TWO SIMPLE BINDING ASSAYS FOR MONOCLONAL ANTIBODIES AGAINST INTERNAL ANTIGENIC DETERMINANTS

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1. Introduction

The production of monoclonal antibodies by hybridoma cells is a powerful technique for analyzing the cell surface. Hybridoma cells producing antibodies against external determinants are usually identified by an indirect binding assay where the monoclonal mouse immunoglobulin is first bound to the cell surface and the number of monoclonal antibodies bound is titrated by binding a second radiolabelled anti-mouse immunoglobulin antibody [1,2]. This procedure is very simple as whole cells can be centrifuged at low speeds. To identify internal determinants the cell must be disrupted or made 'leaky' so that the whole cell can still be used as a target. In this paper I present conditions in which membrane proteins can be covalently coupled to sepharose and used in a simple binding assay to identify monoclonal antibodies against internal determinants of cell surface proteins. The 'inside' antibodies were then used to establish conditions where whole cells can be permeabilized and used directly in a binding assay. Simple screening procedures are essential in the identification of rare antibodies. Both of these screening procedures may be used to identify monoclonal antibodies against antigenic determinants which are hidden in the whole cell system.

2. Materials and methods

2.1. *Cell lines and tissues*

An EB virus transformed cell line (LKT) was derived from human peripheral B lymphocytes (M. Thomson, Rigshospitalet, Copenhagen). Primary human fibroblasts were explanted from ovarian teratomas and kindly provided by Dr Sue Povey, Galton Lab., London. The mouse myeloma line used for fusion was the 8-azaguanine non-secreting derivative of MOPC21, P3-NS1/1-Ag4-1 [3]. All these cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, penicillin (100 U/ml) and streptomycin (50 μ/ml). Placentae were obtained either from term pregnancies or at an earlier stage after termination of pregnancy. Foetal rat lung fibroblasts were provided by Dr John Holborrow, London Hospital.

2.2. *Immunization and cell fusion*

Balb/c mice were immunized with:

(i) Crude membrane fraction prepared by hypotonic cell lysis and differential centrifugation [4];
(ii) Purified plasma membrane glycoproteins from the lymphoblastoid cell line, Bristol 8 as in [5,6] (these mice were immunized by Dr M. J. Crumpton, National Institute for Medical Research, London);
(iii) Human adult haemoglobin (Sigma).

Subcutaneous injection was administered at least 3 times over 2 months and finally the mice were primed for fusion by a tail-vein immunization 5 days before fusion. Cell fusion was performed as in [7].

2.3. *Covalent coupling to Sepharose*

Sepharose 4B (Pharmacia Ltd) was washed several times with distilled water and activated with cyanogen bromide as in [8,9]. The activation was carried out at pH 10.5 at 15°C and terminated by the addition of ice and copious washing with 0.1 M carbonate buffer (pH 9.0). The activated beads were finally resuspended in 0.1 M carbonate buffer (pH 9.0), 0.5% deoxycholate (sodium salt, Calbiochem Ltd). Appro-

priate amounts of the activated Sepharose was added to the protein to be coupled in the carbonate–deoxycholate buffer (CD buffer). The beads were kept in suspension by gentle mixing for 24 h. The amount of protein coupled was monitored optically.

2.4. *Binding assay*

An aliquot of sepharose with the appropriate coupled protein in CD buffer was incubated with an equal volume or less of hybridoma cell supernatant or purified antibody. This ensures that the detergent level does not fall to <0.25%. The incubation was performed at room temperature with continuous or intermittent mixing. The beads were then washed by centrifugation with at least 3-times 6 vol. CD buffer. The binding of antibody to the beads was detected using iodinated rabbit anti-mouse IgG. The rabbit anti-mouse IgG was purified by affinity chromatography against mouse IgG pepsin degraded and the fragments separated by gel filtration (a gift of Dr A. F. Williams [10]). The Fab fragments were iodinated by the chloramine T procedure to a spec. act. $\sim 10^7$ cpm/μg.

