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Dot-Based ELISA and RIA: Two Rapid Assays that Screen Hybridoma Supernatants against Whole Live Cells

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Two assays are described that are suitable for the screening of large numbers of hybridoma supernatants as well as for the screening of a wide range of tissues for the distribution of a given cell surface antigen. These assays use whole live cells as targets, avoiding fixation or extraction, which could alter the antigenic structural profile. The assays are rapid, simple and inexpensive. Their advantages and applications are discussed.

Key words: *hybridoma screening – ELISA – radioimmunoassay*

Introduction

The attempt to raise monoclonal antibodies (MoAb) to cell surface antigens has created a unique screening problem. Many researchers screen hybridoma supernatants against cell extracts or fixed cells. One cannot, however, overlook the risk that extraction or fixation procedures expose intra-cellular antigens or, more seriously, alter the structural configuration of the membrane antigens. It is possible that MoAb raised to the in vivo configuration on whole live cells may fail to recognize the altered one in vitro screening.

In an attempt to circumvent this potential problem we have developed 2 related screening assays that use whole live cells as targets, thereby obviating concern about preservation of the antigenic profile. We have also made these assays sparing of cells, using as few as 3000 cells/supernatant screened; particularly useful when the desired target cells are scarce or difficult to obtain. In both assays small drops of a target cell suspension are applied to nitrocellulose filters in a manner similar to that used by Hawkes et al. (1982). These filters then serve as carriers for either an ELISA or a

Abbreviations: ELISA, enzyme-linked immunosorbent assay; RIA, radioimmunoassay; BSA, bovine serum albumin; PBS, phosphate-buffered saline, pH 7.2; SPA, staphylococcal protein A.

RIA. The use of 96-well filtration plates that fit on a vacuum holder (both by Millipore Corp.) permits us to perform all washes by suction, considerably reducing the amount of time required for each assay. We find these assays to be rapid, versatile, and yielding results in excellent agreement with those of a tube-based assay long in use in our lab.

Materials and Methods

Nitrocellulose or fluorocarbon 96-well filtration plates (Millipore Corp.) with 0.22 μm poresize are soaked 1–12 h in phosphate-buffered saline (PBS) containing 1% BSA (pH 7), 0.1% polyoxyethylene sorbitan monolaurate (Tween-20; Sigma Chemical) to block non-specific binding sites. Excess detergent is removed by repeated washings with PBS containing 1% BSA (PBS/BSA). Target cells are washed in PBS/BSA, pelleted, and resuspended at the desired concentration; 3000–50,000 cells/μl will give satisfactory results. Using the vacuum holder (Millipore Corp.), mild suction is applied to the plate while 1 μl of the cell suspension is dotted into the center of each well. The mild suction prevents the cell suspension from spreading out, but harsh suction should be avoided at all times.

After a single wash with PBS/BSA the plate is lightly blotted on the bottom to prevent seepage of reagents through the filter membrane by capillary action. Hybridoma supernatants are added (50 μl/well is sufficient), and the plate is incubated. All incubations are for 30–60 min at 25 °C, the supernatants are removed by suction, the plate is washed 3 times with PBS/BSA, and is lightly blotted before addition of the second antibody. The second antibody used depends on the way the assay is to be completed (i.e., d-ELISA or d-RIA).

d-ELISA

The avidin-biotin-peroxidase complex method (Vectastain ABC kit, Vector Laboratories) as described by Hsu et al. (1981) is used. All reagents are diluted in PBS/BSA. A biotinylated anti-mouse antibody is applied and allowed to incubate. Following 3 washes with PBS/BSA, the avidin-peroxidase complex (ABC) is added and the plate is incubated. After a final set of 3 washes with PBS/BSA the developer is prepared as follows: 4-chloro-1-naphthol is dissolved in methanol (3 mg/ml). While this solution can be kept in the cold in the dark for approximately 2 weeks, it should be discarded when it begins to turn yellow. Shortly before use 5 vols. of PBS are added and this solution is brought to 0.01% v/v H_2O_2. This developer is applied to the plate (50–75 μl/well) and allowed to incubate long enough for the chromogenic reaction to be complete (5–15 min). A positive reaction will give a dark purple dot. The plate is washed once with distilled water and allowed to dry. The color of positive reactions will fade over time. This process can be slowed considerably by keeping the plates in the dark; for permanent records we suggest photographing the results within a month of the assay's completion.

