AN IMPROVED METHOD FOR DETECTION AND TITRATION OF ANTIBODIES CYTOPHILIC FOR MACROPHAGES

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A new microtechnique for detection and titration of antibodies cytophilic for homologous macrophages is introduced and experiments designed to determine the optimum conditions for its performance reported. The technique employs a series of small chambers formed on a microscope slide by the application of a plastic film in which holes have been punched. The procedure uses $5 \times 10^4$ cells and 25 μl of serum per chamber, and each test can be completed in 2 h. A permanent preparation results which can be examined as and when convenient.

INTRODUCTION

In 1960, Boyden and Sorkin first observed that normal rabbit spleen cells, when incubated with a rabbit anti-human serum albumin, became coated with antibodies that resisted removal by washing. These cells were shown to have antibody on their surface by their capacity specifically to adsorb $^{131}$I-HSA. However this method did not identify which type of spleen cell was adsorbing the antibody. Boyden (1964) showed that glass adherent peritoneal cells (i.e. macrophages), after exposure to anti-sheep red blood cell serum and washing, would form rosettes with sheep red blood cells (SRBC). It was evident that antibodies had become adsorbed to the macrophage surface and these he termed 'macrophage cytophilic antibodies'. Boyden carried out his procedure in a culture chamber consisting of a coverslip set in a hole in a thick perspex slide. Modifications of Boyden's culture chamber have been described in several reports of studies on macrophage cytophilic antibodies. Berken and Benacerraf (1966) used lucite rings fixed onto 22 mm square glass coverslips. Tizard (1969) modified this technique by forming the monolayer on a microscope slide within a wax ring. Mittal (1972) carried out the test on cavity slides.

This paper introduces a microtechnique for the detection of macrophage

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cytophilic antibodies that enables a large number of sera to be screened for these antibodies in identical conditions on one slide, or the titration of several sera to be carried out in identical conditions at one time. The technique also lends itself to investigation of the optimum conditions for the detection of these antibodies and some investigations to this end are also reported here.

MATERIALS AND METHODS

Microscope slide chambers

Twelve chambers are formed on each microscope slide (acid washed) by the application of a suitable sized piece (70 X 30 mm) of self-adhesive plastic film, into which 5-mm diameter holes have been punched (fig. 1). The plastic film found appropriate for this purpose was Fablon ‘design 115’ (Commercial Plastics Ltd., London, England). Other types of film, by the same and other manufacturers, and other materials, e.g. self-adhesive paper labels, nail polish rings, reinforcements, siliconization of a surface of the slide with wax resistants, etc., all allowed spreading of the cell suspension and sera so that interaction occurred between wells. The formation of single wells with paraffin wax on microscope slides (Tizard, 1969) was found to use large volumes of serum; also immediate microscopic observations were necessary as permanent preparations could only be made by extensive manipulation.

Serum

Guinea pig anti-SRBC serum was used in the experiments done to determine optimum conditions for the method reported here. Three groups each consisting of three guinea pigs were immunized with a single dose of $6 \times 10^8$ SRBC per ml either in Freund’s complete adjuvant containing *Mycobacterium tuberculosis* strain C, Weybridge, 1 mg/ml; or in Freund’s complete adjuvant containing *Corynebacterium parvum* 1383 (kindly provided by G.J. O’Neill, Department of Bacteriology and Immunology, Glasgow), 500 μg/ml;
or in Freund's incomplete adjuvant. The adjuvant was prepared by the method described by Herbert (1973). Each guinea pig received 0.5 ml of the emulsion intramuscularly. Serum was collected 7, 21 and 28 days later. On the 22nd day the animals were boosted by an intradermal injection of 0.1 ml of 1 × 10^7 SRBC per ml in 0.85% NaCl solution. Normal serum was collected from the experimental animals before antigen injection.

