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A Simple Method for Detection of Monoclonal Isotypes

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ABSTRACT

A simple rapid method of enzyme labeled anti-isotype assay (ELIA) for detection of monoclonal isotype on hybridoma cells is proposed. This alternative method was first carried out on hybridoma cell lines 147C11 and 257C11 produced against *Trypanosoma cruzi* and male accessory secretion of *Panstrongylus megistus,* respectively. The monoclonal antibodies produced by these hybridoma were characterized by this method as IgM (147C11) and IgG1 (257C23) isotypes, allowing evaluation of isotype without having to wait until the concentration of antibody present in the supernatant itself rises. Results were confirmed by Ouchterlony immunodiffusion. The proposed method offers the advantages of a permanent rapid procedure for light microscopy.

Index Entries: Monoclonal isotypes; characterization of monoclonal isotypes; monoclonal antibody.

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INTRODUCTION

Kohler and Milstein (*1*) demonstrated that the fusion of antibody-secreting B lymphocytes with a mouse myeloma cell line can result in a hybridoma that continuously secretes monoclonal antibodies of predefined specificity. Since then, the hybridoma approach has been extensively used for research and diagnostic purposes. Immunoparasitology provides a good example of the use of this technology (*2–5*).

According to Nisonoff (*6*) some of the most important uses of hybridomas are:

1. To obtain large amounts of antibody that would otherwise be available only in small quantities;
2. To obtain monospecific antibodies, which are directed against a single epitope on an antigen molecule;
3. To obtain pure antibodies against antigens which cannot be purified;
4. To identify or isolate cells by virtue of antigens present on their surface, cytoplasm, and nucleus;
5. Not only can one obtain pure antibody from an unpure antigen, but the monoclonal antibody thus obtained can be used to purify the antigen; and
6. The method provides a continual source of antibodies with invariant properties for the quantitative immunological assays of a variety of antigens.

Therefore, monoclonal antibodies provide investigators with valuable and powerful tools for solving problems previously unapproachable using conventional immunological techniques.

Determination of monoclonal isotype is usually performed by analyzing the supernatant of the clone by either the immunudiffusion method (*7*) or enzyme linked immunosorbent assay-ELISA (*8*). Direct immunofluorescence with conjugated antisera against different isotypes on cytospin preparations from the hybridoma is also used (*9*).

In this report we describe a rapid and easy alternative method (enzyme labeled anti-isotype assay-ELIA) for the detection of monoclonal isotypes utilizing direct immunoperoxidase staining on the clone cells secreting immunoglobulins.

MATERIALS AND METHODS

Hybridomas

Hybridomas were produced using the mouse plasmacytoma cell line Sp2/0-Ag 14 (*10*) with a modification of the fusion method mentioned previously (*1*).

Hybridoma 147C11 was produced by fusing Sp2/0-Ag 14 hybridoma cells with activated spleen cells from BALB/c mice immunized with freeze-thawed and sonicated *Trypanosoma cruzi*, Y strain (*11*), the etiological agent of Chagas' disease.

Hybridoma 257C23 (*12*) was produced by fusing Sp2/0-Ag 14 cells with activated spleen cells from BALB/c mice immunized with the secretory products of the male accessory glands of *Panstrongylus megistus*, the vector of Chagas' disease.

The cell line Sp2/0-Ag 14 a nonsecreting immunoglobulin plasmacytoma cell line was used as a control.

Detection of Antibody Isotypes

Enzyme Labeled Anti-Isotype Assay (ELIA)

Hybridoma cells were harvested and centrifuged at 300g for 10 min, the cells were then resuspended in Tris buffered saline, TBS (10 m*M* tris-HCl pH 7.6 containing 0.15*M* sodium chloride) to 10^6 cells/mL. Fifteen μL of this suspension was applied to each well of a 8 wells slide (INLAB)-that had been previously treated with a gelatine solution (3% gelatine, 0.05% chrome alum in distilled water) and allowed to dry. This solution adheres the cells to the slides. After drying at room temperature (RT), the cells were fixed with acetone (Merck) for 2 min and transferred to TBS pH 7.6. The peroxidase conjugated antibodies against the various isotypes (IgG1, IG2a, IgG2b, IgG3, CALTAG; IgA and IgM, SIGMA) were diluted 1:20 in TBS pH 7.6. Twenty μL of each dilution were added to each well and incubated for 30 min at RT in a humidity chamber. After washing for 3×5 min each, the slides were incubated for 15 min at RT with 0.03% 3,3'-diaminobenzidine (DAB, Sigma), 0,01% H_2O_2 in 0.1*M* PBS pH 7.3. Counterstaining with hematoxylin was optional. Finally, the slides were washed, dehydrated, cleared, and mounted. The results of the immunochemical staining were evaluated by light microscopy.

