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Chemical and Immunological Studies of Cell Surfaces From Normal and Transformed Cells

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Immunological and chemical studies of cell surfaces from normal and transformed BALB/c fibroblasts have shown alterations associated with transformation. The cells studied include normal lines which do not cause tumors when injected into BALB/c mice, viral transformants, and spontaneous transformants which cause tumors that either regress or grow progressively, killing the host. The spontaneously transformed progressors include cell lines which are immunogenic and nonimmunogenic as determined by the ability of tumor excision to protect an animal from subsequent rechallenge by tumor cells. Tumor-bearing mice produce lymphocytes which are nonspecifically cytotoxic for all the normal and transformed lines. Some of the cell lines induce specific antibody formation in BALB/c hosts. Antisera have been prepared in rabbits which are specific for the transformed cell lines. These antisera can be used to determine specific surface changes on the transformed cells. Chemical studies have shown glycolipid alterations between the normal cells and some, but not all, of the transformants. Glycoproteins labeled by lactoperoxidase-^{125}I or [^{3}H]glucosamine were compared by SDS gel electrophoresis. Results from these studies do not show changes associated with malignancy. Individual glycoprotein regions from gels were treated with pronase, and the glycopeptides compared by Sephadex G-50 chromatography. Alterations in glycopeptides from several cellular glycoproteins are the only changes which appear to be associated with malignancy.

Key words: **cell surface, transformed cells, glycolipids, glycoproteins**

The cell surface is believed to play a central role in both growth regulation by cell-cell and cell-hormone (growth factors or chalones) interactions and cell adhesion. While it has been widely recognized that changes in cell surface components could be fundamental to cancer (for reviews see Refs. 1–3), there is no evidence that any of the reported changes detected in viral, carcinogen, or spontaneously transformed cells are primary to a transformation event. The problem has been that we lack both a biological assay for determining function and a chemical understanding of cell surface components important in cellular interactions.

The immunological response made by animals bearing tumors offers a possible approach to defining cell surface change in cancer. A number of investigators have reported evidence that a variety of both carcinogen induced and virally caused tumors

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possess unique antigens (4, 5). It has been suggested that the release of these antigens (tumor specific or viral) in the form of free antigen or antigen-antibody complexes could prevent a successful rejection response (6–11).

There has been no definitive work to link results from immunological studies to chemical alterations which have been shown to accompany the transformation of a normal to a malignant cell. These reported chemical changes include altered carbohydrate compositions, a decrease in the level of complex glycolipids, loss of a 240,000 mol wt surface glycoprotein (LETS), and alterations in profiles of cellular derived glycopeptides chromatographed on molecular sieving columns (12–20). In the work reported here, we present our preliminary chemical, biological, and immunological studies of normal, spontaneously transformed, and virally transformed cells derived from BALB/c mice. We are attempting to correlate surface change with transformation, immunogenicity, and malignancy.

MATERIALS AND METHODS

Cell Lines and Cell Culture

All cell lines were derived from BALB/c mice. A_{31} is a cloned line of BALB/c 3T3 fibroblasts and was a gift from Dr. G. Todaro of NIH. c5 is a transformed cell line cloned from A_{31} as previously described (21, 22). c5T was isolated from a tumor caused by injecting c5 cells into a BALB/c mouse. MSC was derived from a Moloney strain murine sarcoma virus induced tumor in a BALB/c mouse, and was the gift of Dr. S. Russell of Scripps Clinic and Research Foundation. 3T12T cells were isolated from a tumor caused by injecting 3T12 cells (a gift from Dr. Todaro) into a BALB/c mouse. PBC cells are early passage BALB/c embryo fibroblasts (22). Cells were grown in monolayer cultures in antibiotic free Dulbecco's modified minimal essential medium supplemented with 10% fetal calf serum (Grand Island Biological Company, Berkeley, California). Routine tests for mycoplasma contamination were performed (23, 24). Cultures testing positive were not used for any experiments and were discarded.

Preparation of Lymphocytes

Spleens were excised from tumor bearing or immunized mice and placed in 5 ml Dubecco's modified minimal essential medium (DMM) in 60-mm plastic culture dishes. Lymphocytes were teased from spleens and adherent cells removed by incubating the suspension for 45 min at 37°C. Cells were then collected by sedimenting the suspension at 1,000 × g for 10 min. Erythrocytes were removed from the single cell suspension by one addition of 5 ml containing 0.75% NH_4Cl in 0.016 M Tris, pH 7.4. Lymphocytes were washed twice more in DMM and suspended in DMM-10% heat inactivated fetal calf serum at a concentration of 2×10^6 cells/ml. Such preparations retained cytotoxic reactivity for at least 24 h when stored at 25°C.

Cytotoxicity was determined by 2 methods. Following the procedure described by Hellstrom (25), target cells were plated at concentrations of 100–200 cells/well in 100 μl DMM containing 10% heat inactivated fetal calf serum. Following an overnight incubation to allow target cell attachment, 100 μl of the lymphocyte suspension was added and the plates incubated for 48 h. Lymphocytes and dead cells were removed by 2 washes with saline, and the remaining live cells stained with crystal violet and counted. In the majority of experiments, the procedure described by McKhann (26) was followed. Target cells were first plated in 35-mm tissue culture dishes at a concentration of 2×10^5 cells/dish. [^{3}H] Uridine (50 μCi at a specific activity of 29 Ci/mM) was added and the cells

incubated overnight at 37°C. The labeled monolayer was then rinsed twice with sterile saline and the cells trypsinized and suspended at a concentration of 500 cells/100 μl in DMM containing 10% heat inactivated FCS. For the lymphocyte mediated killing assay, 100 μl of target cells were mixed with 100 μl of the lymphocyte suspension (usually from 1×10^4 to 2×10^5 lymphocytes) and placed in Microtest II wells. Following a 48 h incubation, the medium was removed and attached live cells rinsed twice with saline. The labeled cells were removed by 2 rinses with 200 μl of 0.5 M NaOH and placed in scintillation vials. The aqueous volume was made up to 1.0 ml by adding 0.5 ml of 20% glacial acetic acid, and radioactivity was determined using a counting solution containing 960 ml Triton X-114, 2,900 ml xylene, and 125 ml Liquiflor (New England Nuclear Corporation, Boston, Massachusetts).

Antisera

New Zealand white rabbits were inoculated subcutaneously with freeze-thaw disrupted c5T or 3T12T cells. The inoculations were at multiple sites on a bimonthly schedule over an 18-month period. The rabbits were bled by venous puncture and sera prepared in standard fashion. The sera were heat inactivated at 56°C for 30 min and stored as 0.5 ml aliquots at −70°C.

