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Release of Monoclonal Antibody-defined Antigens by Human Colorectal Carcinoma and Melanoma Cells[1]

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ABSTRACT

Many but not all of the cell surface antigens studied were released into tissue culture medium of human colorectal carcinoma and melanoma cells maintained *in vitro*. Two groups of monoclonal antibody-defined antigens are described. One group is composed of molecules that are released easily and in large quantities into the tissue culture milieu. In this group, four glycolipid antigens of colon carcinoma, including a monosialoganglioside, and five glycoproteins of melanoma were detected in tissue culture supernatants. Antigens of the second group could not be detected in tissue culture supernatants. This group included one colorectal carcinoma antigen, the nature of which is unknown, and a glycoprotein of melanoma cells.

INTRODUCTION

Monoclonal antibodies which detect tumor-associated antigens expressed on human melanomas or colorectal carcinomas have been described previously (10, 13, 14, 19). These antigens are expressed on the surfaces of tumor cells, and some have been characterized by immunoprecipitation with monoclonal antibodies after solubilization of radiolabeled cells (14, 17-19). Earlier observations indicated that the antigens may not be confined to the cell surface but may also be secreted into tissue culture medium. This report describes studies designed to detect antigens in tissue culture medium by means of a selected panel of monoclonal antibodies.

MATERIALS AND METHODS

Cell Lines. Colorectal carcinoma and melanoma cell lines designated SW were established by Leibovitz (15) at Scott and White Clinic, Temple, Texas. Melanoma cell lines designated WM were established at the Wistar Institute (9, 20). Other cell lines are described elsewhere (9, 13, 14). The cells were maintained in L15 medium (15) supplemented with 10% FCS.[3]

SSFM. L15 medium was supplemented with insulin (Sigma; 5 μg/ml) transferrin (Sigma; 5 μg/ml) and 1% nonessential amino acids, as described elsewhere (3).

Preparation of SSFM Supernatants. Confluent monolayer cultures were washed with phosphate-buffered saline [g/liter: NaCl, 8.0; KCl, 0.20; KH_2PO_4, 0.20; Na_2HPO_4, 1.15; $CaCl_2$ (anhydrous), 0.10; $MgCl_2 \cdot 6H_2O$, 0.10] and incubated in SSFM for 8 days at 37°. Supernatants were collected, and cells and cell debris were removed by centrifugation at 1500 × g (15 min/4°); PMSF was added to make the solution 2 mM, and aliquots were stored at −70°.

The 3 M KCl Tumor Cell Membrane Preparation. Detailed description is presented elsewhere.[4] Briefly, cells were collected by trypsinization, washed 3 times in a 50-fold volume of 0.15 M NaCl, resuspended in 10^{-3} M $ZnCl_2$ containing 2 mM PMSF at a concentration of 2×10^7 cells/ml, and incubated for 30 min at room temperature followed by 5 min at 4°. The cells were then disrupted in a homogenizer and centrifuged at 200 × g for 5 min at 4°. The supernatant was centrifuged at 30,000 × g (1 hr, 4°), and the sediment was resuspended in 3 M KCl containing 2 mM PMSF, stirred for 20 hr at 4°, and centrifuged again at 100,000 × g for 1 hr at 4°. The supernatant was dialyzed and centrifuged at 15,000 × g for 20 min. The preparation was aliquoted and stored at −70°.

Monoclonal Antibodies. Anti-melanoma antibodies included in this study have been described previously: 691-13-17 (antibody 13-17); 691-19-19 (antibody 19-19); and 691I5Nu-4-B (Nu4B) (13). Antibodies 3723, 3724, and 3727 were kindly supplied by Dr. S. Ferrone of Scripps Clinic and Research Foundation, La Jolla, Calif. ACRC included in this study were 1116-NS-3a-22 (NS-3a-22), 1116-NS-10 (NS-10), 1116-NS-19-9 (NS-19-9), 1116-NS-33a (NS-33a), 1116-NS-52a (NS-52a) (14), and antibody 1083-17-1A (17-1A) (10). The binding specificities of antibodies used in this study are summarized in Table 1.