d-RIA

A polyclonal anti-mouse antibody is applied and allowed to incubate. After the

incubation the plate is washed 3 times with PBS only (pH 8) and ^{125}I-labeled staphylococcal protein A (SPA) is added (100,000 cpm in 50 μl/well). BSA is eliminated from this and subsequent washes to prevent foam from forming and possibly spreading across the bottom of the plate during the suction washes. Such spreading foam, of no concern in the d-ELISA, could carry radioactivity with it, which would cause non-specific contamination. The pH of the wash buffer is chosen because SPA binds better to IgG1 at pH 8 than at pH 7 (Ey et al., 1978). For this same reason the proper dilution of SPA should be made up in PBS at pH 8. Following the incubation and a final set of 3 washes with PBS alone (pH 8), the plate is allowed to dry, the edges of the plate are broken off to allow close plate-film contact, and the plate is placed in a film cassette on XAR film (Kodak) with an underlying calcium tungstate intensifying screen (DuPont). Exposure time is 2–12 h at $-70\,^{\circ}$C. Alternatively, single emulsion X-ray film may be used and the exposure time lengthened to 10–24 h.

Tube-based assay (Shaw et al., 1980)

Five milliliter polystyrene tubes are filled with PBS/BSA and are allowed to sit for 20 min to block non-specific binding sites. The buffer is then poured out and the last drops are aspirated. Target cells are washed in PBS/BSA (pH 7), pelleted and resuspended at a concentration of 10,000 cells/μl. Fifty microliters of this suspension are added to each of the tubes with the exception of the first one, which receives 50 μl buffer instead. This blank will eventually provide a measure of the background counts. Hybridoma supernatants are added (50 μl/tube), the tubes are gently shaken and incubated in a shakerbath at 37 °C for 1 h. Upon removal from the shakerbath 3 ml PBS/BSA (pH 8) are added, the tubes are gently shaken and centrifuged at $800 \times g$ for 10 min. The supernatant is aspirated carefully without disturbing the pellet. ^{125}I-labeled SPA is added (100 ng/tube in 250 μl PBS/BSA (pH 8)), the cells are resuspended and incubated on a shaker at 25 °C for 1 h.

During this incubation 400 μl soft conical polyvinyl chloride centrifuge tubes (West Coast Scientific) are filled with PBS/BSA and allowed to sit 20 min to block non-specific binding sites. The buffer is aspirated and 200 μl of a Percoll (Pharmacia) solution is added, taking care to avoid all bubbles. The desired concentration of Percoll varies with cell size, larger cells requiring lower concentrations. For small cell carcinoma of the lung cells we have used 15% Percoll in normal saline (v/v).

The SPA-reacted cells are triturated briefly and 100 μl aliquots of each cell suspension are carefully layered onto the surface of 2 Percoll tubes. The tubes are centrifuged at $10,000 \times g$ for 4 min; this centrifugation pellets the cells while the free ^{125}I-SPA remains above. The tips of the tubes are cut off above the pellet, and the cell-associated ^{125}I-SPA is counted in a gamma counter.

Results

The assays described above are versatile and can be used not only to screen hybridoma supernatants for the presence of MoAb, but also to determine tissue

distribution of the antigen against which the MoAb was raised. We give examples of both types of assays.

We generated and maintained hybridoma cultures by the usual methods (Köhler and Milstein, 1975), using intact cells from a variety of tumors as immunogens. The supernatants from these cultures were screened for the presence of specific antibody using either the d-ELISA or the d-RIA and comparing the results with those obtained by the tube-based assay.

Agreement between d-ELISA and the tube-based assay was 85% (263/336 samples), while agreement between d-RIA and the tube-based assay reached 92% (260/282 samples). In both assays the 'false negatives' (i.e., a positive result with the tube-based assay but a negative result with either of the dot-based assays) were rare, constituting approximately 4% of the samples (15/336 for d-ELISA, 12/282 for d-RIA). 'False positives' (i.e., a negative result with the tube-based assay but a positive result with either of the dot-based assays) were more common with d-ELISA than with d-RIA (10% and 6% respectively). A typical d-ELISA screening supernatants is shown in Fig. 1.

