

A RAPID METHOD FOR THE DETECTION OF ANTIBODIES TO CELL SURFACE ANTIGENS: A SOLID PHASE RADIOIMMUNOASSAY USING CELL MEMBRANES

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Cell membranes isolated from murine lymphocytes or ascites tumors bind tightly to the surface of flexible plastic microtiter plates in the absence of additional proteins. This allows the detection of membrane associated molecules by specific antibodies and thus forms the basis for a rapid and sensitive radioimmunoassay for antibodies to membrane-bound components. The assay compares favorably with a variety of methods currently used to detect antibodies to cell surface antigens. The assay detects a variety of well characterized murine cell surface antigens (H-2, I-A, T-200, Thy-1.2, Ig). The level of antibody binding to membranes on plates correlates well with antigen density on intact cells. A modification of the assay involving competition between cross-reacting antibodies allows detection and resolution of closely spaced antigenic determinants.

INTRODUCTION

The detection of antibodies specific for cell surface antigens is among the most important techniques of cellular immunology. Such antibodies are useful in determining the role of membrane-bound molecules in immune function, delineating subpopulations of lymphocytes and defining cell surface differentiation markers (Katz, 1977; Williams et al., 1978). In addition, the possibility of monoclonal antibody production (Kohler and Milstein, 1975) enables very detailed study of the structure and organization of cell surface molecules (Mescher, 1979; Goding and Herzenberg, 1980). It is, therefore, important to have a sensitive, rapid, convenient and reproducible assay for antibodies to cell surface antigens.

Several assay methods are currently used widely, including cytotoxicity (Gorer and O'Gorman, 1956; Hammerling et al., 1978), radioimmunoassay (RIA) of antibody binding to whole cell targets (Tsu and Herzenberg, 1980), and fluorescent activated cell sorter (FACS) analysis (Loken and Herzenberg, 1975). Each has its particular advantages (see Discussion), but each has rate limiting steps such as the preparation of target cell suspensions and subsequent manipulations during the course of the assay. A technique was

sought which would reduce the time spent in these phases of the assay without sacrificing sensitivity, reproducibility or specificity. We describe a sensitive new assay, the membrane binding assay, which is ideally suited for rapid detection of antibodies reacting with membrane antigens. Its distinct advantages are that while preserving the sensitivity, specificity, and reproducibility of the other methods, it is very rapid and simple. The assay is based on the fact that purified plasma membranes bind to the surface of flexible polyvinylchloride (PVC) microtiter plates in the absence of additional proteins. Subsequent washing with a protein-containing buffer does not displace the membranes. This allows antibodies against membrane determinants to be detected by an appropriate radiolabeled 'second step' reagent.

MATERIALS AND METHODS

Mice

CKB (i.e. C3H.Ig^b, H-2^k, Ig^b) and CSW (i.e. C3H.SW, H-2^b, Ig^a) mice were obtained from the breeding colonies of H.O. McDevitt or L.A. Herzenberg, Stanford University. Young mice (4–6 weeks) were used as a source of thymuses whereas either young mice or retired breeders were used as a source of spleens.

Monoclonal antibodies

Mouse allogenic monoclonal antibodies used were produced by Oi et al., 1978. The mouse monoclonal lines and their corresponding specificities are: 11-4.1, H-2K^k; 10-2.16, I-A^k; 10-4.2, Ig-5^a). The rat xenogeneic monoclonals (antibodies produced from fusion of immune rat anti-mouse spleen cells with a murine myeloma line) are described elsewhere (Ledbetter and Herzenberg, 1979). The rat monoclonals used and their corresponding specificities are: 30-H12, Thy-1.2; 30-G12, T-200; 53-6.7, Lyt-2; 53-8.1, T-30; 53-7.3, Lyt-1; 49-H4, ThB; 30-H11, specificity not yet determined.

Second and third step reagents

[¹²⁵I]Protein A and [¹²⁵I]rabbit anti-mouse IgM were prepared according to the method of Tsu and Herzenberg (1979). In the case of mouse monoclonals, [¹²⁵I]Protein A was used to detect antibody binding. In the case of rat monoclonals the Protein A binding fraction of mouse anti-rat Ig serum (Goding, 1978) was followed by [¹²⁵I]Protein A in order to detect antibody binding. The mouse anti-rat reagent was prepared in SJL/J mice. It was titered out to the greatest dilution which gave maximum specific binding.

Membrane preparation

The simple procedure for isolation of plasma membranes in the form of membrane sheets is based on the method of Mehdi and Nussey (1975). For completeness it will be described in detail here. All plastic or cellulose nitrate tubes used in the course of the isolation were soaked overnight in 1 mM EDTA, pH 7.0, then rinsed 10 times with doubly distilled water.