Binding Assay. Indirect RIA was performed as described previously.[4] RIA on live cells was performed using 5×10^5 cells/well. In solid-phase RIA, 50 μl of 3 M KCl extract per well corresponding to the same number of cells (150 mg protein per ml) or, similarly, 50 μl of concentrated SSFM per well, containing 80 mg protein per ml, were used. For indirect solid-phase RIA, soft polyvinyl chloride plates (Cooke Engineering, Alexandria, Va.) were coated with antigen preparations by drying overnight at room temperature followed by 2 hr at 37°. The plates were then washed twice and incubated for 2 hr at room temperature with 1% gelatin in 0.01 M sodium borate, pH 8.0. Antibodies were then added, and plates were incubated overnight at 4° and then washed 3 times; binding was detected with ^{125}I-labeled rabbit anti-mouse $F(ab')_2$. After an overnight incubation followed by 3 washes, the bound radioactivity was counted in a Packard Auto-Gamma spectrometer.

Inhibition Assay. Gelatin-coated microtiter plates were used to mix previously determined dilutions of monoclonal antibody in equal amounts with dilutions of inhibitor. After incubation (18 hr at 4°), the mixtures were transferred to wells of polyvinyl chloride plates precoated with SSFM tissue culture supernatant

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[3] The abbreviations used are: FCS, fetal calf serum; SSFM, supplemented serum-free medium; PMSF, phenylmethylsulfonyl fluoride; ACRC, anticolorectal carcinoma; RIA, radioimmunoassay.

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[4] M. Herlyn, H. F. Sears, Z. Steplewski, and H. Koprowski. Specific antigens to colorectal carcinoma in sera of patients are detected by monoclonal antibodies, submitted for publication to *Cancer Research*, 1980.

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Table 1

Summary of the binding specificities of anti-melanoma and ACRC monoclonal antibodies used for the analysis of antigens

	Ratio of cultures binding antibodies secreted by											
	Anti-melanoma clones						ACRC clones					
Target cells	13-17	19-19	Nu4B	3723[a]	3724[a]	3727[a]	NS-3a-22	NS-10	NS-19-9	NS-33a	NS-52a	17-1A
Melanoma	12/17	13/17	17/17	14/17	17/17	14/17	0/10	1/10	0/10	0/10	0/10	0/10
Colorectal carcinoma	0/6	0/6	0/6	0/4	0/4	0/4	6/8	7/8	5/8	8/8	5/8	8/8
Astrocytoma	5/7	4/7	6/7	2/3	2/3	2/3	0/2	0/2	0/2	0/2	0/2	0/2
Lung carcinoma	0/3	0/3	0/3	0/1	0/1	0/1	0/3	0/3	0/3	0/3	0/3	0/3
Breast carcinoma	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
Burkitt's lymphoma	2/2	0/2	0/2	0/2	0/2	0/2	0/1	0/1	0/1	0/1	0/1	0/1
Fibroblasts[b]	0/7	0/7	0/7	0/4	4/5	0/4	0/5	0/5	0/5	0/5	0/5	0/5
Lymphocytes[c]	8/8	0/8	0/8	NT[d]	NT	NT	0/6	0/6	0/6	0/6	0/6	0/6

[a] Antibodies kindly supplied by Dr. S. Ferrone, Scripps Clinic and Research Foundation, La Jolla, Calif., and described at the Workshop on Monoclonal Antibodies to Melanoma, Bethesda, Md., March 26 to 27, 1981.
[b] Fibroblasts: fetal, 2; adult, 2; and SV40-transformed, 3.
[c] Peripheral blood lymphocytes: freshly isolated, 3; Epstein-Barr virus transformed, 5.
[d] NT, not tested.

of SW 1116 colorectal carcinoma cells as a target. Following the second incubation (18 hr at 4°), the plates were washed, and binding of free unbound monoclonal antibody was detected as described above; the percentage of specific inhibition of binding was calculated from the mean values of triplicate wells. Details of the assay are described elsewhere.[4]