Indirect Immunofluorescence Assay

Surface specificities of rabbit antisera were determined by the indirect immunofluorescence assay as modified from Baldwin and Barker (28). Basically, monolayers of fibroblasts were rinsed and harvested by incubation for 10 min with 10^{-2} M EDTA/ solution. A (NaCl, 8 g/liter; KCl, 0.2 g/liter; Na_2HPO_4, 1.12 g/liter; KH_2PO_4, 0.2 g/liter; pH 7.4). The harvested cells were washed 3 times in Dulbecco's modified minimal essential medium (DMM) by centrifugation (300 × g, 5 min) and the final pellet readjusted to 10^6 cells/ml. The 10^6 cells were pelleted in 1.5-ml plastic conical centrifuge tubes and then resuspended in 50 μl of the appropriate heat inactivated antiserum for a 30-min incubation at 37°C. These cells were then washed 3 times with 0.2 ml DMM and resuspended in 50 μl of fluorescein isothiocyanate conjugated goat antirabbit globulin (Grand Island Biological Company) which had previously been absorbed against the target cell lines and diluted 1:3 with DMM to remove nonspecific cell surface fluorescence. Incubation was for 30 min at 37°C after which the cells were washed twice with DMM and finally resuspended in a glycerol: Solution A (50:50 vol/vol) solution. Cells were examined on a Nikon fluorescence microscope using the appropriate filters and the dark field condenser.

Dye Exclusion Cytotoxicity

This technique was performed precisely as outlined by Hilgers et al. (27) using a noncytotoxic complement source from New Zealand white rabbits.

Radioactive Labeling

Cell surface proteins were labeled by the lactoperoxidase-^{125}I method as described (16). Approximately 10^6 cells were plated on 60-mm culture dishes and incubated for 24 h in normal growth medium. The monolayers were then washed 3 times with phosphate buffered saline (PBS; 0.1 M NaCl, 2.7 mM KCl, 15.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.2), and labeled for 10 min at room temperature in 0.5 ml/plate of PBS containing 5 mM glucose, 20 μg/ml lactoperoxidase (Calbiochem, La Jolla, California), 0.1 U/ml glucose oxidase (Calbiochem), and 250 μCi/ml Na^{125}I (17.0

Ci/mg). The reaction was stopped by adding PBS containing 1.47 mg/ml cold NaI and the monolayers were washed 3 times with the same solution. The cells were then scraped from the plates into PBS containing 2 mM phenylmethylsulfonyl fluoride and pelleted by centrifugation at 2,300 rpm. The labeled pellets were stored at $-70°$. In some experiments the cells were subjected to mild trypsin treatment immediately after labeling. Such cells were washed as above and incubated for 10 min at room temperature with 10 μg/ml trypsin in a buffer consisting of 0.046 M Tris, 0.015 M $CaCl_2$, 0.088 M NaCl, pH 8.1. Control plates were incubated in the buffer alone. Control and trypsinized cells were then harvested and stored as above. Labeling of glycoproteins or glycolipids with $[^{14}]$- or $[^{3}H]$glucosamine was performed by incubating cell monolayers for 24–48 h in DMM containing 10% fetal calf serum and either 5 μCi/ml $[^{3}H]$glucosamine (10.74 Ci/mmole) or 2 μCi/ml $[^{14}C]$ glucosamine (237.7 mCi/mmole). The labeled cells were washed 3 times with Solution A (calcium and magnesium free PBS), and removed from the flask with one round of freeze thawing in Solution A containing 2 mM phenylmethylsulfonyl fluoride. The cells were then homogenized (Dounce), centrifuged at 1,300 rpm for 5 min to remove nuclei and large pieces, and the crude mixture of labeled membrane fragments remaining in suspension was pelleted by centrifugation at 100,000 × g for 90 min. The pellet was collected and stored at $-70°C$.

SDS Polyacrylamide Gel Electrophoresis

Labeled membrane samples were dissolved in a solution containing 20% sucrose, 1% sodium dodecyl sulfate (SDS), 0.05% dithiothreitol, 0.054 M Tris, pH 6.1. The samples were boiled in the above solution and applied to either slab or tube gels. The complete electrophoresis system was as described by Neville (29) and consisted of the following: upper reservoir buffer: 0.04 M boric acid, 0.041 M Tris, 0.1% SDS, pH 8.64; lower reservoir buffer: 0.031 N HCl, 0.424 M Tris, pH 9.19; stacking gel: 3% acrylamide in 0.027 M H_2SO_4, 0.054 M Tris, 5% glycerol; resolving gel: 7.5% acrylamide in lower reservoir buffer containing 10% glycerol. The ratio of acrylamide to N,N'-methylene-bisacrylamide was 25:1 in all gels. Stacking and resolving gels were polymerized by the addition of 0.08% ammonium persulfate and 0.05% TEMED. Slab gels were dried and radioactivity was detected by autoradiography on Kodak RP/R-14 film. Tube gels were sliced into 1-mm sections and glycoproteins eluted by shaking in 1% SDS overnight. Radioactivity was determined in a Beckman liquid scintillation counter in a mixture containing 2,900 ml xylene, 960 ml Triton X-114, and 125 ml Liquiflor (New England Nuclear Corporation).

Pronase Digestion

Labeled membrane preparations or SDS gel sections containing labeled glycoproteins were incubated in a solution of 0.1 M Tris, 20 mM $CaCl_2$, pH 7.8, and pronase (Calbiochem) for 5–8 days at 37°C. Routinely, 100 μg pronase was included on day 0 and 50 μg added every other day. A few drops of toluene were added to inhibit bacterial growth.

Gel Chromatography

Pronase glycopeptides were analyzed on a Sephadex G-50 column (1.5 × 90 cm). Samples were applied and eluted with 0.05 M potassium phosphate, 0.02% sodium azide, pH 7.2. Two-milliliter fractions were collected, and radioactivity was determined in a Beckman liquid scintillation counter.

