Proc. Nat. Acad. Sci USA
Vol. 72, No. 3, pp. 1012–1016, March 1975

Immunological Escape Mechanism in Spontaneously Metastasizing Mammary Tumors

(glycocalyx/surface antigen solubility/5′-nucleotidase)

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Communicated by Jacob Furth, December 22, 1974

ABSTRACT **Immunological and biochemical studies of spontaneously metastasizing and nonmetastasizing rat mammary carcinomas and their plasma membranes indicated that: (*i*) all spontaneously metastasizing tumors have little or no demonstrable glycocalyx, while all nonmetastasizing tumors have a thick glycocalyx; (*ii*) there is a direct relationship between the glycocalyx and immunogenicity, and an inverse relationship with the metastasizing capacity of tumor cells, properties which can be quantitated by levels of the plasma membrane marker enzyme 5′-nucleotidase (EC 3.1.3.5; 5′-ribonucleotide phosphohydrolase) activity; (*iii*) the absence of glycocalyx from the metastasizing tumor cell surface seems to result from its dissociation from plasma membranes, for solubilized cell surface antigen is readily found in the blood of metastasizing tumor bearing rats, while there was no detectable tumor cell surface antigen in the blood of the nonmetastasizing tumor hosts tested; (*iv*) both metastasizing and nonmetastasizing mammary tumors appear to have a common soluble cell surface antigen; (*v*) in addition to this common antigen, there is another membrane-bound antigen in the nonmetastasizing, immunogenic tumor cell surface which presumably is the tumor specific transplantation antigen; and (*vi*) this antigen is immunobiologically unique, but seems to be immunochemically related to the common soluble antigen. It is postulated that the lack of an immunogenic coat and/or the presence of solubilized tumor cell surface antigen in the blood may provide an immune escape mechanism for tumor cells by interfering with cell-mediated immune response of tumor hosts, leading to their dissemination.**

In an earlier report one of us (U.K.) (1) described the induction of spontaneously metastasizing mammary carcinomas (MT) in a highly inbred strain of W/Fu female rats by feeding 3-methylcholanthrene in splenectomized, thymectomized, or both splenectomized and thymectomized hosts, and subsequently subjecting the developing tumors to "immunoselection" *in vivo*. Such tumors maintained their metastasizing capacity in normal syngeneic rats generation after transplantation generation, indicating that the metastasizing capacity is an intrinsic property of tumor cells. It was further demonstrated that the metastasizing tumor cells were nonimmunogenic while the ordinary methylcholanthrene-induced nonmetastasizing mammary carcinomas in normal female rats were highly immunogenic, as tested by immunization of the hosts with an equal number of radiation-killed tumor cells, followed by a challenge with a counted number of live tumor cells.

In order to study the relationship between tumor cell immunogenicity and metastasizing capacity, two spontaneously metastasizing, non- or weakly-immunogenic mammary carcinomas (SMT-2A and TMT-50), induced in the manner described above, and a spontaneously metastasizing mammary carcinoma induced with 7,12-dimethylbenz(a)-anthracene in a BCG (bacille Calmette–Guérin) inoculated rat (BCG-MT) were selected. They were matched according to the degree of structural differentiation and growth rate with three nonmetastasizing, immunogenic mammary carcinomas (MT-W9B, MT-W9A, and MT-91) also induced with methylcholanthrene and maintained in the same strain of female rats. Tumor transplantation was performed by inoculation of 0.1 ml of fine tumor mince suspended in Medium 199 at a 1:1 ratio, into the right inguinal mammary fat pad. When the transplanted tumors reached 1–2 cm in average diameter, the rats were killed with ether and fresh tumors were promptly collected for the morphological, biochemical, and immunological studies described below.

