Epidem. Inf. (1988), **100**, 101–109
Printed in Great Britain

Red-cell IgM-antibody capture assay for the detection of *Mycoplasma pneumoniae*-specific IgM

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(*Accepted 15 September 1987*)

SUMMARY

A red-cell IgM-antibody capture assay has been developed for detecting *Mycoplasma pneumoniae*-specific IgM, which is based on the adsorption or 'capture' of IgM from patients' sera onto so-called 'inagglutinable' bovine red cells, chemically linked with anti-human μ. When *M. pneumoniae* antigen is added to the system, the red cells agglutinate in the presence of *M. pneumoniae*-specific IgM.

The test was compared with the μ-capture ELISA described by Wreghitt & Sillis (1985), and was found to give comparable results. The two tests had similar sensitivity and specificity and could detect *M. pneumoniae*-specific IgM for a similar time (up to 6 months) after proven *M. pneumoniae* infection.

However, the red-cell antibody capture assay is a much more simple and rapid test, taking only 1 h to perform (compared to 24 h for μ-capture ELISA). The red-cell IgM-antibody capture assay is therefore amenable to rapid diagnosis of *M. pneumoniae* infection and the institution of early appropriate antibiotic therapy.

INTRODUCTION

IgM-antibody capture assays were first described in 1979 for the detection of hepatitis A and rubella virus-specific IgM (Duermeyer, Wieland & Van der Veer, 1979; Flehming *et al.* 1979; Krech & Wilhelm, 1979). Since then this technique has been applied to the diagnosis of many microbial infections including *Mycoplasma pneumoniae* (Price, 1980). Wreghitt & Sillis (1985) developed a μ-capture ELISA for detecting *M. pneumoniae*-specific IgM, and found it to be a sensitive, reliable and specific test. In another study employing this assay, Wreghitt & Sillis (1987) studied the IgM response to *M. pneumoniae* infection in patients during the epidemic of 1983.

In this paper we describe a different antigen-specific μ-capture test, based on the adsorption or 'capture' of IgM, from a patient's serum, onto so-called 'inagglutinable' bovine red cells (Coombs, Gleeson-White & Hall, 1951; Uhlenbruck, Seaman & Coombs, 1967) chemically linked with anti-μ. No

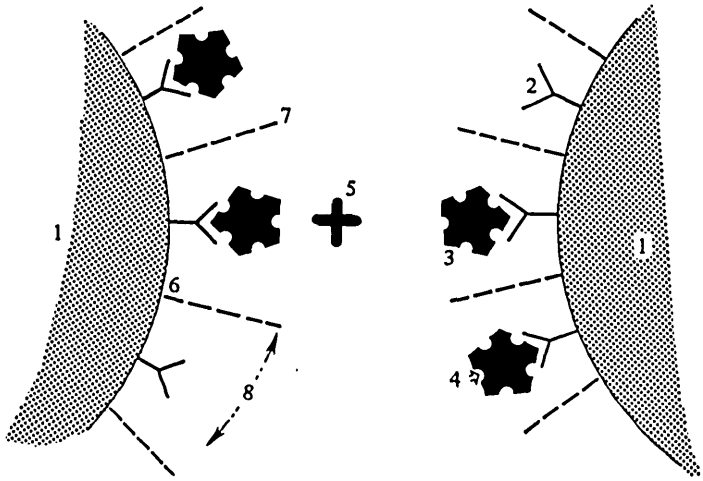

Fig. 1. Diagrammatic representation of antigen-specific IgM red-cell capture assay. 1, Bovine (E6) red cells of the moderately agglutinable class. 2, Sheep anti-human μ coupled to the red cell by chromic chloride. 3, Captured IgM from patient's serum with antigen-specific combining sites (4). 5, *Mycoplasma pneumoniae* antigen. 6, Protein backbone of the red cell membrane. 7, Mucopeptide chains with attached sialic acid rendering cells relatively inagglutinable. 8, Effective periphery of the red cell membrane.

haemagglutination (actually reverse passive haemagglutination or RPH) occurs at this stage because the captured IgM molecule cannot bridge across and bind cells together due to the long muco-peptide chains extending from the protein backbone of the red cell membrane where the anti-μ is coupled. Only on addition of a further antigenic molecule (*M. pneumoniae* antigen in this case) reacting specifically with the captured IgM does agglutination (or rather RPH) occur, the molecular chain presumably now being of a dimension to reach beyond the 'effective periphery' of the cell membrane and thus being able to bridge cell to cell.