2.5. *Antisera and antibodies*

Aside from the genesis of monoclonal antibodies described here a purified anti-HLA monoclonal antibody identified and characterized by Barnstable et al. [2] was used as well as the supernatant of cells producing this antibody (W632). Dr Alan Williams kindly supplied a monoclonal antibody against a rat cell surface protein (W3-13) and Ms Frances Brodsky a monoclonal antibody against β-2 microglobulin (BB5).

Rabbit antiserum against calf brain tubulin was prepared using tubulin purified by 3 cycles of polymerization [11] and was a gift from Dr R. A. Badley, Unilever Res., Sharnbrook, England. Dr Badley also provided rabbit antiserum against chicken gizzard desmin prepared as in [13] with the proteolysis inhibitors [14].

2.6. *Immunofluorescence and electron microscopy*

Fibroblasts were grown on coverslips in RPMI medium supplemented with 10% foetal calf serum. The cells were fixed in acetone at –20°C for 15 min. Washed in PBS and incubated with the appropriate antibody in a moist atmosphere for 30 min. Excess first antibody was removed by washing in PBS and the cells were incubated in fluorescent second antibody. Fluorescenated rabbit antimouse IgG was purchased from Miles Labs. and fluorescenated sheep anti-rabbit IgG from Wellcome. The coverslips were mounted in PBS–glycerol (1:9) and viewed in a Leitz Ortholux epifluorescent microscope using a X63 objective. Photographs were taken by exposing Pan X film (Kodak) for 1 min. Rabbit skeletal muscle fibres were glycerinated and prepared for electron microscopy by Ms Belinda Bullard as described in [15]. Rabbit skeletal muscle actin and myosin were purified by Ms Bullard by the procedures in [16,17].

3. Results and discussion

3.1. *Immobilization of membrane proteins*

Proteins can be immobilized in many different ways. One of the most common procedures is covalent coupling to cyanogen bromide-activated Sepharose [8,18]. This paper describes conditions where membrane proteins may be coupled to Sepharose. Deoxycholate was chosen as the detergent to disrupt the membrane fragments because of the low micelle size and the planar structure of the bile salt [19]. These two factors might be expected to minimize the amount of proteins hidden by the detergent molecule and unavailable for coupling. The activated Sepharose was immediately mixed with the proteins in carbonate–bicarbonate buffer (pH 9.0), deoxycholate at 1% and stirred at 4°C for 18 h. Immediately after binding the residual imidocarbonate groups gave very high backgrounds, these decline over several days, but can be reduced more quickly by incubating the Sepharose with 1 M Tris–HCl (pH 8.0) or 1 M glycine. The coupling efficiency in the presence of detergent membrane proteins or soluble proteins is high. At least 7 mg membrane protein can be bound to 1 ml Sepharose. The activated Sepharose has a high background in the binding assay which is not present before activation (table 1a). This background is independent of the coupled protein and may be due to ionic interactions with charged groups formed on activation. These interactions are not disrupted by 1.2 M NaCl. The possibility that the M_r 3×10^6 exclusion limit of Sepharose 4B physically traps molecules is ruled out by the low background on unactivated Sepharose. Table 1b shows that membrane proteins coupled to Sepharose give increased binding of known monoclonal antimembrane antibodies over the haemoglobin–Sepharose control. This binding is specific for human proteins and as glycolipids will not be coupled

excludes antibodies against the carbohydrate of glycolipids. This binding can be inhibited by preincubating the antibody with excess antigen (table 1b, 10). The kinetics of binding (not presented) show that the reaction is complete in <1 h with intermittent mixing. The sensitivity (fig.1) of this assay is certainly adequate for monitoring the supernatant medium of hybridoma cells but requires a greater absolute amount of target protein than the whole cell binding assay. Each point shown in fig.1 and table 1 used 30 μl settled bed volume of Sepharose 4B at 1 mg/protein/ml, equiv. 10^7 cells. The whole cell binding assay requires 10^6 cells/point. Thus in the presence of detergent membrane proteins may be coupled to Sepharose and are a suitable target for monoclonal antibodies.