RESULTS

Cell Growth in SSFM. Preliminary experiments indicated that the presence of calf serum in medium inhibited detection of soluble antigen. We therefore attempted to grow tumor cells in the absence of FCS. SW 1116 cells cultured in SSFM grew for up to 10 days of culture (Chart 1). Analysis of various tumor cells in culture showed that colorectal carcinoma cell lines SW 1222 and SW 403 were able to multiply in SSFM (Chart 2). Melanoma cell lines SW 691 grew only during the first 3 days and reached 5×10^5 cells/2.5 sq cm. Further studies have shown that all colorectal carcinoma cells will multiply in SSFM for 10 days, while melanoma cells will multiply for only the first 3 days but will stay alive up to 10 days. There was no striking change in the ratios of viable and dead cells under serum-free conditions after 8 days in culture.

Release of Surface Antigens by Colorectal Carcinoma Tumor Cells. SSFM collected from 8-day-old cultures of tumor cells was assayed for the presence of monoclonal antibody-defined antigens. Colorectal carcinoma-specific antigens were released by the majority of tumor cell lines (Table 2). Antigens detected by ACRC antibodies NS-10, NS-52a, NS-19-9, NS-33a, and NS-3a-22 were all released by the cell line SW 1116 which had previously been used to immunize mice for the production of the hybridomas (14); however, monoclonal antibody 17-1A failed to detect antigens in SSFM. Cell lines SW 403 and SW 948 also secreted antigens into the medium which could be detected by all antibodies, except antibody 17-1A. Culture SW 1463 secreted only the antigen detected by one antibody (NS-10). We have found that, in the presence of FCS, the amount of detectable antigens was always much lower and sometimes not detectable at all (results not shown) in tissue culture medium.

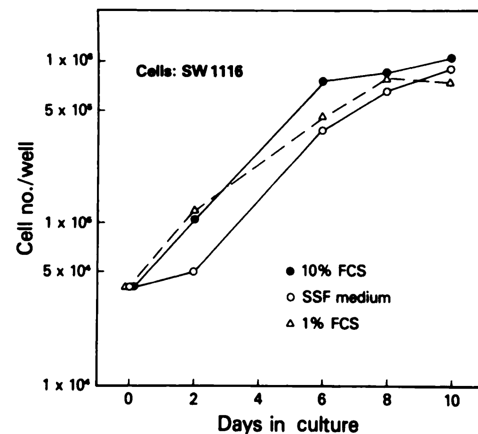

Chart 1. Growth of colorectal carcinoma cells (SW 1116) in SSFM. Human colorectal carcinoma cells (SW 1116) were plated at 4×10^4 cells/2-ml Linbro well (24-well plate), in minimal essential medium supplemented with 10% FCS. After overnight incubation, the monolayer cells were washed 3 times with phosphate-buffered saline and refed with fresh medium containing 10% FCS, 1% FCS, or SSFM containing transferrin (5 μg/ml) and insulin (5 μg/ml). At 2, 6, 8, and 10 days in culture, the number of viable cells per well was established. Standard deviations for triplicate cell counts were generally less than 10%.

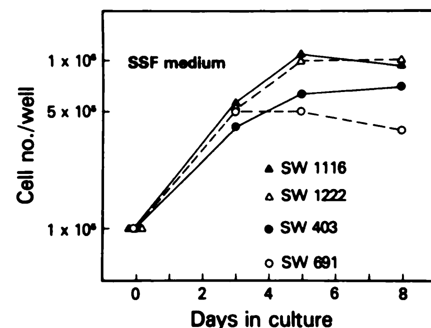

Chart 2. Effect of serum-free medium on various cell lines. Human carcinoma cell lines SW 1116, SW 1222, and SW 403 and human melanoma cell line SW 691, were plated at a density of 1×10^5 cells/well in minimal essential medium supplemented with 10% FCS. After overnight incubation, the monolayers were washed 3 times with phosphate-buffered saline and refed with fresh SSFM (*SSF medium*) containing transferrin (5 μg/ml) and insulin (5 μg/ml). At Days 3, 5, and 8, the number of viable cells per well was calculated. Standard deviations for triplicate cell counts were generally less than 10%.

