Simplified Chromatographic Separation of Immunoglobulin M from G and Its Application to *Toxoplasma* Indirect Immunofluorescence

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The indirect immunofluorescent (IIF) antibody technique for the detection of *Toxoplasma gondii* immunoglobulin M (IgM) often gives false negative results, probably due to the competition between IgG and IgM. We therefore adapted a gel filtration procedure for the separation of IgG and IgM to a routine diagnostic test capable of handling at least 10 sera per day and requiring only 50 μ l of serum. The results from 108 sera having positive complement fixation titers for *Toxoplasma* showed that 17 were IgM positive when the whole serum was tested by IIF compared with 55 positive when the IgM fraction was used. Sera with anti-deoxyribonucleic acid titers do not give false positive results after fractionation, and the removal of IgG eliminates false positive results due to rheumatoid factor. A prospective study showed that *Toxoplasma* IgM may persist up to 9 months.

The indirect immunofluorescence (IIF) test for the demonstration of specific immunoglobulin M (IgM) antibodies (14) is simple to perform and widely used. However, the serum of patients suffering from acute or early convalescent toxoplasmosis often contains specific IgG to toxoplasmosis, which can rapidly saturate the binding sites on the *Toxoplasma* antigen and thus inhibit the binding of the specific IgM macroglobulins (4, 5, 7). Thus, the most reliable assay for specific *Toxoplasma* IgM in patient serum requires the separation of IgM from IgG. Sucrose density gradient centrifugation (2, 15) and column chromatography (3, 8, 13) are the most common methods used to separate the IgM fraction from the whole serum. These two techniques limit the number of serum that can be handled, requiring more time and expensive equipment than the procedure we have developed.

In this report a gel filtration procedure using Bio-Gel A-5m for the separation of IgM and IgG has been adapted to a routine diagnostic test of *Toxoplasma gondii*-specific IgM.

MATERIALS AND METHODS

Test sera. A total of 108 serum samples were obtained from patients having clinical symptoms of toxoplasmosis and possessing complement-fixing (CF) antibodies >1:30 and IIF total antibodies >1:256 against *T. gondii* antigen. Lyophilized sera from 11 systemic lupus erythematosus patients were kindly supplied by K. Schopfer. A total of 100 sera from healthy blood donors, with ages ranging from 20 to 67

years, were obtained as negative controls from the blood transfusion service of the St. Gallen Cantonal Hospital.

Fractionation of sera. Bio-Gel A-5m (200 to 400 mesh) (BioRad Laboratories GmbH, Munich) equilibrated with 0.05 M tris(hydroxymethyl)amino-methane-hydrochloride, 0.15 NaCl, and 0.03% NaN₃, pH 7.2 (0.45- μ m membrane filtered [Millipore Corp.]) was poured into the barrel of a 5-ml polypropylene concentric disposable syringe (Becton, Dickinson AG, Basle, Switzerland). A column (1.1 by 3.5 cm) was formed over a nylon net in the bottom of the syringe; drops were collected from a 2-cm piece of polypropylene tubing (1.0-mm inner diameter) inserted into the syringe outlet. When the syringe is filled with buffer, a flow rate of 8 to 12 drops (1 drop = 33 μ l) per min is obtained. A flow rate giving near optimal resolution in this column 4 to 6 drops per min, which can be achieved by applying an adjustable screw clamp on the outlet tubing. The void volume is about 1.2 ml.

A 100- μ l amount of serum was thoroughly mixed with 5 μ l of Dextran blue 2000 (Pharmacia Fine Chemicals, Uppsala, Sweden) (50 mg/ml), of which 50 μ l was applied to the top of the gel bed under the supernatant buffer. The void volume of 1.2 was collected and discarded, and the subsequent 10 drops were pooled (Fig. 1) as the Dextran blue began to elute and served as the IgM fraction. The column was rinsed with 4 ml of buffer before the next sample was applied. The fractionation of one sample thus requires about 20 min.

The columns were run at room temperature. They can be used normally for 40 to 50 tests. Uneven migration of the Dextran blue is the best measure of a contaminated or otherwise malfunctioning column.

Rapid qualitative analysis of immunoglobulin separation. To quickly check that the separation of

FIG. 1. Separation of IgM from IgA and IgG on the mini-column. Pooled normal blood donor serum (100 μ l) was chromatographed as described in the text. A void volume (1.2 ml) was discarded, and subsequently two-drop (60 to 70 μ l) fractions were collected, and IgG, IgM, and IgA were quantitated. The shaded area represents the 10 drops following the first drop containing Dextran blue normally used as the IgM fraction. $\circ$, IgM; $\square$, IgG; $\triangle$, IgA.

