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Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies directed against intracellular antigens

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For investigations involving monoclonal antibodies (mAbs) against cellular antigens cell binding assays are routinely used to determine the immunoreactive fraction after radiolabeling. In general, surface antigens are targets for radioimmunodetection, but recent studies indicate that intracellular determinants may also prove useful for this purpose. Thus, there is a need to adapt the regular cell binding assay for use with antibodies directed against cytoplasmic antigens. Here we describe a fixation method which permits such mAbs to bind to cell surfaces as well as to intracellular determinants. Moreover, the procedure may be used for antigens that are sensitive to the commonly used aldehyde fixatives. The method is illustrated with two human IgM mAbs 16.88 and 28A32, which recognize cytoplasmic antigens. Human colon cancer cells in suspension were fixed with either acetone or glutaraldehyde. Intracellular antigens appeared to be best exposed for antibody binding after fixation with acetone as determined by immunofluorescent staining and flow cytometry. An antibody directed against the cell surface antigen HLA class I showed similar binding with both live cells and acetone-fixed cells. Double-inverse plots of the binding of radiolabeled 16.88 or 28A32 antibody with acetone-fixed cells gave reliable immunoreactive fraction values. Acetone-fixed cells stored at 4°C could be used for immunoreactivity assays for at least 6 months without loss of performance.

Key words: Monoclonal antibody; Cell fixation; Immunoreactive fraction; Cytoplasmic antigen

Introduction

Radiolabeled monoclonal antibodies (mAbs) with specificity for tumor cells are being used for

in vivo detection and treatment of cancer. Most of the antibodies selected are directed against cell surface antigens because of their presumed greater accessibility to antibodies. However, recent studies have shown that antibodies against intracellular antigens can specifically localize tumor lesions (Dairkee and Hackett, 1988; Epstein et al., 1988; Steis et al., 1990; Boven et al., 1991). This localization is most probably associated with the presence of antigen released by necrotic cells.

For clinical applications of a radiolabeled mAb preparation it is important that unbound radionuclide is removed and that the conjugation

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Abbreviations: BSA, bovine serum albumin; EDTA, ethylene diaminetetraacetic acid; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HSA, human serum albumin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; TCA, trichloroacetic acid.

procedure does not extensively impair the binding capacity. The immunoreactive fraction of the final product is generally determined according to the method described by Lindmo et al. (1984). The principle of this procedure is based on the binding of the antibody to increasing concentrations of cells. Binding under conditions representing infinite antigen excess can subsequently be calculated by linear extrapolation in a double-inverse plot. In this assay live cells or cells fixed with glutaraldehyde are employed. As an alternative, cells may be fixed in paraformaldehyde (Beaumier et al., 1986). Neither method of fixation allows for binding of antibody to intracellular antigen.

The present report describes a fixation technique which enables mAbs to bind to both cell surface and intracellular antigens. The method is applied in the cell binding assay to determine the immunoreactive fractions of two human IgM mAbs recognizing cytoplasmic antigens. Moreover, the number of binding sites for both antibodies may be calculated in cells expressing the relevant antigen.

Materials and methods

Cells

The human colon cancer cell line WiDr (Noguchi et al., 1979) was grown as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS. Cells were harvested with 0.2% EDTA in PBS. Single cell suspensions were obtained by pipetting and cell suspensions were washed twice with PBS before fixation.

Fixation

Cells were fixed with glutaraldehyde by the addition of an equal volume of 0.2% glutaraldehyde in PBS to a cell concentration of approximately 10^6 cells/ml, after which the mixture was incubated with gentle stirring for 30 min at 0°C. For acetone fixation cells were pelleted, resuspended in 10 ml ice-cold acetone and incubated for 10 min with gentle stirring. The fixed cells were then centrifuged, washed twice with PBS and resuspended in PBS containing 1% BSA and

0.01% sodium azide. If not used immediately, the cells were stored at 4°C.