Electron Microscopic Study of the Tumor Cell Surface. The glycocalyx of the tumor cells was demonstrated by staining this structure with ruthenium red by the method of Luft (2, 3), with a Siemens Elmiskop IA. Ruthenium red is an inorganic dye said to bind specifically with the acid mucopolysaccharides of the cell surface coat, bringing out the glycocalyx as an intensely electron dense layer over the luminal surface of glandular epithelial cells by catalyzing the precipitation of osmium in the fixative. A thick glycocalyx was found on the surface of all nonmetastasizing MT cells (Fig. 1A), but not on the spontaneously metastasizing cells (Fig. 1B). The acid mucopolysaccharide or glycoprotein coat was not only present over microvilli of the luminal surface, but also around the entire cell. A similar observation has been made by Gasic *et al.* and others (4, 5). These workers used Mowry's modification of Hale's fresh colloidal ferric oxide staining and concluded that local invasiveness and metastatic behavior of tumor cells seemingly depended on the quantity and nature of the cementing substance originating in tumor cells. In later papers, Gasic and Berwick (6) and Dermer and Kern (7) further characterized the cementing substance as sialic acid-containing mucins. However, in the present study the ruthenium red-positive glycocalyx on the immunogenic tumor cell surface was not removed by incubating one million cells with 25 units/ml of *Vibrio cholerae* neuraminidase (General Biochemicals, Chagrin Falls, Ohio) at 37° for 1 hr. Electron micrographs of these cells showed no appreciable change in the thickness of the glycocalyx after the neuraminidase treatment. Thus, the ultrastructural study suggests that (*i*) the highly immunogenic, nonmetastasizing MT cells have an acid mucopolysaccharide coat on their surface, which cannot be entirely removed by neuraminidase, while the non-immunogenic, spontaneously metastasizing cells lack such a coat, and (*ii*) the relative thickness of the glycocalyx demonstrated by ruthenium red appears to

Abbreviations: MT, mammary carcinoma(s); 5′-AMPase, 5′-nucleotidase; CEA, carcinoembryonic antigen.

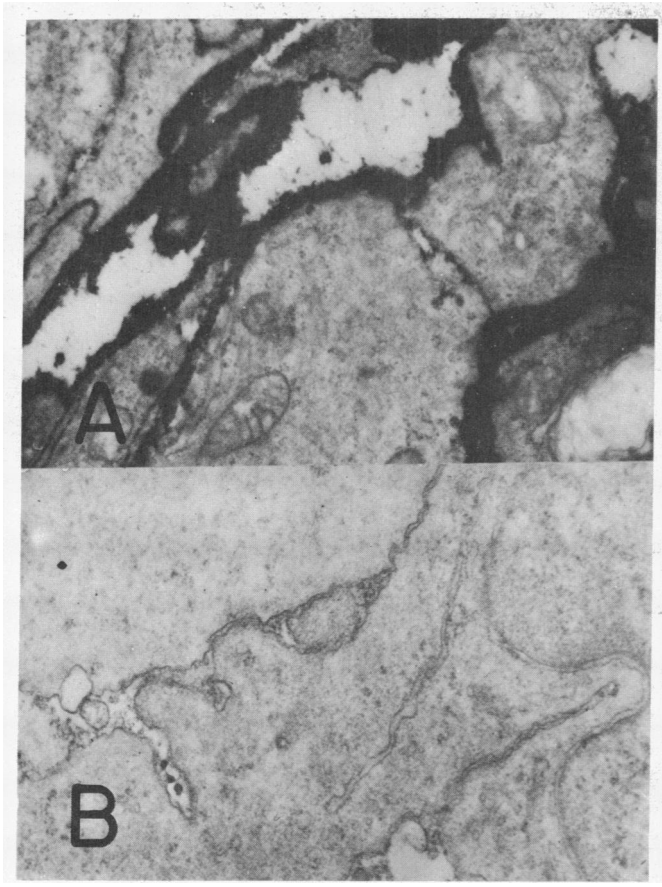

FIG. 1. Demonstration of glycocalyx by ruthenium red staining in the metastasizing and nonmetastasizing rat mammary carcinomas. Magnification ×19,500. (A) Electron micrograph of nonmetastasizing, immunogenic tumor MT-W9B. Note the thick glycocalyx on the luminal surface, as well as cell-to-cell borders. (B) Electron micrograph of spontaneously metastasizing, non-immunogenic tumor STM-2A. Note the absence of ruthenium-positive material on the cell surface.