**Peritoneal cells**

A normal, unimmunized, guinea pig was bled out under ether anaesthesia. The peritoneal cavity was then rinsed with 50 ml of tissue culture medium, kept at room temperature, containing 100 µg streptomycin/ml, 100 units penicillin/ml, and 10 units heparin/ml (Evans Medical Ltd.). Standard tissue culture medium consisted of Dulbecco's medium with 1% MEM non-essential amino acid solution, 0.5% 200 mM L-glutamate solution (Bio-cult Laboratories Ltd.), 0.4% glucose, and brought to pH 6.8 with 4% NaHCO₃. The cell suspension obtained was collected in siliconized containers held in an ice-bath, and then centrifuged at 250g for 10 min. The cells were re-suspended in 1.0 ml of medium and held in an ice-bath whilst a cell count was made. For most experiments the suspension was then adjusted to contain 2 × 10^6 cells per ml.

**Macrophage monolayers**

Each chamber was filled with 25 µl of cell suspension, then placed in a moist atmosphere (Petri dish with damp filter paper) and incubated at 37°C in an atmosphere of air and 5% CO₂. After 30 min incubation the supernatants were removed with a Pasteur pipette and each chamber washed once with tissue culture medium at room temperature.

**Binding of cytophilic antibodies to macrophages**

Neat serum or dilutions of the serum were added to the macrophage monolayers in volumes of 25 µl. The chambers were then incubated in a moist atmosphere at 4°C for 30 min in air. The supernatant was then removed and the cells washed five times with medium. Chilled medium was used for the first washing followed by medium at room temperature.

**Rosette formation**

Each chamber was filled with 25 µl of a 0.5% (PCV) suspension of SRBC in medium. They were then incubated in a moist atmosphere in air at room temperature for 30 min. After incubation they were thoroughly washed by dipping the whole slide into a 250 ml beaker containing medium followed by a beaker containing phosphate buffered saline (PBS) pH 7.2, 0.15 M. The
plastic film was then removed and discarded. The cells were washed again with PBS to remove SRBC that might have been trapped at the edges of the wells. The slides were then fixed either in absolute alcohol for at least 1 h (or overnight) or in 2% glutaraldehyde in PBS for 1 h and stained with Giemsa or Leishman's stain for 30 min. Finally the microscope slide was covered with a thin layer of mounting medium and a 22 × 60 mm coverslip applied so that all 12 monolayers were covered.

It is important to employ care with regard to certain technical points in the method described: the cells should be washed with medium that is at the same temperature as that at which the cells are incubated. They must be covered with fluid at all times during the procedure, to maintain optimal conditions and to avoid non-specific spreading or death.

Scoring the results

Two hundred macrophages were examined in each chamber under the high power of the microscope (40X). The results of the test were expressed in terms of the percentage of macrophages participating in the cytophilic antibody activity (CA%), i.e. showing complete or partial rosettes. The total number (divided by 2) of individual SRBC found to be attached to the 200 macrophages was referred to as the 'cytophilic antibody index' (CAI). The methods by which this index may be obtained is discussed later and shown

<table>
<thead>
<tr>
<th>Group: (SRBC attached to macrophage)</th>
<th>Number of macrophages examined</th>
<th>SRBC adhering to the macrophages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal serum</td>
<td>Test serum</td>
</tr>
<tr>
<td>0—1 (0) *</td>
<td>199</td>
<td>77</td>
</tr>
<tr>
<td>2—4 (3) **</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>5—7 (6)</td>
<td>37</td>
<td>55</td>
</tr>
<tr>
<td>8—10 (9)</td>
<td>18</td>
<td>222</td>
</tr>
<tr>
<td>11+ (11)</td>
<td>8</td>
<td>88</td>
</tr>
<tr>
<td>Total</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>CAI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCAI</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCAI (log 10)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Multiplication factor used is 0, thus random attachment of single cells is ignored.
** Factor (i.e. mean number of cells (SRBC) attached to a macrophage) used to multiply the number of macrophages examined so as to obtain total adherent SRBC. CAI = cytophilic antibody index, SCAI = specific cytophilic antibody index.
in table 1. ‘Specific cytophilic antibody index’ (SCAI) was the ratio of CAI in the test serum to CAI in the control serum. The following formulae therefore apply: \( \text{CA\%} = \left( \frac{\text{number of macrophages with SRBC rosettes}}{\text{total number of macrophages counted (200)}} \right) \times 100 \); \( \text{CAI} = \frac{\text{total number of SRBC attached to 200 macrophages}}{2} \); \( \text{SCAI} = \frac{\text{CAI test serum}}{\text{CAI control serum}} \). It has been found convenient to express the SCAI in the \( \log_{10} \) form.