The scheme of the reaction is illustrated in Fig. 1 and it is compared in Fig. 2 with the steps involved in the μ-capture ELISA. This paper reports preliminary experiments to find the optimal conditions for this red-cell IgM-antibody capture assay. The method is assessed by comparing results on patients' sera with those obtained on the same sera using a μ-capture ELISA.

MATERIALS AND METHODS

Serum samples

A total of 50 samples of serum from 41 patients with clinical features that suggested *M. pneumoniae* infection was selected. All the samples had *M. pneumoniae* complement fixation (CF) antibody titres of > 64 or were corresponding acute phase sera with lower titres. A serum with *M. pneumoniae* CF titre > 256 and with the highest *M. pneumoniae*-specific IgM titre was chosen as the reference positive control serum in the μ-capture ELISA. This was assigned

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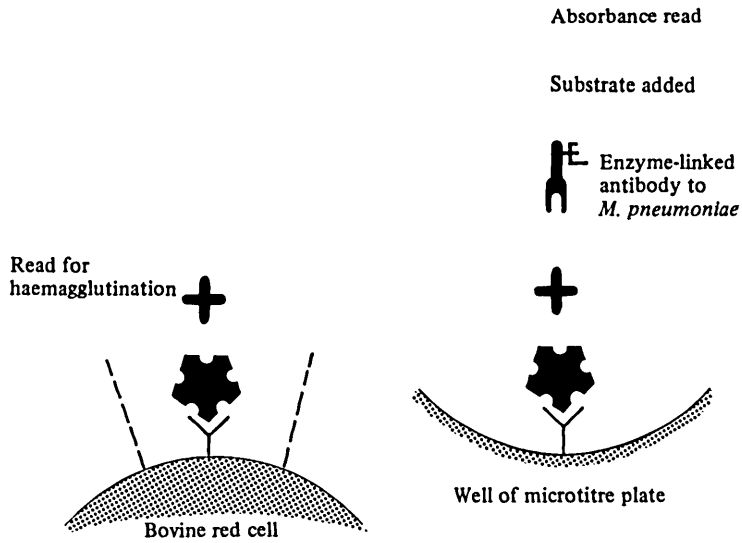

Fig. 2. Comparison of red-cell IgM-antibody capture assay (left) with μ-capture ELISA test (right). Symbols as in Fig. 1 and ⊀ indicates enzyme-linked antibody to *M. pneumoniae*.

an arbitrary value of 100 units of *M. pneumoniae*-specific IgM per 0·1 ml. This positive control serum was diluted in negative control serum to give dilutions containing 33, 10, 3·3, 1·0, 0·33 and 0·1 units.

Thirty samples of serum from 10 patients taken at intervals of up to 9 months after culture-proven *M. pneumoniae* infection and kindly made available by Dr M. Sillis, Public Health Laboratories, Norwich, were also studied to see how long after infection the specific IgM could be detected.

μ-capture ELISA

This was performed as described by Wreghitt & Sillis (1985); sera with $> 0{\cdot}33$ arbitrary units of *M. pneumoniae*-specific IgM per 0·1 ml were regarded as positive.

Mycoplasma pneumoniae *antigen*

This was prepared as described by Wreghitt & Sillis (1985) and kindly made available by Dr M. Sillis, Public Health Laboratory, Norwich. Briefly, the FH strain of *M. pneumoniae* was grown in 8 ml of modified Hayflick's broth with 1% glucose and phenol red as indicator (Leach, 1973). The culture was grown to form an adhered monolayer in a 4 oz 'medical flat' on its side during incubation at 37 °C until an acid colour developed. The spent broth was regularly replaced and the bottle reincubated until the monolayer was confluent, when the broth was decanted, and the monolayer gently washed with FBS pH 7·6. The antigen was then scraped from the glass, resuspended in PBS, and then frozen and thawed once, sonicated at maximum setting for 10 min, and stored in 1 ml aliquots at −20 °C until required.

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Selection of the 'inagglutinable' bovine red cells

For the present tests the appropriate red cell is the more agglutinable red cell of the bovine species, which displays red cells of varying degrees of inagglutinability. The red cell was selected by being relatively agglutinable with the Paul–Bunnell antibody both on direct agglutination and in an antiglobulin test (Edebo, Coombs & Binns, 1980).

