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Antibodies to Tumour Eluates React Preferentially with Non-Lymphoid Tumours

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Summary. *Rabbit antisera raised against eluates from a murine fibrosarcoma were characterised using a ^{125}I-protein A assay and a wide variety of target cells. The sera bound preferentially to rodent tumours of non-lymphoid origin, whereas monkey and human cells did not react. Murine lymphoid cells and macrophages (normal or transformed) and normal liver and kidney cells all bound low amounts of the antibody, while embryonic cells were intermediate in reactivity.*

Target cell treatments indicated that the surface antigens being detected were sensitive to proteolysis and calcium depletion. In addition actively growing cells bound more antibody than resting cells. Double binding assays with sera specific for plasma membrane components suggested the eluate antigens may play a structural role. Immunofluorescent studies demonstrated that surface antigens detected by the antisera capped and were lost and this was followed by synthesis and surface re-expression.

Sera such as these, which can distinguish between normal and malignant cells in the rodent, have obvious applications in many aspects of tumour-related investigations.

Introduction

Previous studies have shown that host immunoglobulin is associated with certain solid tumours in vivo and attempts were made to recover the immunoglobulin with the aim of determining its specificity [3]. The procedure adopted was that of acid citrate elution, which had been shown to remove immunoglobulins from tumour cells [5]. However, in addition to immunoglobulin, the acid citrate eluates were found to contain major non-immunoglobulin components with M_r 33,000 and 36,000, which were also present in eluates from cultured tumour cells [3]. These proteins appeared to be less prevalent in comparable eluates from normal tissue, with the exception of peritoneal exudate cells, and their possible uniqueness to the malignant phenotype seemed worthy of further investigation.

One approach adopted was an attempt to raise rabbit antisera against acid citrate eluates prepared from a cultured murine fibrosarcoma. All the antisera obtained were found to bind strongly to murine fibrosarcoma cells but not to spleen cells. The present paper describes in detail the cell binding characteristics of one of these sera.

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Materials and Methods

Eluates were prepared from cultured cells derived from a methylcholanthrene-induced tumour (CCH1) of CBA origin [7] by incubating 10^8 cells for 10 min at 0° C in 1 ml 0.12 *M* acid citrate buffer pH 3.5. The supernatant obtained was stabilised by addition of 10 µl kallikrein inactivator/ml (Calbiochem), passed through a Millipore 0.22 µm filter, and dialysed against PBS pH 7.2. Further kallikrein inactivator was added and the eluates stored in liquid nitrogen.

New Zealand white rabbits were immunised four or five times with 100–175 µg protein mixed with an equal volume of Freund's complete adjuvant for the initial injection (SC), incomplete Freund's adjuvant for the second injection (SC), and alum subsequently (IP) over a period of 6 or 12 months. Sera were obtained 3 weeks after the final injection. All the rabbits produced antibodies which reacted with CCH1 tumour cells. Pre-immunisation sera were also collected. All sera were passed through a fetal calf serum-CnBr Sepharose 4B column before use.

Rabbit antisera specific for actin, clathrin and tropomyosin were kindly donated by Dr M. J. Owen (ICRF, London), Dr M. C. Willingham (NIH, Bethesda) and Dr J. J. C. Lin (Cold Spring Harbor, NY).

Normal tissues were obtained from inbred CBA/Ca mice of either sex, aged 6–12 weeks. Suspensions of mouse splenocytes and thymocytes were prepared by mechanical disruption in a ground-glass homogeniser. Peritoneal exudate cells were harvested by peritoneal lavage with heparinised (10 U/ml) RPMI 1640. Cultures of CBA mouse kidney and liver were prepared by digestion with a mixture of trypsin (0.1 mg/ml: Sigma), collagenase (0.01 mg/ml, grade A: Sigma), and deoxyribonuclease I (0.04 mg/ml: Sigma). The cells were cultured in either RPMI 1640 or William's Medium D containing 10% FCS, in a gas incubator.

