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Purified Membrane Fractions from Mammary Tumor Cells Elicit Biological Reactivity in *In Vitro* Cell-mediated Immune Reactions*

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Abstract—*Lithium chloride (LiCl) aggregation followed by centrifugation through a sucrose step gradient was used to obtain purified plasma membranes from two sublines of mouse mammary adenocarcinoma. These two tumor lines were chemically induced by treatment of a hyperplastic nodule with 7,12-dimethylbenzanthracene (DMBA). One line, D1-DMBA-3, has been found to be immunogenic to the host of origin, while the other, D1-DMBA-2, does not elicit specific tumor immunity as previously tested in* in vivo *and* in vitro *immune reactions. The purified membrane fractions were assayed for protein, DNA and sialic acid content as well as enzymatic markers of membrane purity. When tested in blastogenesis and cytotoxicity reactions, membrane-containing fractions from the D1-DMBA-3 immunogenic tumor were found to be stimulatory to spleen cells of D1-DMBA-3 tumor bearers over a ten-fold protein concentration. Spleen cells from normal mice do not respond in these reactions to the various tumor fractions. No reactivity was observed when non-membrane-containing preparations were used as stimuli in the cell-mediated immune reactions. The specificity of these reactivities was further demonstrated by the lack of responses when fractions from the non-immunogenic D1-DMBA-2 tumor were tested in parallel in our* in vitro *assays. The data presented indicate that the procedure employed is useful for the isolation of membrane-associated, tumor-specific antigens which can be easily quantitated and still retain biological activity in* in vitro *tests of cell-mediated immunity.*

INTRODUCTION

CELL-MEDIATED immune reactions against tumor-associated antigens have been studied in diverse model systems [1, 2]. In many of these reports the antigenic preparations used consist of intact tumor cells or crude antigenic extracts [3, 4]. In recent years several laboratories have devoted great attention towards the purification and characterization of tumor-specific and tumor-associated antigens [5–7]. Some investigators have been able to isolate purified preparations of antigens from various tumor sources, both of human and animal origin. The antigenicity of these purified proteins is often assessed by serological reactivity with xenogeneic or allogeneic antisera [8, 9].

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Solubilized membrane preparations have been used in some cell-mediated immune assays. Optimal reactivity for these assays usually requires protein concentrations between 50 and 1500 μg/ml [10, 11]. In some cases when viral-induced tumors [12] or solubilized allogeneic extracts [13] are used, positive reactions have been obtained with much lower levels of protein. However, with solubilized antigenic preparations it is difficult to determine the possible contribution(s) of the membrane substrate to the antigenic potential.

In the present report we describe a procedure to purify and characterize membranes of tumor cells capable of stimulating lymphocytes from tumor-immune animals to undergo blastogenic transformation and participate in cytotoxic reactions triggered by tumor-associated antigens. This method not only yields large amounts of purified mem-

brane but is capable of producing fractions that elicit biological activity at concentrations as low as 0.25 μg protein/ml. Furthermore, this reactivity is retained in preparations stored at −70°C for periods of up to a year.

MATERIALS AND METHODS

Animals and tumors

Ten to twelve-week-old BALB/c virgin mice bred in our laboratories by sister–brother matings were used throughout the studies. Two transplantable mammary adenocarcinomas were employed. These tumors were derived from a non-viral, non-carcinogen-induced preneoplastic alveolar nodule in a BALB/c mouse after treatment with 7,12-dimethylbenzanthracene (DMBA) [14]. The tumor line designated D1-DMBA-3 is immunogenic for the host, while D1-DMBA-2 is non-immunogenic [15]. Both were transplanted by subcutaneous injections of 0.1 ml of minced suspensions.