Results shown in Chart 3 illustrate the binding of a panel of monoclonal antibodies to colorectal carcinoma antigens present on the surface of SW 1116 cells, in 3 M KCl extracts and in SSFM from SW 1116 cells. In all cases, the binding to antigens in SSFM and in 3 M KCl membrane preparations by monoclonal antibodies NS-10 (Chart 3*A*), NS-19-9 (Chart 3*B*), NS-33a (Chart 3*C*), and NS-52a (Chart 3*D*) was higher than binding to live cells grown in the presence of serum or under serum-free conditions. This was also found to be true for all other monoclonal antibodies tested (results not shown).

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Table 2
Comparison of binding of monoclonal ACRC antibodies to live cells and to SSFM of colorectal carcinoma cells

Cell line	Target	cpm in RIA for following hybridoma antibodies					
		Ns-3a-22	NS-10	NS-19-9	NS-33a	NS-52a	17-1A
SW 403	Live cells[a]	2220	2490	3690	650	3620	5230
	SSFM[b]	180	4230	1220	4250	4730	0
SW 837	Live cells	780	1610	440	1110	130	3550
	SSFM	NT[c]	870	0	0	0	0
SW 948	Live cells	1990	1250	2240	180	3470	3330
	SSFM	650	4970	420	3470	1550	0
SW 1083	Live cells	350	620	200	490	360	4680
	SSFM	0	710	0	340	0	0
SW 1116	Live cells	2350	1700	2290	1530	5220	3360
	SSFM	2170	5750	2830	4480	7600	0
SW 1222	Live cells	2330	3010	2290	2270	5210	4270
	SSFM	0	270	170	250	660	0
SW 1345	Live cells	0	0	0	1610	0	5120
	SSFM	0	270	0	0	0	0
SW 1463	Live cells	4430	2260	2320	2180	3200	4580
	SSFM	0	1390	0	0	0	0

[a] $\times 10^5$/well.
[b] μl/well.
[c] NT, not tested.

Release of Surface Antigens by Melanoma Cells. The melanoma antigens which were detected in SSFM from 8-day-old cultures are presented in Table 3. The DR antigen detected by antibody 13-17 (9, 17) was present in the medium from a majority of DR-positive melanoma cell lines. Significant secretion of antigens detected by antibodies 19-19 and Nu4B was detected only from melanoma cell lines WM 35 and WM 115. Antigens detected by antibodies 3723, 3724, and 3727 (supplied by Dr. Ferrone) were detected in supernatants of almost all melanoma cell lines (Table 3).

ACRC monoclonal antibodies did not bind to SSFM tissue culture supernatants from melanomas or from other tumor and normal cell lines, and none of the anti-melanoma monoclonal antibodies bound to SSFM supernatants from colon carcinoma cell lines or from normal human fibroblasts (results not shown).

Antigens released into tissue culture medium were detected not only by direct binding but also by the binding inhibition assay. As shown in Chart 4, binding of monoclonal antibody NS-33a to SSFM target was gradually inhibited by increasing concentration of either SSFM or 3 M KCl extract from SW 1116 colorectal carcinoma cells but not by extracts from control WM 9 melanoma cells. Similar binding inhibition results were obtained for antibodies NS-3a-22, NS-10, NS-19-9, and NS-52a.

DISCUSSION

In this study, we have found that many but not all of the cell surface antigens of colorectal carcinoma and melanoma cells are released into SSFM of *in vitro*-cultured cells. Antigens detected by ACRC monoclonal antibodies NS-3a-22, NS-10,[5] and NS-33a are released by the majority of colorectal carcinoma cells. The 3 antigens defined by these antibodies are glycolipids which can also be detected in the patient's serum samples.[4] Two other antibodies, NS-19-9 and NS-52a, detect antigen(s) characterized as a monosialoganglioside (16) and are present not only in SSFM of colorectal carcinoma cells but also in sera of patients with colorectal carcinoma (12).