Antibodies and radiolabeling

The human IgM mAbs, 16.88 and 28A32, were provided by Biotechnology Research Institute (Rockville MD, USA). Both antibodies were developed from peripheral blood lymphocytes of colorectal cancer patients participating in an active specific immunotherapy trial for treatment of microscopic disease after surgical resection (Hoover et al., 1985). The antibodies recognize intracellular antigens (Haspel et al., 1985; Starling et al., 1988). Presently, these antibodies are used in clinical trials for tumor detection in colon cancer patients (Steis et al., 1990; Boven et al., 1991). Normal human IgM (Sigma, St. Louis, MO) was used to measure non-specific binding of the IgM in flow cytometry studies. The murine mAb W6/32 which is reactive with a framework determinant of the HLA class I antigen (Dakopatts, Glostrup, Denmark) was used as a marker for a cell surface antigen in immunofluorescence studies.

Labeling of the human mAbs with ^{125}I was performed according to the one-vial method (Haisma et al., 1986). Briefly, 500 μg of antibody were mixed with 1 mCi (16.88) or 2 mCi (28A32) of ^{125}I in a vial coated with 250 μg iodogen. After 10 min of incubation at room temperature with gentle shaking, a sample was removed to determine the amount of incorporated iodine. 1 ml of AG1-X8 resin (BioRad, Richmond, CA) mixed with 1% HSA in PBS was added to the vial to absorb unbound iodine. This reaction mixture was then filtered through a 0.22 μm filter to remove the resin and sterilize the product. In the case of 28A32, the incubation mixture was combined with an equal volume of 10% NaI before addition of resin to remove non-covalently bound iodine from the antibody. Specific activities were approximately 1 mCi/mg antibody. Precipitation with 10% TCA indicated that >95% of the radioactivity was protein bound in the final preparations.

Immunofluorescence

Binding of antibodies 16.88 and 28A32 was examined by flow cytometry of immunofluores-

cence-stained live or fixed cells. Cell suspensions were incubated with 16.88, 28A32, normal human IgM or W6/32 at 10 $\mu\text{g/ml}$ for 45 min at 0°C. After washing with PBS, cells were incubated with FITC conjugated rabbit anti-human μ chain or rabbit anti-murine Ig (Dakopatts, Glostrup, Denmark) for 45 min. After a further wash, antibody binding was analyzed using a flow cytometer (FACStar^{plus}, Becton Dickinson).

Immunoreactive fraction

The binding assays for the antibodies 16.88 and 28A32 were set up using a concentration of ^{125}I -labeled antibody of 20–40 ng/ml and five serial 1/2 dilutions of live or fixed cells starting with a cell concentration of 5×10^6 cells/ml in PBS with 1% BSA. Nonspecific binding of antibody was determined at the lowest cell dilution to which unlabeled antibody was added at a final concentration of 100 $\mu\text{g/ml}$ in order to saturate binding sites. After rotating the tubes for 18 h at room temperature, the cells were pelleted and half of the volume was removed and added to separate tubes. After counting the tubes with the cell pellet (*P*) and the tubes with the supernatant (*S*), bound and total radioactivity was calculated by the following formula:

$$\text{Bound (B)} = P - S; \text{Total (T)} = P + S$$

The immunoreactive fraction was calculated by plotting T/B versus $1/[\text{cell concentration}]$ and extrapolating a fitted straight line to its intercept with the ordinate. The fraction of immunoreactive antibody was determined as the inverse of the intercept value. Non-specific binding of the antibodies was calculated with the same formula shown above.

Scatchard analysis

Live, glutaraldehyde- or acetone-fixed cells were prepared at 1×10^6 cells/ml in 1% BSA in PBS. Two aliquots of cells were set up for each cell preparation. To one of these aliquots, unlabeled antibody was added to a final concentration of 100 $\mu\text{g/ml}$ in order to saturate binding sites for subsequent measurement of non-specific binding. Ten serial 1/2 dilutions of the radiolabeled antibodies were made up in 1% BSA in

PBS starting at 50 $\mu\text{g/ml}$. An equal volume of radiolabeled antibody solution was added to a constant number of cells and incubated for 18 h at room temperature with gentle rotation. Total and cell-bound radioactivities were determined as described for the assay of the immunoreactive fraction. The number of sites per cell was calculated by plotting specific binding versus the ratio of specific binding and reactive free antibody. From the intercept value at the abscissa the binding capacity per cell was determined and from the slope of the line the association constant.

