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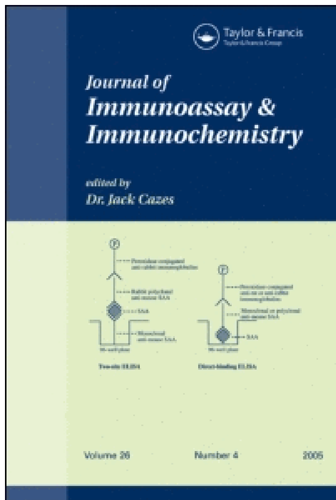

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IDENTIFICATION OF IMMUNOGLOBULIN CLASS AND SUBCLASS OF MOUSE MONOCLONAL ANTIBODIES TO HUMAN CELL SURFACE ANTIGENS BY MIXED HEMADSORPTION ASSAY

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Key Words: Monoclonal Antibodies
Ig Class and Subclasses
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ABSTRACT

A microassay is described to determine immunoglobulin Ig class or IgG subclass of mouse monoclonal antibodies by mixed hemadsorption assay. Monoclonal antibody bound to adherent target cells is reacted with serial dilutions of a panel of class or subclass specific rabbit anti-mouse Ig antisera and binding of the latter is traced by anti-rabbit globulin-coated indicator erythrocytes. The class or subclass of the bound monoclonal antibody is revealed by preferential binding of the corresponding rabbit antibody. Unlike gel immunodiffusion analysis, the mixed hemadsorption assay may be performed with unconcentrated hybridoma culture supernatants.

INTRODUCTION

Monoclonal antibodies produced by hybridomas resulting from fusion of mouse myeloma cells and antibody producing splenocytes (1) are currently widely used in many laboratories. Extensive immunochemical studies of mouse heavy chain classes have led to the association of particular biological

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activities with each Ig class or subclass (2). For this reason it may be desirable to determine or even select for Ig class or IgG subclass during the early stages of cloning and establishment of hydridomas.

We have recently established a number of mouse hybridomas producing monoclonal antibodies against various human melanoma surface antigens (3,4,5). In this report, we describe an assay modified from the conventional mixed hemadsorption technique (6-8) to determine Ig class or IgG subclass using minute amounts of unconcentrated culture supernatants of the hybridomas. This assay is simple, reproducible and sensitive, and is independent of the ability of Ig to bind complement or protein A.

MATERIALS AND METHODS

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Mouse Monoclonal Antibodies

Seven mouse monoclonal antibodies recently developed in our laboratory by the hybridoma system (1) were used in this study. The designations and serological specificities as well as the titres against the immunizing melanoma cell line, CaCL 78-1, determined by the direct mixed hemadsorption assay (3,9) of the first six monoclonal antibodies are listed in Table 1. The methods used for the production of these antibodies have been described elsewhere (3-5). The seventh monoclonal antibody 214.1AP1 is the product of a hybridoma formed by fusion of the mouse P3-NS1-Ag4 (see below) and splenocytes from a BALB/c mouse immunized with cultured human erythroleukemia cells (K-562). This antibody is directed against a determinant present on fetal calf serum (FCS).

Cell Lines and Culture Methods

The human melanoma cell line, CaCL 78-1, was established in our laboratory from a lymph node metastasis and maintained as a monolayer in Eagle's minimum essential medium (MEM) containing 10% heat-inactivated FCS (10).

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TABLE 1

Mouse Monoclonal Antibodies Used in this Study

Monoclonal antibody	Titre (reciprocal) against immunizing human melanoma line CaCL 78-1[a]	Specificity
87.20	640	Species specific antigen
7.51	2,560	Fetal neuroectodermal antigen
7.60	320	Fetal neuroectodermal antigen
140.72	51,200	Melanoma/carcinoma cross-reacting oncofetal antigen
140.240	2,560	Melanoma-restricted oncofetal antigen
140.96	1,280	Species specific antigen
214.1AP1	2,560	Fetal calf serum related antigen

[a] Supernatants of hybridoma cultures were tested by mixed hemadsorption direct assay (3).