3.2. *Monoclonal antibodies against internal determinants*

Assaying hybridoma cell supernatants with the whole cell binding assay only detects antibodies against the cell surface. The antibodies are also detected by membrane proteins bound to Sepharose (fig.2,3,▪). Supernatants which are negative in the whole cell binding assay may:

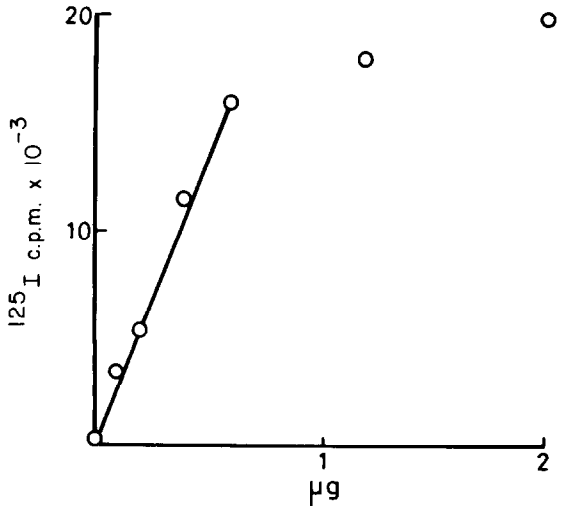

Fig.1. Binding of pure monoclonal anti-HLA IgG to membrane proteins coupled to Sepharose. Membrane proteins were prepared from human lymphoblastoid cell line by hypotonic lysis [5] and coupled to Sepharose (1 mg protein/ml settled bed vol. (sbv) of Sepharose; 30 μl sbv were used for each point). The cpm bound to membrane–Sepharose minus cpm bound to a negative control, 30 μl sbv haemoglobin–Sepharose (1 mg/ml) is shown for increasing amounts of pure anti-HLA in the first incubation.

Table 1
Nature of binding

a.	Second antibody: Iodinated rabbit anti-mouse Ig cpm bound to	
First antibody	Unactivated Sepharose	Human haemoglobin –Sepharose
1. Culture	512	1865
medium	411	2439
2. Cell supernatant	856	7551
α HLA-ABC	825	8078
3. Pure α HLA-ABC	750	7259
	524	6331
4. Pure α HLA-ABC	670	6752
1.2 M NaCl	339	5604

b.	Human haemoglobin –Sepharose	Human membrane protein –Sepharose
5. Culture medium	4413	3944
6. Monoclonal α rat antigen	6086	5893
7. Monoclonal α glycolipid	5304	5671
8. Monoclonal α HLA-ABC	9245	16 461
9. Monoclonal $\alpha\beta_2$ microglobulin	4450	7178
10. Monoclonal $\alpha\beta_2$	4647	4839
inhibited with	4573	5433
pure β_2	4729	5524

(i) Lack antibodies against human membrane proteins, or
(ii) Contain antibodies directed against hidden internal determinants.

Membrane proteins bound to Sepharose can distinguish these two classes. The △ in fig.2,3 are results of binding assays on supernatants which were negative in the whole cell binding assay. It is clear that many of these supernatants contain antibodies directed against hidden antigenic sites not accessible in the whole cell assay. The hybridoma cells in fig.3 are derived from mice immunized with lectin purified plasma membrane glycoproteins so these 'inside antibodies' are very probably directed against the internal cytoplasmic regions of these transmembrane proteins. Because of the interest in cytoskeletal association with the

