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Demonstration and Partial Purification of Common Melanoma-Associated Antigen(s)*

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Abstract—*Sera from melanoma patients, patients with other tumours, non-cancer patients and healthy donors were screened for antibody against NK*1-4 *melanoma cells by indirect membrane immunofluorescence. Approximately* 8% *of melanoma patients and* 8% *of patients with other tumours reacted against NK*1-4 *cells, but only the melanoma patients were positive against other melanoma cell lines. After establishing that they did not react with cell ines from other types of tumour, two positive sera from melanoma patients were used to detect melanoma-associated antigen(s) in* 3 M KCl *extracts of melanoma and other tissues. Absorption with melanoma extracts inhibited the immunofluorescence activity of these sera, but extracts of other tumours, normal skin or embryos failed to absorb antibody. Melanoma extract was fractionated by chromatography on a specific immunoadsorbent column prepared with one of the reactive melanoma sera. Material bound to the immobilised antibody was eluted with* 3 M NaSCN *and tested for antigenic activity in comparison with the unbound material and the unfractionated* 3 M KCl *extract. Melanoma-associated antigen was found in the bound fraction, and was estimated to be concentrated approximately* 10-*fold (per unit of protein) compared with the unfractionated extract. The protein profile of the bound fraction was much less heterogeneous than those of the unbound or unfractionated materials and it is suggested that immunoadsorbent columns could be used as an important step in the isolation of highly-purified tumour-associated antigen preparations.*

INTRODUCTION

ANTIGENS associated with human malignant melanoma have been demonstrated in many laboratories using *in vitro* techniques for assaying cell-mediated immunity [1] and humoral antibody against melanoma cells [2–7]. The types of antigen identified have included individual tumour-specific antigens and separate common melanoma associated antigens [4, 6], and also melanoma-related foetal antigens [8, 9]. Most studies have been directed towards identifying these antigens and characterising their specificity and distribution on intact melanoma cells by means of lymphoid cells or sera from autologous or allogeneic melanoma patients. In order to understand the biochemical nature of melanoma antigens, however, it is desirable to isolate the antigens in soluble form, and this has been reported by several groups [10–14]. In most cases relatively crude extracts have been prepared and analysed. The following is a report of demonstration of a common melanoma-associated antigen in 3 M KCl extracts of melanoma, followed by its partial purification by specific immunoadsorbent chromatography in order to obtain antigenic material amenable to further chemical analysis.

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MATERIALS AND METHODS

Target cells

Target cells were long-term cultured tumour cell lines. Most were maintained as monolayer cell cultures in Eagle's Minimum Essential Medium (MEM), supplemented with foetal calf serum (10%), penicillin (200 IU/ml) and streptomycin (100 μg/ml). They were grown in 25 cm^2 Corning tissue culture flasks and were routinely harvested with 0.25% trypsin (Difco 1 : 250) in Hanks' balanced salt solution (HBSS). Several cell lines were donated by workers in other institutions, and are listed in Table 1. Most of

Table 1. List of cell lines used in membrane immunofluorescence tests

Cell line	Tumour of origin	Donor
NK1-4	Melanoma	Dr. J. E. De Vries, Amsterdam
Mel-2a	Melanoma	Dr. C. Sorg, Münster
Mel-57	Melanoma	Dr. C. Sorg, Münster
Mel-Swift	Melanoma	Dr. C. Sorg, Münster
MeWo	Melanoma	Dr. C. Sorg, Münster
RPMI-5966	Melanoma	Dr. C. Sorg, Münster
163	Melanoma	Dr. R. H. Whitehead, Melbourne
364	Melanoma	Dr. R. H. Whitehead, Melbourne
9812	Lung carcinoma	Dr. J. Fogh, New York
T24	Bladder carcinoma	Dr. M. Moore, Manchester
EB33	Prostate carcinoma	Prof. F. Schöder, Rotterdam
HT29	Colon carcinoma	Dr. L. M. Franks, London
EB2	Burkitt lymphoma	Prof. M. A. Epstein, Bristol
HS578T	Breast carcinoma	U.S. Naval Biomedical Research Center
HS788T	Osteogenic sarcoma	U.S. Naval Biomedical Research Center
HS791T	Osteogenic sarcoma	U.S. Naval Biomedical Research Center
HeLa	Cervical carcinoma	Obtained commercially