Anti-human μ-Fc reagents

These were a gift from Dr A. W. Bradwell of the Department of Immunology, University of Birmingham. These antisera were raised in sheep and as IgG-rich fractions were rendered μ-specific and tested for isotype-specificity as described by Coombs *et al.* (1978).

Coupling bovine red cells with polyclonal sheep anti-μ reagents

The anti-μ reagents were coupled to bovine red cells, without previous enzyme treatment, with the chromic chloride procedure (Siddle *et al.* 1984). The effectiveness of the coupling procedure was checked by making a similar coupling to enzyme-treated sheep red cells. The coupling onto bovine red cells is not so easily checked because of the relative inagglutinability of the red cells. Coupling onto bovine red cells can be checked by performing a capture test as described in this paper.

The specificity of the two IgM-capturing antibodies Z368F and Z631V was established by coupling them onto chymotrypsin-treated sheep red cells and using these in reverse passive haemagglutination tests against two monoclonal human IgM anti-rhesus antibodies and two IgG monoclonal antibodies kindly supplied by Dr N. Hughes-Jones (MRC Group on Mechanisms in Tumour Immunity, MRC Centre, Hills Rd, Cambridge). They reacted only with the human IgM monoclonal antibodies.

Performance of the red-cell IgM-antibody capture assay

Human sera (preferably absorbed with bovine red cells, but not necessary so) were titrated from a 1 in 10 dilution with ten- or fourfold dilution steps, the last tube having PBS diluent alone. Bovine red cells coupled with anti-human μ were added as a 1% suspension in equal volume. After 15 min incubation at room temperature the cells were deposited by light centrifugation, given one wash and resuspended to a 1% suspension. The washed cells (30 μl) were set out in two rows in a microtitre plate. To one row was added 30 μl of a 1 in 10 dilution of the *M. pneumoniae* antigen (found to be optimal) and to the other 30 μl of PBS diluent.

The plates were shaken and read for haemagglutination (RPH) when the red cells settled.

Stabilization and freeze drying of the anti-μ coupled bovine red cells

Stabilization was performed with a 0·01% solution of glutaraldehyde as described by Cranage, Gurner & Coombs (1983). The mixture for freeze-drying was PBS containing 1·5% sucrose (w/v) and 1% bovine plasma albumin (batches first tested for suitability).

RESULTS

Preliminary investigations on the antigen-specific IgM red-cell capture assay

Two polyclonal μ-specific sheep IgG anti-human IgM reagents (Z631 and Z368 kindly supplied by Dr A. W. Bradwell) were usd in these preliminary experiments. The IgM specificity of these reagents was shown in tests reported by Coombs *et al.* 1978. Coupling these Ig reagents at 2·0, 1·0 or 0·5 mg/ml with chromic chloride to chymotrypsin-treated sheep red cells gave a positive RPH reaction with a 1 in 40000 dilution of human serum, measuring its IgM content.

Similar coupling (at 2·0 mg/ml) to non-enzyme-treated bovine red cells (E6, in the more agglutinable class, Edebo, Coombs & Binns, 1980) resulted in coupled red cells which did not agglutinate (RPH) when added to serial dilutions of human serum. However, the IgM in the serum was adsorbed onto the bovine red cells. This could be shown by washing the red cells with adsorbed IgM and mixing with enzyme-treated sheep red cells coupled with the same anti-IgM reagent, in a sort of mixed two-stage RPH. With the anti-globulin (μ-specific)-linked E6 bovine red cells, IgM captured from human serum could (after washing) be shown to a titre of 5000, which (see above) was a lower titre than found in the usual homogeneous RPM using the same antibody coupled to enzyme-treated sheep red cells.

The next experiment showed that in the acute stage of *M. pneumoniae* infection, some of the sera IgM which could be captured by anti-IgM coupled E6 bovine red cells had antigen (i.e. *M. pneumoniae*) specificity. This was shown by adding an *M. pneumoniae* antigen preparation to the cell suspensions with captured IgM. In performing a test, anti-μ coupled E6 bovine red cells were added to dilutions of patients' sera, from 1 in 10 in a tenfold dilution series. After a short incubation, the red cells were centrifuged, washed once in PBS and then added as a 1% suspension to the *M. pneumoniae* antigen. Strong haemagglutination was seen up to a 1 in 10000 dilution of the serum. The optimal *M. pneumoniae* antigen dilution was 1 in 10; but agglutination was found up to a dilution of 1 in 1000. No agglutination was found in the absence of antigen.