The antigen detected by monoclonal antibody 17-1A was not found in supernatants of any of the cell lines tested or in the sera of colorectal carcinoma patients. This antigen seems to be confined to the cell surface; such an antigen(s) is ideally suited as a target for monoclonal antibodies to which cytotoxic agents could be conjugated (6). Interestingly, 17-1A antibody of γ2a isotype is reactive in antibody-dependent cell cytotoxicity (7) and inhibits human colorectal carcinoma tumor growth in nude mice (8).

Monoclonal antibody-defined antigens of melanoma cells also seem to separate into 2 categories. One consists of antigens released in large quantities, *i.e.*, detected by antibodies 3723, 3724, and 3727 and by antibodies Nu4B and 13-17. Antibody 19-19, in contrast, seems to detect an antigen that is released by only a very few cell lines.

It is worth stressing that much more antigen was released when serum was omitted from tissue culture media. We have also shown here that tumor cells maintained in very simple serum-free medium supplemented only with insulin (5 μg/ml), transferrin (5 μg/ml), and 1% nonessential amino acids (3) were able to multiply for 3 to 10 days. Using these SSFM supernatants as a target for binding of a panel of monoclonal antibodies (10, 13, 14) for comparison with other sources of target antigens, we have found them to be comparable to live cells or 3 M KCl membrane extracts. Such target antigens are very useful for the establishment of diagnostic assays such as antibody-binding inhibition assay (Chart 4) which can easily be adapted for the detection of tumor antigens in the serum of patients (12). A major advantage of using SSFM from carefully chosen tumor cell lines is their potential for producing large quantities of a high concentration of uniform material. This has advantages not only for use as a target for RIA's but also for the isolation of the antigens against which the monoclonal antibodies are directed.

Although the mechanism of release of surface macromolecules by tumor cells is unknown, it is well established that such release takes place, at least in tissue culture. Histocompatibility antigens of cultured murine lymphoma cells (5), human melanoma cell surface antigen (1, 2), and transplantation antigens of rat fibrosarcoma (4) are rapidly released into tissue culture medium. Bystryn *et al.* (2) found no difference in the proportion of released macromolecules by melanoma cells and normal fibroblasts. The rapid release of membrane antigens seems to

[5] Note added in proof. Antibody NS-10 detects Le[b] antigen (M. Brochhaus, personal communication).