Table 2. Membrane immunofluorescence against NKl-4 melanoma cells harvested by different methods

Method of cell harvesting*	Fluorescence index† with serum from Healthy donor (A1325)	Melanoma patient (A69)
PBS, 20 min	0.00	0.71
EDTA 0.02%	0.00	0.87
Dispase 0.05%	0.00	0.86
Trypsin 0.25%	0.02	0.80
Scraping	0.00	0.81

*Cell harvesting methods are described in the text.
†Fluorescence indices were calculated with reference to medium controls.

the work was carried out with melanoma cell line NKl-4.

Sera

Sera were obtained from melanoma patients, patients with tumours other than melanoma, patients hospitalised for non-cancer illnesses, and from healthy volunteer donors. All sera were stored in small aliquots at −20°C.

Sera from hospital patients were provided by various hospitals in the Nottingham area. Some of the melanoma patients had taken part in a trial of specific active immunotherapy [15].

Membrane immunofluorescence test

Cells were harvested from monolayer culture with 0.25% trypsin and suspended in HBSS. Several other methods of harvesting were evaluated and after preliminary tests (Table 2); trypsinization was adopted because this was the method routinely used for subculturing and it did not appear to damage antigens detected in the immunofluorescence test.

Aliquots of 5×10^5 cells were washed twice in HBSS in 35×5 mm plastic tubes and the cell pellets suspended in 50 µl undiluted serum and incubated at room temperature for 15 min. The cells were washed by centrifugation 4 times in HBSS and incubated for a further 15 min in 50 µl of FITC-conjugated sheep anti-human IgG (Burroughs Wellcome) diluted 1/20 in HBSS. After 4 more washes the cells were suspended in 50 µl 1: 1 (v/v) glycerol: phosphate buffered saline (PBS, pH 7.3), and examined on a fluorescence microscope using transmitted ultraviolet illumination and a darkground condenser. Cells with staining of the complete cell membrane or segments of the cell membrane were scored as positive. A pool of AB serum was used as a standard

negative control. The results were expressed as a fluorescence index (FI) defined as follows:

$$FI = \frac{\%\text{ cells unstained by Ab serum} - \%\text{ cells unstained by test serum}}{\%\text{ cells unstained by AB serum}}.$$

An FI of between 0.30 and 1.00 was taken as a positive reaction [16]. The AB serum gave a completely negative reaction compared with HBSS, with all cell lines.

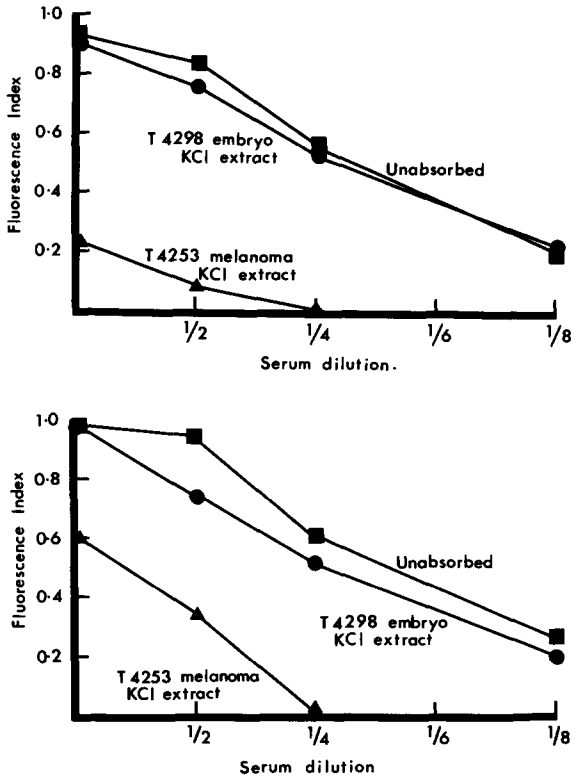

Fig. 1. Absorption of melanoma patient's sera at various dilutions with 3 M KCl *extracts of melanoma* (*T*4253, 1.6 mg/50 µl *serum*) *and* 19 week *embryo* (*T*4298, 2.0 mg/50 µl *serum*). (*a*) *Serum A*69; (*b*) *Serum S*9321.