The mouse myeloma cell line P3-NS1-Ag4 (NS1), originally established by Dr. C. Milstein (Medical Research Council Laboratory, Cambridge, England) was kindly obtained from Dr. R. Kennett, University of Pennsylvania, Philadelphia, PA. The culture was maintained in alpha-medium containing 10% heat-inactivated FCS (3).

Rabbit Antisera to Mouse Ig Classes and Subclasses

Rabbit anti-IgG_1, anti-IgG_{2a}, anti-IgG_{2b}, anti-IgG_{2b}, anti-IgG_3, anti-IgG and anti-IgM were obtained from Litton Bionetics Inc., Kensington, MD. These antisera were prepared by immunizing rabbits with highly purified antigens as evaluated by polyacrylamide gel disc electrophoresis for biophysical homogeneity and immunoprecipitation analysis for antigenic purity. The antisera were rendered "monospecific" by solid phase immunoadsorption to yield products free of soluble immune complexes and unwanted immunoadsorbent.

Mouse Immunoglobulins

Mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, and IgM purchased from Litton Bionetics Inc. were prepared from purified mouse myeloma proteins.

Mixed Hemadsorption Assay

Monoclonal antibodies to adherent target cells were detected by the mixed hemadsorption assay adapted for small volumes of reagents (9). The binding of antibody to the target cells is traced by adherence of sheep red blood cells (SRBC) coated with rabbit anti-mouse Ig antibody (polyspecific). For identification of Ig class or IgG subclass of the bound monoclonal antibody, the procedure has been modified as outlined in Figure 1.

Mouse monoclonal antibody in 10 µl aliquots at a predetermined dilution (4 doubling dilutions above that giving 50% positive cells in mixed hemadsorption tests against the immunizing human melanoma line, CaCL 78-1) was added to duplicate wells of microtest plates (No.3034, Falcon Plastics, Oxnard, CA) and incubated for 1 hour at room temperature. Each well had been seeded with sufficient cells to yield 100 adherent target cells at the end of

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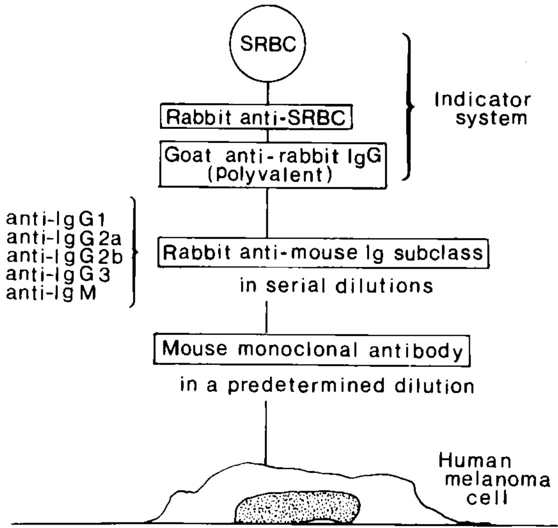

Figure 1. Principle of the mixed hemadsorption assay for identification of Ig class or IgG subclass of mouse monoclonal antibody. A complex chain of antigen-antibody reactions represented diagramatically is produced in order to detect the second link from the target cells in the chain.

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18-24 hours of incubation at 37°C. After three washes with PBS containing 0.2% gelatin, 10 µl of serially diluted rabbit anti-Ig subclass antiserum was added to each well and incubated for 1 hour at room temperature. After washing as above, 10 µl of sensitized SRBC suspension was added to each well and incubated for 1 hour at room temperature. Unbound SRBC were washed off and the plates examined microscopically and scored for the percentage of target cells with 5 or more adherent SRBC.

Preparation of the indicator SRBC for the detection of antibody of rabbit origin has been detailed in a previous communication (8).