RESULTS

Optimum conditions for formation of the macrophage monolayer

A monolayer was required in which the cells were well separated from each other so that rosettes formed round each could be easily distinguished and enumerated. On the other hand, sufficient cells were required to ensure that a statistically adequate count could be made. Investigations to determine a suitable concentration of cells in suspension and time to be allowed for attachment to the glass were therefore carried out.

Four different concentrations of cells, increasing by ten-fold steps, from \( 2 \times 10^5 \) cells per ml to \( 2 \times 10^8 \) cells per ml were examined. Two chambers for each concentration at each time interval were prepared. A total of 10 chambers were filled with each concentration of cells (i.e. 2 chambers on each of 5 microscope slides), and incubated at 37°C in a moist atmosphere with 5% \( \text{CO}_2 \). At intervals of time up to one hour one of the slides was removed from incubation and the monolayers washed, fixed and stained. The appearance of each monolayer and its suitability for rosette formation was then scored on an arbitrary scale. High concentrations of cells and long incubation times produced unsuitable multilayered clumps of cells. However, when similar high concentrations were incubated for a short period, only a small proportion of the cells present became attached to the glass, forming a monolayer of the type desired. Conversely, low concentrations of cells incubated for 60 min also produced a good monolayer. For convenience in all subsequent experimentation a concentration of cells that gave a suitable monolayer after 30 min incubation was chosen, i.e. \( 2 \times 10^6 \) cells per ml.

Determination of the optimum time intervals required for cytophilic antibodies to bind to macrophage monolayers and for rosettes to form

Thirty macrophage monolayers were incubated with guinea pig anti-sheep red blood cell serum at 4°C. After an initial 20 min incubation and every 10 min thereafter up to 60 min, five monolayers were removed. These were then washed, a suspension of SRBC applied to each, and incubated at room temperature. After each 10 min of incubation one monolayer was removed, washed, fixed and stained. Macrophages bearing SRBC were first observed after 30 min incubation with serum and 10 min incubation with SRBC (fig.
2A). Maximum rosette formation occurred after 30 min incubation with serum and 30 min incubation with antigen (fig. 2B and 2C). Incubation for longer periods than these did not materially increase the CAI as shown in fig. 3.

Determination of the 'specific cytophilic antibody index' (SCAI)

Twenty-four macrophage monolayers were incubated with the test serum, or normal serum, or medium (two monolayers per sample), and rosettes formed in the manner described above. Two methods of scoring the results (one faster than the other) were compared; 200 macrophages in each monolayer being counted. The macrophages were grouped, by the number of SRBC rosetting, either in 11 groups, i.e. SRBC adhering to a single macrophage, or in 5 groups as indicated in table 1. The percentage of macrophages showing rosettes in each group was obtained and the CAI was found by obtaining the total number of SRBC adhering to the two hundred macrophages

Fig. 2. Macrophages bearing sheep red blood cells via specific cytophilic antibodies. Rosettes were formed on macrophage monolayers by incubating the cells with guinea pig anti-SRBC serum for 30 min, and incubation with the antigen (SRBC) for 10 min (A), or 30 min (B, and C). × 1500.
examined. The ratio of the CAI in the test serum to the CAI in the normal serum was expressed as the SCAI. If, in the normal serum, no SRBC rosettes are observed, then the CAI is equal to the SCAI in the test serum.

Similar results for the SCAI were obtained whether the rosettes were counted in 11 groups \( \log_{10}\text{SCAI} = 1.477 \pm 0.24 \) or in 5 groups \( \log_{10}\text{SCAI} = 1.493 \pm 0.233 \) for eight different guinea pig anti-SRBC sera. Although counting the macrophages in 5 groups gave slightly higher SCAI, the differences were not significant at 5% level \( P > 0.05 \) by Student's 't' test. Therefore to save labour the method of scoring the macrophages in 5 rosetting groups, i.e. 0—1, 2—4, 5—7, 8—10 and 11+, attached red cells, was adopted.