Serum absorption

After preliminary tests (Fig. 1) absorptions were performed on serum diluted 1 in 4 with HBSS. Aliquots of 50 µl diluted serum were incubated for 2 hr at 37°C with a defined quantity of absorbing tissue extract. The mixture of serum and extract was then used in the membrane immunofluorescence test against NKl-4 cells, with unabsorbed serum as a positive control. Neutralization of antibody was expressed as the percentage reduction in the Fl obtained with absorbed serum, compared with the FI obtained with similarly diluted unabsorbed serum.

Preparation of soluble tissue extracts

Tumour tissues were mostly obtained from hospitals in the Nottingham area. Surgical specimens were trimmed free of necrotic areas and soluble extracts were prepared either by 3 M KCl treatment [17] or by papain treatment [18].

For 3 M KCl extraction, chopped tissue was homogenized in 3 M KCl in an Ultraturrax homogenizer, using 4 ml of the salt solution per g tissue. The homogenate was stirred at 4°C for 18 hr and centrifuged at 78,000 ***g*** for 60 min. The supernatant was dialyzed overnight against distilled water and precipitated nucleoproteins were removed by centrifugation at 78,000 ***g*** for 60 min. The soluble supernatant was dialyzed against PBS for 6–12 hr. After final clarification by centrifugation at 165,000 ***g*** for 15 min extracts were stored at −20°C.

Papain extraction was carried out on an extranuclear tumour membrane (ENM) fraction sedimented from the 600 ***g*** supernatant of tumour homogenates by centrifugation at 78,000 ***g*** for 60 min. The membrane fraction was suspended in 5 mM Tris–PO_4 buffer (pH 7.8), L-cysteine was added to a concentration of 5 mM, and the suspension warmed to 37°C. Papain (Sigma Chemical Co.) was added in the proportion of 1 mg enzyme per 30 ENM protein, followed by incubation at 37°C for 60 min. The soluble material resulting from this digestion was dialyzed against 5 mM Tris–PO_4 buffer and applied to a DEAE-cellulose ion exchange column equilibrated with the buffer. Papain was eluted from the column with 5 mM Tris–PO_4, then the solubilized membrane proteins were eluted with 0.5 M Tris–PO_4 (pH 7.8). The extract was concentrated and dialyzed against PBS, and stored at −20°C.

In addition to tumour extracts, 3 M KCl extracts were prepared from normal non-melanomatous skin, and several aborted foetuses. Protein determinations were made by the method of Lowry *et al.* [19].

Immunoadsorbent purification of melanoma extract

Two millilitres of serum from a melanoma patient (serum A69 shown to be reactive against NKl-4 cells) was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsälä) at 10 mg per ml gel. Before use, the immunoadsorbent was washed with 3 M NaSCN followed by PBS. A soluble 3 M KCl extract (T4503) of melanoma tissue was applied to the column and incubated for 12 hr at 4°C.

Unbound material was removed by washing with PBS and material bound to the immobilised antibody in serum A69 was eluted with 3 M NaSCN. The eluate was passed directly over Sephadex G25 to separate the bound material from the salt. After concentration, the isolated bound and unbound fractions were examined for antigenic activity by their ability to neutralise antibody in patients' sera reacting against NKl-4 cells.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed using 10% acrylamide gels (7.0 × 0.5 cm) according to the method of Weber and Osborn [20]. After staining proteins with Coomassie Brilliant Blue, densitometric scans of gels at 550 nm were obtained using a Gilford 250 spectrophotometer.

