Estimation of IgM rheumatoid factors by fluorimetry

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SUMMARY Sheep red cells coated with rabbit IgG antibody may be used to detect IgM rheumatoid factors either by agglutination or microscopically by the fluorescence of antihuman IgM antibody conjugate bound to rheumatoid factors on the cell surface. By substituting for red cells the plastic surface of a filter disc coated with rabbit IgG it was possible to elute the bound conjugate and measure the fluorescence in a fluorimeter. The results of both the sheep cell agglutination test and the fluorescence test agreed quite well for the majority of sera; both tests seemed to reflect IgM rheumatoid factor concentrations in these sera. The difficulties encountered in attempting to define and measure accurately rheumatoid factor concentrations in a serum are emphasised.

The sensitivity and apparent simplicity of agglutination tests make them deservedly popular for the detection of abnormal levels of antibody. The clinical specificity of the sheep cell agglutination test (SCAT) renders it a valuable diagnostic aid, despite the fact that only high molecular weight IgM rheumatoid factors (RFs) are detected and the reagents are labile and difficult to standardise. Rabbit IgG bound to a hydrophobic latex gives agglutination titres with RFs which are related to SCAT titres (Griehle *et al.*, 1969; Bach and Schmidt, 1972; Marcolongo and Saletti, 1974).

Following the procedure described by Estes *et al.* (1973), Hettenger and Muller (1975) used fluorescent antiglobulin reagents to titrate RFs bound to rabbit IgG-coated sheep cells microscopically and were able to distinguish between SCAT positive and SCAT negative sera. Since elution of antibodies from a plastic surface, using detergent (Salmon *et al.*, 1969), is easily achieved we therefore adapted the microscopic fluorescence titration procedure to measure, in a conventional fluorimeter, labelled anti-human IgM antibody bound to a plastic surface bearing RF-rabbit IgG complexes.

Materials

Sera were obtained from patients attending clinics or admitted to the rheumatology ward and were stored immediately at -20°C . SCAT tests were performed within a few days and fluorescence tests within a few weeks whenever possible. Fluorescein-labelled antisera prepared by Dakopatts A/S were

obtained from Mercia Diagnostics Ltd. Rabbit Cohn FII and Tween 20 were supplied by Koch-Light Ltd. Chemicals of analytical grade were used when available. Polypropylene discs, 9 mm in diameter were punched from P & S no. 12 filter cloth (kindly donated by P & S Textiles Ltd, Haslingden, Rawtenstall, Lancs.). 2.5 ml flat-bottomed autoanalysis cups were used as containers.

Stock borate saline buffer (Kotoulas and Moroz' 1971) containing 0.15 M sodium chloride and 0.15 M boric acid was adjusted to pH 8.6 with sodium hydroxide and used as a 10% solution. Stock potassium phosphate buffer pH 7, 1 mol/l containing sodium azide (2 g/l) was diluted to 0.1 mol/l for use and Tween 20 (0.05%) was added to reduce non-specific binding effects (Engvall and Perlmann, 1972). A stock solution (10 mg/ml) of rabbit FII in phosphate buffer was prepared and filtered for use. It was stored at -20°C . Alkaline sodium dodecyl sulphate (SDS) was prepared using 2 g/100 ml 0.1 N NaOH.

Method

Using a 1 cm diameter quartz cell in a Locarte digital fluorimeter with 13 stage PM tube and zinc lamp, the optimum sensitivity was achieved with a primary filter combination 805 Q (Ilford) and BG 12/2 mm (Schott) and secondary filters 805 Q, OG 515/2 mm, and Wratten 55 (Kodak). The instrument response was linear, turbidity interference minimal, and stability within experiments and between days high after a few minutes' warm-up.

After treatment with ethanol and hot 40% caustic soda solution, the plastic discs were rinsed

thoroughly and packed loosely into a plastic tube. The discs were mixed with rabbit FII solution (2 μ l per disc) in borate saline (30 μ l per disc) for about 6 hours at room temperature. Each batch of 40 to 60 discs was rinsed, washed overnight in Tween phosphate buffer (50–100 ml), rinsed, and drained. The patient's serum under investigation, diluted with Tween phosphate buffer to 100 μ l, was added to a new autoanalysis cup and an FII-coated disc was put in, avoiding bubbles. The racks containing these cups were shaken gently, covered, and kept at room temperature for about 6 hours. Each disc was washed three times by filling the cup with Tween phosphate buffer and draining.

From a freshly prepared solution of anti-IgM conjugate and filtered normal rabbit serum in buffer (10 μ l and 40 μ l respectively in 1 ml) a portion of 100 μ l was added to each cup and disc, and after gentle agitation the covered racks were kept overnight at room temperature. Three washes removed excess conjugate and bound conjugate was dissolved by adding 1 ml alkaline SDS solution to each cup. Fluorescence values of the eluates were read in an instrument standardised at 1.000 with 1 ml SDS solution containing a 5 μ l portion of conjugate/serum in buffer, using a blank of SDS solution.