**Screening for cytophilic antibodies**

One of the main reasons for developing the method described here was to apply it for routine screening for cytophilic antibodies in the sera of either immunized or infected experimental animals. An example is reported in table 2. For this purpose, 24 macrophage monolayers on two microscope slides were incubated with neat sera from guinea pigs that had been immunized with SRBC in different adjuvants. The procedure followed was that described in the Materials and Methods section. From the results obtained (table 2) it is apparent that only three sera, i.e. those with a SCAI \( \log_{10} \) greater than 2.0 have high levels of antibody and are worth examining further. The experiment was valuable as it rapidly enabled us to show the
TABLE 2
Screening for cytophilic antibodies in sera of guinea pigs immunized with sheep red blood cells a.

<table>
<thead>
<tr>
<th>Adjuvant used</th>
<th>Guinea pig number</th>
<th>Specific cytophilic antibody index ($\log_{10}$) e</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7 day serum</td>
</tr>
<tr>
<td>M. tb. b</td>
<td>6B</td>
<td>1.77</td>
</tr>
<tr>
<td></td>
<td>6R</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>6W</td>
<td>0.00</td>
</tr>
<tr>
<td>C.p. c</td>
<td>7B</td>
<td>2.60</td>
</tr>
<tr>
<td></td>
<td>7R</td>
<td>1.79</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>FIA d</td>
<td>8B</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>8R</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>8W</td>
<td>0.00</td>
</tr>
</tbody>
</table>

a The guinea pigs received an intramuscular injection of SRBC emulsified in a water-in-oil emulsion containing either b Mycobacterium tuberculosis or c Corynebacterium parvum in the oil phase, or d in Freund's incomplete adjuvant (details indicated in Methods section).
e The SCAI ($\log_{10}$) is the mean of duplicate determinations of undiluted serum.

Early appearance, at 7 days, of macrophage cytophilic antibodies in the sera of guinea pigs immunized with SRBC emulsified in water-in-oil emulsion containing Corynebacterium parvum.

The titration of macrophage cytophilic antibodies

The technique is suitable for titration of macrophage cytophilic antibody in a given serum, by obtaining the end-point of its activity in a dilution series. Tests were therefore carried out, in duplicate, by exposing a series of macrophage monolayers to doubling dilutions of the three guinea pig anti-SRBC sera noted above. After subsequent exposure to the antigen the SCAI was calculated for each chamber. The serum titre was taken as the serum dilution with SCAI ($\log_{10}$) of 1.7 to 2.0, i.e. hatched area in fig. 4.

When the results were plotted the slopes did not indicate significant deviation from linearity (fig. 4) as shown by the least squares regression lines. It is probable that the slopes of the curve depend on the avidity with which the antibodies can bind to the macrophages. This is therefore a suitable method for titrating cytophilic antibody activity as it is probable that saturation of macrophage receptors occurs at high concentration.

DISCUSSION

The present study was undertaken in an attempt to improve existing techniques for studying antibodies cytophilic for macrophages. The experiments
reported enabled optimum cell concentrations for formation of the macrophage monolayers to be determined and also suitable periods of time for coating the cells with antibody and allowing the rosettes to form (fig. 3).

This microtechnique has been found to produce macrophage monolayers of very consistent composition. This is probably because cells sedimenting in so small a volume all come into contact with the glass surface at about the same time. Thus they are either exhibiting a similar degree of attachment at the time of the first wash, or a consistent sub-population of the total population of peritoneal cells is selected.

The technique lends itself to the screening of a number of sera for macrophage cytophilic antibody, and to the titration of one or more sera and determination of the avidity of the binding of the antibodies to the macrophages. Each slide has sufficient chambers for these purposes and all are treated consistently throughout all the operations necessary, so that random variations from chamber to chamber are largely obviated.

The use of the technique for the detection of macrophage cytophilic antibodies to sheep erythrocytes is reported here. It has also been used to study cytophilic antibodies in animals of different species, immunized with soluble antigens, or suffering from experimental listeriosis (R. Vasquez, in preparation) and trypanosome infections (Vasquez et al., 1975). Additionally it has been used in the detection of antibodies cytophilic for human leucocytes (R. Vasquez, in preparation).

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REFERENCES

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