RESULTS

The biological properties of the cell lines are summarized in the data of Table I. A_{31} is a clone of BALB/c 3T3 cells, and has normal properties including a low saturation density, the inability to grow in soft agar, lack of agglutinability by Concanavalin A, and failure to cause tumors when injected into either BALB/c or athymic nude mice. PBC are low passage BALB/c embryo fibroblasts. 3T12T cells are prepared from tumors caused by injecting 3T12 cells into BALB/c mice as described (21, 22). BALB/c mice receiving injections of from 10^3 to 5×10^5 3T12T cells will be killed by the tumor within 30 days. MSC cells are derived from a tumor caused by inoculating BALB/c mice with the Moloney pseudotype of murine sarcoma virus. MSC cells have been shown to produce virus, and can cause tumors which grow progressively or regress depending on several parameters, including the initial cell dose injected into animals (30). c5 is a spontaneously transformed clone derived from A_{31} cells and causes tumors which regress in immunocompetent BALB/c mice. Injecting c5 into nude mice leads to the development of progressively growing tumors that kill the mice within 60 days. c5T is a transformed cell line derived from a tumor caused by injecting c5 cells into BALB/c mice (prior to regression). Mice receiving 5×10^6 c5T cells will be killed by their tumor within 60 days. The 3T12T line can be classified as nonimmunogenic, since injecting irradiated tumor cells or tumor excision does not protect mice from subsequent challenge by 3T12T cells. MSC and c5T are immunogenic since the same procedures protect animals from rechallenge. Interestingly, mice which have had c5 tumors that regress are protected from subsequent rechallenge by c5T cells, indicating shared antigens between c5 and c5T cells. The immunogenicity of c5, c5T, and MSC cells is paralleled by their stimulation of antibody production in tumor bearing mice.

Immunological Studies

We studied lymphocyte cytotoxicity as a possible assay for specific cell surface alterations on tumor cells. Lymphocytes prepared from spleens and lymph nodes of mice injected 10 to 20 days previously with tumor cells were cytotoxic for cultured cells. Cytotoxic lymphocytes could be detected in mice bearing tumors caused by inoculating any of the transformed cell lines. However, while we could demonstrate killing, the lymphocytes from tumor bearers did not show any specificity (Table II). Animals bearing tumors caused by either c5T or 3T12T cells had lymphocytes capable of killing A_{31}, c5T, or 3T12T cells. In other experiments (data not shown), similar preparations of lymphocytes were cytotoxic for PBC and even Swiss 3T3 cells. Similar results were found in 10 separate preparations of lymphocytes from tumor bearing animals. Varying the ratios of lymphocytes to target cells did not change the observed pattern of a lack of specificity for killing by lymphocytes.

Kall and Hellstrom (31) recently published experiments describing methods for sensitizing lymphocytes from normal BALB/c mice in vitro. Following their procedures, we incubated lymphocytes from BALB/c mice on a monolayer of mitomycin C treated A_{31}, 3T12T, or MSC cells. The results from these experiments are shown in the data of Table III. Lymphocytes cocultivated with A_{31} cells were not activated for killing either A_{31} or any of the transformed lines. In contrast, activation of lymphocytes against 3T12T cells resulted in the production of lymphocytes which were cytotoxic for 3T12T cells and the other normal and transformed lines. Activation against virus producing MSC cells resulted

TABLE I. Biological Properties of BALB/c Cell Lines

Cell line	Growth in methocel	Con A agg. (500 μg/ml)	Tumorigenicity in nude mice	Tumorigencity in BALB/c	BALB/c tumor bearer fate	Immunogenicity (protection from re-challenge after immunization)	Antibody formation in tumor bearers
PBC	–	–	–	–			
A_{31}	–	–	–	–			
MSC	+	+	+	+	Regressed	+	+
c5	+	+	+	+	Regressed	+	+
c5T	+	+	+	+	Killed	+	+
3T12T	+	+	+	+	Killed	–	–

Procedures for determining the ability of cell lines to grow in methocel and the technique for measuring Concanavalin A agglutinability have been described (22). Tumorigenicity was determined by the subcutaneous injection of up to 5×10^6 cells into 3-week-old BALB/c mice or athymic nude mice. "Regressed" means that a vascularized tumor was produced which disappeared over a 1-month period. Such tumors failed to return in the next 6 months to 1 year. "Killed" means that the mice were killed by the tumor, usually within 1–2 months following the injection of 5×10^3 to 5×10^6 cells. Mice were immunized either by 4 weekly injections of 5×10^6 irradiated (5,000 rads) tumor cells or by excising tumors which had reached 1–2 cm in diameter. "Antibody" circulating in tumor bearer serum was determined as described in Materials and Methods.

TABLE II. Specificity of Cell Mediated Cytotoxicity by Lymphocytes From Mice With Tumors Caused by c5T and 3T12T

Lymphocyte source	Target cells	CPM remaining	% Cytotoxicity[a]
None	A_{31}	21,695 ± 1,071	
Control	A_{31}	11,037 ± 1,396	–
c5T bearer	A_{31}	7,499 ± 689	32 < 0.01[b]
3T12T bearer	A_{31}	5,840 ± 473	42 < 0.01
None	c5T	9,898 ± 779	
Control	c5T	4,727 ± 527	–
c5T bearer	c5T	3,134 ± 481	34 < 0.01
3T12T bearer	c5T	2,321 ± 270	51 < 0.01
None	3T12T	54,244 ± 4,709	
Control	3T12T	31,846 ± 2,415	–
c5T bearer	3T12T	18,702 ± 1,818	41 < 0.01
3T12T bearer	3T12T	16,902 ± 1,835	47 < 0.01

[a] $\frac{\text{CPM test lymphocytes}}{(1 - \text{CPM control lymphocytes})} \times 100$

[b] Calculated by the Student T test

Lymphocyte preparation and cell mediated killing determined by the [^{3}H] uridine assay were performed as described in Materials and Methods. Lymphocytes were prepared from tumor bearing animals while the tumors were from 1 to 2 cm in diameter (usually days 18–26).

TABLE III. In Vitro Lymphocyte Sensitization and Cytotoxicity

Sensitizing monolayer	Target[a] cell	No. surviving cells	% Cytotoxicity	
A_{31}	A_{31}	48 ± 11	–	
3T12T	A_{31}	18 ± 6	66	< 0.01
MSC	A_{31}	45 ± 5	15	N.S.
A_{31}	3T12T	83 ± 4	–	
3T12T	3T12T	8 ± 1	90	< 0.01
MSC	3T12T	72 ± 12	13	N.S.
A_{31}	MSC	24 ± 5	–	
3T12T	MSC	14 ± 2	42	< 0.01
MSC	MSC	13 ± 2	46	< 0.01

[a] 1×10^{4} lymphocytes added per target well

In vitro activation. Cells for lymphocyte activation were incubated at 3×10^{6} cells/ml in Dulbecco's modified minimal essential media (DMM) containing 20 μg of mitomycin C/ml for 30 min at 37°C. The cells were rinsed 2 times in DMM and 1×10^{6} cells added to a 60-mm plate (20 cm^{2} growing area). Lymphocytes were produced from spleens as described in Materials and Methods, and suspended at a concentration of $1-3 \times 10^{7}$ cells/5 ml in sensitizing medium RPMI 1640, with 15% FCS as described (31). Five milliliters of cell suspension was added and the plates incubated for 6 days at 37°C. The activated lymphocytes were washed in DMM containing 10% fetal calf serum and cell mediated cytotoxicity determined.

