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Separation of Immunoglobulin M (IgM) Essentially Free of IgG from Serum for Use in Systems Requiring Assay of IgM-Type Antibodies Without Interference from Rheumatoid Factor

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The proposed method was designed to replace the tedious and difficult separation of immunoglobulin M (IgM) from IgG by sucrose gradient sedimentation. In this method, a 250-μl portion of serum diluted 20-fold was passed through a small column of quaternary aminoethyl-Sephadex A-50 ion exchanger. IgG was not retained, but additional washes were required to remove all but 5%. A second buffer-eluting fluid recovered an average of 80% of the original IgM in a defined dilution. The entire operation took 15 min. The efficiency of this process was evaluated by the following: (i) radial immunodiffusion measurements of IgG and IgM; (ii) recovery studies of isohemagglutinins; and (iii) demonstrated removal of interference by the rheumatoid factor. The method was applied successfully to distinguish rubella IgM antibody.

The assay of specific immunoglobulin M (IgM) antibodies can aid in the diagnosis of early infection and may obviate a paired convalescent serum sample in acute virus infections. This concept is not new, but no present system has met the qualities of reliability, ease of performance, low cost, and adaptability to subsequent uses.

IgG antibodies to the same antigen interfere with the assay of specific IgM antibodies by excluding the less avid IgM antibodies when the quantity of antigen is marginal (9) and by providing an attachment for any IgM-rheumatoid factor (RF), thereby causing bound IgM to lose specificity (15). Thus, the removal of IgG from IgM is necessary in those systems which do not discriminate the final immunoglobulin interacting with antigen (e.g., hemagglutination inhibition assay) and vastly improves the specificity of systems which can identify the bound immunoglobulin.

Serum IgM and IgG have been separated by sucrose gradient sedimentation, which requires an ultracentrifuge (5), staphylococcal protein A binding of IgG (13, 16), gel exclusion chromatography (3, 8, 17), ion exchange (17, 18), and solid-phase immunological entrapment of IgM (12). A comparison of results obtained before and after treatment of the serum with 2-mercaptoethanol has been made (2, 5).

We present here a method for obtaining serum IgM "free" of IgG by ion exchange. This method was adapted from a previously reported method for isolating IgG from sera (11). The procedure presented here used 250 μl (or 100 μl) of serum, with separation in 15 min on a small column of quaternary aminoethyl (QAE)-Sephadex A-50. Recoveries of IgM averaged better than 70%.

We optimized the method by monitoring recoveries of isohemagglutinins and IgM, IgG, and IgA. We used the absorbance at 280 nm to determine the amount of wash required.

Finally, we examined 10 serum samples from the Center for Disease Control, Atlanta, Ga., for IgM antibodies to rubella. For these assays we prepared a Cooke microtiter plate (96 wells, flat bottom; Dynatech Laboratories, Inc., Alexandria, Va.). Rubella virions suspended in 0.01 M carbonate buffer (pH 9.5) were aliquoted to yield 3 μg of protein (14) per well. After drying in a vacuum desiccator, the plate was treated with 20% acetone in saline for 30 min at room temperature, followed by three 3-min washes with a saline solution containing 0.5% Tween 20 and 0.5% bovine serum albumin (Sal/T/BSA). The samples at 1:8 to 1:64 dilutions in Sal/T/BSA were added to duplicate wells in 50-μl aliquots and incubated with gentle rocking for 1 h at room temperature. Known positive and negative controls were included. After 3 washes with Sal/T/BSA, goat anti-human IgM coupled to glucose oxidase at a 1:80 dilution in Sal/T/BSA was distributed to all wells in 50-μl aliquots. After a 1-h incubation at room temperature, the plate was washed with saline containing 0.05% Tween 20. A 200-μl amount of a solution pre-

TABLE 1. *Recovery of IgM and isohemagglutinin activity after QAE-Sephadex treatment*

Serum Sample	IgM (mg/dl[a])	Isohemagglutinin titer[b]	IgG (mg/dl[a])
Specimen L			
Untreated	120 (100)	1:40	
Treated	99 (83)	1:32	54
In wash	23 (20)		
Specimen M			
Untreated	150 (100)	1:128	
Treated	115 (77)	1:64	20
In wash	30 (20)		

[a] Corrected for dilution. Numbers in parentheses represent the percentage of the total amount of IgM recovered.