Blocking Experiments

Rabbit anti-mouse Ig subclass antiserum in 100 µl aliquots at a predetermined dilution (see above) was absorbed with increasing amounts of each purified IgG subclass or IgM protein at room temperature for 1 hour and

at $4^{o}C$ for 2 hours. Absorbed as well as unabsorbed rabbit antisera were added to wells containing target cells and mouse monoclonal antibody, and bound rabbit antibody detected as described above.

RESULTS

The Ig subclass of seven mouse monoclonal antibodies was determined by the preferential binding of one of the rabbit antisera specific for mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 or IgM to target cells precoated with the monoclonal antibody. In each case, one of the rabbit antisera to mouse IgG subclasses gave 16- to 640-fold greater reactivity than the rest, and the most reactive rabbit anti-IgG subclass antiserum reacted with the monoclonal antibody to a similar titre as the rabbit anti mouse IgG (polyvalent) (Table 2). In addition, this assay is capable of detecting monoclonal antibodies of the IgM class. Normal rabbit serum in place of rabbit anti-mouse Ig failed to show any reactivity (data not shown). When myeloma NS1 culture supernatant or normal mouse serum in place of monoclonal antibodies was similarly tested, no binding of the rabbit antisera was observed. It is concluded therefore that monoclonal antibody 87.20 belongs to IgG_1, 140.96, IgG_1; 7.51, IgG_1; 7.60, IgG_{2a}; 140.72, IgG_1; 140.240, IgG_{2a} and 214.1AP1, IgM.

To confirm the specificity of the isotype antisera, the rabbit anti-mouse IgG subclass antibody was absorbed with increasing amounts of different purified mouse IgG subclass or IgM proteins prior to testing on monoclonal antibody bound to the target cells. Representative results are shown in Figure 2a, b and c. Only the mouse IgG subclass corresponding to the specificity of the previously determined most reactive rabbit subclass antiserum efficiently eliminated the reactivity of that antiserum.

DISCUSSION

The present studies have shown that the mixed hemadsorption assay can be used for the identification of Ig class and IgG subclass of mouse monoclonal

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TABLE 2

Determination of Ig Class or IgG Subclass of Mouse Monoclonal Antibodies By Mixed Hemadsorption Assay[a]

Monoclonal Antibody[b]	Antibody titre (reciprocal) of rabbit anti-mouse Ig Subclass Antiserum Bound to Mouse Monoclonal Antibody					
	Anti-IgG_1	Anti-IgG_{2a}	Anti-IgG_{2b}	Anti-IgG_3	Anti-IgM	Anti-IgG
87.20	102,400[d]	100	800	100	<100	102,400
7.51	102,400	100	50	50	<50	102,400
7.60	20	320	20	<5	<5	NT[c]
140.72	1,638,400	100	800	800	<100	409,600
140.240	100	1,638,400	12,800	800	<100	819,200
140.96	204,800	100	800	100	<100	102,400
214.1AP1	<5	5	20	20	2,560	20
Normal mouse serum	<5	<5	<5	<5	<5	<5

a Tested against the human melanoma line CaCL 78-1

b Culture supernatant at a predetermined dilution (See Materials and Methods)

c Not tested

d Underline indicates Ig class or IgG subclass designation of monoclonal antibody

antibodies. The results obtained by direct testing were confirmed by gel immunodiffusion (data not shown). It must be stressed that precipitation lines in gel immunodiffusion can only be achieved with some of the mouse monoclonal antibodies following 15- to 20-fold concentration of culture supernatants. Similarly not all antibodies derived from ascites fluid gave precipitation bands in gel immunodiffusion with rabbit anti-mouse subclass antisera. The ability to identify the Ig class or IgG subclass of the monoclonal antibody at an early stage in the screening process will be useful, when antibody of a particular class or subclass is being sought for its biological function, eg. IgM and IgG_{2a} for complement fixation.