Using in vitro activated lymphocytes, 1×10^{4} cells were added to each well containing target cells as described in Materials and Methods. The number of surviving cells was counted for sight wells, and the average is shown ± the standard deviation. Lymphocytes activated by culture on mitomycin C treated A_{31} cells did not show any cytotoxicity relative to experiments where either no lymphocytes or control lymphocytes were added to target cells. The number of surviving cells from experiments where mitomycin C treated A_{31} cells are incubated with lymphocytes are thus used as a control in calculations of lymphocyte cytotoxicity.

in lymphocytes with greatest cytotoxicity for MSC cells. The process of activation appears to be able to detect some basic difference between A_{31} and 3T12T cells, but once activated the lymphocytes show no specificity for the target cells they will kill.

On numerous occasions, we tested for the ability of sera from tumor bearers to block the killing of target cells by either in vitro activated or tumor bearer lymphocytes. Results from these experiments were uniformally negative. c5T or 3T12T bearer sera added to the lymphocyte killing assay did not affect target cell survival.

In another immunological approach to studies of cell surface change, we inoculated New Zealand white rabbits with freeze-thaw disrupted 3T12T or c5T cells. After absorption against A_{31}, sera from rabbits injected with 3T12T could be shown to react with transformed lines (c5, c5T, or 3T12T) only. Using antisera from rabbits injected with c5T cells, we were able to prepare antisera specific for the immunogenic (c5 and c5T) transformed cells (Table IV). We have subsequently shown that the antisera specifically precipitates a unique surface glycoprotein found only on the surfaces of c5 and c5T cells.

Chemical Studies

Glycolipids labeled in cells incubated with $[^3H]$glucosamine were compared by thin layer chromatography. The percent of label in the various glycolipid species are summarized in the data of Fig. 1. Growing or confluent A_{31} and the malignant nonimmunogenic 3T12T cells are very similar, with the majority of the label comigrating with GM_2 and G_{D1a}. Very little globoside or G_{M3} is detected in extracts from either 3T12T or A_{31} cells. The immunogenic lines show glycolipid compositions which are altered relative to A_{31} or 3T12T cells. MSC cells show increased G_{M3} and globoside and decreased G_{M2} and G_{D1a}. c5 and c5T are very similar and show increased G_{m3} and decreased G_{M2} compared with A_{31}. The results from glucosamine labeling are similar to earlier studies where ganglioside compositions were determined by measuring sialic acid in various glycolipid species in normal and transformed BALB/c cells (32). The surface labeling patterns of proteins on A_{31}, PBC, and 3T12T cells labeled by lactoperoxidase-^{125}I were indistinguishable when preparations were resolved by SDS gel electrophoresis. Figure 2 shows ^{125}I profiles for the 3 spontaneously transformed cell lines before and after mild trypsinization (10 μg/ml for 10 min at room temperature). c5 shows nearly identical patterns before and after trypsin treatment, as does c5T. Virtually no labeling is seen in the LETS region, while a very heavily labeled band of about 105,000 mol wt appears in both these cell lines. 3T12T is strikingly different from the other 2 transformed lines, with the heaviest label appearing in a band of 240,000 mol wt, analagous to the LETS glycoprotein. This band is sensitive to mild trypsinization, as has been reported for the LETS glycoprotein. 3T12T also shows much greater labeling of several bands of about 165,000 (which are not trypsin sensitive) and about 135,000 mol wt (which are trypsin sensitive) than do c5 and c5T. There is no labeling of a 105,000 mol wt band in 3T12T.

Membrane Glycoproteins

Crude membrane glycoproteins were labeled with $[^3H]$glucosamine and prepared as described in Materials and Methods. The labeled preparations were analyzed by SDS-polyacrylamide gel electrophoresis with the results shown on Fig. 3. Three labeled regions were found in all the cell lines tested: region I, about 220,000–250,000; region II, about 105,000–165,000; and region III, appearing about 70,000 mol wt. There were virtually no differences in region I patterns between any of the cell lines. Region II labeling patterns

TABLE IV. Indirect Immunofluorescence Using Anti-c5T Rabbit Antisera

	Target cell line				
Serum type	A_{31}	3T12T	c5T	c5	MSC
control serum	–	–	–	–	–
immune serum	+	+	+	+	+
A_{31} absorbed immune serum	–	–	+	+	–

Antiserum was raised in New Zealand white (NZW) rabbits against the c5T cell line as outlined in Materials and Methods. 0.5 ml of this crude antiserum (termed "immune serum") was absorbed at 4°C for 8 h against the 80,000 × g pellet (crude membrane fraction) of 5 × 10^7 homogenized A_{31} cells. The crude membranes were then pelleted and the absorption repeated for a total of 3 times. The resultant antiserum was termed "A_{31} absorbed immune serum." "Control serum" was obtained from nonimmunized NZW rabbits. The cell surface specificities of the above sera were determined by the indirect immunofluorescence assay as outlined in Materials and Methods. Negative surface fluorescence ("–") means that none of the cells showed positive surface fluorescence. Positive surface fluorescence ("+" means that 95–100% of the cells demonstrated ring or cap fluorescence.

vary between the cell lines tested. A_{31} and 3T12T cells appear to possess at least 2 glycoproteins in this region, IIa and IIb. c5, c5T, and MSC show these same glycoproteins as well as an additional glycoprotein, IIc, of about 105,000 mol wt. Region III again appears similar among all the lines. This consists of a broad peak that actually separates into a doublet on some gels. MSC also shows labeling of a group of bands between regions II and III. In comparing each region it was found that A_{31} always shows relatively lower labeling of region II and III than do the transformed lines.

