patients recovering from other viral infections, 68.4% of sera from patients with rheumatoid arthritis, and 5.6% of sera from normal blood donors gave false-positive results by the indirect measles IgM enzyme immunoassay (EIA). By the indirect mumps IgM EIA, 9% of sera from other viral infections, 70.1% of sera from patients with rheumatoid arthritis, and 5.6% of sera from normal blood donors gave false-positive reactions. The reverse test system for measles IgM gave false-positive results in 1.5% of sera from the group with other viral infections, and the reverse mumps EIA gave false-positive results in 0.9% of the patients. Other sera groups did not react in either measles or mumps reverse IgM assays. The results indicated that although nonspecific reactions are frequent in indirect IgM tests for viral antibodies, such reactions are rarely encountered when reverse IgM EIA tests are employed.

The presence of specific immunoglobulin M (IgM) class antibodies is considered to be indicative of ongoing or recent viral infections (22). The detection of specific IgM antibodies can therefore be a useful tool in the early and rapid diagnosis of infections. When virus isolation or antigen detection is difficult, a simple, rapid IgM test with high specificity and sensitivity is required.

Both radioimmunoassay and enzyme immunoassay (EIA) methods have been widely applied for demonstration of viral IgM antibodies (1, 7, 9, 11, 17, 19, 21, 28, 29). Although these types of tests are suitable for routine serodiagnosis, the presence of rheumatoid factor (RF) may give misleading results (12, 21, 26, 28, 31). This interference is based on the IgM class character of some RF, which has antibody activity against the Fc portion of the IgG molecule (10). RF can frequently be detected in serum specimens from patients with rheumatoid arthritis (RA) and occasionally in small quantities in sera from normal individuals (30, 31). Its production may also be activated during infectious diseases (2), as in rubella (21), Epstein-Barr virus (18), mumps (12), and measles (26) infections.

This report describes a comparison of nonspecific binding reactions in indirect (26) and reverse (8) assay systems for measles and mumps IgM antibodies. The results suggest that most of the nonspecific results in the indirect IgM assays are due to the presence of RF in serum samples and, furthermore, that the reverse-type test largely avoids the interference of RF.

MATERIALS AND METHODS

Antigens. Monolayers of Vero cells were infected with a wild-type strain of measles or mumps virus at an input multiplicity of 0.001 PFU per cell (27). Infected cells were harvested when the cytopathic effect reached 100% and were washed three times in phosphate-buffered saline (PBS) (pH 7.4). Suspended cells (20% [vol/vol]) in PBS were homogenized (R Omnimixer; Ivan Sorvall, Inc., Norwalk, Conn.), and the particulate material was pelleted in an ultracentrifuge (model L3-50; Beckman Instruments, Fullerton, Calif.) with a refrigerated SW 27 rotor at 22,000 rpm for 1 h. The pellet was suspended in PBS, and the antigens were stored at 4°C.

Control antigens were prepared in the same manner as the virus antigens from uninfected Vero cells. The protein concentrations were measured by the method of Lowry et al. (14), with bovine serum albumin powder fraction V obtained from bovine plasma (Armour Pharmaceutical Co. Ltd., Eastbourne, England) used as the protein standard.

Solid-phase test equipment. Sets of nine polystyrene cuvettes (Labsystems Oy, Helsinki, Finland) were used as the solid phase. The intensity of the endpoint color was measured vertically (24) in an FP-9 Analyzer (Labsystems Oy). The results were computed with a programmable Hewlett-Packard model 9815 S calculator connected to the FP-9 Analyzer.

Antisera. Rabbits (weight, ca. 3.5 kg) were immunized intradermally with purified wild-type measles virus, isolated from a patient with measles (27), or purified mumps virus of the Enders strain, obtained from K. Penttinen (Department of Virology, University of Helsinki, Helsinki, Finland). Both viruses were purified by the method of Vainiopää et al. (27).

The animals were immunized four times at 3-week intervals with 200 μg of virus suspension mixed with Freund incomplete adjuvant. A booster dose of 100 μg was given intravenously 4 weeks after the fourth intracutaneous dose. Five days after the booster dose, the rabbits were exsanguinated, and the blood was collected.