Results

Fixation

Two different fixation methods were explored, glutaraldehyde and acetone, for WiDr colon cancer cells. Glutaraldehyde fixation tended to clump the cells, but this could be prevented by keeping the cells well suspended during fixation. Acetone fixation, but not glutaraldehyde fixation, made the cells permeable to macromolecules as shown by trypan blue dye exclusion.

Flow cytometry

Flow cytometry of immunofluorescence-stained WiDr cells was used to study the binding of mAbs 16.88 and 28A32. Both antibodies showed negligible binding to live cells. Glutaraldehyde-fixed cells gave high background staining due to autofluorescence of the glutaraldehyde. Therefore, this fixation method could not be evaluated by immunofluorescence. Fixation of the cells with acetone resulted in a dramatic increase in immunofluorescence of the cells stained with either 16.88 or 28A32. This binding appeared to be specific, because normal human IgM showed no binding with acetone-fixed cells. Binding of the anti-HLA class I antibody which was used as a general marker for cell surface antigens was similar for both live and acetone-fixed cells (Fig. 1).

Immunoreactive fraction

The immunoreactive fractions of ^{125}I -labeled 16.88 and 28A32 were determined by the use of WiDr cells, either live or after glutaraldehyde or acetone fixation. For both antibodies, binding to

live cells was less than 5% and judged to be non-specific in view of the binding in the presence of excess antibody. Glutaraldehyde-fixed cells were reactive with radiolabeled 16.88, but not with 28A32. Acetone-fixed cells showed good reactivity with both antibodies.

A plot of the reciprocal of cell concentration against the reciprocal of the fraction of bound and total applied antibody was drawn for both radiolabeled antibodies with acetone-fixed WiDr cells and for radiolabeled 16.88 with cells fixed using glutaraldehyde (Fig. 2). From the ordinate intercept value, representing the presence of infinite antigen, the immunoreactive fraction of the radiolabeled antibodies was calculated. The im-

Fig. 1. Flow cytometry analysis of WiDr cells stained with monoclonal antibodies 16.88 (·····), 28A32 (-----) and W6/32 (-·-·-); normal human IgM (—) was used as a control. Live cells (A) or cells fixed with acetone (B) were incubated with 16.88, 28A32 or W6/36 followed by FITC-conjugated rabbit anti-human IgM (16.88 and 28A32) or rabbit anti-murine Ig (W6/32).

Fig. 2. Immunoreactive fraction plot for the binding of ^{125}I -labeled 16.88 (○ and ●) and 28A32 (□) monoclonal antibody to acetone- (open symbols) or glutaraldehyde-fixed (closed symbols) WiDr colon cancer cells. The lines were determined by linear regression analysis.

munoreactive fraction of mAb 16.88 was calculated to be 0.90 using cells fixed with glutaraldehyde. When acetone-fixed WiDr cells were used, a similar immunoreactive fraction value was obtained. The immunoreactive fraction of radiolabeled 28A32 could only be evaluated with acetone-fixed cells and was 0.99. Nonspecific binding was less than 5% in all instances and was therefore not used to correct immunoreactivity values.

The binding of both antibodies to acetone-fixed cells and the binding of 16.88 to glutaraldehyde-fixed cells was specific, as shown by inhibition experiments. Binding of ^{125}I -labeled 16.88 could be blocked with an excess (100 $\mu\text{g}/\text{ml}$) unlabeled 16.88, but not with normal IgM or 28A32, whereas binding of radiolabeled 28A32 could only be blocked with an excess of 28A32 (data not shown). Acetone-fixed cells were stored at 4°C for 6 months and antibody-binding capacity was preserved.