Established cell lines were cultured in the appropriate medium, e.g., RPMI 1640, Ham's F10, Dulbecco's MEM, containing FCS or newborn calf serum (10% or 5% v/v), L-glutamine (2 m*M*), penicillin (100 U/ml), and streptomycin (100 µg/ml) (Gibco Europe Ltd).

Cultured cells were harvested by exposure to either 0.02% EDTA at 20° C or 0.05% trypsin, 0.02% EDTA at 37° C for 10–20 s.

After washing, the cells were resuspended at 5×10^5/ml in PBS containing 5% FCS (PBS–FCS) for the protein A assay. The viability of all cell preparations was routinely assessed by nigrosin dye exclusion and normally exceeded 90%.

Protein A (1 mg/ml: Pharmacia) was labelled with ^{125}I by the chloramine T method [2], free iodine being removed by passage through a Sephadex G25 column. To assess rabbit antibody binding, 10^5 cells were added, in triplicate, to plastic 'Removawells' (Dynatech) and incubated for 1 h at 0° C with 200 µl antiserum dilutions in PBS-FCS (usually 1 : 100). After three washes in PBS-FCS, 100 µl ^{125}I-protein A containing 1×10^5 cpm was added to each well and incubated for 45 min at 0° C. The cells were washed four times and counted. On every occasion when other cell types were being tested, CCH1 and spleen cells were included as positive and negative controls, respectively. In addition in many cases assays were repeated on different occasions.

For immunofluorescent staining, cytocentrifuge smears were acetone-fixed and standard indirect staining procedures were employed using fluorescein-labelled goat anti-rabbit IgG (Hoechst U. K. Ltd). Cell suspensions were reacted on ice with the same reagents.

Results

Anti-eluate binding was determined for a large variety of cells of murine, rat, hamster, monkey, and human origin, including normal epithelial, fibroblast, and lymphoid cells, embryonic fibroblasts, and cell lines derived from tumours which either were virally or chemically induced or occurred spontaneously. All the rabbit anti-sera reacted with CCH1 tumour cells and bound little to splenocytes or thymocytes. The binding characteristics of one of these sera will be presented in detail and Fig. 1 illustrates typical binding data from the ^{125}I-protein A assay in which the low level of reactivity with lymphoid and normal cells can be seen. Spleen cells were always tested at a ten-fold concentration (i.e., 10^6/well) to allow for their smaller surface area compared with most tumour cells. Standard deviations of the counts bound were normally less than 10% of the total.

To simplify the comparison of anti-eluate binding between the many cell types tested, a semi-quantitative method of presentation was devised (see Table 1). All the rodent tumours of non-lymphoid origin tested were found to bind anti-eluate antibody, but the degree of binding could not be correlated with their mode of transformation or tissue origin. Lymphoid cells, whether normal or transformed, displayed little reactivity. Macrophages (normal, elicited or transformed) and cultured liver (murine) and kidney (murine, hamster) cells exhibited low but consistent amounts of binding, whereas rat and murine embryonic fibroblasts were intermediate in reactivity (Fig. 1). In addition, there was a clear-cut species specificity in that monkey and human cell lines bound virtually nothing above background (Fig. 1).

Visual assessment of the degree of fluorescent staining of the cells tested above was in agreement with the protein A assay results. Staining of fixed cells was cytoplasmic and in CCH1 cells consisted of an eccentric, perinuclear ring of speckled fluorescence.