Serum specimens. A total of 486 serum samples were tested for measles IgM antibodies by both types of tests. The serum panel consisted of paired sera from 15 patients with measles, paired sera from 119 patients convalescing from other viral infections, 57 single sera from patients with RA, and 161 single sera from blood donors. The diagnoses of the infections were based on at least fourfold increases in the complement fixation titers of serum pairs. The first specimens were obtained at or immediately after the onset of the

disease symptoms, and the second specimen was obtained from 7 to 14 days afterward.

Mumps IgM antibodies were assayed in 475 serum samples. These consisted of 98 paired sera from mumps patients, 213 sera of patients recovering from other viral infections, 57 single sera from patients with RA, and 107 single sera from blood donors.

All the sera from patients with viral infections and RA were obtained from the serum collection of the Department of Virology, University of Turku. Blood donor sera were obtained from the Finnish Red Cross Volunteer Blood Donor Center, Turku, Finland (31). All the sera were stored at −20°C until tested. A reference measles IgM-positive pool was prepared from 18 measles IgM-positive sera, and a reference measles IgM-negative pool was prepared from 18 measles IgM-negative sera. A positive mumps IgM control pool was prepared from 20 mumps IgM-positive sera, and a negative pool was prepared from 15 mumps IgM-negative sera. The seronegative pools were selected from complement fixation-negative normal sera and the IgM-positive pools from sera of patients with acute measles or mumps. The seropositivity and seronegativity of these pools for measles or mumps IgM antibodies were confirmed by the indirect EIA tests.

EIA for RF. RF levels were determined in a solid-phase enzyme immunoassay for the IgM-class RF as described previously (32).

Absorption of sera with latex particles. IgG-coated latex particles (Behringwerke AG, Marburg, Federal Republic of Germany) were used to remove RF from sera as described previously (26).

EIA procedures. (i) Indirect measles and mumps IgM EIAs. The indirect measles and mumps IgM EIAs were developed from an EIA protocol published previously (26). Measles and mumps antigens were passively adsorbed onto polystyrene cuvettes. Both virus (I) and control (C) Vero antigens were used at a protein concentration of 50 μg/ml in PBS. One block of nine cuvettes was used for the assay of one serum sample. Three cuvettes in each block were sensitized with 100 μl of viral antigen, and the other six cuvettes were sensitized with control antigen. After overnight incubation at room temperature, the cuvettes were washed once with PBS and saturated with diluent. The diluent consisted of 20% heat-inactivated pig serum–2% Tween 20 (Koch-Light Laboratories Ltd., Colnbrook, Bucks, England)–0.1 mM thimerosal (Thiomersal; BDH Chemicals Ltd., Poole, England) in 0.1 M Tris-hydrochloride buffer, pH 8.0 (PTT).

Triplicate samples, diluted 1:200 in PTT at a volume of 200 μl, were tested on the virus and control antigens. The remaining three cuvettes coated with control antigen were used as diluent controls. The samples were incubated for 1 h at 37°C, after which time the cuvettes were washed twice with tap water. IgM antibodies were demonstrated with horseradish peroxidase-conjugated swine antibodies against human IgM. This was diluted 1:2,000 in PTT and added at a volume of 200 μl. Cuvettes were incubated as indicated above and rewashed twice with tap water. The substrate for the enzyme reaction consisted of *o*-phenylenediamine (3 mg/ml; Koch-Light Laboratories) plus 10 μl of 30% hydrogen peroxide in 15 ml of 0.1 M citrate disodium-hydrogen phosphate buffer (pH 5.5). A volume of 500 μl of the substrate was added to each cuvette, and the enzyme reaction was carried out for 1 h in reduced light. The enzyme reaction was terminated with 1 N HCl, and the color intensity was measured at 492 nm in an FP-9 Analyzer. The results were calculated by subtracting the mean optical density (OD) value of the C antigen cuvettes from the mean OD value of the I antigen cuvettes. The I − C value was the basis for the calculations of the final results. Results were expressed as a ratio between the sample I − C and the negative control pool I − C values. Specimens with an I − C value higher than three times the I − C value of the IgM-negative pool were considered positive.