Glycopeptides

We next compared glycopeptides from these cell lines. [^{14}C] and/or [^{3}H]glucosamine labeled membrane fragments were digested exhaustively with pronase and the resulting glycopeptides analyzed using Sephadex G-50 chromatography. Figure 4 shows the co-chromatogram of [^{3}H]glucosamine labeled A_{31} and [^{14}C]glucosamine labeled 3T12T membranes after pronase digestion. It can be seen that 3T12T is enriched in higher molecular weight glycopeptide species compared to A_{31}, confirming the earlier work of Meezan et al. and Buck et al. (18, 19). If the A_{31}-3T12T glycopeptide mixture was treated with neuraminidase prior to Sephadex G-50 chromatography, the elution patterns of glycopeptides from the 2 cell lines appeared very similar. 3T12T glycopeptides also showed an enrichment in faster eluting species when compared to PBC glycopeptides. Similar results were obtained when c5T derived glycopeptides were compared with A_{31} cells. There were no differences detected between glycopeptides of growing and confluent A_{31} cells.

The individual glycoprotein regions were separated from [^{3}H]glucosamine labeled A_{31} and [^{14}C]glucosamine labeled 3T12T membranes by electrophoresis in a large (1.5 × 15 cm) tube gel. Portions of the gel corresponding to regions I, II, and III were separated, digested exhaustively with pronase, and analyzed on Sephadex G-50. The results are presented on Fig. 5. In both cell lines, the glycopeptides from region I appear to be smallest in size, those from region II are of intermediate size, and those of region III are largest. 3T12T region I and II glycopeptides appear larger than A_{31} region I and II glycopeptides,

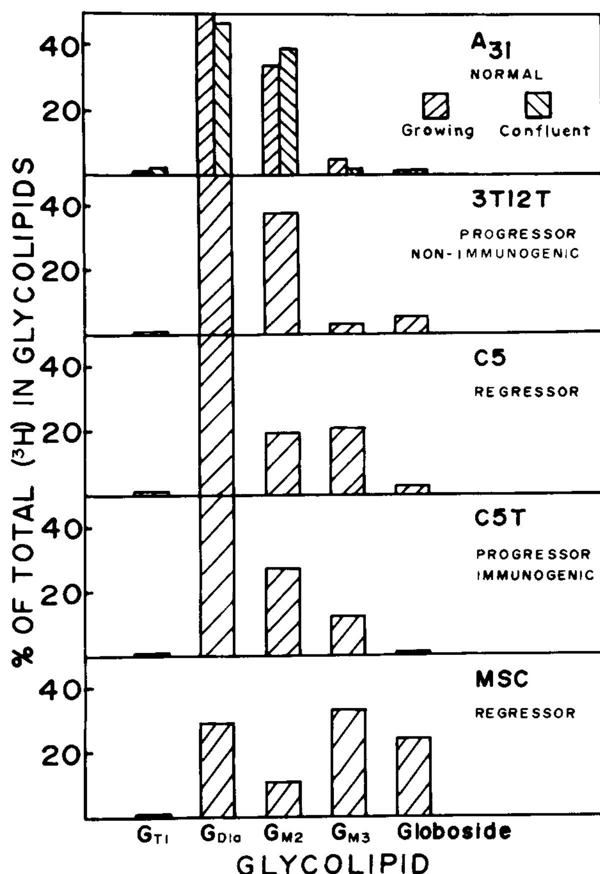

Fig. 1. Cells were labeled and membranes prepared as described in Materials and Methods. Labeled membrane fragments were extracted 3 times with $ChCl_3$:MeOH (2:1) and the extracts were pooled and dried. Radioactive samples as well as authentic glycolipid standards were applied to silica gel G-25 20 × 20 cm × 0.25 mm plates (Brinkman Instruments, Westbury, New York) and developed with chloroform:methanol:0.25% $CaCl_2$ (60:35:8). The plates were then dried and standard glycolipids were detected by reaction with iodine vapor. Labeled glycolipids were detected by cutting the developed TLC plate into 0.5-cm strips which were then placed into scintillation vials for radioactivity determinations.

respectively. Region III glycopeptides from both cell lines have similar elution profiles. There is a multiple cause for the enrichment of large glycopeptides in pronase digests of crude 3T12T membrane preparations. 3T12T has several glycoproteins (regions I and II) that produce larger glycopeptides than do the corresponding glycoproteins from A_{31}, and 3T12T shows consistently more glucosamine incorporation into glycoproteins that contain the largest glycopeptides from both cell lines (region III). If the separated region I, II, and III glycopeptides are treated with neuraminidase prior to Sephadex G-50 chromatography, the migration of all 3 regions is shifted to a smaller molecular weight. The size order of region III > region II > region I remains, however, after neuraminidase treatment. In addition, 3T12T region I and II glycopeptides remain larger than A_{31} regions I and II glycopeptides after neuraminidase treatment.