Spleens or thymuses were dissected out into tubes kept on ice. All operations were at 0–4°C. The organs were minced finely into particles approximately 1 mm². To the minced tissue, 3–6 vol. (w/v) homogenization buffer (10 mM sodium phosphate buffer, pH 7.4, containing 1 mM MgCl₂, 30 mM NaCl, 5 μM phenylmethylsulfonyl fluoride (PMSF) and 0.02% NaN₃) was added and the tissue homogenized using a Polytron homogenizer (Brinkmann) at a power setting of 7. A total of 2–5 bursts, 5 sec long, each separated by 1–2 min of cooling was sufficient to break the cells. It should be noted that ascites tumor cells, spleen cells, or thymocytes in a suspension of 10⁷–10⁸ cells/ml could be homogenized as described above for plasma membrane preparation and in such cases 2 μg/ml DNase should be included in the homogenization buffer. Cell breakage was monitored by light microscopy. The homogenate (28–30 ml) was layered over 10 ml of 41% sucrose in homogenization buffer and centrifuged at 95,000 × *g* for 2 h using Beckmann rotor SW 27. Membranes forming the white band at the interface of the homogenate and sucrose solution were aspirated using a Pasteur pipette. To dilute the sucrose, 2–3 vols. of the homogenization buffer was added and the membranes were pelleted by centrifugation at 95,000 × *g* for 20 min. Membranes were washed twice by resuspension in PBS, pH 7.4, containing 1 mM MgCl₂ and 0.02% NaN₃ followed by centrifugation. The final membrane pellet was stored at –70°C or in liquid nitrogen until use. Membrane protein was measured by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard.

Membrane binding assay

Membranes were diluted to 0.1 mg/ml protein in PBS, pH 7.4, containing 1 mM MgCl₂ and 0.02% NaN₃ (incubation buffer) and 20 μl were added to each well of a polyvinylchloride microtiter plate (Cooke). The plates were kept for 1 h (or overnight) under refrigeration at 4°C. Unbound membranes were removed by aspirating the liquid from each well using a plastic pipette and washing the plate 3 times with incubation buffer containing 1% BSA (Sigma, RIA grade or FCS obtained from GIBCO). Residual wash-buffer could be removed effectively by slapping the plate face downward on an absorbent surface. The appropriate antibody was diluted in assay buffer and 20 μl added to each well. After incubation for 1 h at 22–24°C, the plates were washed again 3 times as described above. Twenty μl of the second step reagent ([¹²⁵I]Protein A or mouse anti-rat antibody as required)

was added to each well and the plates incubated for a further 45 min. After carefully removing the liquid in each well the plates were washed again 3 times. If mouse anti-rat antibody was used as a second reagent, 20 μ l of [125 I]Protein A was added and the plates incubated for a further 45 min, the liquid aspirated carefully from each well and the plates washed as usual. The plates were dried under a heat lamp. Wells were cut out with scissors or hot wire and counted in a gamma counter.

The cell binding assay is virtually identical to the membrane binding assay, except that plates are centrifuged after each wash and the supernatants aspirated carefully (Tsu and Herzenberg, 1980).

FACS

The methodology and instrumentation used for fluorescence activated cell sorting (FACS) (Bonner et al., 1972) have been described in detail by Herzenberg and Herzenberg (1978).

Cross-blocking assay

The cross-blocking assay is carried out by adding unlabeled antibody to membranes bound to plates. After a half-hour incubation, 20,000 cpm of 125 I-labeled antibody was added. After a further hour's incubation the plates were washed, cut and counted as described above. Since the cross-blocking assay is most sensitive at limiting antigen concentration, membranes were titered out to determine the appropriate level of antigen, i.e., the concentration of membranes giving a 60% of maximum binding for a given antigen.

RESULTS

Binding of membranes to plates

The plasma membrane fraction of murine splenocytes binds tightly to PVC microtiter plates. Serial dilutions of CKB spleen plasma membranes in incubation buffer were bound to the wells of U-bottomed microtiter plates. After removing unbound membranes and washing, rabbit anti-mouse IgM or anti I-A^k was added in order to detect membranes binding to plates. Fig. 1 shows the dilution curve for the membranes. The membranes could be diluted to 0.1 mg/ml and still give good antibody binding (60% of maximum). The optimal coating concentration will vary according to the surface antigen density on the membranes. Experiments using 125 I-labeled membranes reveal a maximum binding of 0.1 μ g protein per well (data not shown).