RESULTS

Antibody reactions against melanoma cells

Preliminary tests were carried out to determine optimum methods of cell harvesting for preservation of antigenicity of melanoma cells, as judged by membrane immunofluorescence. The target cell line, NKl-4, was easily induced to detach from the substrate by a 1 min exposure to 0.02% EDTA, 0.05% Dispase (Boehringer Corporation) or 0.25% trypsin, by 20 min exposure to PBS free of Ca^{2+} and Mg^{2+}, or by scraping with a rubber policeman. Cells obtained by all these methods were tested with a known positive serum from a melanoma patient (A69) and a normal donor serum in the membrane immunofluorescence test (Table 2). Fluorescence indices (FIs) obtained with either serum were similar on cells harvested by each method, indicating that none of the treatments degraded cell surface antigens or unmasked "concealed" antigens to any significant degree. Trypsin was used routinely for subculturing cells because it produced no discernible long-term damage to the cells, whereas some of the other treatments lowered their plating efficiency. For convenience and because it did not adversely affect cell surface antigens, trypsin was therefore routinely used to disperse cells used in the immunofluorescence test.

Sera from melanoma patients, patients with other forms of cancer and non-cancer controls were tested for reactivity against NKl-4 cells, as summarized in Table 3. No positive reactions were obtained with healthy donors, and only one of 30 sera from non-cancer hospitalised patients was reactive. However, reactivity by melanoma patients was also low, only 4 of 48 patients tested reacting positively against these cells. Repeated serum samples from these donors were all reactive, whereas repeated samples from all other melanoma patients were consistently negative. The positive patients conformed to no particular pattern with respect to stage of disease or the therapy they had received. Patients with other tumour types showed a similar level of reactivity, 4/50 samples from different patients producing positive reactions. It thus appeared from the preliminary screening against NKl-4 cells that reactivity by melanoma patients might not be tumour-related.

Sera which were positive for NKl-4 cells were then tested against 7 other melanoma cell lines as shown in Table 4. Sera A69, S9321 and S6975 derived from different melanoma patients were reactive against at least one other melanoma cell line. In comparison, 4 sera from other cancer patients and one non-cancer patient serum which were positive for NKl-4 cells, failed to react significantly to give FI > 0.30 with any other melanoma cell line. The NKl-4 reactive antibody in sera from melanoma patients was thus more closely associated with melanoma cells than that in sera from other cancer patients. Three NKl-4 reactive melanoma sera were further tested for reactivity against cell lines from unrelated tumours (Table 5). One of these sera, S6975, reacted against breast carcinoma cell line HS578T, and all three were positive against

Table 3. Membrane immunofluorescence against NKl-4 melanoma cells by sera from different donors

Serum donors	No. donors + ve / No. tested	No. sera + ve / No. tested
Melanoma patients	4/48	8/104
Patients with other tumours	4/50	4/50
Non-cancer patients	1/30	1/30
Healthy donors	0/30	0/48

Table 4. Membrane immunofluorescence against various melanoma cell lines by NK1-4 positive sera

Melanoma target cells	Mean fluorescence index with sera from: Melanoma patients A69	S9321	S6975	Other cancer patients A715*	S8634†	S5994‡	S5955‡	Non-cancer donor A6
NK1-4	0.89	0.75	0.59	0.92	0.39	0.33	0.61	0.81
Mel-2a	0.00	0.02	0.00	0.00	0.01	0.04	0.00	0.00
Mel-57	0.00	0.43	0.00	0.00	0.00	0.00	0.00	0.00
Mel-Swift	0.01	0.00	0.00	0.00	0.00	0.01	0.02	0.04
MeWo	0.37	0.10	0.02	0.05	0.04	0.02	0.07	0.17
RPM1-5966	0.00	0.53	0.00	0.00	0.00	0.00	0.00	0.00
163	0.82	0.18	0.34	0.00	0.04	0.00	0.00	0.06
364	0.03	0.84	0.20	0.25	0.05	0.00	0.00	0.28

*Lung carcinoma patient.
†Breast carcinoma patient.
‡Ovarian carcinoma patients.