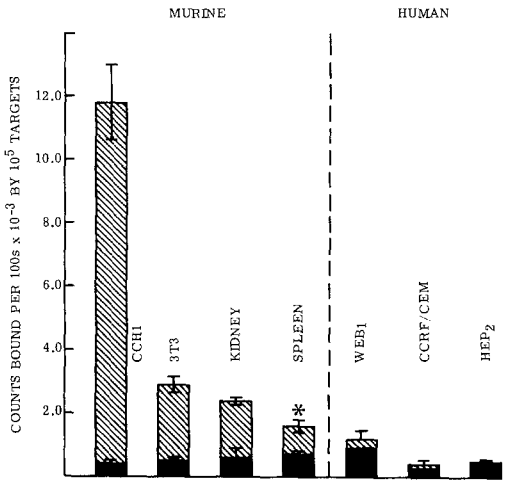

Fig. 1. The results of a typical ^{125}I-protein A binding assay: antisera were diluted to 1 : 100. Standard deviations are indicated as *vertical bars*. (■) Pre-immune serum; (▨) anti-eluate serum; (✳) 10^6 target cells

Table 1. Summary of the binding activity of anti-tumour eluate antibody to tumour and other targets

Target cells	No. of lines	No. of experiments	Binding[a]
Mouse and rat fibrosarcomas	5	33	+ + +
Mouse melanoma	1	1	+ + +
Rat hepatoma	1	4	+ + (+)
Mouse and rat mesotheliomas	3	5	+ + +
Virus-transformed cells from mouse, rat and hamster	6	12	+ +
Mouse and rat mammary carcinoma	2	3	+ +
Rat pleoblastocytoma	1	1	+ +
Mouse liver cell line	1	1	+ +
Mouse and rat embryonic fibroblasts	3	3	+ (+)
Normal, elicited, and transformed mouse macrophages	6	10	+
Normal mouse and hamster kidney	2	5	+
Normal mouse liver	1	3	+
Mouse splenocytes, thymocytes	2	15	− (+)
Mouse and human B cell lines	12	15	−
Human T cell lines	2	2	−
Human embryonic fibroblasts	2	4	−
Human synovial epithelial cells	1	2	−
Human carcinomas (bladder, nasopharyngeal)	10	14	−
African green monkey kidney	1	2	−

[a] The ratio of the mean counts bound by anti-eluate to that bound by the pre-immune serum was determined for each cell type. Ratios less than 2 were assigned −; 2–4, +, 4–8, + +; > 8, + + +

Table 2. Effect of serum absorption[a] on binding of anti-tumour eluate antibody to target cells

Cell type	No of cells/ml	% Reduction of binding to			
		CCH1	Spleen	Kidney	Liver
CCH1	5×10^6	77	+ 100[b]	3	+ 28
Spleen[c]	10^8	0, 5	47, 45	23, 18	ND, + 5
	5×10^7	0, + 8	16, 13	19, 17	ND, + 7
Kidney	3.3×10^6	+ 8	+ 30	20	16
Liver	2×10^6	1	3	14	21

[a] Sera diluted 1:100 in PBS-FCS were incubated for 2 h at 0° C with the number of cells stated and the supernatant stored frozen until tested
[b] Increased binding
[c] Two sets of results shown
[d] Not done

Absorption of the anti-eluate with different cell types tended to be tissue-specific (Table 2). Tumour cells removed anti-tumour activity without affecting binding by spleen cells and the converse was also found. Absorption with freshly prepared murine kidney and liver cells did not apparently remove any of the reactivity with tumour or spleen cells. The effect of serum absorption with intact CCH1 cells on cytoplasmic fluorescent staining was also investigated. Absorption was confirmed by the absence of detectable surface staining while the cytoplasmic staining of fixed cells was greatly reduced.