Ouchterlony Immunodiffusion Method

Ouchterlony immunodiffusion was the method used to confirm the results obtained by the ELIA method. Briefly, acetate membranes (Gel bound, FMC) mounted on slides were precoated with 3 mL 0.9% agarose for electrophoresis (Merck) dissolved in veronal buffer pH 8.2. The gels were punched, making 3 rosettes per slide. Each rosette was composed of one central well and 6 peripheral ones. The distance between central and each peripheral well was 6 mm and the diameter of each well was 2 mm. Five μL of the supernatants of each monoclonal (147C11 and 257C23) and the supernatant of Sp2/0 cell, sixfold concentrated, were distributed in each central well. Five μL of each anti-isotype (IgG1, IG2a, IgG2b, IgG3, IgM, and IgA, SIGMA) diluted 1:4 were distributed in the peripheral wells. After incubation for 48 h at RT in a humidity chamber the slides were washed for 24 h in 0.15*M* NaCl. The slides were then covered

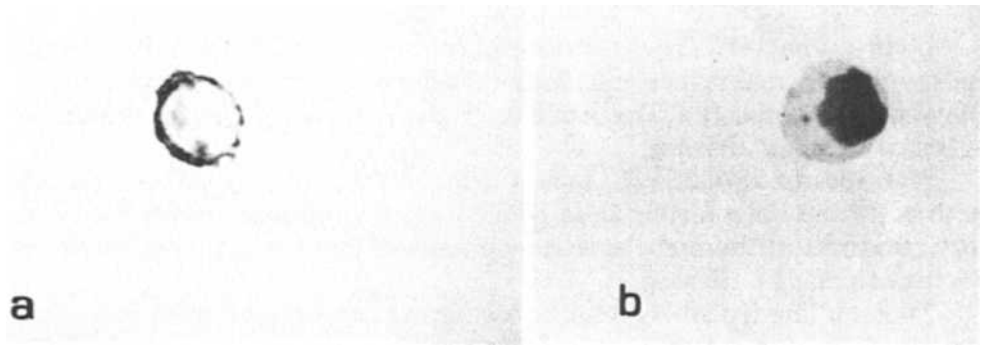

Fig. 1. Immunoperoxidase staining reaction by ELIA method of hybridoma 147C11 line cells. **a** positive cytoplasmic reaction against anti-isotype IgM; **b** positive cytoplasmic reaction was not observed in cells to which antibodies against the others isotypes had been applied.

with filter paper and dried overnight at 50°C. The acetate membranes were removed and stained for 15 min with black starch solution (1 g black starch (Merck), 100 mL methanol (Merck), 20 mL acetic acid (Merck), and 80 mL distilled water). Five percent acetic acid was used for destaining.

RESULTS

Hybridoma 147C11 showed a strongly positive cytoplasmic reaction for IgM indicating the presence of this isotype. The nucleus was not visible (Fig. 1a). This brown cytoplasmatic reaction product was not observed in cells to which antibodies against the other isotypes had been applied (IgG1, IgG2a, IgG2b, IgG3, and IgA)). Following counterstaining hematoxylin the nucleus became visible in both positive and negative cells (Fig. 1b).

In hybridoma 257C23, cytoplasm reacted with anti-isotype IgG1. Figure 2 shows the positive peroxidase reaction alone where the nucleus was not visible (Fig. 2a). This reaction product was not observed when others anti-isotypes were applied (Fig. 2b).

Cells from Sp2/0 cell line treated as above proved consistently negative with all antibodies. Each hybridoma was assayed fourfold, giving consistent results showing good reproducibility.

These results were confirmed by Ouchterlony immunodiffusion, which gave a precipiting line to IgM (hybridoma 147C11) and to IgG1 (hybridoma 257C23) (Fig. 3a,b). A precipitin line was not visualized when the Sp2/0 cell control was used (Fig. 3c).