Preparation of tumor cell membranes

The method employed is an adaptation of one described by Kornfeld and Siemers [16], further modified by Claflin *et al.* [17]. Tumor tissue was obtained from BALB/c mice bearing either D1-DMBA-3 or D1-DMBA-2 tumors. Two hundred grams of each were collected, trimmed of necrotic and hemorrhagic pieces, and frozen at −70°C until use. The frozen tissue was then shredded with a Kitchen Magic vegetable cutter (Popeil Brothers, Inc., Chicago, IL) fitted with an 8-inch plate with 3/32 × 3/8 inch cutting slits. The finely shredded, still-frozen tissue was then suspended in four volumes of cold 0.25 M sucrose containing 0.05 M Tris–HCl, 0.001 M EDTA, pH 7.5, and homogenized in an Oster blender for 5 sec at low speed and 5 sec at high speed. The resulting crude homogenate was then centrifuged at 980 ***g*** for 10 min. The supernatant was separated from the nuclear pellet and treated with 3 M LiCl to a final concentration of 0.1 M LiCl. The treated supernatant was centrifuged for 90 min at 30,000 ***g*** in a Sorvall RC-2 centrifuge at 4°C. This step yielded a crude membrane pellet and a soluble supernatant which was not further processed. This crude membrane was homogenized five times in a glass homogenizer and resuspended in a 0.005 M Tris buffer with 0.001 M EDTA and 0.100 M LiCl, pH 7.5 (220 ml/kg), then mixed with an equal volume of 80% sucrose. This preparation was layered onto an equal volume of 30% sucrose and centrifuged at 50,000 ***g*** for 4 hr. Each fraction obtained was washed five times in 0.05 M Tris, pH 7.5, sedimenting at 45,000 ***g*** for 1 hr between each wash. Aliquots were removed for determination of protein, sialic acid, enzyme activity and DNA, and the rest stored at −80°C.

Analytical methods

Protein was measured as described by Lowry *et al.* [18], using bovine serum albumin as standard. DNA was determined using the diphenylamine reaction as described by Giles and Myers [19], using calf thymus DNA as standard. Samples and standards were hydrolyzed in 7.5% perchloric acid at 70°C for 15 min before addition of the diphenylamine reagent. The thiobarbituric acid method of Warren [20] was used for the measurement of sialic acid. Hydrolysis in 0.25 M H_2SO_4 for 1 hr at 80°C resulted in the release of sialic acid from the subcellular fractions. The sialic acid was separated from contaminating deoxyribose and sucrose by adsorption and elution from Dowex 1-X8 (formate), as described by Spiro [21]. The column was washed with water and the sialic acid eluted with 0.3 N formic acid. The eluate was lyophilized and then analyzed for sialic acid. An aliquot of a standard solution of *N*-acetylneuraminic acid (Pierce Chemical Company) was carried through the entire procedure and used as the standard in the colorimetric assay.

Marker enzyme assays

The 5′-nucleotidase activity, a plasma membrane marker enzyme, was assayed as described by Aronson and Fouster [22]. The β-*N*-acetylglucosaminidase activity, a marker for lysosomes, was measured as described by Li and Li [23].

Preparation of spleen cells

Spleens were removed aseptically from normal and D1-DMBA-3 tumor-bearing mice. The cells were passed through a stainless steel mesh with siliconized rubber stoppers and washed through with 10 ml of Hanks' balanced salt solution (HBSS). The resulting single cell suspension was centrifuged and the cell pellet subjected to a hypotonic water shock for elimination of contaminating red blood cells. A cell count was preformed to determine the number of lymphocytes. With this procedure the final cell population contained mainly T (44% Thy 1.2-positive) and B (45% SIg-positive) lymphocytes.

Lymphocyte transformation assay

The spleen cell preparations were adjusted to a concentration of 1×10^6 cells/ml in the growth medium described by Click *et al.* [24]. Various concentrations of the different membrane fractions were added in 25 μl volumes, as shown in the graphs and tables. Cultures were maintained for 3 days in micro-culture plates (Linbrow, Flow Labs, Rockville, MD) with 2×10^5 cells per well and were given an 18-hr pulse of tritiated thymidine (0.5 μCi/well, Amersham Searle, Arlington Heights, IL). The cells were collected on glass fiber filter paper with distilled water in a Skatron multiple automated sample harvester. The filters were counted for radioactivity in a Packard TriCarb liquid scintillation counter and results were expressed as counts per minute.

Cytotoxicity assay

An 'innocent bystander' cytotoxicity reaction triggered by tumor-associated antigens in immune tumor bearers was employed as previously described [25]. The [^{51}Cr]-labeled chicken red blood cells (CRBC) used as targets were prepared as described by Perlmann and Perlmann [26]. Membrane preparations of varying protein concentrations were added in 0.1-ml aliquots to 0.4 ml of spleen lymphocyte suspensions from normal and tumor-bearing mice at 3×10^6 cells/ml. [^{51}Cr]-Labeled CRBC were added in volumes of 0.25 ml at a concentration of 2×10^5 cells/ml. Cultures were incubated for 20 hr at 37°C in a 5% CO_2 atmosphere. At the end of the incubation period the cultures were centrifuged for 10 min at 400 ***g*** at 4°C. Both the supernatant and pellet were individually collected and counted in a Scientific Products gamma counter. Results are expressed as percentage of chromium release.