(ii) Reverse EIA tests for measles and mumps IgM antibodies. Anti-human IgM serum (lot no. D-732, 1 Dec 82, FM 97; Orion Diagnostica, Mankkaa, Finland) was diluted 1/1,000 in PBS, and 200 μl of this dilution was used for coating each cuvette. After overnight incubation at room temperature, the fluid was removed, and the cuvettes were washed once with 500 μl of PBS. These anti-IgM-coated cuvettes were saturated with a solution consisting of 0.5% bovine serum albumin–0.5% Tween 20 in PBS for 30 min at 37°C. In measles IgM tests, serum samples were diluted 1/200 in 0.1 M Tris-hydrochloride buffer (pH 8.0) supplemented with 3% heat-inactivated pig serum–1% Tween 20–0.1 mM thimerosal. This buffer was used as the diluent throughout, and the volume of reagents was 200 μl per cuvette in every step of the immunoassay. After serum incubation for 1 h at 37°C, cuvettes were washed twice with tap water, and control Vero and measles Vero antigens diluted to 5 μg/ml were added. The cuvettes were incubated for 1 h at 37°C and rewashed. Rabbit serum to purified measles virus diluted 1/10,000 was pipetted into the cuvettes and incubated as described above. The cuvettes were rewashed, and horseradish peroxidase-conjugated swine antibodies against rabbit IgG (lot no. D-901, 1 May 82, GE 38; Orion Diagnostica) diluted 1/3,000 were added. After incubation for 1 h at 37°C, the conjugate was washed away as described earlier. The substrate reaction was carried out as described above (26).

The reverse EIA test for mumps IgM antibodies was basically the same as the reverse assay for measles IgM antibodies, except for the following minor differences. The diluent used throughout the mumps IgM immunoassay consisted of 20% fetal bovine serum (GIBCO Europe Ltd., Middlesex, England)–2% Tween 20–0.1 mM thimerosal in PBS. Serum samples were incubated on anti-IgM immunoglobulins for 2 h at 37°C, and the antigen dilutions were incubated overnight at 4°C. Rabbit serum against purified mumps virus was used at a dilution of 1/1,000.

The I − C differences were calculated as described for the indirect tests. The final results were expressed as enzyme immunoassay units (EIU), which were calculated in a linear I − C scale, with 0 EIU used for the diluent control and 100 EIU used for the seropositive control pool. The cut-off value

TABLE 1. Effect of Tween 20 detergent concentrations on the specificity in the reverse test for measles IgM antibody

Tween 20 concn (% [vol/vol])	OD_{492} for[a]:		
	Blank value	IgM serum pool with the following results:	
		Negative	Positive
0	0.230	0.510	1.430
0.03	0.200	0.470	1.370
0.3	0.150	0.270	1.300
1	0.010	0.060	1.040
3	0.020	0.130	0.630
10	0.000	0.020	0.510
30	0.000	0.025	0.690

[a] Results are expressed as the difference of OD at 492 nm (OD_{492}) values obtained with virus and control antigens (I – C).

TABLE 2. Occurrence of IgM antibodies in measles or mumps and other viral infections, RA, and normal material tested with indirect and reverse measles IgM and with indirect and reverse mumps IgM EIAs

Sera from patients with:	No. of patients per total no. tested with IgM antibodies obtained by the following indirect EIAs:				No. of patients per total no. tested with IgM antibodies obtained by the reverse EIAs
	Antibody tests	No absorption with latex-IgG	One absorption with latex-IgG	Three absorptions with latex-IgG	
Measles infections	Measles	30/30	30/30		30/30
Mumps infections	Mumps	98/98	98/98		98/98
Other viral or mycoplasma infections	Measles	15/238	10/15	0/15	3/238
	Mumps	19/213	8/19	0/8	2/213
RA	Measles	39/57	15/39	2/8	0/57
	Mumps	40/57	15/40	2/8	0/57
Blood donors	Measles	9/161	0/9		0/161
	Mumps	6/107	0/6		0/107

for positivity was set to be three times that of the seronegative pool I – C value, which in most cases was about 10 EIU.