A variety of target cell treatments was undertaken and acid citrate elution, fixation, and the action of certain enzymes was found to reduce anti-eluate binding by CCH1 cells (Table 3). The effect of cell cycle on anti-eluate binding was also investigated and the effectiveness of using a low FCS concentration to synchronise the cultures was demonstrated by a rat sarcoma, Walker 2, which grows in suspension. A culture

Table 3. Effect of various target cell pretreatments on the binding of anti-tumour eluate antibody

Treatment	% Increase (+) or decrease (−) in binding of anti-eluates
Elution with acid citrate	− 36
Glutaraldehyde fixation[a]: high concentration (0.1%)	− 27
low concentration (0.0001%)	+ 51
Formaldehyde fixation[a] (3.5%)	− 55
Synchronisation in growth phase[b]	+ 66
Pre-incubation with trypsin, pronase, neuraminidase or Dispase	− 70; − 75; − 59; − 76
Pre-incubation with EDTA, periodate or PBS[c]	− 19, + 10; − 8

[a] Cell suspensions were mixed for 20–30 min at 20° C on a rotator, followed by at least four washes with PBS-FCS
[b] Cells were incubated in medium containing 0.25% FCS for 24 h, followed by 18 h in 10% FCS
[c] Cell suspensions were treated for 15 min at 37° C (5 min at 20° C for EDTA) with the following reagents (maximum concentration), followed by two washes in PBS-FCS: trypsin (0.1 mg/ml: Sigma), pronase (2.5 mg/ml: Calbiochem), neuraminidase (10 U/ml: BDH), Dispase (0.1 mg/ml: Boehringer), EDTA (0.02%: BDH), sodium periodate (10 m*M*: BDH) or Dulbecco A PBS

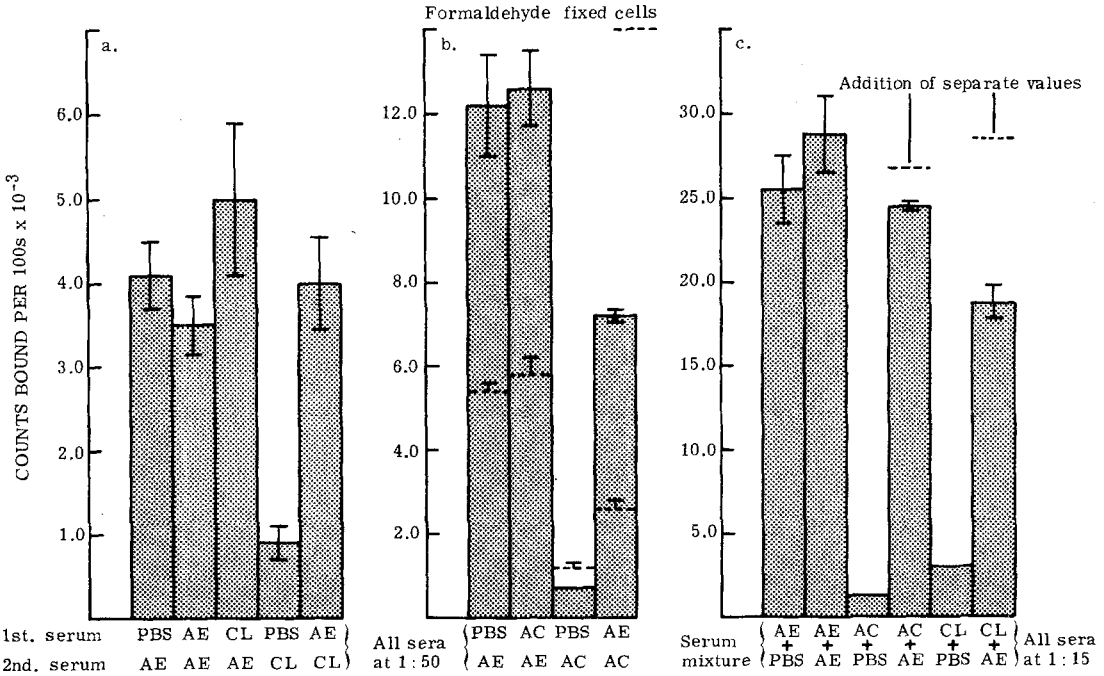

Fig. 2a–c. Effect of anti-clathrin (CL) and anti-actin (AC) on anti-eluate (AE) binding to CCH1. **a, b** Unfixed CCH1 cells were incubated with the first serum followed by unlabelled protein A, then the second serum followed by ^{125}I-protein A, with washings at each stage. In **b** formaldehyde-fixed cells were also used. **c** Unfixed CCH1 cells were incubated with the mixture of antisera shown followed by ^{125}I-protein A. Standard deviations are indicated as *vertical bars*