IgM from IgG was obtained in each column fractionation, a fast qualitative immunodiffusion test was devised. A longitudinal slit was cut with a sharp scalpel on a microscope slide of 1% Noble agar. Wells (2.5-mm diameter, about 5 to 10 on each side of the slit) were cut and filled with 10 μ l of the fractions to be tested. On duplicate slides, 10 μ l of either anti-human IgM or anti-human IgG was pipetted into the slits. The slides were incubated at 37°C in a moist chamber for 3 h. Precipitation lines for the presence of IgG can be seen within 30 min, whereas those of IgM require 2 to 3 h.

Toxoplasma IIF. The lyophilized *T. gondii* antigen was resuspended in 5 ml of distilled water, and 25 μ l of the resuspended antigen was dropped into each well of IF-40 plates (11). Wetting the IR-40 plate with distilled water helped to spread the antigen evenly in the wells. The plates were dried in air at room temperature, fixed for 10 min in cold absolute methyl alcohol, dried, sealed singly in plastic bags, and stored at -20°C until ready for use.

Serial dilutions of the whole serum and of the IgM fraction after separation through the Bio-Gel column were made in phosphate-buffered saline (PBS). Each dilution (75 μ l) was placed into the appropriate well of the plate, incubated for 45 min at 37°C in a moist chamber, and then washed twice in PBS for 10 to 15 min. Thereafter the plate was blotted on a flat surface with the wells facing downwards. Fluorescein isothiocyanate-labeled anti-human total immunoglobulin or IgM (50 μ l) was placed in each well, and the plate was further incubated for 30 min at 37°C and finally washed with PBS as previously described, followed by

a quick wash in distilled water. Naphthalene-black was incorporated at a concentration of 1 mg/ml in the PBS diluent for the conjugate.

A Wild-Heerbrugg incident light microscope equipped with a xenon lamp, exciter and barrier filters, and $\times 40$ objective was used. Fluorescence was scored as - to +++.

Other techniques. IgA, IgM, and IgG were quantitated by rocket immunoelectrophoresis. Antinuclear antibodies were titrated on cryostat sections of rat liver. Rheumafactor was quantitated by latex agglutination (Behringwerke AG, Marburg, Germany).

Reagents. The following reagents were used: PBS (Dulbecco A, pH 7.4) *T. gondii* antigen, killed and lyophilized, batch no. 0638,8976 (F. Hoffman-La-Roche, Inc., Basle, Switzerland). Fluorescein isothiocyanate conjugated goat antihuman IgM (μ -chain specific, batch no. 2211A001AB) was used at a dilution of 1:20 (Hyland Division of Travenol Laboratories, Inc., Costa Mesa, Calif.). Fluorescein isothiocyanate conjugated antihuman total immunoglobulin and IgG were also obtained from Hyland.

RESULTS

Gel filtration of serum. The resolution of IgM from IgG and IgA by the short Bio-Gel A-5m column is shown in Fig. 1. As little as 50 μ l of serum can be used (see above), and equally good resolution is obtained with as much as 150 μ l of serum. The IgM began to elute with the Dextran blue. The IgM fraction, which is routinely collected as 10 drops together, was separated by 10 to 12 drops from IgG and IgA. The recovery of IgM in the peak fractions (0.3 ml) was about 40% of the original serum sample and, hence, about eightfold diluted from the original serum. We normally use this IgM fraction as a 1:10 starting dilution in subsequent tests.

For routine work it is easiest to collect a 10-drop fraction when the Dextran blue begins to elute, which is easily detected by eye. The absence of IgG (and presence of IgM) in this fraction is quickly verified by the immunodiffusion test (see above). Routinely one person can thus fractionate, qualitatively check for IgG contamination, and assay by IIF at least 10 sera per day for the presence of specific IgM.

Application of the method to *Toxoplasma* IIF. In our laboratory a CF titer of $\geq 1:30$ or an IIF anti-total immunoglobulin of $\geq 1:256$ is considered to be suggestive of a past or recent infection, in which case *Toxoplasma*-specific IgM is determined. A total of 100 normal blood donor sera were tested as controls (Table 1). Of the 15 with low IIF titers (including three with a CF titer of 1:10), only one IgM fraction had a low positive titer (1:10). On the other hand, many high titers of specific *Toxoplasma* IgM were observed when 108 sera with CF titers of 1:30 and IIF-Ig titers of 1:256 or more against *Toxoplasma* were tested (Table 2 presents 10

representative sera). Of the 108 sera, only 17 were positive for IgM by IIF on whole serum. Of these, five were positive only at high serum dilutions as seen in Table 2. After chromatographic separation of IgG and IgM, however, 55 sera were positive in IgM-IIF, thus suggesting a diagnosis of a recent *Toxoplasma* infection.