Results

Several variables of the assay procedure were examined. Dilute conjugate in Tween phosphate buffer lost activity slowly and therefore all protein solutions were diluted immediately before use to reduce the effect of denaturation in the assay. No significant fading of fluorescence of SDS solutions was detected after exposure for 30 minutes in the fluorimeter or after standing for 2 hours at room temperature. No more conjugate could be recovered from discs after SDS treatment. FII-coated discs bound approximately 0.1% of the conjugate under assay conditions and coated discs saturated with RFs about 5% of the conjugate. Substitution of 1% crystalline bovine plasma albumin for Tween 20 gave only a marginal improvement in these values.

Six coated discs were saturated with RFs by identical treatment with one serum in large excess. Treatment in the same assay with two anti-IgM conjugates standardised by the manufacturer but with different batch numbers gave different mean fluorescence values. Such variability is a characteristic of antisera to multivalent antigens. The amount of conjugate eluted from a disc is therefore not an exact measure of the amount of IgM bound to the disc.

Incubation for at least 5 hours with antibody at each stage was required for the highest sensitivity.

Equal amounts of conjugate were bound by RF-coated discs rinsed three times or washed for 8 hours and loss of antibody from discs was therefore negligible. Fluorescence measurements showed that in the assay saturation of discs with RFs occurred at serum volumes of about 2 μ l or more. The volume/fluorescence curves were, as expected, nonlinear. Sera gave different plateau values of fluorescence, indicating that the RFs bound were probably not all of the IgM class, although IgM molecules differ in the amount of conjugate they can bind (de Bruyn and Klein, 1976). The maximum amount of conjugate bound represented about 5 ng human IgM bound to a disc.

After 1 μ l samples of serum (titre 128) were treated with one, two, or three coated discs, IgM RFs were readily detectable in all the supernatants. The total amount of RF bound to a disc was therefore dependent upon the affinity as well as the overall concentration of RFs in the serum.

When a variety of solid absorbents, including polystyrene tubes, were tested the plastic filter discs gave better precision. One serum sample gave 20 replicate values with a standard deviation of 4% of the mean value in one experiment. However, the lower precision achieved in routine assays made it necessary to use three replicate discs for every serum dilution. Volumetric errors in the preparation of dilutions were insignificant. The complexity of the assay system meant that a simple correction factor could not apply to results from different experiments.

Five SCAT-positive sera were assayed at one level (0.8 μ l) for IgG RFs, by using fluorescent anti-IgG conjugate, and gave fluorescence values between 0.27 and 0.355, which seemed unrelated to their SCAT titres. Similar serum samples heated at 56°C for 15 minutes gave consistently higher values, 0.256 to 0.583. There was no way of deciding which of the two procedures gave a better measure of IgG RFs in these sera. In contrast, no effect of heating the sera was evident when anti-IgM conjugate was used.

From the manufacturer's data sheet on the fluorescein conjugate 100 μ g IgM precipitates 1 ml of fluorescent antiserum under test conditions. In the assay, therefore, 5 ng IgM RF would bind conjugate represented by a fluorescence value of 1.0, without considering mass action and steric effects, and the contribution of low MW IgM RFs. Our fluorescence values of 0.4 to 1.0 which are commonly given by seropositive sera therefore represent the binding of 2 to 5 ng conjugate, values similar to those reported by Hay *et al.* (1975). Because our absorption results suggest that the major proportion of RFs in a serum remain in solution under assay conditions it seems likely that seropositive sera frequently have IgM RF

concentrations greatly in excess of 5 $\mu\text{g/ml}$, considering rabbit-specific RFs only.

A series of SCAT-positive and SCAT-negative sera from patients and normal individuals were assayed at a sample volume of 1 μl . The results of two experiments are shown in Figs. 1 and 2. Each experiment was composed of determinations done on several days. Within each experiment no significant change in sensitivity occurred, as shown by reassaying each day four representative sera. An unexpected combination of high fluorescence value and low SCAT titre was given by serum from a patient with myeloma and mixed connective tissue disease. Although the determination of both fluorescence values and SCAT titres were subject to experimental error, it was evident that a relationship existed between the titre of a serum and the amount of IgM in 1 μl serum, which was bound to immobilised rabbit IgG under assay conditions.

By constructing a standard curve using one standard serum at different volumes the fluorescence values for test sera at a fixed volume (0.8 μl) could be expressed in terms of the equivalent volume of standard determined at the same time. This approach was found inadequate as few sera could be examined in each experiment.