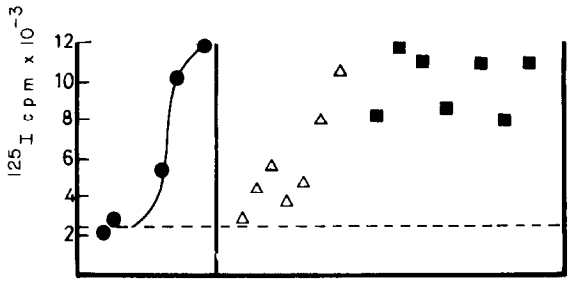

Fig.2. The cpm of ^{125}I-labelled anti-mouse IgG bound to membrane Sepharose (30 μl sbv) incubated with increasing amounts of pure anti-HLA (●); hybridoma supernatants. Shown to be positive by the whole cell binding assay (■); hybridoma cell supernatants shown to be negative by the whole cell binding assay (△). One of the lower group assaying at 2 × 10^3 counts above background was subsequently shown to decorate the microfilament system (see fig.4). The mice were immunized with a crude membrane fraction from human placenta.

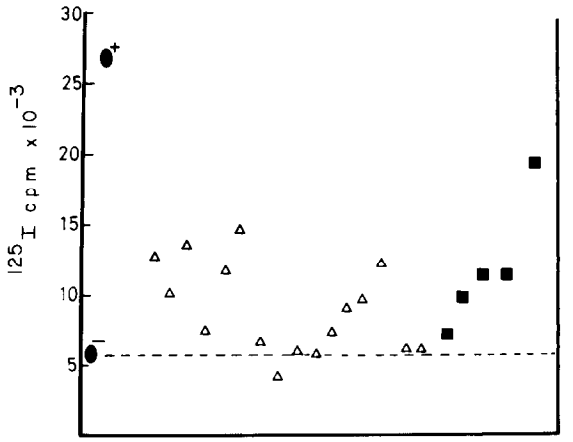

Fig.3. Membrane proteins coupled to Sepharose were used to assay the supernatants of a second hybridoma fusion. In this case the mice were immunized with the lentil lectin bound glycoproteins from a human lymphoblastoid cell line plasma membrane preparation. In the absence of a first antibody 5 × 10^3 cpm of ^{125}I-labelled rabbit anti-mouse IgG are bound (−). Addition of 2 μg pure anti-HLA IgG/100 μl (30 μl sbv) assay gave >25 × 10^3 cpm of anti-mouse IgG bound (+). Hybridoma supernatants bound intermediate amounts if they had been proved positive in the live whole cell binding system (■). Negative supernatants in the whole cell assay contained many positives by the membrane–Sepharose criterion (△).

plasma membrane the putative inside antibodies shown in fig.2 were screened by immunofluorescence for their cellular location. One of these antibodies gave strong staining of microfilament bundles on primary human fibroblasts (fig.4). This staining was eliminated by cytochalasin B (5 μg/ml, 3 h) but unaffected by colcemid (1 μg/ml, 3 h). The staining pattern can be seen to differ from the microtubule network stained with rabbit anti-tubulin antiserum. The microfilament bundles are thought to contain many proteins but myosin and actin may be the major components [20–22]. No association was found between the anti-microfilament antibody and rabbit striated muscle F-actin, acetone denatured actin or precipitated myosin. To test the antibody further foetal rat lung fibroblasts were stained by indirect immunofluorescence. Antisera from individuals with chronic active hepatitis gave clear microfilament bundles but the hybridoma supernatant did not. Nor does the hybridoma supernatant stain glycerinated rabbit striated muscle fibres by either electron microscopy or immunofluorescence. It appears then that the antibody is specific for the human form of the protein, we do not yet know whether the antibody will stain human muscle cells. The characterization of the anti-microfilament antibody shows quite clearly that anti-inside antibodies can be detected by the Sepharose assay system. We used this antibody and the anti-HLA monoclonal antibody to determine if whole cells may be made permeable and used to detect anti-inside antibodies.