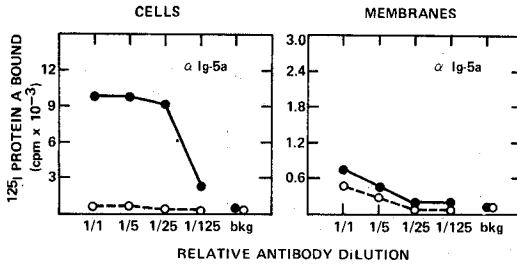


Fig. 3. Binding of monoclonal anti-Ig-5a to cells (CKB, \circ - - - - \circ ; CSW, \bullet - - - - \bullet) and membranes (CKB, \circ - - - - \circ ; CSW, \bullet - - - - \bullet) from thymus. The initial antibody concentration was approximately 20 $\mu\text{g}/\text{ml}$. Other conditions are identical to those used in Fig. 2.

almost as many counts as the cells when anti-T-200 was used, but fewer counts when anti-Thy-1.2 was used (Fig. 4). The specific binding was excellent in both cases.

Correlation of membrane binding assay with relative surface antigen density

Relative surface antigen density on thymocytes was revealed by quantitative immunofluorescence detected by FACS using rat monoclonals and a

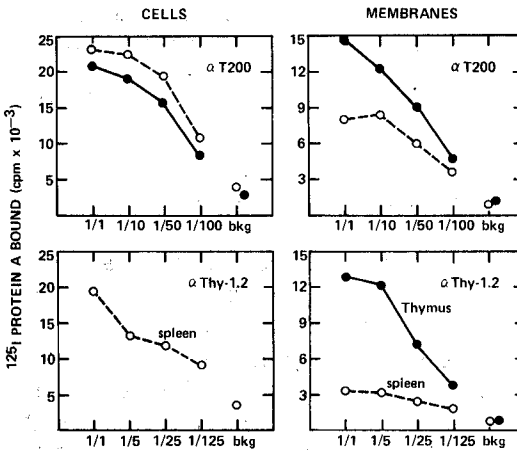


Fig. 4. Binding of rat monoclonal antibodies to membrane determinants on cells or purified plasma membranes. (α -T200 to CKB, \bullet - - - - \bullet ; or CSW, \circ - - - - \circ . α -Thy 1.2 to CSW thymus, \bullet - - - - \bullet ; or CSW spleen, \circ - - - - \circ .) Bkg denotes that RIA buffer was used instead of antibody. The approximate initial concentration of all antibodies was 20 $\mu\text{g}/\text{ml}$. Twenty μl SJL mouse anti-rat second step was used at a 1/150 dilution and 20 μl [^{125}I]Protein A at 1000 cpm/ μl . The values expressed represent the means of three determinations. The standard errors were in all cases less than 10% of the mean value.

TABLE 1

Comparison of membrane plate binding assay to quantitative membrane immunofluorescence for measurements of relative antigen density

Monoclonal antibody	Membrane binding assay ^a	Immunofluorescence ^b
Anti-Thy-1.2 (30-H12)	29	133
Anti-T200 (30-G12)	11	97
Anti-Lyt-2 (53-6.7)	9.0	92
Anti-T30 (53-8.1)	6.9	72
Anti-Lyt-1 (53.7.3)	6.2	18
Undetermined (30-H11)	3.7	9.8
Anti-ThB (49-h4)	3.1	9.6
Bkg (second step alone)	1.0	0.7

^a Results are expressed as the ratio of (specific counts bound)/(background counts bound) measured on thymocyte membranes as described in the text.

^b Geometric mean fluorescence of thymocytes with a FACS-II using a fluorescein conjugated mouse anti-rat Ig second step reagent.

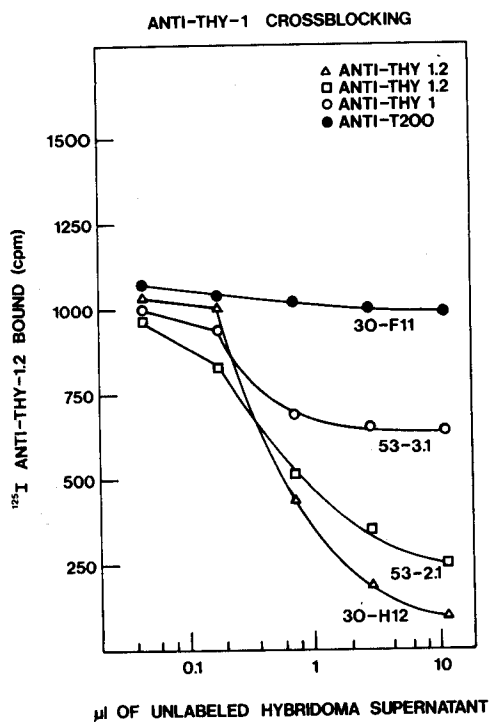


Fig. 5. Anti-Thy-1 cross-blocking detected on SJL thymocyte membranes. Details of the assay are given in the text (from Ledbetter and Herzenberg, 1979).

fluorescein-conjugated anti-rat Ig reagent. Using the same monoclonals the degree of antibody binding to membranes was determined using the membrane binding assay. Results from these experiments are tabulated in Table 1, where it can be seen that the specific binding correlates with the relative antigen density as determined by fluorescence measurements. Thus, the degree of binding is indicative of the relative surface antigen density on the intact cell membrane.