[b] 1:40 and 1:32, anti-B titers; 1:128 and 1:64, anti-A titers.

TABLE 2. *Challenge of QAE-Sephadex fractionation system or solid-phase human IgG to remove RF interference in the rubella IgM antibody assay*

Serum treatment	Absorbance at 642 nm as evidence of IgM antibody for:		
	Serum sample 1	Serum sample 2	Positive control
Diluted[a] 1:8	0.046	0.043	0.130
Spiked with RF[b]			
Titered for RF[c]	1:1,280	1:2,560	
Diluted[a] 1:8	0.203	0.222	
Diluted[a] 1:16	0.110	0.193	
Fractionated by QAE[d]	0.011	0.002	0.125
Adsorbed with solid-phase human IgG[e]	0.180	0.150	

[a] Diluted with Sal/T/BSA.

[b] Monoclonal IgM with antibody activity toward human IgG was added.

[c] Calbiochem-Behring kit.

[d] Yields a 1:8 dilution.

[e] See reference 4.

pared from 3 ml of 18% aqueous glucose (well equilibrated)-2 mg of 2,2-Azino-di-[3 ethylbenzthiazolin-sulfonate (6)] diammonium salt (Boehringer Mannheim Corp., Indianapolis, Ind.)-1 ml of 20 mg% peroxidase-25 ml of 0.1 M phosphate buffer (pH 6) was distributed to all wells and incubated for 30 min. The solution in the wells was quickly transferred to numbered tubes pooling duplicates, and absorbances at 642 nm were obtained. Enzymic activity ceased on transfer.

MATERIALS AND METHODS

Sera used included some obtained from laboratory personnel. Some sera with high titers for the RF were gifts from Michael Catalano, Allergy-Immunology Research Laboratory, Research Institute of Scripps Clinic. Some sera preassayed for IgM antibodies to rubella were obtained from Kenneth Herrmann, Center for Disease Control, Atlanta, Ga. A human monoclonal IgM antibody to human IgG was a gift from Hans Spiegelberg, Department of Immunopathology, Research Institute of Scripps Clinic. Low protein-concentration radial immunodiffusion plates for assaying IgG and IgA were obtained from Calbiochem-Behring, San Diego, Calif. A similar product to determine low concentrations of IgM was obtained from Kallestad Laboratories, Inc., Chaska, Minn. Blood-typing cells A and B were obtained from Biological Corporation of America, Port Reading, N.J. QAE-Sephadex A-50 was obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Small chromatography columns were adapted from the 2.5-ml Sarpette tip, obtained from Walter Sarstedt, Inc., Princeton, N.J. These columns are similar to small chromatography columns obtainable from Evergreen Scientific, Los Angeles, Calif.

We prepared and used a goat anti-human IgM coupled to glucose oxidase as previously described (10). This antiserum was extensively adsorbed with pooled purified human IgG covalently bound to Sepharose 4B (4) before coupling. Rubella virions were gifts from F. C. Jensen, Research Institute of Scripps Clinic. These had been grown in BHK-21 cell cultures, isolated from that milieu by microfiltration, and purified by gradient sedimentation onto a 60% sucrose cushion after concentration.

Method for obtaining serum IgM nearly free of IgG.

A. Preparation of Reagents
   1. Diluting and wash solution
      Add 2.88 g of ethylenediamine and 4.38 g of glacial acetic acid to deionized water to make 1 liter at pH 7.0. Keep at 5°C.
   2. Eluting solution
      Add 15.65 g of glacial acetic acid, 10.6 g of sodium acetate·$3H_2O$, and 20 g of sodium chloride to deionized water to make 1 liter at pH 4.2.
   3. QAE-Sephadex A-50 Suspension
      Combine 10 g of QAE-Sephadex powder with ca. 200 ml of diluting/wash solution and let swell overnight. Decant the supernatant and add enough wash solution to yield about twice the settled volume of the exchanger.
   4. Column preparation
      Use disks from glass fiber filters (GF/A; Whatman) at the base of the column as the exchanger support and add sufficient exchanger suspension to make 1 ml of bed volume (±10%).

B. Procedure
   1. Dilute 250 μl of serum with 5 ml of diluting/wash solution and add to the column.
   2. Discard the solution coming from the column.
   3. Wash the column with 20 ml of diluting/wash solution and discard all washes.
   4. Add 1.5 ml of eluting solution and collect all fluid from the column. This solution contains a 1:8 dilution of the original serum IgM and other proteins but nearly no IgG.