Table 5. Membrane immunofluorescence by sera from NK1-4 positive melanoma patients against cell lines from other tumours

Target cells Line	Tumour type	Mean fluorescence index with serum: A69	S9321	S6975
HS578T	Breast carcinoma	0.00	0.08	0.60
9812	Lung carcinoma	0.00	0.00	0.00
T24	Bladder carcinoma	0.00	0.01	0.06
EB23	Prostate carcinoma	0.02	0.00	0.10
HeLa	Cervical carcinoma	0.04	0.01	0.03
HT29	Colon carcinoma	0.91	0.82	1.00
HS788T	Osteogenic sarcoma	0.00	0.00	0.00
HS791T	Osteogenic sarcoma	0.00	0.01	0.00
EB2	Burkitt lymphoma	0.20	0.00	0.00

colon carcinoma line HT29. However, further experimentation showed that about 90% of human sera from various types of donor react with this cell ine (although the AB control serum was negative) so that reactivity against HT29 was not considered relevant from the point of view of establishing specificity. Apart from these reactions, the melanoma sera were negative against other tumour types. As a result of these initial studies melanoma sera A69 and S9321 were selected as having antibody activity directed preferentially towards melanoma cells, and were used as "indicator" sera to search for melanoma associated antigens in tumour extracts.

Antigen activity in tumour extracts

Antigenicity related to melanoma cells was tested by the ability of tumour extracts to absorb antibody from sera A69 and S9321 which were tested against NKl-4 melanoma cells. Conditions for absorption were established as shown in Fig. 1. Sera A69 and S9321 (50 μl) were absorbed at various initial dilutions with identical volumes of a 3 M KCl extract of pooled melanoma tissue (T4253, 1.6 mg) or a 3 M KCl extract of a 19 week human embryo (T4298, 2.0 mg). The amounts of antigen were not varied so that the volume would be constant, thus avoiding differences in overall serum dilution. The undiluted sera strongly stained the majority of NKl-4 cells, and both titrated to 1/8 dilution where they gave borderline reactivity (FI around 0.30). At higher dilutions no significant immunofluorescence staining was obtained. When preabsorbed with melanoma extract T4253 the FIs obtained with undiluted sera were much reduced, and at 1/4 dilution the absorbed sera were completely negative. Both sera were positive at 1/4 dilution unabsorbed, and the FIs were only slightly lowered following absorption with embryo extract T4298. Since a serum dilution of 1/4 gave complete absorption by the melanoma extract and hence the clearest indication of specificity, this dilution was therefore taken as standard for subsequent absorption tests.

Table 6. Neutralization of anti-NKl-4 antibody in melanoma patients' sera by 3 M KCl extracts of human tissues

No.	3 M KCl extract Tissue	mg/50 μl†	% reduction of reactivity of serum*: A69	S9321
T4253	Melanoma	1.6	98	100
T4299	Melanoma	1.8	13	55
T4305	Normal skin	1.6	2	16
T4297	Breast carcinoma	2.3	12	12
T4302	Breast carcinoma	2.0	5	0
T2035‡	Colon carcinoma	1.5	15	—
T4304	Colon carcinoma	2.0	0	5
T3132	Lung carcinoma	1.6	5	—
T4265	14 week embryo	1.7	15	20
T4264	15 week embryo	1.9	13	11
T4263	16 week embryo	1.6	13	0
T4298	19 week embryo	1.8	6	5

*% reduction of reactivity is the % reduction of fluorescence index against NK1-4 cells following absorption, compared with unabsorbed serum.
†Concentration of extract in terms of mg protein used to absorb 50 μl Of 1/4 diluted serum.
‡T2035 was a papain extract of colon carcinoma tissue. All other extracts were 3 M KCl solubilised.

Table 7. Comparative absorption of NK1-4 positive melanoma and non-melanoma sera with melanoma extracts

Serum	No.	Absorbing extract Extraction	mg/50 μl	% reduction of serum reactivity
A6	T4299	3 M KCl	1.8	0
(Non-cancer donor)	T4518	Papain	1.6	21
A715	T4299	3 M KCl	1.8	0
(Lung carcinoma)	T4518	Papain	1.6	12
S9709*	T4299	3 M KCl	1.8	85
(Melanoma)	T4518	Papain	1.6	79
S9321	T4299	3 M KCl	1.8	48
(Melanoma)	T4518	Papain	1.6	78

*Serum S9709 was obtained from the same donor as serum A69 in Tables 3–5, and gave comparable reactions with NK1-4 cells.