The tissue distribution of several antigens against which we have produced MoAb is shown in Fig. 2. This assay does not test a range of antibodies against a single antigen; instead it employs a range of antigens, in this case single cell suspensions of

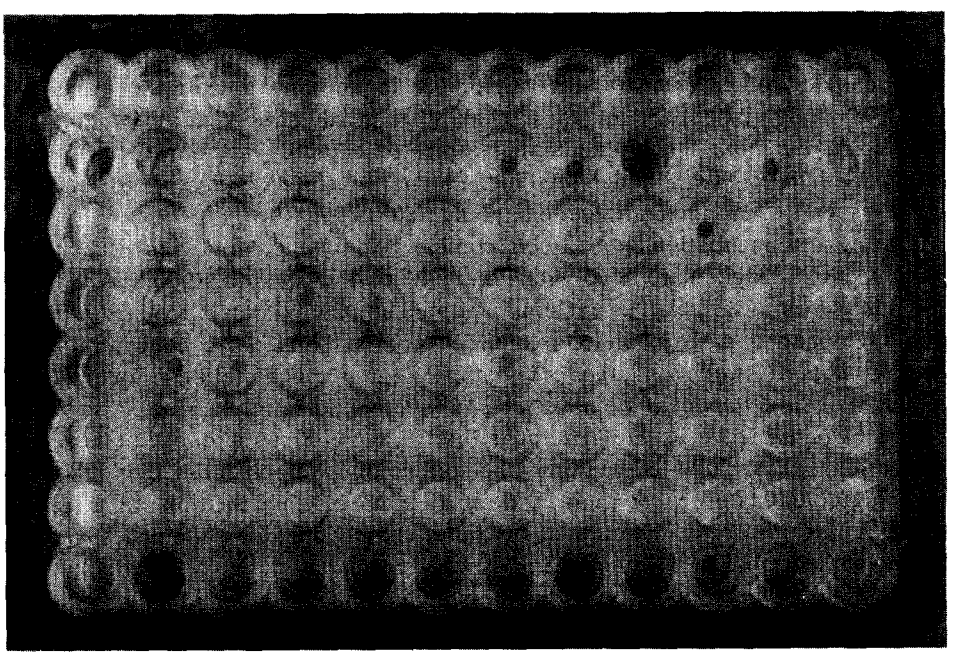

Fig. 1. A d-ELISA assay screening hybridoma supernatants against a single cell type (50,000 cells/well). Dark dots show positive reactions. Row A contains no cells, row G contains cells but received no antibody (negative controls). B1 and B9 received 1:10 dilutions of 2 different sera, the stained background is typical of such high protein concentrations. We recommend diluting sera at least 1:100.

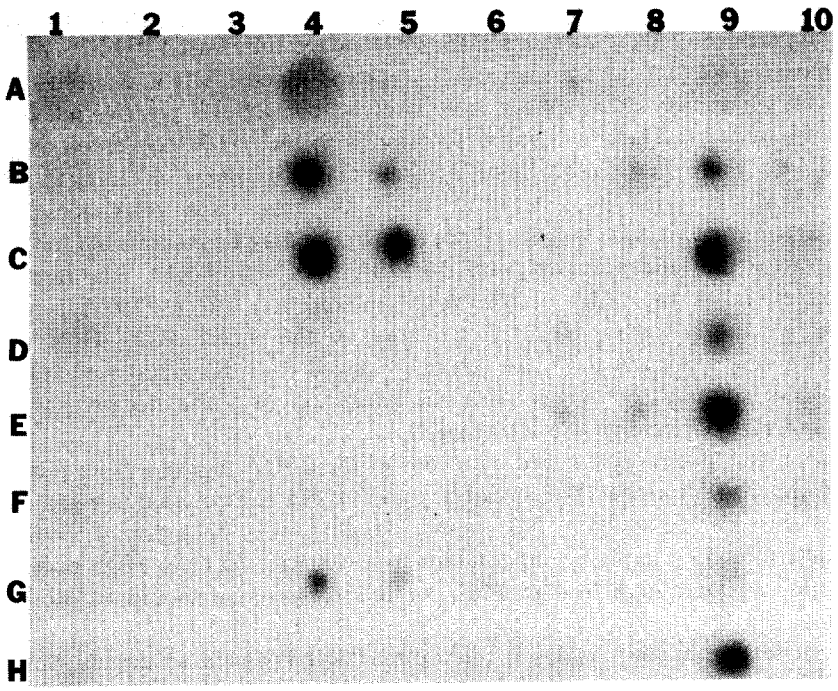

Fig. 2. A d-RIA assay screening 2 sera, 1 ascites, 1 purified antibody and 2 hybridoma supernatants against 5 cell types. Row A: no cells (negative controls). Rows B and C: a choriocarcinoma cell line at 5000 and 50,000 cells/well respectively. Rows D and E: a small cell lung carcinoma cell line at 5000 and 50,000 cells/well respectively. Row F: ovarian carcinoma cells from a biopsy, 5000 cells/well. Row G: mononuclear cells from peripheral blood, 50,000 cells/well. Row H: melanoma cells from a resected tumor, 50,000 cells/well.
Columns 1, 2, 3: normal mouse serum diluted 1:10, 1:100, and 1:1000 respectively. Columns 4, 5, 6: anti-choriocarcinoma antibody diluted 1:10, 1:100 and 1:1000 respectively. Columns 7, 8: 2 undiluted hybridoma supernatants. Column 9: undiluted ascites. Column 10: serum of mouse immunized against ovarian carcinoma, diluted 1:10.