Note: If 100 μl of serum were treated with the same other volumes as above the collected fluid would be a 1:20 dilution.

RESULTS AND DISCUSSION

This procedure depends on the following: (i) sufficient capacity of the ion exchanger to retain IgM; (ii) adequate removal of the IgG by washing; and (iii) recovery of the IgM in a low known dilution of the original serum. The method was optimized by following changes in the procedure with measurements of IgM and IgG (6), recovery of isohemagglutinins, and 280-nm absorbancies of effluents. The data in Table 1 indicate the efficiency finally achieved. The measurement of IgM by radial immunodiffusion lacks good precision under ideal conditions and can be less reliable at low concentrations. In this procedure the eluates were 1:8 dilutions of the original serum, and the washes, reconcentrated to 0.5 ml, were 1:2 dilutions of the original serum but nearly depleted in IgM. We followed the recovery of isohemagglutinins, normally IgM-class antibodies, to corroborate the uncertain radial immunodiffusion values. To avoid our bias we enlisted blood bank personnel to determine the anti-A and anti-B titers. The 20 ml used for the wash may seem excessive but was dictated by the trailing removal of IgG as shown by 280-nm absorbancies. The amount of IgG finally contaminating the recovered IgM was about 5% of the original (Table 1).

TABLE 3. *Removal of IgG antibodies by column treatment in five sera positive for rubella IgG*

Serum sample no.	IgG antibody titer	
	Before column treatment	After column treatment
34	1:8	<1:8
37	1:64	<1:8
46	1:16	<1:8
49	1:16	<1:8
57	1:32	<1:8

TABLE 4. *Reproducibility of recovery of IgM depleted of IgG*[a]

Immunoglobulin	Recovery (mg/dl)			Antibody titer (mg/dl)	
	Original serum	Eluate	Wash	Serum	Eluate
IgM	240	232 ± 67	16 ± 8	1:16	1:16
IgG		44 ± 6		1:16	<1 to 8

[a] Study of eight separate column treatments on a serum known to be positive for both IgM and IgG rubella antibodies.

The RF of the IgM class can produce false evidence for the presence of an IgM antibody toward a specific pathogen in immunofluorescent and enzyme-linked assays which use a labeled anti-IgM antibody. The RF interference occurs when IgG antibodies bind to the pathogen and provide binding sites for the RF. The RF-IgM becomes indistinguishable from pathogen-specific IgM. We determined the efficiency of our ion-exchange column treatment to remove RF interference by the following experiment. Two sera free of RF, with IgG rubella antibodies but no apparent IgM rubella antibodies, were assayed for rubella IgM and then "spiked" with RF and reassayed before and after column treatment. The RF used here was pure IgM isolated from myeloma serum and having the unusual property of showing antibody activity to IgG. The data from this experiment are shown in Table 2. The greater the absorbancy at 642 nm was, the greater the presence of bound IgM.

The data in Table 3 reaffirm the removal of IgG by column treatment. The data in Table 4 indicate reproducibility, but the radial immunodiffusion values probably overestimate the recovery of IgM.

Practical application of this procedure was indicated by an assay for rubella antibody of 10 proficiency test-type sera from the Center for Disease Control, Atlanta, Ga. The sera were from a mixture of individuals immune to or convalescing from rubella infection. With the ion-exchange procedure we correctly identified seven sera with rubella-specific IgM and therefore from convalescents.

We have made no attempt to compare the proposed method with any of the other reported methods by parallel treatments with the same sera. Sugg et al. (17) compared and found batch ion exchange with diethylaminoethyl-Sephadex A-50 and agarose gel filtration better able to separate IgM and IgG than dextran or polyacrylamide filtrations or sucrose gradient ultracentrifugation. Absorption of IgG with staphylococcal protein A can remove all but IgG3, but the method is expensive because 66 to 75 mg is required (1, 7) to remove 1 mg of human IgG. Also, all of the above methods do not easily provide the final IgM in either a convenient or known dilution.

The proposed method provides, in 15 min, adequate separation of IgM from IgG. A total of 10 or more sera can be treated in individual columns at the same time with no loss in efficiency.

Caution should be observed, however, as the method will not remove IgG aggregates which occur more frequently in samples held frozen for

long periods or subjected to multiple freeze-thaw cycles or both.

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