A series of absorption tests with extracts from a variety of normal and tumour tissues were carried out, as shown in Table 6. The absorption with extracts T4253 and T4298 were different experiments from those shown in Fig. 1. Again melanoma extract T4253 removed virtually all anti-NKl-4 antibody from sera A69 and S9321. Absorption with a second melanoma 3 M CK1 extract, T4299, was less effective but significantly reduced reactivity by serum S9321. A further extract of pooled melanoma tissue (T4518) was also shown to remove NKl-4 reactive antibody from melanoma sera (Table 7) indicating that the antigen(s) responsible were a property of more than one melanoma pool. Similar extracts of other tumour tissues or normal skin at concentrations equivalent to or greater than the melanoma extracts had no significant antibody neutralization effect (Table 6). Also, a series of human embryo extracts had no effect, suggesting that antibody absorption was due to antigen(s) primarily associated with malignant melanoma. Table 7 shows absorption by melanoma extracts of non-melanoma sera which cross-reacted with NKl-4 cells, compared with absorption of melanoma sera. Non-cancer patient serum A6 and lung carcinoma patient serum A715 were strongly positive against NKl-4 unabsorbed (Table 4)

*Table 8. Neutralization of NK*1-4 *antibody in melanoma sera by an immunoadsorbent-purified fraction of melanoma extract T*4253

Fraction	mg/50 μl	% reduction of reactivity by serum: A69	S9321
Whole extract	1.6	98	100
	0.4	39	0
Unbound on immunoadsorbent column	1.2	0	19
Bound and eluted from immunoadsorbent column	0.2	52	51

and following absorption with melanoma extracts T4518 most of their reactivity remained. In contrast, absorption of melanoma sera S9321 and S9709 by these melanoma extracts removed anti-NKl-4 antibody in 3 of the 4 combinations tested. These results support the view that antibody against NKl-4 cells in cross-tested sera was probably not directed against melanoma associated antigens, but the major antibody activity in the melanoma sera was melanoma-associated.

Partial purification of melanoma-associated antigen

Melanoma extract T4253 was fractionated on an immunoadsorbent column prepared from NKl-4 positive melanoma serum A69, known to have antibodies reacting with T4253 (Fig. 1a, Table 6). Material unbound after 12 hr was eluted with PBS, followed by elution of bound material with 3 M NaSCN. Both fractions were tested for their ability to neutralise reactivity of sera A69 and S9321 in comparison with the unfractionated 3 M KCl extract. As shown in Table 8, the unbound material did not significantly inhibit reactivity of these sera against NKl-4 cells although it was used at a concentration approaching that of the unfractionated extract. In contrast, the material which was eluted after binding to the column was able to reduce the reactivity of both sera even though the low yield of this fraction meant that it was used at a protein concentration of only 0.2 mg/50 μl serum. At a similar concentration the unfractionated extract was ineffective, indicating that the antigenic activity with respect to protein concentration was significantly concentrated in the immunoadsorbent-bound fraction.

Gel electrophoresis on sodium dodecyl sulphate (SDS) showed that the bound fraction was much less heterogeneous than the unbound or unfractionated material (Fig. 2). The bound fraction contained less than 10% of the original protein and produced only 5