Table 1 records the results of red-cell IgM-antibody capture tests with known positive and negative sera and six sera tested blind. Results using the μ-capture ELISA are also recorded and show good agreement. Also shown are results of assays of total IgM in the eight sera using both the mixed two-stage RPH described above and the usual homogeneous RPH assay. It is clear that the antigen-specific IgM detected is not related to the assay for total IgM (non-antigen-specific).

Comparative tests using the red-cell IgM-capture system and ELISA μ-capture assay

Following these encouraging results, stored sera previously tested by the μ-capture ELISA (Wreghitt & Sillis, 1987) were tested by the red-cell IgM-antibody capture-assay at dilutions of 1 in 40, 1 in 160, 1 in 640, 1 in 2500 and 1 in 10000. The results obtained with the two test procedures are shown in Fig. 3, where it can be seen that there is good agreement between the results of the two assays, and that the two tests have similar sensitivity.

There were three serum samples taken from one patient at various times after

Table 1. *Preliminary 'blind test' on six serum samples for* Mycoplasma pneumoniae*-specific IgM using the red-cell IgM-antibody capture assay*

	μ-Capture ELISA test antibody units	Red-cell IgM-antibody capture assay titre	Usual homogeneous RPH† titre	Mixed two-stage RPH* titre
Serum 1	> 33U	> 10000	640000	10000
Serum 2	> 3.3U	1000	160000	2500
Serum 3	0	< 10	640000	10000
Serum 4	0	< 10	160000	2500
Serum 5	0	< 10	10000	160
Serum 6	> 3.3U	500	640000	2500
Known negative	0	< 10	40000	1000
Known positive	> 33U	> 10000	160000	2500

* RPH, Reverse passive haemagglutination for IgM in serum. The usual RPH where antibody-linked enzyme-treated sheep red cells are added to dilutions of the ligand is a homogeneous reaction with no washing step. The two-stage test was where antiglobulin (μ-specific)-linked enzyme-treated sheep red cells were added to E6 bovine red cells with 'captured' IgM and which had also been washed. This test was done to correspond to the red-cell IgM antibody capture assay.

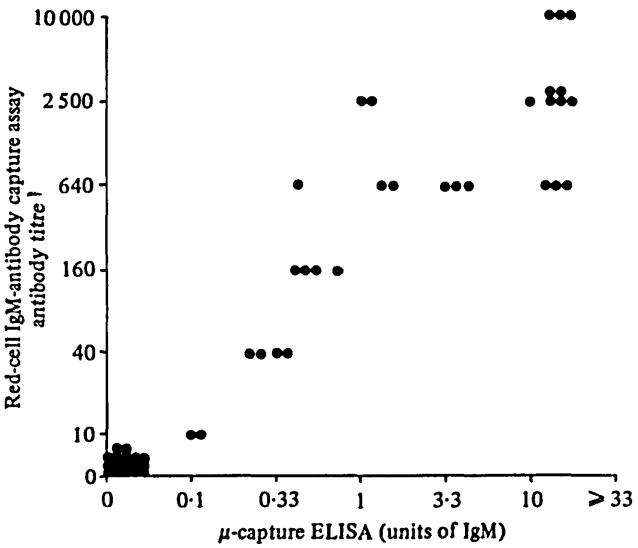

Fig. 3. Correlation between red-cell IgM antibody capture titre and μ-capture ELISA units of IgM for 47 sera from patient with clinical features that suggested *M. pneumoniae* infection.

M. pneumoniae infection, which agglutinated the control cells to a titre of 640 (where PBS was used instead of antigen). These sera were excluded from Fig. 3 because their true titre could not be established.

The results of μ-capture ELISA and red cell IgM antibody capture tests on sera taken at various times after proven *M. pneumoniae* infection are shown in Fig. 4.