DISCUSSION

In this work we propose a simple, rapid, and easy method for detection of monoclonal isotypes by using the direct immunoperoxidase technique on the hybridomas secreting immunoglobulins. Direct immuno-

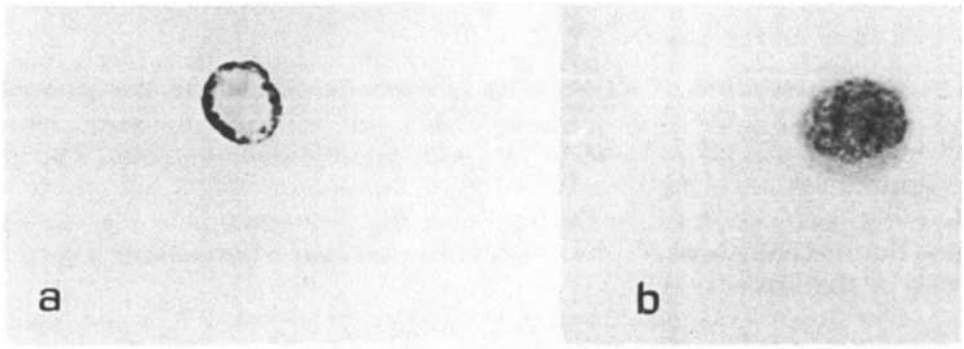

Fig. 2. Immunoperoxidase staining reaction by ELIA method of hybridoma 257C23 line cells. **a** positive cytoplasmic reaction against anti-isotype IgG1; **b** positive cytoplasmic reaction was not observed in cells to which antibodies against the other isotypes had been applied.

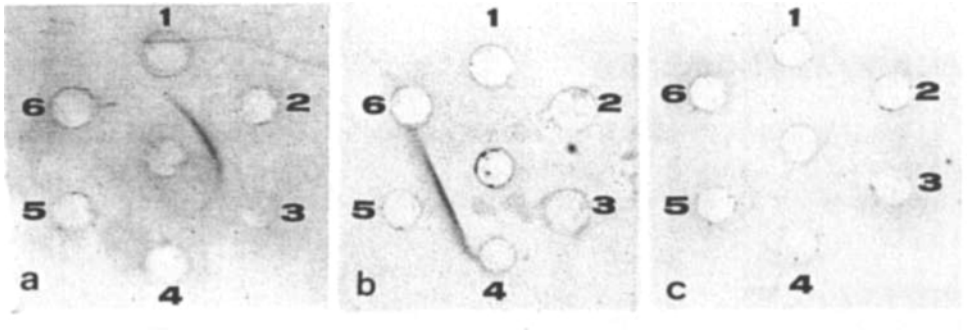

Fig. 3. Immunodiffusion tests of monoclonal antibodies (concentrated culture media) using antisera against mouse Ig isotypes. Peripheral wells **a, b,** and **c: 1** anti-mouse IgG2b serum; **2** anti-mouse IgM serum; **3** anti-mouse IgA serum; **4** anti-mouse IgG3 serum; **5** anti-mouse IgG1 serum; **6** anti-mouse IgG2a serum. Center well: **a** 147C11; **b** 257C11; **c** Sp2/0.

peroxidase staining involves the use of antibodies conjugated with the enzyme horseradish peroxidase. Peroxidase is commonly used for several reasons (*13*):

1. Its small size will not hinder the binding of antibodies to adjacent sites:
2. It is easily obtainable in highly purified form so that the chance of contamination is minimized;
3. It is very stable, and therefore will remain unchanged during manufacture, storage, and application;
4. Only small amounts are present in tissue specimens, and this endogenous peroxidase activity is easily quenched;
5. There is a wide availability of chromagens that can be acted upon by peroxidase to form a colored reaction product that will precipitate at the site of the antigen–antibody interaction; and

6. It is inexpensive.

In the detection of monoclonal isotypes, supernatants are generally used employing the immunodiffusion (*7*), and ELISA (*8*) methods. However, the results given by these methods are obtained after 24 h. The immunofluorescence method (*9*) is more rapid but results are prone to fadding, subsequently. As the reaction is not permanent it is necessary to use fluorescent photography in order to maintain a permanent record of isotype determination.

Our proposed method can be performed in approx 1:30 h and results can be preserved indefinitely. In addition, it can be used on the thawed clones secreting known isotypes to verify if they are still productive. Furthermore, it is not necessary to wait several days for a cell line to develop until the antibody concentration in the supernatant rises enough to use other current methods.

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