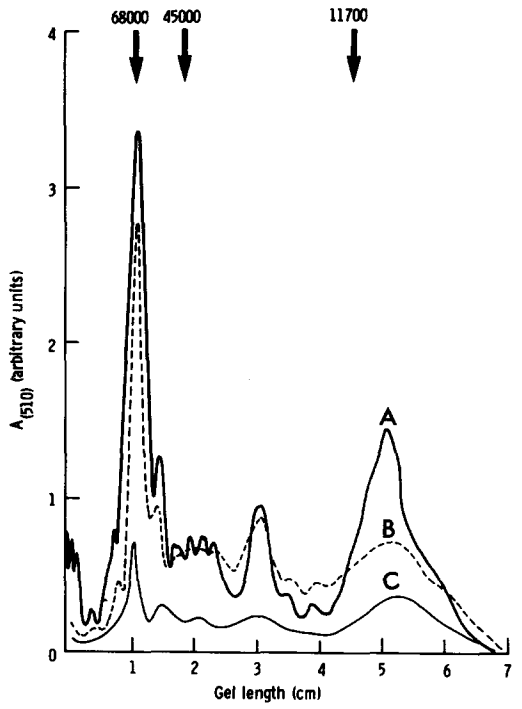

*Fig. 2. SDS gel electrophoresis of immunoadsorbent-fractionated and unfractionated melanoma extract T*4253*; protein profiles superimposed. A—whole T*4253 3 M KCl *extract; B—material unbound by immunoadsorbent column prepared from serum A*69*; C—material bound to and eluted from immunoadsorbent column prepared from serum A*69.

major peaks on the SDS gel, whereas the unbound material gave a complex electrophoresis profile similar to that of the unfractionated 3 M CKl extract. Passage on the immunoadsorbent column thus removed most of the non-relevant protein, leaving a partially purified fraction containing essentially all of the antigenic activity.

DISCUSSION

A variety of serological methods have been used to detect membrane antigens associated

with malignant melanoma, including complement-dependent cytotoxicity [2], antibody-dependent cellular cytotoxicity [21], immunadherence [3, 6], precipitation assays [10] and immunofluorescence [4, 7, 22]. It is thus firmly established that serologically defined tumour associated antigens exist on the melanoma cell surface. Melanoma antigens with a similar distribution to those defined serologically have also been demonstrated by assays for cell-mediated immunity, both *in vitro* [1], and *in vivo* in the form of delayed cutaneous hypersensitivity to melanoma extracts [11, 12]. Some reports, in which autologous combinations of target cells and serum as well as allogeneic combinations were studied, have shown that at least two classes of antigen exist on melanoma cells. These are individual antigens recognised mainly by autologous serum, and common antigens detected by cross-reaction with sera from other melanoma patients [4, 6]. In some cases, cross-reactivity has been shown to be due to common foetal antigens on the melanoma cell surface [8, 9, 23]. It has been reported that melanoma-associated antigens may cross-react with antigens on Bacille Calmette-Guérin (BCG) so that serum donors who have been treated with BCG may react against melanoma cells as a result of BCG immunization [24]. However, no such association between BCG treatment of serum donors and antibody reacting with melanoma cells was observed in the present studies, and Roth *et al.* [12] reported that there was no particular association with BCG treatment and delayed cutaneous hypersensitivity ro melanoma extracts. The melanoma cell line NKl-4 probably has a low level of cell surface antigen related to foetal calf serum which was used to supplement the culture medium [25], but this appears to be quite separate from the melanoma-associated surface antigen. Similarly, Gupta *et al.* [26] have shown that human osteogenic sarcoma cells gained a heterologous antigen component during growth in medium containing foetal calf serum, but this component was independent of tumour-associated antigen.

The proportion of melanoma patients reactive against NKl-4 cells in our studies was lower than in some other reports in which melanoma cells have been tested against allogeneic sera by membrane immunofluorescence [7, 9, 22]. In these reports the cells were used as adherent monolayers and were not exposed to enzymes immediately before testing. However, it was shown that NKl-4 cells harvested with trypsin reacted just as strongly with positive sera as those which had not been exposed to enzymes (Table 2). Also, trypsinized rat tumour cells have been used as target cells in immunofluorescence studies for many years in our laboratory, without evidence of tumour antigen loss [16]. Recent results using an ^{125}I-labelled protein A assay on adherent NKl-4 cells confirmed the negative reactivity of the majority of sera initially screened by immunofluorescence (unpublished results) so it is likely that the low reactivity observed was due to the choice of target cells rather than due to the technique employed. Many of the NKl-4 negative sera might have proved positive against some of the other melanoma cell lines listed in Table 1, but this possibility has not yet been explored. A further complication of using cultured cell lines has been suggested by Sorg *et al.* [7]. These authors showed that cultured melanoma cells can undergo clonal changes in antigen expression so that the spectrum of antigens recognised by different sera may differ from one clone to another. This shift in antigen expression was sufficient to allow the selection of positive and negatively reacting sublines from a single parent cell line. If clonal variation is a universal property of cultured cell lines, antigenic drift would be an important and difficult to control variable affecting attempts to detect or define tumour antigens by serology.