In order to use a purified, stable rabbit IgG antigen in place of Cohn fraction II, ovalbumin-coated discs were treated with rabbit antiovalbumin serum. Both antibody treated and ovalbumin control discs bound negligible amounts of fluorescent antihuman IgM conjugate under the usual assay conditions. The fluorescence values for two human sera were 0.1 and 0.3 with the ovalbumin-coated control discs, suggesting that these sera contained sufficient antiovalbumin antibodies to interfere with the RF assay.

Discussion

The SCAT and related red cell and latex tests are the only ones generally available for the demonstration of RFs. The disadvantage of these agglutination tests are well known and many attempts have been made to introduce new, quantitative tests which are claimed to be specific, accurate, and clinically valuable. Whereas immunoassay techniques for determining the concentration of homogeneous antigens and simple chemicals may be judged in terms of specificity, accuracy, and precision, greater problems arise in the measurement of heterogeneous antigens, such as parathyroid hormone and serum immunoglobulins. Highly selective antisera

Fig. 1

Fig. 2

Figs. 1 and 2 show the results of experiments 1 and 2 respectively. Both show the SCAT titres of sera and their IgM fluorescence values determined at one concentration.

are required (Grubb, 1973; Lea and Ward, 1975) and the results may not be quantitative, though clinically of value (Schmidt-Gayk *et al.*, 1976).

The quantitation of antibody presents similar problems. The antibody population in an antiserum raised to a specific antigen is rarely homogeneous, and antibodies of all immunoglobulin classes may be present. The antigen may have many different determinants, each with its own specific antibody population and antibodies of low affinity may be present in large amounts (Kim *et al.*, 1974). Even the simplest approach to the quantitation of one antibody (IgE) in a serum requires several measurements to be made to take account of the equilibrium between free antibody and that bound to allergen (Bongrand *et al.*, 1976). The amount of one antibody bound to an insoluble antigen is thus influenced by the experimental conditions chosen and is not determined solely by the total amount present.

Clinical observations suggest that the SCAT test provides the most useful evidence for the presence of IgM RFs in a serum. We have made a basic assumption that the titre of a serum reflects closely the concentration of those IgM RFs able to react with rabbit IgG determinants on the sheep cell surface. Moreover our results could not be judged by any independent criterion other than SCAT titres. On this stated basis the evident relationship between SCAT titre and fluorescence value, measured by our technique, leads us to believe that the fluorescence value of a serum is also a satisfactory index of IgM RF concentration.

Norberg (1976) emphasised that independent evidence is needed to prove valid any assay procedure which purports to reflect IgG RF concentrations. Lacking such evidence we were unable to decide if either of the fluorescence assay procedures using heated or unheated serum samples and anti-IgG conjugate provided a satisfactory index of IgG RF concentrations.

Compared to the radioimmunoassay technique described by Hay *et al.* (1975) and Nineham *et al.* (1976), the technique we developed has several advantages. In the radioimmunoassay 50 μ l of patient's serum in a total volume of 0.5 ml was used. As only 1 μ l serum in 100 μ l total volume is required for the fluorescence assay, the interference by dissociable immune complexes (Abruzzo and Heimer, 1974) is reduced. Our choice of longer reaction times likewise not only increased sensitivity, with a reduction in serum concentration, but also allowed equilibrium to be approached.

For routine purposes the preparation and purification of radiolabelled antibody is tedious and demanding. Not only are the techniques for the critical testing of specificity well established for

fluorescent antisera (Chantler and Haire, 1972) but the availability of these antisera fractionated before and after labelling reduces the differences between assays arising solely from differences between conjugate preparations. It is important to note that techniques using labelled antihuman IgM antibody do not distinguish between human agglutinating high MW IgM antibodies and the non-agglutinating IgM antibodies of low MW.

Addition of unlabelled normal rabbit serum to the fluorescent conjugate was essential to reduce non-specific binding and the unwanted specific binding of labelled rabbit IgG as antigen to the bound RF on the plastic discs. These precautions may account for the much lower background uptake of conjugate by plastic discs than was found, for example, in the fluorescence assay of Cukor *et al.* (1976) who used cyanogen bromide-treated paper loaded with pollen allergen.

In conclusion, we suggest that the fluorescence test provides an alternative method to the sheep cell agglutination test for assaying sera containing rabbit-specific IgM RFs, the results of the two tests comparing quite well. The values obtained are influenced by the equilibrium between bound and free RFs and the test cannot be quantitative, as chemical estimations are, but the technique does seem to be valid. In its present form it still suffers from the disadvantage of day-to-day variability, common to many 'solid-phase' tests, but appears to be better in this respect than the SCAT test. A major cause of this variability is probably the low affinity of RFs for rabbit IgG. Fluorescence tests of a similar kind may be satisfactory alternatives to many latex agglutination tests and to others using radiolabelled or enzyme-labelled reagents.

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