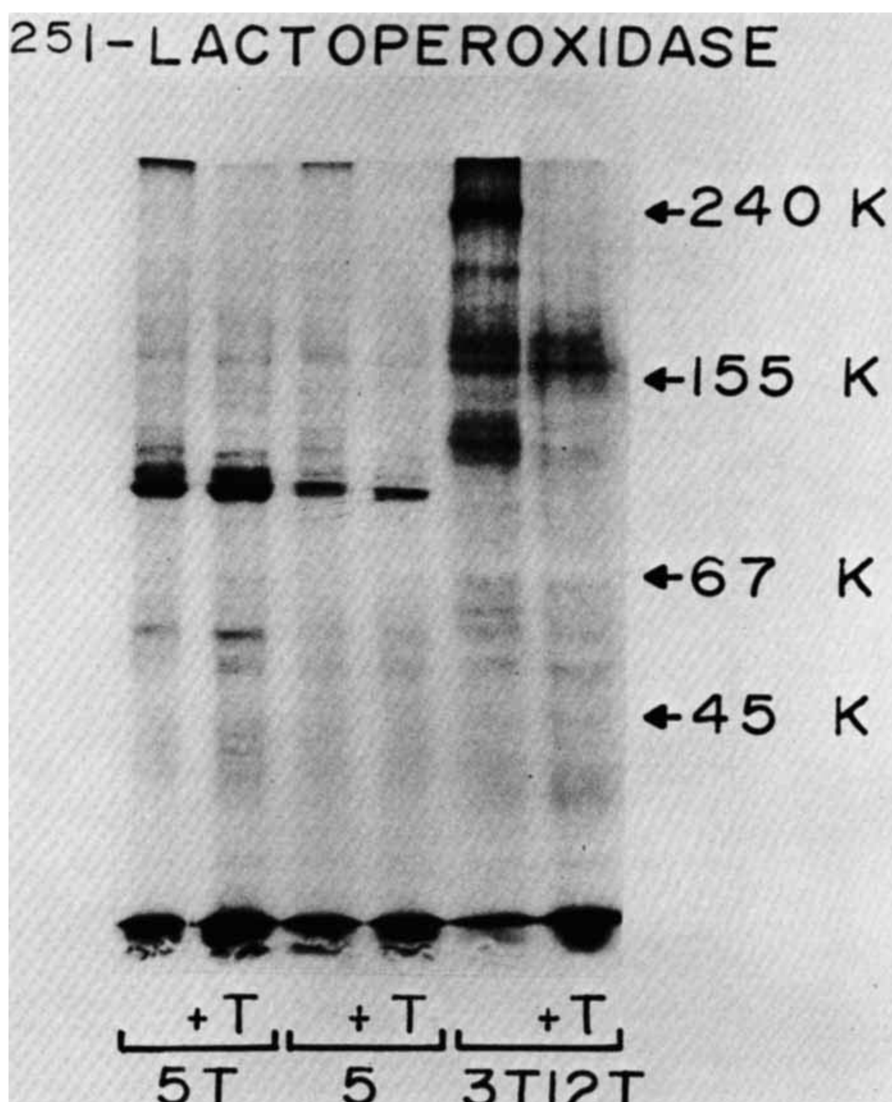

Fig. 2. SDS-polyacrylamide gel electrophoresis of ^{125}I labeled membranes from c5T, c5, and 3T12T. Approximately 50,000 cpm of each labeled cell material was applied to a 7.5% slab gel and electrophoresed at 150 V for approximately 2 h. The gel was dried and exposed on x-ray film. Molecular weight markers used include: spectrin (large band), 240,000; goat IgG, 155,000; bovine serum albumin, 67,000; hen egg albumin, 45,000. "+T" means that the ^{125}I labeled monolayer was treated with 10 $\mu g/ml$ trypsin for 10 min at room temperature prior to harvest.

DISCUSSION

We want to emphasize the necessity of studying transformed cells which have well-defined tumorigenic and malignant properties. Spontaneous transformants also appear to be an important addition to the more usual experiments which included only viral or

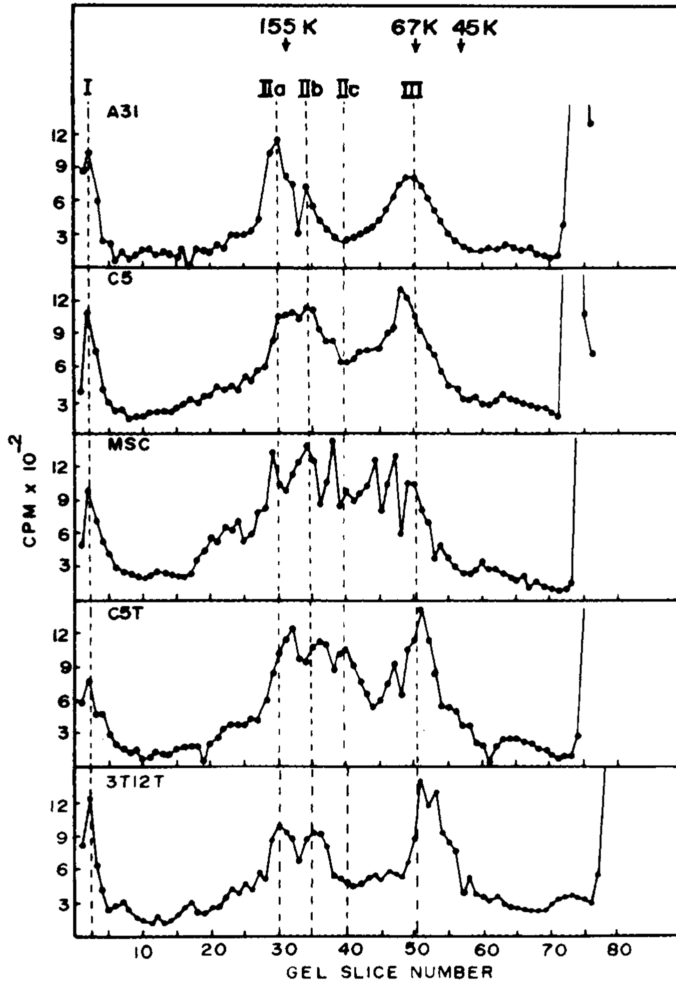

Fig. 3. SDS-polyacrylamide gel electrophoresis of [^{3}H]glucosamine labeled glycoproteins. 60,000 cpm of each labeled membrane preparation was applied to 7.5% gels in 6.0 × 120 mm glass tubes. Gels were electrophoresed at 3 mA per tube for approximately 3 h. Gels were rimmed from the glass tubes, frozen, and cut into 1-mm slices. Slices were shaken overnight in 2 ml 1% SDS to elute glycoproteins and the eluted material was counted in a Beckman liquid scintillation counter. Molecular weight markers used include: goat IgG, 155,000; bovine serum albumin, 67,000; hen egg albumin, 45,000.

carcinogen transformed cells. For example, numerous studies have shown that virally transformed cell lines have lost the ability to synthesize some of the complex glycolipids (2). In contrast, malignant spontaneous transformants frequently do not have these alterations (33). That carcinogens lead to numerous secondary changes is also not a new concept. Carcinogen transformed cells are known to be antigenically distinct, while spontaneous transformants are frequently nonimmunogenic (34–36).

The immunological response by tumor bearers offers an opportunity to detect and therefore assay for unique tumor cell antigens. The Hellstroms and others studying a number of animal model systems have developed the concept that tumor bearers make an