RESULTS

Effect of certain technical factors on test results. During the development of the reverse measles IgM test, different concentrations of Tween 20 detergent were employed. It was found that a relatively high content of Tween 20 was needed to reduce the background to an acceptable level and to retain good specificity in the test (Table 1). The final diluent used for each phase of the reverse measles IgM immunoassay consisted of 0.1 M Tris-hydrochloride supplemented with 3% heat-inactivated pig serum–1% Tween 20–0.1 mM thimerosal. The most satisfactory diluent for mumps reverse assay consisted of PBS with 20% heat-inactivated fetal calf serum–2% Tween 20–0.1 mM thimerosal. Only a few of several batches of fetal calf serum tested could be used in the mumps reverse IgM assays because most of them were found to give high background OD values.

Individual batches of antigens produced during 1 year were tested with IgM-positive and -negative serum control pools diluted 1/200 and were found to have very similar antibody binding capacities, with less than 10% variation in OD values. Antigens were used at a dilution of 50 μg/ml in both of the direct assays, whereas in the reverse assays, the concentration of 5 μg/ml was found to be optimal. The average day-to-day coefficient of variation was 7%, and the within-assay coefficient of variation was 6.4% for both of the reverse assays. Variations in both of the indirect IgM assays were at the same levels as have been observed previously (26).

Determinations of measles and mumps virus IgM. All the 30 sera from measles patients and 98 sera from mumps patients were positive for homologous IgM antibody in both assay systems (Table 2). Both the acute- and convalescent-phase specimens were positive for each patient.

False-positive reactions in sera from patients convalescing from other viral infections and in sera from patients with RA. Approximately 6% (15 of 238) of sera from patients with mycoplasma infections or virus infections other than measles were positive in the indirect measles IgM assay, but only 1.3% (3 of 238) were positive in the reverse assay. Among RF sera and blood donor sera, 39 of 57 (68.4%) and 9 of 161 (5.6%), respectively, gave false-positive results when tested in the indirect assay, but none of the specimens in these two groups were positive in the reverse test (Table 2).

Almost 9% (19 of 213) of sera from patients convalescing from mycoplasma infections or virus infections other than mumps were positive in the indirect mumps IgM assay, but in the reverse test, only 0.9% (2 of 213) were positive. By the indirect assay, 40 false-positive results were detected in sera from patients with RA (70.1%) and in 6 of 107 (5.6%) normal sera. None of the sera in these two groups were positive in the reverse test (Table 2).

Nonspecific reactions in indirect assays were most frequent in serum samples from influenza A and B and *Mycoplasma pneumoniae* infections (4 of 47 in measles and 9 of 39 in mumps assays). Only one of these serum samples reacted in the reverse measles test, and one reacted in the reverse mumps test. Serum specimens (2 of 16) from patients recovering from a respiratory syncytial virus infection reacted in indirect IgM assays for both viruses, but only one was positive in the reverse test for mumps-specific IgM. Sera from rubella patients (2 of 18) had nonspecific reactions in both measles and mumps indirect IgM tests. These two sera also gave positive reactions in the reverse measles EIA test. Three varicella-zoster-specific sera which were positive in indirect IgM tests were negative in reverse IgM tests. Sera (two of seven) from parainfluenza 3-infected patients reacted positively in mumps IgM indirect assay, but not in the reverse test.

RF levels in specimens. The RF levels were measured in the sera showing false-positive reactions in the indirect tests, in the negative serum samples in the group of other viral or *Mycoplasma pneumoniae* infections, and in blood donor sera. Results were read from a standard curve. Negative serum samples from other infections gave a mean value of 14.4 ± 4.7 IU/ml. This differed significantly from the RF value of normal blood donors (8.8 ± 5) and from the RF values of false-positive samples from patients recovering from other viral infections (47 ± 33) and RA sera (62 ± 45) (the Mann-Whitney U-test; $P < 0.001$ in both comparisons).

Latex absorption was used to remove the false-positive reactivity in the specimens. Virus-specific IgM was assayed before and after latex absorption (Table 2). The false-positive sera behaved similarly with sequential latex absorptions as shown previously in measles indirect assays (26).