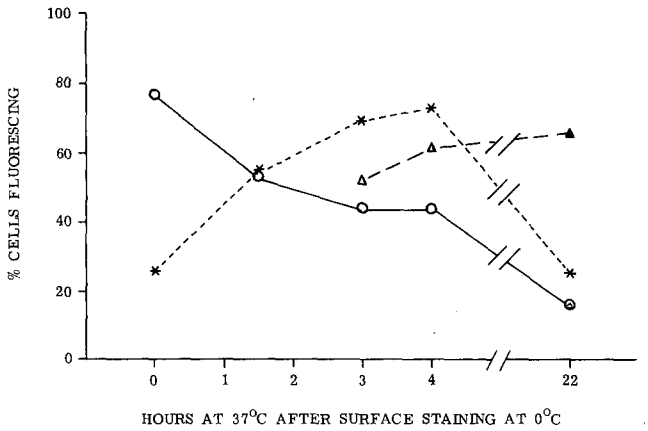

Fig. 3a–c. Resynthesis of antigen by CCH1 cells following capping with anti-eluate antibody. CCH1 cells were incubated in suspension for 1 h on ice with anti-tumour eluate antibody, washed, and transferred to 37° C. At times indicated, cells were **a** stained with fluorescein conjugate (– ○ –); **b** re-incubated with anti-tumour eluate antibody, then fluorescein conjugate (– △ –); **c** prepared as smears, fixed, and incubated with anti-tumour eluate antibody, then rhodamine conjugate (– ✲ –). The percentage of cells with surface (**a, b**) or cytoplasmic (**c**) staining was estimated. Staining: surface (○–○); (△- -△) surface (restained); cytoplasmic (✲ – ✲)

containing 5×10^6 cells in 0.25% FCS had increased to 7.5 $\times 10^6$ cells after 18 h in 10% FCS, whereas the control culture remained static. Anti-eluate binding by the 'growth phase' cells was 171% of the stationary culture. CCH1 cultures treated in this way gave similar results (166%).

In an attempt to determine the nature of the surface antigens detected, a series of double binding assays was devised which explored the interaction at the cell surface of anti-eluate with antisera specific for other membrane antigens. The target cells were incubated first with antiserum or PBS-FCS followed by unlabelled protein A, then with the second antiserum followed by ^{125}I-protein A. In these experiments the sera were not present at saturating concentrations. Alternatively, the sera were incubated with the cells as a mixture at saturation. Using rabbit antibody specific for clathrin, a protein associated with coated vesicles in the plasma membrane [4], it was found that pre-incubation of CCH1 with this antibody had little effect on subsequent anti-eluate binding. Pretreatment with anti-eluate only slightly reduced further binding by the same antiserum (Fig. 2a). In contrast, anti-clathrin binding was increased more than four-fold after pre-incubation of the cells with anti-eluate. When, however, the anti-eluate and anti-clathrin sera were added as a mixture there was a marked reduction in binding (Fig. 2c).

These experiments were repeated using a rabbit anti-actin serum. Binding by anti-eluate was unaffected by pretreatment with anti-actin but conversely pre-incubation with anti-eluate increased the anti-actin binding ten-fold (Fig. 2b). Formaldehyde fixation of the cells increased anti-actin binding but reduced anti-eluate binding (Fig. 2b). Fixation also interfered with the increased binding by anti-actin which had occurred in the unfixed cells following anti-eluate binding. When, however, these two sera were incubated with the cells as a mixture, the counts bound were similar to the addition of their separate results (Fig. 2c).