RESULTS

Isolation of membrane fragments

Figure 1 shows schematically the fractionation procedure for the isolation of purified plasma membranes. Aliquots of all fractions obtained at each step were collected. The sucrose gradient yielded three fractions: (1) a slightly turbid band identified as 'top'; (2) a clear supernatant region termed 'middle'; and (3) a solid pellet. These three fractions were collected and, along with aliquots of other fractions, were characterized by analytical procedures and for enzymatic markers.

Analytical determinations

The chemical composition of the subcellular fractions from tumor cells was investigated with analytical techniques. DNA, protein and sialic acid contents were determined in the fractions obtained from both the immunogenic D1-DMBA-3 tumor and the non-immunogenic D1-DMBA-2 tumor. In Table 1 it can be seen that the protein recovered in the top and middle fractions represents 0.039% of the total protein of the D1-DMBA-2 tumor and 0.048% for the D1-DMBA-3 line. Only trace amounts of DNA are present in these fractions. Sialic acid, a membrane marker, was increased 10.7-fold in the D1-DMBA-3 tumor and 13.6-fold in the D1-DMBA-2 tumor in the top sucrose gradient fraction.

Marker enzyme activity

Two enzymes, 5'-nucleotidase and β-*N*-glucosaminidase, were assayed respectively as positive and negative markers of purity. In Table 2 it can be seen that 5'-nucleotidase, a plasma membrane marker, was increased 7.7-fold in the D1-DMBA-2 tumor and 8.4-fold in the D1-DMBA-3 line. The residual activity found in the pellet may be due to remnants of other membranes. Levels of β-*N*-acetylglucosaminidase, a lysosomal enzyme, indicate little contamination with lysosomes or associated structures in the highly purified membrane fraction. In both tumor sublines an approximate 5-fold reduction was achieved in the top fraction of the sucrose gradient when compared to the crude membrane fraction. The low levels of this enzyme in the purified fractions may represent the mechanical disruption of lysosomes during the procedure.

Lymphocyte transformation assays

The potential for eliciting biological activity of the various fractions obtained was assessed in lymphocyte transformation assays. Positive blastogenic reactions were observed with all concentrations of crude homogenate tested. The 1.0 μg concentration and 2.5 μg of crude membrane were stimulatory at higher levels than the crude homogenate. Maximal responses were observed using the most highly purified preparation, i.e. the top fraction of the sucrose gradient step. High levels of [^{3}H]-thymidine incorporation were obtained in cultures stimulated with all three concentrations of this tumor antigen preparation. An apparent dose–response curve was observed with this fraction and the highest incorporation was seen in cultures of lymphocytes from tumor animals exposed to a concentration of 1 μg of antigenic protein, as was shown in Fig. 2.