The serum samples which needed several latex absorptions to remove nonspecific reactivity were two rubella, one *Mycoplasma pneumoniae*, one RSV, and one influenza B. These had the highest RF levels and were also positive in the reverse assays.

RF standards of 0, 14.3, 28.6, 64.5, 86, and 143.3 IU/ml were assayed in both types of mumps IgM assays. In indirect tests, the 28.6-IU/ml standard gave the first positive result, and I – C values of RF standards increased linearly. The highest RF standard had an I – C value about the same as

that of the mean of the mumps IgM-positive pool. In reverse assays, all the standards reacted below the cut-off level for positivity. Similarly, RA sera gave a mean of 8.7 ± 2.3 EIU in the reverse assay. Although these results suggested that the IgM RF was directly related to false-positive reactivity in the tests, one herpes simplex-positive serum sample with reactivity in the indirect measles IgM assay, but with a normal RF level (4.6 IU/ml), was from a patient with a known history of systemic lupus erythematosus.

DISCUSSION

The demonstration of virus-specific IgM by indirect EIA with one serum dilution can be a valuable tool in the diagnosis of recent virus infections. In the case of mumps, diagnosis has usually been based on symptoms, but other viral diseases can mimic the clinical picture of mumps (5, 13). Furthermore, clinical identification of mumps infection can be unreliable, for example, in cases of meningitis or encephalitis without evidence of parotitis.

Both indirect and reverse tests gave positive results in homologous tests on sera which were known to be obtained from patients with acute measles or mumps infections. Indirect IgM tests for both the viruses gave false-positive results with almost the same frequency. The results suggested (Table 2) that the RF was common in patients with acute viral or mycoplasmal infections (2, 12, 18, 20, 21, 28). This was especially noticed in patients with influenza and *Mycoplasma pneumoniae* infections.

RF was measured in IgM false-positive and -negative sera from patients with mycoplasmal or viral infections other than measles or mumps. The level of RF was significantly higher in false-positive sera than in blood donor samples and in negative sera from patients with other infections. Thus, false-positive reactivity would appear to be due at least partially to the RF. This is also supported by the fact that it could be removed by absorption with IgG. RF may also have caused the false-positivity in sera from patients with RA. These types of antibodies may be quite common in low titers in chronic diseases of autoimmune nature, as was the case in 68% of our patients with RA. False-positive reactivity may also be due to reactivity with cellular components (3, 15, 25). Therefore, the inclusion of control antigens in any diagnostic IgM test is necessary because of auto- and alloreactivity present in acute viral infections and in autoimmune diseases.

Among the seven sera from patients with parainfluenza 3 infections, the indirect mumps EIA gave two positive IgM reactions. Parainfluenza and mumps viruses are antigenically related myxoviruses, and heterotypic IgG antibody responses are frequent after mumps or parainfluenza infections (4–6, 13, 16). However, the reactivity was abolished by repeated latex absorptions, and it did not occur in the mumps reverse assay. Therefore, the reactivity present in these serum samples was probably due to RF rather than antigenic relationship between the viruses.

The indirect measles IgM assay detected false-positive sera among serum samples from patients with rubella. These were rendered negative by repeated latex absorptions. These sera also showed positive IgM antibody activity against measles in the reverse test. It has been described previously (12) that specific positive rubella IgM sera may show nonspecific IgM activity against measles virus before absorption.

Indirect tests were performed during 1 day. The tests were useful for diagnosis of measles and mumps IgM, but the need for latex absorptions complicates the performance of the tests. The removal of RF may be incomplete (23), or several subsequent absorptions may be required (26). In the present study, the absorption procedures did not affect the specific mumps or measles IgM antibody levels.

The reverse assay (8, 19) diminished the number of false-positive sera by minimizing the effect of the RF. False-positive reactivity may occur even in this type of test. Anti-IgM attached onto the solid phase may bind RF. The subsequent binding of specific IgG or anti-IgG conjugates can cause false-positive reactions (8). However, reverse-type IgM assays offer reliable and sensitive methods for detection of virus-specific IgM.

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