The detection system described in this report clearly identifies the Ig class or IgG subclass of a given antibody preparation. The fact that minimal cross-reactivity is seen among rabbit anti-IgG subclass antisera may reflect minor contamination of these antisera. Thus the method described cannot be used to verify the monoclonality of a hybridoma antibody. The presence of minor contamination of rabbit antisera or mouse subclass proteins may explain the inhibitory activity of IgG_{2b} on IgG_1 antiserum and IgG_{2b} on IgG_{2a} antiserum (Figure 2b and c). Alternatively, minor cross-reactivity among IgG subclasses may indeed exist, based on the analysis of structure of mouse gamma-subclass chains showing that these proteins are more closely related to one another than to other heavy chains (11-13).

The assay procedure described in this paper is simple and reproducible, and requires only minute amounts of unconcentrated supernatant of hybridoma cultures. Furthermore, it offers a number of other advantages over some reported techniques such as antibody mixed hemadsorption (14), anti-C3-mixed hemadsorption (14), immune adherence (14), Staphylococcal protein A assay (14,15) radioimmunoprecipitation (16), and radioimmunoassay (17). Our technique can be performed without consideration of whether the mouse Ig class or IgG subclass has the capacity to bind complement or protein A. In contrast, most of the other assays are based on binding ability of the antibody to complement or protein A, and have disadvantages in that IgG_1 has

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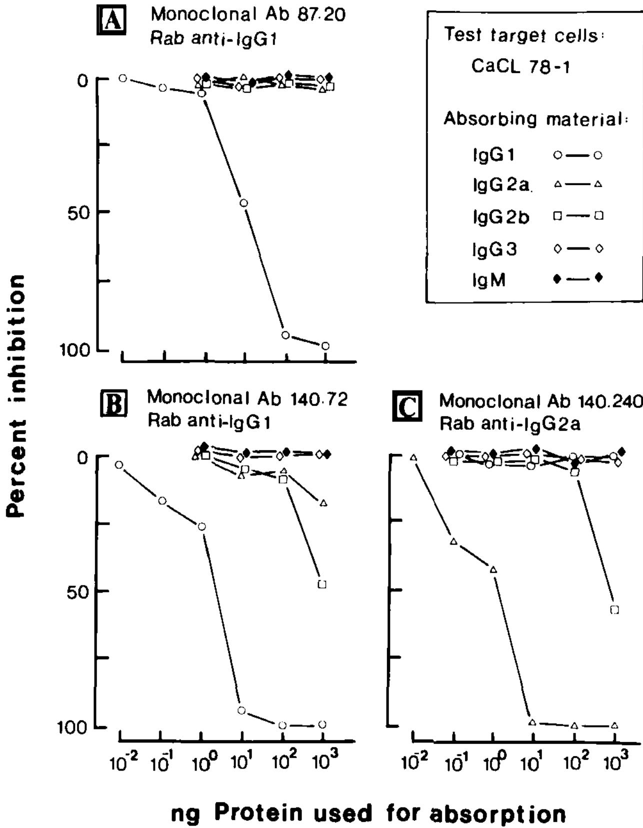

Figure 2. Blocking experiments to confirm the specificity of rabbit anti-mouse IgG subclass antiserum reacting with monoclonal antibody. The amounts of protein for absorption as indicated were absorbed with 100 μl of rabbit anti-Ig 100 μl subclass antiserum, at a predetermined dilution. For anti-IgG in A was 1:5,000, for anti IgG_1 in B, 1:40,000; and for anti IgG_{2a} in C, 1:5,000.

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a low affinity for complement and protein A; IgG_3 has a weak affinity for complement with relatively strong binding to protein A; and IgM has a weak affinity for protein A (2,15). Finally, for investigators who wish to avoid the cost and biohazard of radiolabeled material, our modification of the mixed hemadsorption provides a simple and sensitive alternative system.

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