Detection of spatially related antigenic determinants using the cross-blocking assay

The cross-blocking modification of the assay allows detection of antibodies which completely, or partially, block a known antibody. This is illustrated in Fig. 5 where the ^{125}I -labeled antibody is a rat monoclonal specific for the Thy-1.2 determinant. An anti-T-200 (30-F11) does not significantly block the labeled anti-Thy-1.2, since it reacts with a completely distinct determinant. Another monoclonal (53-3.1) partially blocks the labeled anti-Thy-1.2, suggesting that it reacted with a determinant close to that recognized by the labeled antibody. It was later discovered to be specific for a framework determinant of the Thy-1 antigen, since it reacted with both Thy-1.1 and Thy-1.2.

DISCUSSION

Several assays for detecting antibodies to cell surface antigens are already in use. The complement-mediated cytotoxicity assay is a very sensitive method for detecting cytotoxic antibodies (IgG2a, IgG2b, IgG3, IgM in the mouse) but does not detect non-cytotoxic antibodies (IgG1, IgE and IgA). The effort involved in finding or producing sera which will react well with the antibodies but do not contain high amounts of heterophilic antibody tend to complicate the assay. In addition, the method requires the antigen in question to be present on a large portion of the cells used as targets.

FACS analysis is, perhaps, the most informative assay, as it provides data on surface density and subpopulation distribution of the determinant detected by the antibody. Since it is a direct binding assay it does not rely on complex biological reagents. It also allows for the detection of antigen on a small subpopulation of target cells. Most laboratories, however, do not have routine access to a FACS instrument and it is not well suited for very large assays.

The cell binding assay is closely analogous to the membrane binding assay described here. The time consuming aspect of this assay is that the plates must be centrifuged, and supernatants carefully aspirated between each step. All of the above assays require same-day preparation of target populations. The solid phase plate binding assay (Tsu and Herzenberg, 1980), has proven very useful for detection of proteins which stick to the

surface of PVC microtiter plates. Unfortunately, cells, unless treated with glutaraldehyde or antibody (Stocker and Heusser, 1979), do not bind to the plates. It has previously been demonstrated that solubilized membrane material will stick to the plates and allow detection of antibody binding (Pratt and Comoglio, 1976). The membrane binding assay described here, however, differs significantly from the previous two methods in that the membrane molecules are not subjected to covalent modification by glutaraldehyde or to the high ionic strength necessary for solubilization. This allows the detection of antigenic determinants in a membrane environment close to the one they experience in cells. In our hands the membrane plate binding assay detects most (19 of 20) determinants seen in the cell binding assay. In the one case of poor detection, the determinant involved (IgD) has been shown to be very susceptible to proteolytic attack (Bourgois et al., 1977). Here PMSF serine protease inhibitor was used. Use of other protease inhibitors may preserve highly sensitive determinants.

The membranes bind tightly so all washing steps can be performed as in a solid phase plate binding assay (i.e., plates need not be centrifuged and well contents can be flicked out after each step). This represents a significant saving in time. Since membranes are prepared in relatively large batches and can be stored for at least one year, one membrane preparation can satisfy the needs of a full-time screening effort. Thus the membrane preparation need only be done once for each cell type of interest. This is particularly important to investigators screening for antibodies to determinants on cells which are not available on a continuous basis (i.e., human tissue, embryonic tissue, etc.). The membrane preparation can be performed on fresh samples as they are available and used thereafter. The membrane preparation is general enough to be extended, with modifications, to tissue other than spleen, lymph node or thymus.

The obvious extension of this assay to organelles other than the plasma membranes should make possible the successful screening of antibodies to any subcellular membrane determinant. Preliminary results using rat liver microsomes are promising and suggest that subcellular membrane organelles bind to PVC microtiter wells, using conditions similar to the ones described here (i.e., BSA- and FCS-free dilution buffer). This fact, along with the rapidity and simplicity of the membrane binding should make it very useful in efforts to produce and characterize both classical and monoclonal antibodies to a wide variety of interesting intra- and extracellular antigens.

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