Lymphoblastoid cells fixed with paraformaldehyde (1%), glutaraldehyde (0.2%) or acetone (2 vol.) in the absence of detergent bound anti-HLA strongly but bound little if any anti-microfilament antibody. However, if after 5 min fixation in 0.2% glutaraldehyde in phosphate-buffered saline (PBS) at 22°C the solution was made 1% in Triton X-100 and incubated for a further 5 min, after several washes with PBS these cells remained intact by phase contrast microscopy and did bind anti-microfilament antibody (fig.4). This is a simple alternative assay system for anti-inside antibodies. The glutaraldehyde-fixed, detergent-treated cells have a higher background binding than whole live cells which may be due to glutaraldehyde-mediated crosslinking of the soluble antibody. However, extensive treatment of the cells with the reducing agent sodium borohydride (0.5 mg/ml) did not reduce this background even though this treatment allows immunofluorescent analysis of glutaraldehyde-fixed cells. It appears that this background is not due to covalent interaction with remaining activated groups.

The detection of anti-microfilament antibody in hybridioma cells derived from mice immunized with membranes from a lymphoblastoid cell line provides a

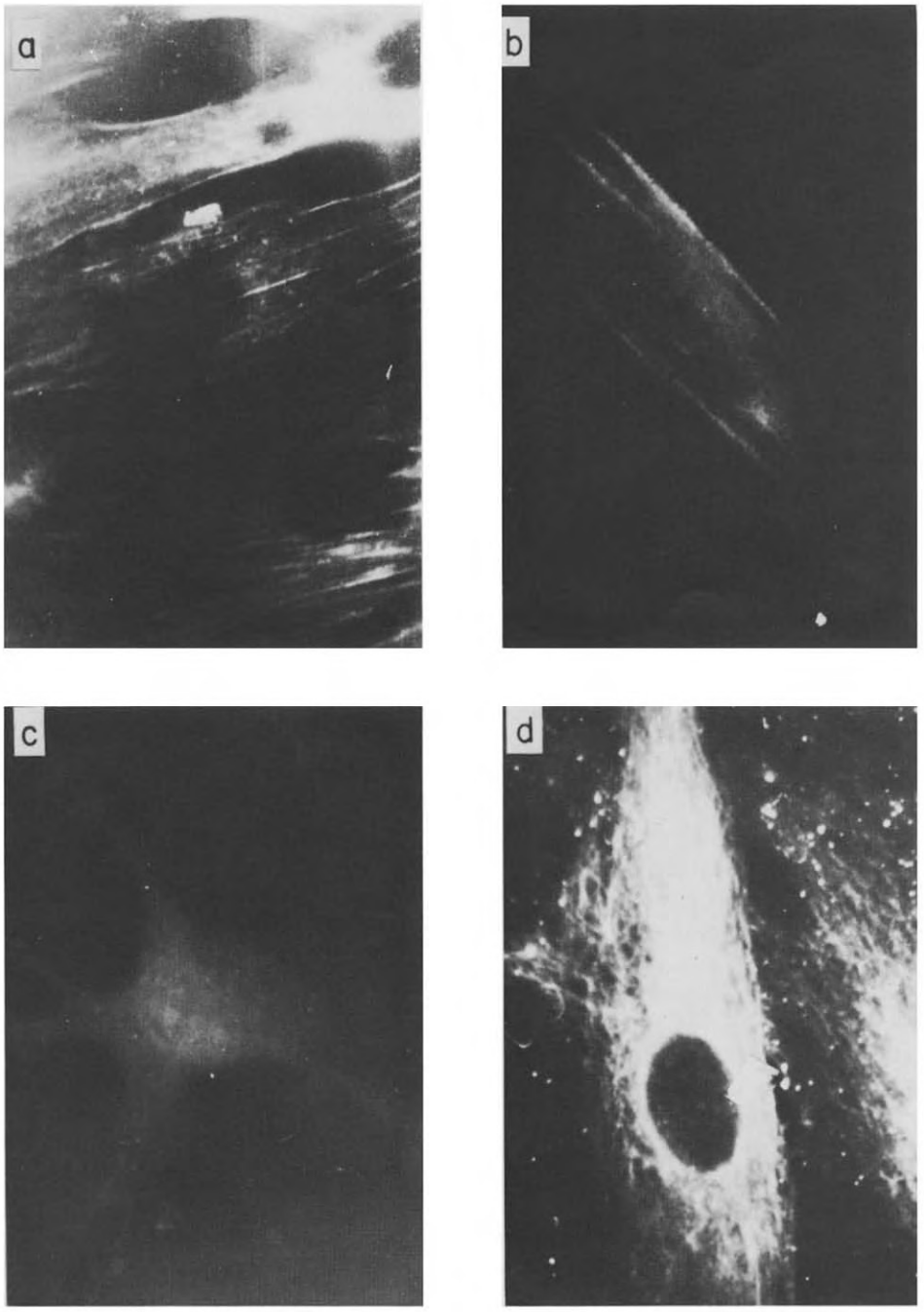

Fig.4. Indirect immunofluorescence on primary human fibroblasts using monoclonal antimicrofilament antibody in (a–c) and rabbit anti-tubulin antiserum in (d). The microfilament bundles in (a,b) are not present in the cytochalasin B treated cell seen in (c). The microtubule stained cell (d) is shown for comparison. The fluorescent second antibody obtained through Miles Labs. was used at a dilution of 1:50. The monoclonal antibody was diluted 1:2 and the rabbit antiserum 1:20 with PBS. The magnification is 1500 diameters. In the absence of first antibody there was no staining.