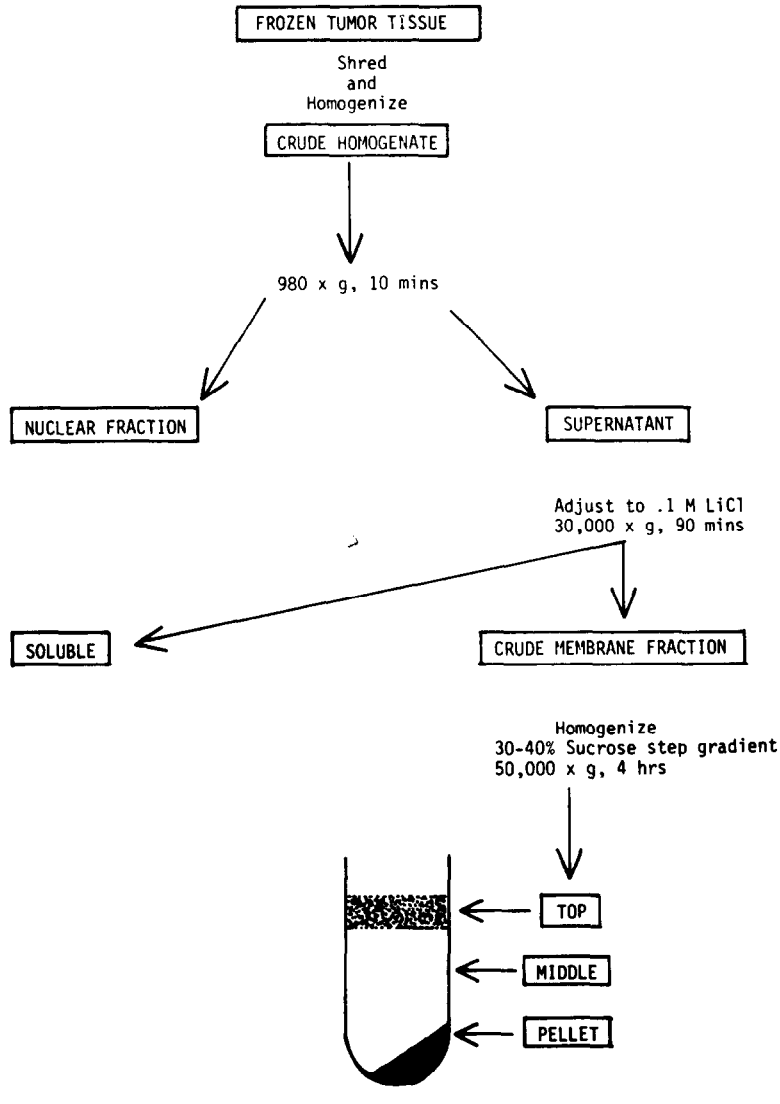

Fig. 1. Flow chart indicating the steps in the preparation of purified plasma membranes from mouse mammary tumors.

Table 1. Chemical composition of the subcellular fractions from the D1-DMBA-3 immunogenic and the D1-DMBA-2 non-immunogenic mammary tumors

Fraction	Protein*		DNA†		Sialic acid†	
	D1-DMBA-3	D1-DMBA-2	D1-DMBA-3	D1-DMBA-2	D1-DMBA-3	D1-DMBA-2
Crude homogenate	14.38	18.71	0.384	0.399	1.462	1.332
Nuclear fraction	20.58	24.74	2.22	2.14	0.457	0.473
Supernatant	N.D.	10.23	N.D.	0.090	N.D.	1.71
Soluble fraction	4.57	4.95	0.070	0.069	0.758	0.899
Crude membrane	3.29	3.56	0.042	0.041	8.92	8.98
Sucrose gradient membranes:						
Top	0.226	0.136	0.007	0.008	15.7	18.2
Middle	0.144	0.156	0.007	0.006	13.2	12.69
Pellet	2.21	N.D.	0.134	N.D.	2.91	N.D.

*mg/ml.
†μg/mg protein.

Table 2. Marker enzyme activities in subcellular fractions from the D1-DMBA-3 immunogenic and the D1-DMBA-2 non-immunogenic mammary tumors

Fraction	5'-Nucleotidase*		β-*N*-Acetyl glucosaminidase*	
	D1-DMBA-3	D1-DMBA-2	D1-DMBA-3	D1-DMBA-2
Crude homogenate	0.122	0.138	0.093	0.061
Nuclear fraction	0.228	0.187	0.176	0.133
Supernatant	N.D.	0.099	N.D.	0.060
Soluble fraction	0.018	0.014	0.160	0.060
Crude membrane	0.626	0.597	0.200	0.206
Sucrose gradient membranes:				
Top	1.47	1.79	0.036	0.039
Middle	1.36	1.87	0.038	0.044
Pellet	0.872	N.D.	0.101	N.D.

*μmol/hr/mg protein.