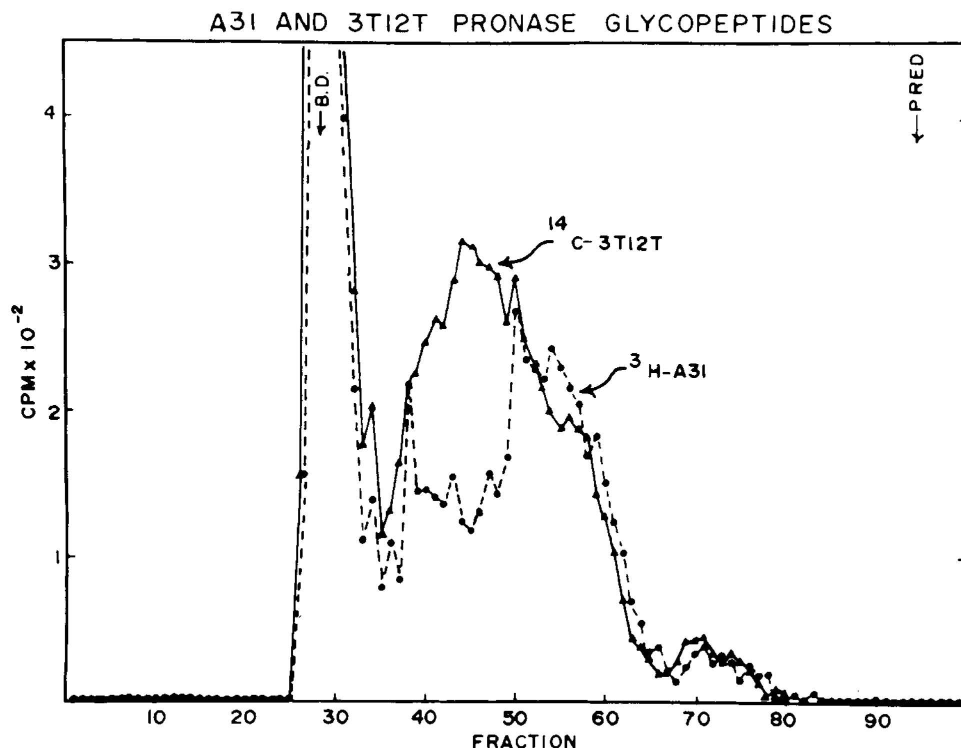

Fig. 4. Sephadex G-50 chromatography of A_{31} (●) and 3T12T (▲) pronase glycopeptides. [^3H] glucosamine labeled A_{31} and [^{14}C]glucosamine labeled 3T12T membrane preparations containing equal amounts of radioactivity were mixed and digested for 5 days with pronase. An aliquot of the digested material was then applied to a Sephadex G-50 column (90 X 1.5 cm). B.D.) blue dextran 2000; p. red) phenol red.

immunological response, but that progression or regression of the tumor depends on whether antigens derived from tumor cells can block lymphocyte mediated cytotoxicity (4, 10). These studies have generally used either viral or carcinogen transformed cells. The situation with human cancers is not clear. Hellstrom originally reported specific cell mediated immunity to human neoplasms of various histological types (36, 37). However, the specificity of lymphocytes from humans for their respective tumors has been questioned (38). Recently, a number of investigators have discussed problems in using microcytotoxicity assays for studies of human neoplasia (39–41).

Results from our studies of lymphocyte mediated cytotoxicity in mice bearing spontaneously transformed immunogenic and nonimmunogenic tumor cells support the concept that an immunological response can be made against progressively growing tumors. However, we were unable to demonstrate the presence of serum blocking factors. The lack of specificity for the cell precluded the use of cell mediated immunity to detect unique tumor antigens on the progressor spontaneous transformants. It is interesting to note that an immunological response can be measured even in animals bearing nonimmunogenic tumor cells. The use of antibodies both from tumor bearers and immunized mice along with specific antisera prepared from rabbits offers the best opportunity for an immunological assay capable of detecting cell surface change. Previously, rabbit antisera directed

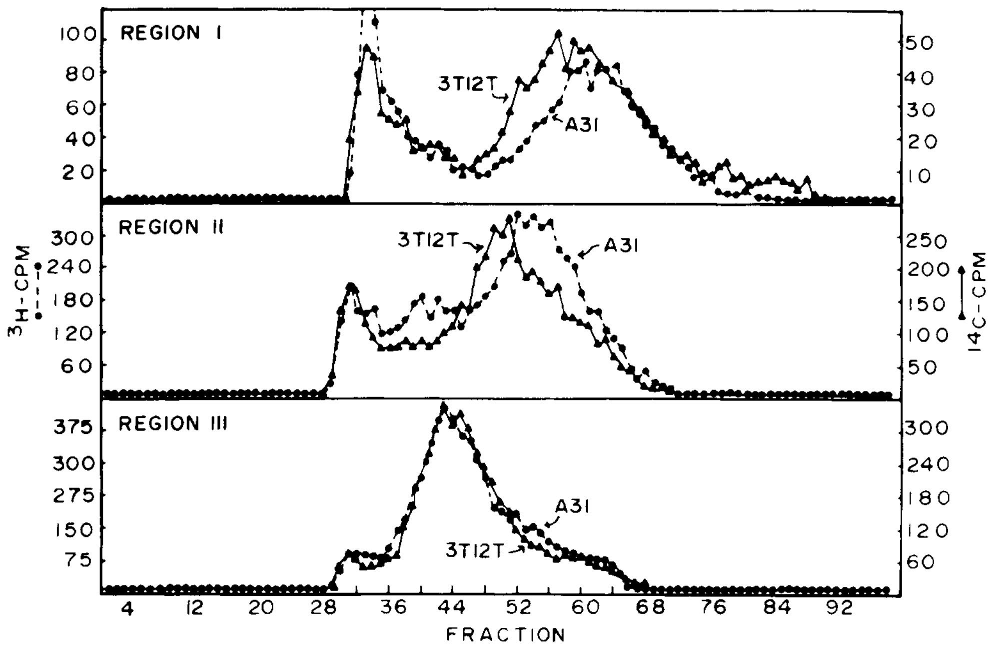

Fig. 5. Sephadex G-50 chromatography of A_{31} (●) and 3T12T (▲) pronase glycopeptides of isolated glycoprotein regions. [^{3}H]glucosamine labeled A_{31} and [^{14}C]glucosamine labeled 3T12T membrane preparations were mixed and coelectrophoresed on a 7.5% tube gel (1.5 × 15 cm). Part of the gel was sliced and counted and portions of the remaining gel corresponding to glycoprotein regions I, II, and III (see text) were excised and incubated for 8 days with pronase. The digested material from each region was then eluted from the gels, lyophilized, and applied to a Sephadex G-50 column (90 × 1.5 cm).

against human leukemic cells were used successfully to isolate a unique tumor cell antigen (42). The rabbit antiserum which reacts specifically against c5 and c5T cells is of special interest. We have now shown that the unique glycoprotein migrating in the IIc region is specifically precipitated by the alloantisera. This provides a one step purification for the unique antigen shared by c5 and c5T.