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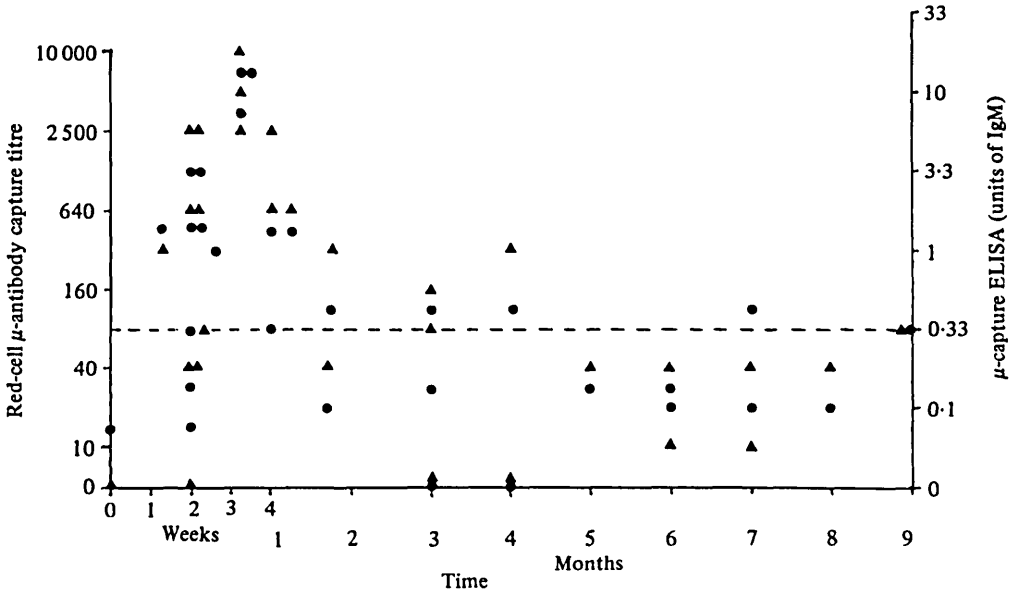

Fig. 4. Amount of *M. pneumoniae*-specific IgM detected by red-cell IgM-antibody capture test (▲) and μ-capture ELISA (●) in sera taken at various times after proven *M. pneumoniae* infection. Dotted line indicates positive cut-off values.

It can be seen that *M. pneumoniae*-specific IgM was detected for a similar time after infection in both assays (approximately 4–6 months).

Capturing antibodies coupled to the red cells were specific for human IgM and as would be expected it could be shown that prior treatment of the patient's serum under test with dithiothreitol (DTT) – a disulphide bond reducing agent – ablated the reaction. The sera were treated with either 30, 20 or 10 mM (DTT) at 37 °C for 40 min. Even the 10 mM concentration removed all activity. Similar treatment of an IgG monoclonal antibody to *Chlamydia psittaci* did not remove its demonstration in a corresponding antigen-specific IgG red-cell capture assay.

The specificity of the reaction was shown with tests on acute stage sera and homologous antigens from patients with mycoplasma infection, psittacosis and toxoplasmosis – the results with the latter two infections will be reported.

DISCUSSION

We have shown that the red-cell IgM-antibody capture test for the detection of *M. pneumoniae*-specific IgM compares very favourably with the μ-capture ELISA described by Wreghitt & Sillis (1985). The tests had comparable sensitivity and detected *M. pneumoniae*-specific IgM for a similar time after proven infection. The amount of *M. pneumoniae*-specific IgM was greatest in the early convalescent period, with amounts of antibody declining up to 7 months after proven infection but only small amounts of antibody could be detected after this period. Thus the amount of *M. pneumoniae*-specific IgM correlated with the length of time after infection. By contrast, it has been found (Van Griethuysen *et al.* 1984; Wreghitt

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& Sillis, 1985) that *M. pneumoniae* IgM can be detected for up to a year after infection by indirect ELISA which makes this latter test less useful diagnostically.

The red-cell IgM-antibody capture test is simple to perform and does not require expensive washing or reading equipment. The test takes approximately 1 h to perform in comparison with the μ-capture ELISA which takes 24 h. Thus, the red-cell IgM-antibody capture test is amenable to rapid diagnosis, which is important since *M. pneumoniae* infection responds to antibiotic therapy.

We have shown that the red-cell IgM-antibody capture test specifically detects only human IgM antibody and that the captured IgM antibody can be inactivated with DTT. We have so far not investigated why the three serum samples from one patient gave non-specific titres of 640 with control cells.

We believe this test may be a valuable asset in the rapid diagnosis of *M. pneumoniae* infection and preliminary tests have shown that the system is amenable to the detection of IgM to other human pathogens such as *Chlamydia psittaci* and *Brucella abortus*. It should, therefore, be possible in the near future to test for specific IgM to a range of microbial pathogens causing human disease in order that rapid antibiotic therapy can be administered.

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