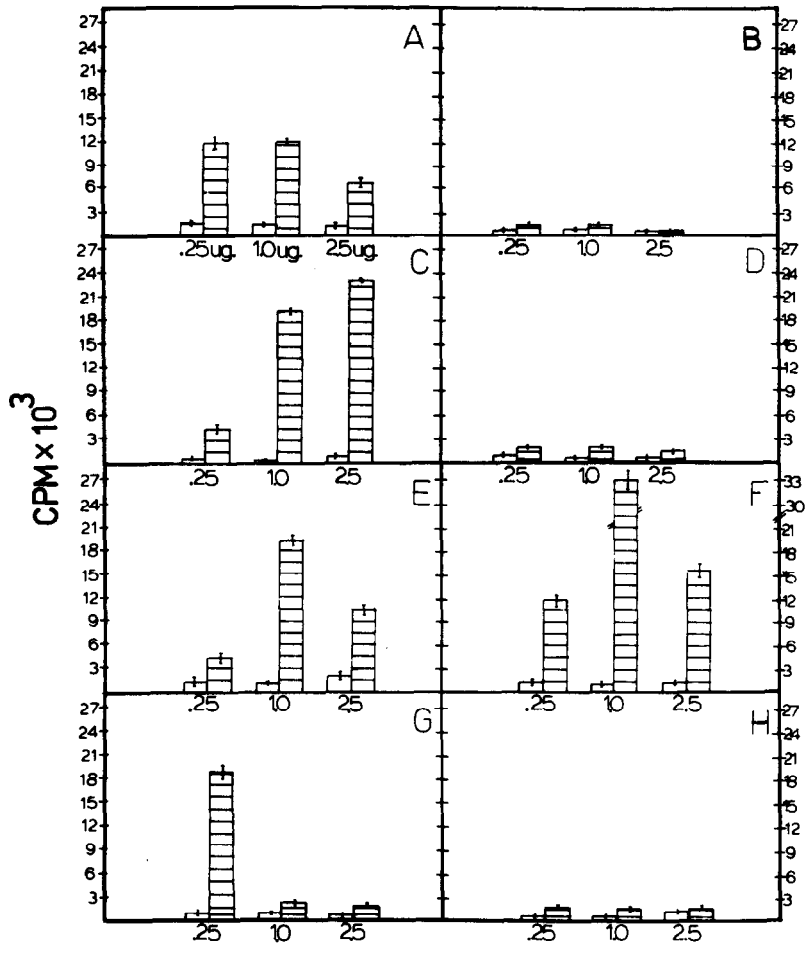

Fig. 2. Uptake of [³H]-thymidine by spleen cells of normal (□) and D1-DMBA-3 tumor-bearing (▤) BALB/c mice. Each panel represents the blastogenic responses to the various fractions derived from the membrane purification procedure. (A) Crude homogenate; (B) nuclear fraction; (C) supernatant; (D) post-LiCl treatment soluble fraction; (E) crude membrane; (F) top of sucrose gradient; (G) middle portion of sucrose gradient; (H) pellet of sucrose gradient. Final concentrations of protein used as stimuli are 0.25, 1.0 *and* 2.5 μg.

Cytotoxicity reaction

The results of the 'innocent bystander' cytotoxicity reactions triggered by the various fractions obtained are shown in Fig. 3. The results closely parallel those obtained in the lymphocyte transformation assay. Thus, all membrane-containing fractions were capable of eliciting the cytotoxic reaction, while the other fractions gave no biological activity. The top sucrose gradient induced a cytotoxic response at all three concentrations tested.

Comparison of the biological reactivities elicited by the D1-DMBA-2 and D1-DMBA-3 tumor fractions

Since one could argue that the technical procedure employed may generate structures that could act as non-specific stimuli, it was deemed necessary to establish the specificity of the reactions obtained. Thus, studies were performed using fractions derived from the D1-DMBA-3 immunogenic and the D1-DMBA-2 non-immunogenic tumor. In Table 3 it can be seen that cultures containing 1 μg of the crude homogenate, the crude membrane and the top sucrose gradient fraction from D1-DMBA-3 were stimulatory in the blastogenesis and cytotoxicity assay when spleen cells were used. However, no responses were obtained when the same spleen cells were exposed to the corresponding fractions of the D1-DMBA-2 tumor. Furthermore, all fractions of D1-DMBA-2 tumor obtained by the purification procedure were tested in other experiments at the same three concentrations used for the D1-DMBA-3. In data not shown it was found that these were also non-reactive, thus attesting to the specificity of the responses and further demonstrating that the procedure employed does not generate stimulatory artifacts.