Scatchard analysis

Scatchard analysis assays were set up to determine the number of antibody molecules bound per cell and the apparent association constant of the binding reaction. The data were corrected for non-specific binding and the immunoreactive fraction of the antibodies. For 16.88 both glutaraldehyde- and acetone-fixed WiDr cells were evaluated. The maximum binding capacity was similar for both fixation methods and calculated

Fig. 3. Scatchard plot of binding of ^{125}I -labeled 16.88 (○ and ●) and 28A32 (□) monoclonal antibody to acetone- (open symbols) or glutaraldehyde-fixed (closed symbols) WiDr colon cancer cells. The lines were determined by linear regression analysis.

to be 0.68 nM for glutaraldehyde-fixed cells and 0.62 nM for acetone-fixed WiDr cells. This indicates that 0.83×10^6 and 0.76×10^6 antibody molecules could be bound per cell at saturation. After glutaraldehyde fixation of WiDr cells 28A32 showed no specific binding, but fixation of the cells with acetone resulted in a maximum binding capacity of 0.26 nM, corresponding to 0.32×10^6 molecules per cell. From the slopes of the lines K_a values were calculated: $K_a = 5.2 \times 10^9 \text{ M}^{-1}$ for 16.88 and $K_a = 5.3 \times 10^9 \text{ M}^{-1}$ for 28A32 (Fig. 3).

Discussion

Acetone was investigated in our experiments as a fixative for cells in suspension in order to expose intracellular antigen. Acetone is widely used for the fixation of frozen sections for immunohistochemical detection of antibody binding. Therefore, for many antigens it is known that fixation with acetone does not diminish antibody binding. We confirmed that target cells are permeabilized after acetone fixation as demonstrated by their inability to exclude trypan blue. Flow cytometry of immunofluorescence-stained cells indicated that intracellular antigen was able to interact with the human IgM mAbs 16.88 and 28A32. Acetone fixation provided good preserva-

tion of cell surface antigen, as was shown for the HLA class I antigen.

No significant specific binding of either monoclonal antibody was observed with live cells, which is in agreement with our data from flow cytometry and with previous reports from other investigators (Starling et al., 1988; Steis et al., 1990). Glutaraldehyde-fixed cells bound radiolabeled 16.88, but not 28A32. This finding was unexpected since 16.88 exhibited no binding to live cells and glutaraldehyde-fixed cells were not permeable to macromolecules such as trypan blue. The antigen recognized by 16.88 is known to be an altered form of cytokeratin with similarities to cytokeratins 8 and 19 (Steis et al., 1990). These are cytoplasmic antigens, which are located close to the plasma membrane. Apparently, fixation of cells with glutaraldehyde externalizes this antigen on the cell surface. Acetone-fixed WiDr cells showed good reactivity with both radiolabeled mAbs. The intracellular location of the antigen recognized by 28A32 is unknown, but it is presumably not exposed on the cell surface after glutaraldehyde fixation. Alternatively, fixation with glutaraldehyde may alter the antigen binding site to such an extent that 28A32 antibody is no longer capable of binding.

Apart from the human mAbs 16.88 and 28A32, we have also studied the binding of two murine mAbs: anti-CEA mAb BW431/26 (Bosslet et al., 1988) provided by Behringwerke (Marburg, Germany) and the pan-carcinoma mAb 323/A3 (Edwards et al., 1986) provided by Centocor (Leiden, Netherlands). CEA is present both intracellularly and on the cell surface, whereas mAb 323A3 binds to a cell surface antigen. Both mAbs were radiolabeled with ^{125}I and binding studies were performed with glutaraldehyde- and acetone-fixed SW1398 colon cancer cells (Rutzky, 1985) which express the relevant antigens. Both radiolabeled antibodies performed similarly with glutaraldehyde- or acetone-fixed cells. The respective immunoreactive fractions for BW431/26 and 323/A3 were 83% and 78% for cells fixed with glutaraldehyde and 75% and 66% for cells fixed with acetone. Non-specific binding was less than 3% in all instances. Binding of anti-CEA mAb BW431/26 was increased approximately two-fold after acetone fixation, indicating that intracellular

CEA could also be bound after acetone-fixation. In conclusion, the acetone fixation technique may be of use in cell binding assays for radiolabeled antibodies that either identify intracellular antigens or recognize antigens that are damaged after fixation with glutaraldehyde or paraformaldehyde.