It is evident (Table 2) that there are many false-negative results in the routine IIF-IgM test for *Toxoplasma* that uses whole sera. That such results are caused by the blocking of IgM by IgG in binding to the *Toxoplasma* antigen is substantiated by sequentially incubating IgG and IgM *Toxoplasma*-specific antibody fractions with the antigen (Table 3). Both the IgG and IgM column fractions are positive to high dilutions using fluorescein conjugate anti-IgG and anti-IgM, respectively (experiments A and B). However, initial incubation with the IgG fraction

followed by the IgM fraction gives a clear positive reaction with anti-IgM only after the IgG fraction has been diluted 160-fold (experiment C). Similar results could be obtained by simultaneous incubation of IgM- and IgG-positive fractions (not shown), thus demonstrating the blocking effect of *Toxoplasma*-specific IgG on specific IgM.

Since antinuclear antibodies may cause false positive results in IIF for specific IgM (1), 11 sera with antinuclear and antideoxyribonucleic acid titers from systemic lupus erythematosus patients were tested for *Toxoplasma* antibodies in CF, by IIF in whole serum (anti-immunoglobulin) and in the IgM fraction (anti-IgM) (Table 4). Two sera (no. 1 and 5) negative in CF (<10) had high IIF titers in whole sera with anti-immunoglobulin and serum no. 1 also with anti-IgM, but the purified IgM fractions of both are negative. Two other sera (no. 3 and 4) also had IIF titers with anti-IgM when tested with whole serum. The IgM fraction of serum no. 3 was weakly positive (titer of 10), which, in the presence of a CF titer of 40, suggests the presence of *Toxoplasma*-specific IgM. Since sera 1 and 4 have detectable rheumafactor titers, it is likely that the positive IgM titer obtained using whole serum reflects the presence of rheumafactor (4).

The test was employed to follow the disappearance of *Toxoplasma*-IgM antibodies after

TABLE 1. *Toxoplasma* serology on 100 healthy blood donors

Whole sera				IgM fraction	
CF		IIF-Ig		IIF-IgM	
Titer	No. of cases	Titer	No. of cases	Titer	No. of cases
<10	97	<64	85	<10	99
10	3	128-512	15	10	1

TABLE 2. *Toxoplasma*-specific IgM in whole serum and IgM fraction

Serum	Clinical diagnosis	Toxo- CF ^a	IIF-Ig	IIF-IgM													
				Whole serum ^b							IgM fraction ^b						
				10	20	40	80	160	320	640	10	20	40	80	160	320	640
1	Lymphadenopathy	160	512	—	—	—	+	—	—	—	+	+	+	+	—	—	—
2	Lymphadenopathy	40	128	—	—	+	+	+	—	—	+	+	+	+	+	—	—
3	Lymphadenopathy	160	512	—	—	—	+	+	+	—	+	+	+	+	+	—	—
4	Subfebrile	160	1024	—	—	+	+	+	+	—	+	+	+	+	+	+	—
5	Lymphadenopathy	80	1024	—	—	—	+	+	—	—	+	+	+	+	+	—	—
6	Fever, lymphadenitis	80	512	—	—	—	—	—	—	—	+	+	+	+	—	—	—
7	Polyadenopathy	80	1024	—	—	—	—	—	—	—	+	+	+	+	—	—	—
8	Toxoplasmosis	120	512	—	—	—	—	—	—	—	+	+	+	—	—	—	—
9	Lymphadenitis	40	256	—	—	—	—	—	—	—	+	+	+	+	+	—	—
10	Death in utero	160	512	—	—	—	—	—	—	—	+	+	+	—	—	—	—

^a Toxo-CF, *Toxoplasma* titer by CF.

^b +, Positive fluorescence; -, no fluorescence.

TABLE 3. Blocking of IgM by IgG

Expt.	Incubation no. ^a		FITC-conjugated antiserum	Dilution of fraction ^b				
	1	2		1:10	1:20	1:40	1:80	1:160
A	IgG fraction ^c	PBS	Anti-IgG	+++	+++	+++	++	++
B	PBS	IgM fraction	Anti-IgM	+++	+++	+++	+	±
C	IgG fraction ^c	IgM fraction	Anti-IgM	±	±	±	±	+

^a Incubation 1 was followed by a 10-min PBS wash, then as described in the text.