DISCUSSION

Over the past several years, increasing emphasis has been placed on the isolation and characterization of tumor-specific antigens [27,

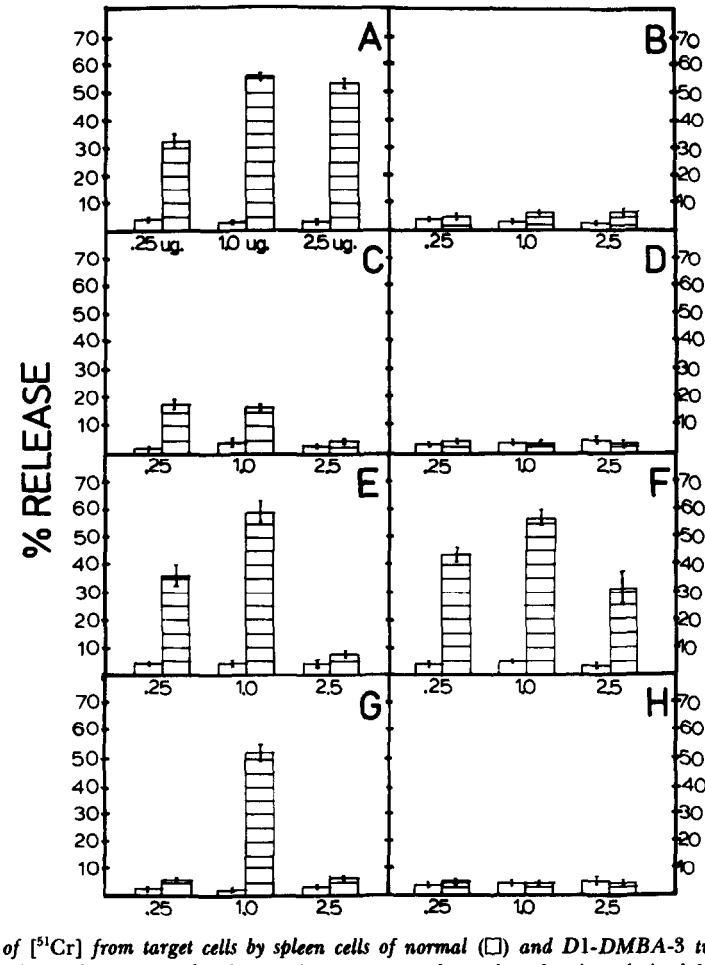

Fig. 3. Release of [^{51}Cr] from target cells by spleen cells of normal (□) and D1-DMBA-3 tumor-bearing (▤) BALB/c mice. Each panel represents the blastogenic responses to the various fractions derived from the membrane purification procedure. (A) Crude homogenate; (B) nuclear fraction; (C) supernatant; (D) post-LiCl treatment soluble fraction; (E) crude membrane; (F) top of sucrose gradient; (G) middle portion of sucrose gradient; (H) pellet of sucrose gradient. Final concentrations of protein used as stimuli are 0.25, 1.0 and 2.5 μg.

Table 3. Biological activities elicited by various subcellular fractions from the D1-DMBA-3 immunogenic and the D1-DMBA-2 non-immunogenic mammary tumors

Stimuli*	Blastogenesis*† Normal mice	Blastogenesis*† D1-DMBA-3 tumor bearers	Cytotoxicity‡ Normal mice	Cytotoxicity‡ D1-DMBA-3 tumor bearers
No stimulant	830 ± 55	1925 ± 76	3.69 ± 0.22	4.41 ± 0.24
D1-DMBA-3 fractions:				
(1) Crude homogenate	950 ± 69	10936 ± 547	3.26 ± 0.23	55.6 ± 2.8
(2) Crude membrane	934 ± 67	18815 ± 527	4.26 ± 0.43	59.4 ± 4.6
(3) Top sucrose fraction	943 ± 81	32774 ± 1540	4.19 ± 0.25	57.26 ± 3.2
D1-DMBA-2 fractions:				
(1) Crude homogenate	284 ± 71	722 ± 180	3.97 ± 1.32	4.8 ± 1.6
(2) Crude membrane	360 ± 90	1096 ± 274	3.57 ± 1.19	6.5 ± 2.2
(3) Top sucrose fraction	302 ± 76	895 ± 224	5.33 ± 1.77	8.8 ± 2.9

*All fractions were tested at 1 μg/ml final concentration.
†Blastogenesis results expressed as cpm/well ± S.E.
‡Cytotoxicity results expressed as percentage cytotoxicity ± S.E.

28]. Various methods have been applied to this problem with differing degrees of success when tested in *in vivo* and *in vitro* assays [29, 30], and in blocking studies [2]. While it is widely assumed that the antigens relevant to tumor immunity reside in the plasma membranes [31–33], preparations used for testing are often poorly characterized in this respect.