References

- Beaumier, P.L., Neuzil, D., Yang, H.M., Noll, E.A., Kishore, R., Eary, J.F., Krohn, K.A., Nelp, W.B., Hellstrom, K.E. and Hellstrom, I. (1986) Immunoreactivity assay for labeled anti-melanoma monoclonal antibodies. *J. Nucl. Med.* 27, 824.
- Bosslet, K., Steinstrasser, A. and Schwarz, A. (1988) Quantitative considerations supporting the irrelevance of circulating CEA for immunoscintigraphic visualization of CEA expressing carcinomas. *Eur. J. Nucl. Med.* 14, 523.
- Boven, E., Haisma, H.J., Bril, H., Martens, H.J.M., Van Lingen, A., Den Hollander, W., Kessel, M.A.P., DeJager, R.L. and Roos, J.C. (1991) Tumour localisation with ^{131}I -labeled human IgM monoclonal antibody 16.88 in advanced colorectal cancer patients. *Eur. J. Cancer*, 27, 1430.
- Dairkee, S.H. and Hackett, A.J. (1988) Internal antigens accessible in breast cancer: implications for tumor targeting. *J. Natl. Cancer Inst.* 80, 1216.
- Edwards, D.P., Grzyb, K.T. and Dressler, L.G. (1986) Monoclonal antibody identification and characterization of a M_r 43,000 membrane glycoprotein associated with human breast cancer. *Cancer Res.* 46, 1306.
- Epstein, A.L., Chen, F.M. and Taylor, C.R. (1988) A novel method for the detection of necrotic lesions in human cancer. *Cancer Res.* 48, 5842.
- Haisma, H.J., Hilgers, J. and Zurawski, V.R. (1986) Iodination of monoclonal antibodies for diagnosis and radiotherapy using a convenient one vial method. *J. Nucl. Med.* 27, 1890.
- Haspel, M.V., McCabe, R.P., Pomato, N., Janesh, N.J., Knowlton, J.V., Peters, L.C., Hoover, H.C. and Hanna, M.G. (1985) In: Reisfeld and Sell (Eds.), *Monoclonal Antibodies in Cancer Therapy*. Liss, New York, p. 505.
- Hoover, H.C., Surdyke, M.G., Dangle, R.B., Peters, L.C. and Hanna, M.G. (1985) Prospective randomized trial of adjuvant active specific immunotherapy for human colorectal cancer. *Cancer* 55, 1235.
- Lindmo, T., Boven, E., Cuttitta, F., Fedorko, J. and Bunn, Jr., P.A.J. (1984) Determination of the immunoreactive fraction of radiolabeled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. *J. Immunol. Methods* 72, 77.
- Noguchi, P., Wallace, R., Johnson, J., Early, E.M., O'Brien, S., Ferrone, S., Pellegrino, M.A., Milstein, J., Needy, C., Browne, W. and Petricciani, J. (1979) Characterization of WiDr: a human colon carcinoma cell line. *In Vitro* 15, 401-408.
- Rutzky, L.P. (1985) The biology of human colon tumor cells in culture. *Adv. Cell Cult.* 4, 47.
- Starling, J.J., Cote, R.J., Marder, P., Borowitz, M.J. and Johnson, D.A. (1988) Tissue distribution and cellular location of the antigens recognized by human monoclonal antibodies 16.88 and 28A32. *Cancer Res.* 48, 7273-7278.
- Steis, R.G., Carrasquillo, J.A., McCabe, R., Bookman, M.A., Reynolds, J.C., Larson, S.M., Smith, J.W., Clark, J.W., Dailey, V., Del Vecchio, S., Shuke, N., Pinsky, C.M., Urba, W.J., Haspel, M., Perentesis, P., Paris, B., Longo, D.L. and Hanna, M.G. (1990) Toxicity, immunogenicity, and tumor radioimmunodetecting ability of two human monoclonal antibodies in patients with metastatic colorectal carcinoma. *J. Clin. Oncol.* 8, 476.