^b Only the IgG fraction was diluted in experiment C. +++, Highly positive; ±, questionable.

^c IgG fraction from a serum without *Toxoplasma* IgM.

TABLE 4. *Toxoplasma* IIF on ANA- and RF-positive sera^a

Serum	Serology			<i>Toxoplasma</i> IIF ^b		
	ANA ^c	RF ^d	<i>Toxoplasma</i> ^e	Whole serum		IgM fraction
				Total immuno-globulin	IgM	
1	320	10	<10	512	60	<10
2	40	ND ^f	40	512	ND	<10
3	80	<10	40	128	40	10
4	320	20	<10	64	40	<10
5	≥160	<10	<10	256	<10	<10
6	80	ND	<10	64	<10	<10
7	320	ND	AC ^g	<32	ND	<10
8	320	ND	240	256	ND	<10
9	320	ND	<10	32	ND	<10
10	640	ND	<10	32	<10	<10
11	320	ND	<10	32	ND	<10

^a All titers are expressed as the reciprocal.^b Whole serum was checked by FITC-labeled anti-human total immunoglobulin and by FITC-labeled anti-human IgM; the IgM fraction was tested with FITC-labeled anti-human IgM only.^c ANA, Antinuclear antibodies.^d RF, Rheumafactor.^e *Toxoplasma*, *Toxoplasma* antibody by CF.^f ND, Not done.^g AC, Anticomplementary.

the initial IIF detection of *Toxoplasma*-IgM. The composite results (Fig. 2) show that high titers (>1:80) are seen in the first 4 weeks corresponding to the time when clinical symptoms suggestive of toxoplasmosis were found. Late convalescent sera taken 3 to 12 months later retain *Toxoplasma*-IgM-positive titers in 6 of 17 patients. This result suggests caution in the interpretation of *Toxoplasma*-IgM-positive titers as reflecting acute or very recent infections.

DISCUSSION

The presence of specific IgM in serum can be obscured by specific IgG (Table 3) (6), which is often found in serum early in the course of *Toxoplasma* infections (5). In these cases, and in others such as suspected cytomegalovirus and rubella infections in pregnant women, it becomes advantageous to separate IgG and IgM to obtain a reliable result. Gel chromatography satisfactorily separates serum IgM, IgG, and IgA (3); in addition, rheumafactor, a frequent cause of false positive IgM staining, does not interfere since IgG is removed (10). The use of a large column (30 to 60 cm long and 1.5 to 2.5 cm diameter), however, requires the application of 1 to 2 ml of serum, overnight elution, and often the subsequent concentration of the eluted IgM-containing fraction. The small column procedure presented in this paper needs only 50 to 150 μ l of serum (especially important with newborns or young infants when cord blood is not available), the column is ready for a new sample every 20 to 30 min (two or three columns can be handled

FIG. 2. Persistence of *Toxoplasma* IgM following acute infection. The *Toxoplasma* IgM titers were determined in successive sera from 17 patients with clinical symptoms of toxoplasmosis during the initial 2 to 4 weeks. At least two follow-up sera from each patient were examined. $\blacktriangle$, *Toxoplasma*-IgM titer in IgM fraction from the mini-column. Shaded area, positive *Toxoplasma*-IgM titer.

simultaneously), and the dilution of the IgM is only about 10-fold.

Several other Bio-Gel, Sepharose, and Sephadex resins were tried, but Bio-Gel A5-m (200 to 400 mesh) gave the best flow rate, separation, and reproducibility. It should be stressed that a flat upper gel bed surface and careful layering of the sample are required for optimal separation. Since the columns are used at room temperature and over a period of several days, the addition of sodium azide to the buffers is essential.

A great advantage of this method is that large and expensive equipment, e.g., ultracentrifuges, column monitors, and fraction collectors, is not required, which should benefit smaller labora-

tories and those in underdeveloped areas.

Protein A from *Staphylococcus aureus* is now frequently used to absorb IgG from serum. Potential problems with the use of protein A are lack of removal of IgG subclass 3, the possible binding of IgM, and the incomplete removal of interference by rheumafactor (4, 9). We have tested several highly positive (160 to 640) rheuma sera and have confirmed that false positives by IIF using whole serum (CF, <10) are eliminated by the use of the column-fractionated IgM fraction (unpublished results).

We are currently using the mini-column technique for the detection of cytomegalo-, rubella-, Epstein-Barr-, and hepatitis-A- virus-specific IgM.

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