Many of the studies evaluate the biological activity of the preparations by their interactions with allogeneic or xenogeneic antisera [34, 35]. Some researchers have successfully utilized *in vivo* assays such as delayed hypersensitivity reactions [36]. Several investigators have employed 3 M KCl extracts in cell-mediated studies [4, 10]. However, this chemical reagent has a profound disaggregating effect on the membranes, leading to a solubilized preparation at the expense of the tertiary structure which may be of importance for the integrity of antigenic determinants. Indeed, in our laboratory [37] we have evidence that purified virions from murine mammary tumor virus (MMTV) stimulate blastogenic responses in splenocytes of BALB/cCrgl mice bearing D1-DMBA-3 tumors. However, in recent work (Lopez *et al.*, unpublished) we have found that these same purified viral antigens do not elicit 'innocent bystander' cytotoxicity, but purified membranes from MMTV expressing mammary tumors syngeneic to their BALB/cCrgl host will induce spleen cells from these mice to lyse labeled target cells. This observation indicates that association with membranes might be necessary for the triggering of some cell-mediated immune reactions, a phenomenon reminiscent of that described by Zinkernagel and Doherty [38].

Furthermore, Raphael and Tom [39] have demonstrated that solubilized membranes of a colon tumor were inactive or only weakly reactive in two *in vitro* assays of cell-mediated immunity. When these solubilized membranes were incorporated into liposomes, reactivity was detected at levels enhanced up to five-fold. Alternate methods of obtaining membranes include the use of cell disruption bombs followed by ultracentrifugation, and various other procedures of membrane solubilization such as treatment with proteases, detergents or other chaotropic agents. Hu *et al.* [35] have recently made a comparative study in a leukemia model system of various solubilization procedures, i.e. papain digestion, detergent treatments by using Triton X-100 or Np-40, and 3 M KCl extractions. This investigator found that this last procedure gave the best preparations as tested in *in vivo* protection experiments. In addition, they further purified their 3 M KCl extracts in Sephadex G-200 columns.

In the present study we describe a technique which allows the simple, rapid isolation of membrane-associated, tumor-specific antigens in good yield from large quantities of tumor. Furthermore, using a series of analytical procedures as well as enzyme markers we have shown that this technique results in highly purified membrane fragments.

The most stringent test of such a preparation, however, is its ability to act as a stimulating antigen in assays of immune function. We have used two *in vitro* cell-mediated immune parameters, i.e. an 'innocent bystander' cytotoxicity reaction and lymphocyte blastogenic transformation, to show that this technique allows for the recovery of tumor antigen pre-

parations which retain full biological activity, even when used at concentrations several fold lower than antigens prepared by other techniques [40, 41].

While the simplicity of this isolation procedure itself is of value, the ability to consistently isolate membrane fragments of high purity with undiminished biological activity is of great importance in the study of tumor immunology. The small amounts necessary to stimulate activity may serve to improve quantitation within tests. In addition, we have found that these tumor antigen preparations are remarkably stable inasmuch as they retain full activity even after 7 months at −80°C (Buessow *et al.*, unpublished results).

In our studies we have made a comparison of two mammary tumor lines of the same origin but presenting differing immunogenic potentials. This parallel study was designed to enable us to determine whether cryptic porteins exposed during the purification procedure could be the cause of an increasing non-specific stimulatory activity in the cell-mediated immune reactions. Previous studies in our laboratories [35, 37] have shown that BALB/c non-immunogenic D1-DMBA-2 mammary tumors possess spleen cells which do not respond to crude extracts from either the homologous D1-DMBA-2 or the immunogenic subline D1-DMBA-3. Conversely, spleen cells from BALB/c mice bearing D1-DMBA-3 tumors react solely to crude extracts from their own tumors while being unresponsive to preparations from the non-immunogenic D1-DMBA-2 and from other syngeneic mammary tumors of viral origin. The results presented herein indicate that the enhanced reactivities seen in cell-mediated immune assays with purified membrane fractions derived from the D1-DMBA-3 tumor are probably due to an increase in the concentration of membrane-associated tumor antigens specific for this tumor. The lack of reactivity to purified membrane preparations from the D1-DMBA-2 tumor further emphasizes the specificity of the antigenic moieties obtained by this procedure.

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