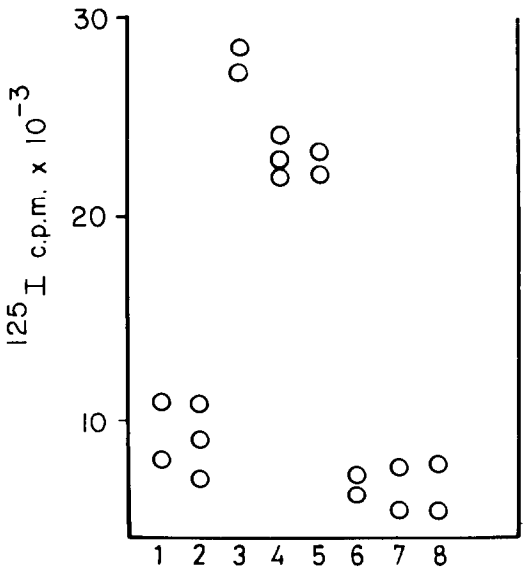

Fig.5. Detergent-treated cell binding assay. Lymphoblastoid human cells fixed in 0.20% glutaraldehyde and treated Triton X-100 are leaky and although the background is relatively high when; no first antibody is used (1); no spurious binding is observed to a monoclonal anti rat antibody (2); a monoclonal antibody against 10 nm filaments (3); and the anti-microfilaments (4) binding as strongly as the anti HLA monoclonal antibody (5); (6–8) confirm that other monoclonal antibodies which bind strongly to rat cerebellum are negative in this assay system.

tool for investigating the relationship of the cytoskeleton and the membrane. The difference in binding of the anti-HLA and anti-microfilament antibodies to whole fixed cells suggests that the microfilament associated antigen is not present on the cell surface. This view is supported by the fact that live attached fibroblasts are not 'patched' by treatment with the anti-microfilament antibody. In contrast treatment of live cells with anti-HLA repeatedly made every cell a mass of aligned fluorescent spots. Patches generated by anti-HLA antibody in [21] aligned over stress fibres. Fig.5 shows that this monoclonal anti-microfilament antibody and a monoclonal antibody against intermediate filaments (identified by C. Barnstable) bind to permeabilized lymphoblastoid cells as detected with iodinated rabbit anti mouse immunoglobulin. This procedure provides a simple test for antibodies against internal cellular determinants.

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