The initial screening against NKl-4 cells suggested that melanoma patients were no more reactive than patients with other tumours. However, when melanoma sera were positive this reactivity was melanoma associated since the same sera showed little or no reactivity against target cells derived from other tumours, but were positive for several different melanoma lines. Conversely, non-melanoma sera which were positive with NKl-4 cells did not react with other melanoma lines. Thus, despite an unpromising initial impression it was possible to identify two allogeneic sera which reacted strongly against NKl-4 cells with primarily melanoma-associated antibody activity, which could be used to detect common melanoma-associated antigen(s) in allogeneic tumour extracts.

Soluble extracts of melanoma have been prepared by sonication of melanoma cells [11], detergent solubilisation [10] treatment of cells or cell membranes with proteolytic enzymes [13, 27] or by 3 M KCl extraction of cells or tissue homogenates [12, 28]. The various methods of preparation appear to

yield antigens with similar biological activities. Thus, for example, extracts prepared by either sonication or 3 M KCl extraction have been used to demonstrate delayed cutaneous hypersensitivity in melanoma patients [11, 12] and both papain-solubilised and 3 M KCl extracts have been shown to react specifically with leucocytes from melanoma patients *in vitro* [27, 28]. In most cases the extracts have been tested with allogeneic melanoma patients (or their sera or leucocytes) and by comparison with unrelated extracts, cancer patients or donors, the general finding is that the soluble extracts contain common melanoma antigen(s). This is confirmed in our studies, since allogeneic melanoma 3 M KCl extracts were able to specifically inhibit reactivity against NKl-4 melanoma cells by positive allogeneic sera, but extracts of other tumours or normal tissues had no significant inhibitory effect at equivalent concentrations. Included among the normal tissues were four embryo extracts, and their lack of inhibitory activity might indicate that the common antigen(s) detected in melanoma extracts were not foetal antigens.

Purification procedures have usually involved chromatography on Sephadex or ion exchange columns, followed by polyacrylamide gel electrophoresis [11–13] but Thomson *et al.* [14] have reported the binding of melanoma-associated antigen (and also breast carcinoma-associated antigen) on an immunoadsorbent column composed of anti-human β-2 microglobulin antiserum bound to Sepharose 4B. However, although β-2 microglobulin is associated with the major histocompatibility locus in humans, apart from the work of Thomson *et al.* [14] it has not been shown to be associated with tumour antigens. In experimental rat hepatomas, β-2 microglobulin appears not to be associated with tumour-specific antigen [29]. We fractionated a crude 3 M KCl melanoma extract on a specific immunoadsorbent column prepared with serum determined empirically to contain antibody reacting with antigenic components of the KCl extract. The material which bound to this antibody and was subsequently eluted was much less heterogeneous than the crude KCl extract in terms of protein constituents resolved by SDS electrophoresis, and contained all of the detectable antigenic activity. It is estimated that on the basis of protein yields and antigenic activity per unit of protein, more than a 10 fold purification of melanoma-associated antigen was achieved by a single passage on the immunoadsorbent column.

Even better purification might be achieved using more specific or more purified antibody in the form of serum fractions from autologous or allogeneic donors. Of particular promise for this technique is the possibility of monoclonal anti-melanoma antibody production by somatic cell hybrids prepared from antibody-producing spleen cells and a mouse myeloma [30, 31]. The use of immunoadsorbents such as ours, but prepared with highly specific monoclonal antibodies could, by removal of most of the non-relevant material, lead to the production of homogeneous and highly active antigenic material of sufficient purity to allow full chemical characterisation.

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