The chemical studies revealed both interesting similarities and differences between the normal and transformed cells. The immunogenic lines showed the simplified glycolipid patterns characteristic of virally transformed cells. In contrast, the most malignant line 3T12T had glycolipid compositions similar to normal cells. It is important to note that our studies only compared glucosamine labeled glycolipids. These methods would not have detected differences in fucolipids or slow migrating glycolipids (43). Similarly, the immunogenic lines c5 and c5T lacked the 240,000 mol wt ^{125}I labeled surface glycoprotein. The nonimmunogenic 3T12T progressor has levels of the LETS protein which are similar to normal cells. The glycoprotein patterns of A_{31} and 3T12T as detected by SDS-polyacrylamide gel electrophoresis were also very similar, with no new major bands appearing. Thus many of the typical transformation specific changes detected in membrane proteins, glycoproteins, and glycolipids of virally transformed cells appear to be secondary in nature and not necessary for expression of the transformed phenotype. It is interesting that the lack of substantial surface alterations in 3T12T parallels the lack of immunogenicity. It appears that a tumor cell that has very similar surface features to normal cells can be more successful in overcoming defenses of the host.

The only consistent difference detected between 3T12T and A_{31} cells was the presence of glycopeptides of higher molecular weight in the transformed cells. These results confirm earlier work using viral transformants (18, 19). Several isolated glycoprotein regions have glycopeptides of different molecular weight. The smaller glycoproteins tend to have higher molecular weight glycopeptides. These differences are retained even after neuraminidase treatment. These results confirm those of Sakiyama and Burge (44) indicating that a number of different kinds of carbohydrate structures are likely to be found on cellular glycoproteins. Glycopeptides prepared from regions I and II of 3T12T glycoproteins are both of higher molecular weight than the corresponding regions from A_{31} cells. It is of interest that the 240,000 mol wt glycoprotein while present in both the normal A_{31} and the spontaneously transformed 3T12T yields glycopeptides which are characteristically altered in the transformed cells. The presumed alteration is therefore occurring on more than one kind of carbohydrate structure. These differences remain after neuraminidase treatment, showing that the change probably involves carbohydrates other than sialic acid.

Further interpretation requires that we compile details of structures of complex polysaccharides of the normal and transformed cells. It is interesting in our system that lack of detectable change in glycolipids and I^{125}-labeled surface proteins is found in the nonimmunogenic progressor. However, 3T12T cells do show alterations in glycopeptides. These results are consistant with recent studies by Smets and co-workers where glycopeptide size profiles were also found to be correlated with malignancy (45).

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REFERENCES

1. Hakomori S: Biochim Biophys Acta 417:55, 1975.
2. Nicholson G: Biochim Biophys Acta 458:1, 1976.
3. Talmadge K, Burger M: In Whelan W (ed): "Biochemistry of Carbohydrates." Baltimore: University Park Press, 1975, p 43.
4. Hellstrom K, Hellstrom I: Adv Immunol 18:209, 1974.
5. Currie G: Biochim Biophys Acta 458:135, 1975.
6. Coggin J, Ambrose K, Dierlam P, Anderson N: Cancer Res 34:2092, 1974.
7. Alexander P: Cancer Res 34:2077, 1974.
8. Currie G, Alexander P: Br J Cancer 29:72, 1974.
9. Epstein L, Knight R: Br J Cancer 31:499, 1975.
10. Baldwin R, Price M, Robins R: Nature (London) New Biol 238:185, 1972.
11. Hellstrom I, Hellstrom K: Int J Cancer 4:587, 1969.
12. Grimes W: Biochemistry 9:5083, 1970.
13. Grimes W, Greegor S: Cancer Res 36:3905, 1976.
14. Hynes R: Proc Natl Acad Sci USA 70:3180, 1973.
15. Hogg N: Proc Natl Acad Sci USA 71:489, 1974.
16. Hynes R, Humphreys K: J Cell Biol 62:438, 1974.
17. Gahmberg C, Hakomori S: Proc Natl Acad Sci USA 70:3329, 1973.
18. Meezan E, Wu H, Black P, Robbins P: Biochemistry 8:2518, 1969.
19. Buck C, Glick M, Warren L: Biochemistry 9:4567, 1970.
20. Ogata S, Muramatsu T, Kobata A: Nature (London) 259:580, 1976.
21. Grimes WJ: In Baserga B, Clarkson B (eds): "Control of Proliferation in Animal Cells." New York: Cold Spring Harbor Laboratory, 1974, p 517.
22. Van Nest G, Grimes W: Cancer Res 34:1408, 1974.

23. Levine EM: Exp Cell Res 74:99, 1972.
24. Peden K: Experimentia 31:1111, 1975.
25. Hellstrom I, Hellstrom K: In Bloom B, Glade P (eds): "In Vitro Methods in Cell Mediated Immunity." New York: Academic Press.
26. McKhann C, Cleveland P, Busk M: Natl Cancer Inst Monogr 37:37, 1973.
27. Hilgers J, Haverman J, Nusse R, van Blitterswijk W, Cleton F, Hageman P, Van Nie R, Calafat J: J Natl Cancer Inst 54:1323, 1975.
28. Baldwin R, Barker C: Br J Cancer 21:793, 1967.
29. Neville P: J Biol Chem 246:6238, 1971.
30. Russell S, Cochrane C: Int J Cancer 13:54, 1974.
31. Kall M, Hellstrom I: J Immunol 114:1083, 1975.
32. Fishman P, Brady R, Aaronson S: Biochemistry 15:201, 1976.
33. Sakiyama H, Robbins P: Fed Proc Fed Am Soc Exp Biol 32:86, 1973.
34. Embleton M, Heidelberger C: Int J Cancer 9:8, 1972.
35. Basombris M, Phren R: Int J Cancer 10:1, 1972.
36. Baldwin R, Embleton M: Int J Cancer 13:433, 1974.
37. Hellstrom I, Hellstrom K, Sjogren H, Warner G: Int J Cancer 7:1, 1971.
38. Takasugi M, Mickey M, Tarasaki P: J Natl Cancer Inst 53:1527, 1974.
39. Herberman R, Oldham R: J Natl Cancer Inst 55:749, 1975.
40. Heppner G, Henry E, Stolback L, Cummings F, McDonough E, Calabrisi P: Cancer Res 35:1931, 1975.
41. Baldwin R: J Natl Cancer Inst 55:745, 1975.
42. Billing R, Terasaki P: J Natl Cancer Inst 53:1635, 1974.
43. Itaya K, Hakomori S, Klein A: Proc Natl Acad Sci USA 73:1568, 1976.
44. Sakiyama H, Burge B: Biochemistry 11:366, 1972.
45. Smets L, van Beek W, van Rosig H: Int J Cancer 18:462, 1976.