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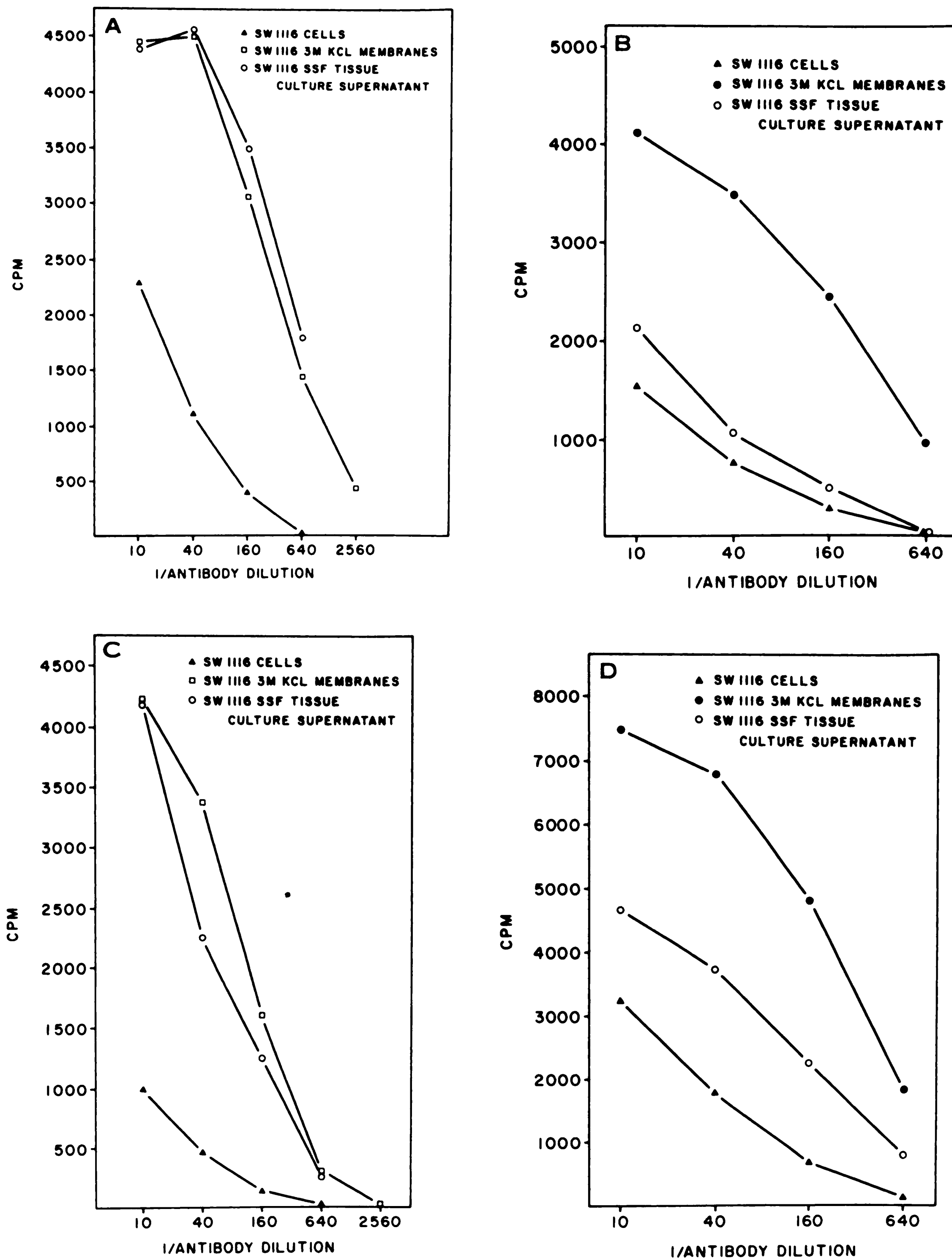

Chart 3. Binding of colorectal carcinoma-specific monoclonal antibodies to different targets. Colorectal carcinoma cell line SW 1116 (2.5×10^5 cells per well), 3 M KCl membrane preparation (7 µg protein per well), and supplemented serum-free spent medium (*SSF*) (4.5 µg protein per well) of SW 1116 cells were used as targets in live-cell or solid-phase RIAs. Specific binding of monoclonal ACRC antibodies 1116-NS-10 (*A*), 1116-NS-19-9 (*B*), 1116-NS-33a (*C*), and 1116-NS-52a (*D*) was tested at different dilutions on all 3 targets. Results represent specific cpm after control counts (P3x63Ag8 supernatant) were subtracted (usually 100 to 300 cpm).

be unrelated to malignant transformation but represents a normal activity of the cell membrane.

Thus, if tumor cells express on their surfaces macromolecules not present on the surface of normal cells, such tumor-associated antigens would be also released and could be detected. A monosialoganglioside detected by monoclonal antibodies NS-19-9 and NS-52a shown here to be easily released by tumor cells was not detected in normal colon tissue or in serum of normal subjects (12, 16). The same antigen was found in colon carcinoma and in meconium (16), suggesting that the molecule is a fetal antigen reexpressed by colon carcinoma cells. Although it is difficult to draw a parallel be-

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Table 3

Comparison of binding of monoclonal anti-melanoma antibodies to live cells and SSFM of melanoma cell lines