several normal and neoplastic human organs, against which a few antibodies are tested. Note that normal mouse serum fails to react with cells although some background binding is present. In contrast, the serum of the immunized mouse does react with cells as indicated by the darker dot in the midst of background binding.

Care is taken to keep the filters and cells moist at all times. Under such conditions immobilization of cells in dots decreases cell viability only slightly (as determined by trypan blue exclusion).

Discussion

We have described 2 assays that are suitable for the screening of large numbers of hybridoma supernatants for antibody reactivity with cell surface antigens as well as

for the screening of a wide range of tissues for the distribution of a given cell surface antigen. Comparisons of these methods with our standard tube-based ^{125}I-SPA/Percoll assay have shown that the 3 assays are of comparable sensitivity and specificity. Discrepancies, where they exist, are far more likely to consist of a positive reaction with the dot-based assays and a negative reaction with the ^{125}I-SPA/Percoll assay ('false positive'), than the reverse. Some of these 'false positives' are due to the presence of IgM and possibly some IgG1, which the tube-based assay does not detect. Endogenous peroxidase activity of the target cells may be responsible for the higher proportion of 'false positives' with d-ELISA than with d-RIA. The rate of 'false negatives' is on the order of 4%. Since, in addition, the d-ELISA is not quantitative, we use it for rapid initial screening of the supernatants. Only the supernatants giving a positive d-ELISA result are tested with the tube-based assay. By this process all negative clones, with the exception of 'false negatives', are eliminated and all positive ones are quantitated.

Quantitation can also be performed by means of the d-RIA. Using a filter punch (Millipore Corp.), well bottoms (filters) can be punched out and counted in a gamma counter. For each sample tested in this manner the amount of background binding should be determined by applying supernatant to a well lacking cells. High protein concentrations can yield high backgrounds, for this reason sera should be diluted before testing.

The special feature of all 3 assays is the use of whole live cells as targets. Since fixation can and does alter some antigens (Baron et al., 1977), there is always the possibility that a MoAb raised to the in vivo configuration of a cell surface antigen may fail to recognize the potentially altered one on a fixed cell. Availability of cells can, however, be a problem and in this respect especially the ^{125}I-SPA/Percoll assay suffers from a serious drawback: it requires 200,000–500,000 cells/supernatant tested. Both dot-based assays can, on the other hand, be performed using as few as 3000 cells/supernatant tested, enabling us to use even small biopsies in our screening assays.

Even where unlimited numbers of cells are available, the dot-based assays are more suitable for the screening of large numbers of samples since they are considerably less demanding of time, equipment, and patience than the tube-based assay. We find that 4–5 96-well plates can be screened in < 4 h. Additionally, the d-ELISA avoids the use of radioisotopes altogether and is, therefore, particularly desirable for the initial screen. Of course, in using the d-ELISA, the usual precautions regarding endogenous peroxidase activity of cells should be observed.

Although the cells need not be applied in a dot (Handley et al., 1982), it should be noted that the use of a dot of cells improves the ability to distinguish a positive reaction even in the presence of background binding since contrast between dot and background aids in detection.

The use of these assays is not limited to the examples already given. Since purified proteins may be dotted onto the filters and used as targets (Hawkes et al, 1982; Horejsi and Hilgert, 1983), these assays can also be used to analyze the molecular nature of the antigen to which a given MoAb binds, e.g., MoAb reactive with neoplastic tissue may be screened against various purified oncofetal proteins. Since

such assays do not require the immobilization of whole live cells, however, economy may dictate that this assay be carried out on sheets of nitrocellulose filter paper rather than in the filter-backed plates.

In summary the d-ELISA and d-RIA, which are modifications of a solid-phase assay, offer one the ability to rapidly and easily screen large numbers of antibody samples against whole live cells. In addition, with equal ease, one type of antibody may be screened against a number of tissues.

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