Immunofluorescent staining of acetone-fixed CCH1 cells by anti-actin and anti-clathrin sera was largely around the membrane, the latter in particular giving an overall speckled appearance. In contrast, anti-tropomyosin faintly stained a fine network of filaments over the whole cell. None of these patterns resembled that given by anti-eluate and no microfilament staining by anti-eluate was seen under suitable fixation conditions, i.e., formaldehyde followed by cold acetone.

The nature of the surface antigens detected was further investigated under capping conditions by immunofluorescence. Capping of the antigens occurred very rapidly after anti-eluate binding and the percentage of cells with surface staining decreased steadily to reach a plateau by 3 h. (Fig. 3). Aliquots of cells were re-incubated with anti-eluate to detect newly expressed antigens, and already by 3 h a small increase in stained cells was evident, rising to approach the original level by 22 h. The proportion of cells with bright cytoplasmic staining showed an immediate rise which preceded re-expression of the surface antigens, returning to the original level by 22 h.

Discussion

These studies have demonstrated that acid eluates of a murine fibrosarcoma have elicited in rabbits antibodies capable of discriminating between normal and malignant cells. Detailed studies of one of these sera showed that it reacted preferentially with rodent tumours of non-lymphoid origin and that it did not recognise human or monkey cells.

From the various target cell treatments it was clear that the reacting eluate components were sensitive to proteolysis and calcium depletion and that their expression may be increased during mitosis. They do not seem to be intracellular proteins simply being released at the cell surface, but are more likely to be membrane-associated proteins, particularly since antigen capping could take place. Since the surface antigen was quickly replaced, a structural or receptor type of function might be envisaged. Further evidence of movement within the membrane was provided by the double binding assays where binding of anti-eluate seemed to cause surface distortion revealing new sites with which both anti-clathrin and anti-actin could react. In contrast membrane fixation greatly reduced this effect.

Further investigation with antisera specific for known structural membrane components failed to suggest that anti-eluate cross-reacted with any of them. The interaction detected between anti-eluate and anti-clathrin when present in high concentrations at the cell surface may have been indicative of steric hindrance rather than antigenic identity.

The absorption studies suggested there may be two broad specificities within the serum, namely anti-tumour and anti-rodent. However, reactivity with normal cells could not be removed completely, indicating some non-specificity in the binding, perhaps via Fc receptors. In contrast, the CCH1 tumour does not express Fc receptors [6]. Attempts at double absorptions using various combinations of normal and tumour cells were confounded by increased binding in some cases, probably as a result of soluble complexes in the preparations. This effect is evident in Table 2. At the present time, there is insufficient evidence for us to say whether the anti-eluate sera are truly specific for malignant cells or whether they are detecting an increased expression of certain antigens. Nevertheless, it must be significant that while 5×10^6 CCH1 cells absorbed 77% of the tumour reactivity, 20 times as many spleen cells removed none.

Preliminary characterisation of the proteins recognised by the anti-eluate sera has been undertaken using two different approaches. First, affinity chromatography studies of ^{125}I-labelled tumour eluates suggest that the antibody reacts with polypeptides of M_r 23,000, 25,000, 34,000, and 36,000. However, these proteins are not readily detected in similar preparations from spleen cells. These results are consistent with our original observations on the electrophoretic patterns of labelled eluate [3]. In contrast, preliminary data obtained by the immunoblot technique on PAGE-SDS gel separated tumour eluates indicate that the antibody interacts with one major component of M_r 46,000–47,000. This protein is also detected, albeit in much reduced amounts, in eluates from normal spleen cells and thymus. These observations serve to emphasise the false impressions that may be gained if only one system is used in antigen characterisation.

Whatever the specificity of these anti-eluate sera, it is apparent that they can distinguish normal from malignant cells in rodents. They should therefore prove useful in oncology research with a wide range of applications in immunochemical studies of tumours, cell sorting by surface immunofluorescence and the investigation of malignant transformation.

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