Cell line	Target	cpm in RIA for following hybridoma antibodies					
		13-17	19-19	Nu4B	3723	3724	3727
WM 9	Live cells	4840	2620	2440	2220	3500	2540
	SSFM	1010	270	740	910	2220	1100
WM 28	Live cells	580	1140	860	860	1930	900
	SSFM	280	190	NT[a]	690	1420	620
WM 28-7	Live cells	910	620	1520	1310	3330	1490
	SSFM	0	150	90	1310	3330	1490
WM 35	Live cells	1630	1720	1220	2550	5080	3280
	SSFM	1970	1500	1740	610	4880	3660
WM 46	Live cells	810	3090	650	3880	6800	1650
	SSFM	0	430	170	1020	2720	1080
WM 47	Live cells	4210	1510	2220	2650	5820	2870
	SSFM	0	0	890	870	2210	1220
WM 47-3	Live cells	4780	3050	4220	4380	7740	4810
	SSFM	0	0	570	520	1290	760
WM 56	Live cells	2900	1730	1140	NT	NT	NT
	SSFM	660	0	280	1140	600	410
WM 98-2	Live cells	4840	2920	1500	2860	6970	2880
	SSFM	3100	0	380	0	370	170
WM 115	Live cells	2510	2800	2530	2360	6960	2790
	SSFM	1320	1750	1860	2400	4600	3610
SW 691	Live cells	3960	2090	2710	NT	NT	NT
	SSFM	2520	840	710	NT	1770	590

[a] NT, not tested.

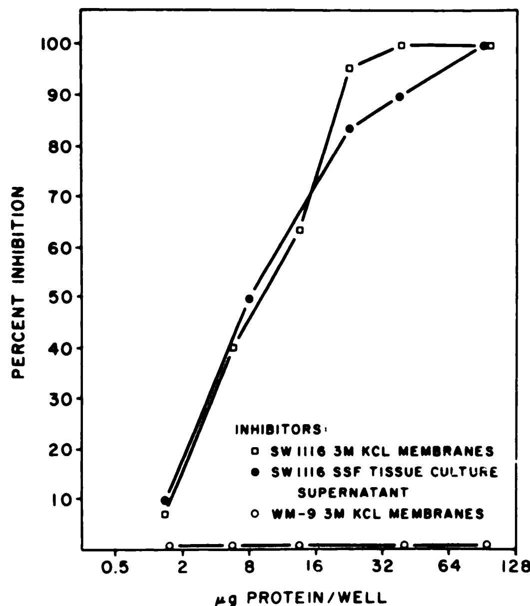

Chart 4. Inhibition of monoclonal antibody 1116-NS-33a binding by different sources of antigen. Target: 1116 SSF tissue culture supernatant. Hybridoma antibody 1116-NS 33a at 1:20 dilution was mixed with different dilutions of SW 1116 colorectal carcinoma cells, 3 M KCl membrane preparation, supplemented serum-free tissue culture supernatant of SW 1116 cells, or WM 9 melanoma cell 3 M KCl membrane preparation. After incubation, the mixture was transferred to a target plate coated with SW 1116 colon carcinoma cell supplemented serum-free supernatant. Percentage of binding inhibition of monoclonal antibody 33a as detected with iodinated rabbit anti-mouse $F(ab')_2$ IgG was calculated from maximum binding of buffer-incubated antibody 33a.

tween tumors grown *in vitro* and *in vivo*, it is possible that confinement of the antigen(s) to the cell surface or its release into the environmental milieu may have a direct bearing on the use of antibodies directed against these antigens for diagnosis or therapy.

In conclusion, our results indicate that some members of our panel of monoclonal antibodies directed against human colorectal carcinoma and melanoma cells can be used to detect antigens released into tissue culture supernatants and that others react with antigens that may be confined to the cell surface and are not released into medium or into the circulation of patients. Other possibilities that may account for our inability to detect immunoreactive antigens in medium, such as denaturation of very labile antigens or destruction of the monoclonal antibody-specific binding site, at present could not be excluded. SSFM collected from tumor cells in culture was found to be an excellent target for inhibition assays used in search for tumor antigens in patient sera.[4] Antigens confined to the tumor cell surface and not released into tissue culture medium which are not present in patients' sera will be ideal for targeting monoclonal antibody cytotoxic conjugates (6) for therapy or isotope conjugates (11) for diagnosis of cancer.

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