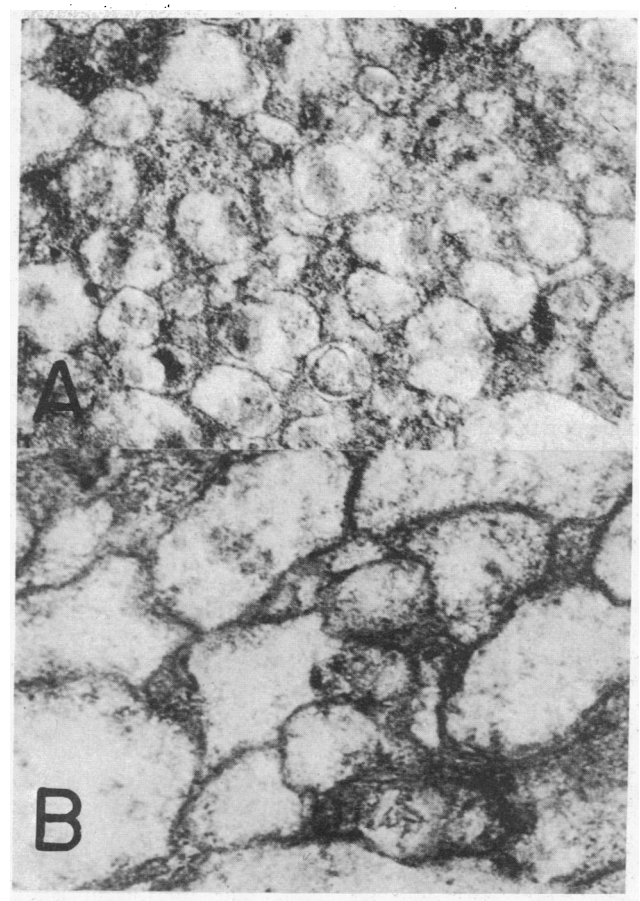

FIG. 2. Electron micrographs of isolated plasma membranes of rat mammary carcinoma SMT-2A stained with uranyl acetate/lead. Note the vesicular forms. (A) ×52,000 magnification; (B) ×87,750 magnification.

be roughly proportional to the relative immunogenicity and loss of metastasizing capacity of the MT cells.

Isolation of Plasma Membranes. The localization of tumor specific antigens on the cell surface is well established (8). In order to isolate tumor specific antigens associated with plasma membrane, MT cells were stripped of their membranes, without allowing cytolysis, by the procedure of Davies (9), in which membranes were eluted by extractions with hypotonic saline solutions. The extent of the membrane stripping, after low-speed centrifugation at 600 × *g* for 10 min, was checked by trypan blue staining of the cells, and the hypotonic treatment was continued until at least 75% of the cells were stained but not lysed. All supernatant fractions were tested for nuclear contamination by measuring the DNA content (10). They were also examined for cytoplasmic contamination by measuring the microsomal enzyme glucose-6-phosphatase (11).

The washed cell sediments and the contamination-free supernatant solutions were pooled for each tumor strain. Each fraction was centrifuged at high speed (105,000 × *g* for 90 min) to obtain a plasma membrane fraction in a pellet form and a clear supernatant fraction, both of which were used for analysis. The plasma membrane of MT cells obtained by this procedure appeared as closed vesicular forms (Fig. 2A and B). Further centrifugation of this supernatant fraction at 200,000 × *g* for 90 min did not produce more membranous materials in the pellet. Instead, it contained amorphous precipitate, as verified by electron microscopy. Thus, formed plasma membrane of the tumors can be precipitated at 105,000 × *g*. The yield of plasma membrane prepared in this manner ranged from 1 to 3% of the original wet weight of the tumor tissue, and from 10 to 15% of the whole cell suspension, as estimated by protein content.

Enzyme Activity Associated with Plasma Membrane. 5′-Nucleotidase (5′-AMPase) (EC 3.1.3.5; 5′-ribonucleotide phosphohydrolase) is a valid marker enzyme for plasma membrane (12–14). This marker enzyme activity was measured by the method of Heppel and Hilmoe (15), with equine muscular 5′-AMP (Sigma Chemical Co., St. Louis, Mo.). The protein content of the various fractions was determined by the procedure of Lowry *et al.* (16). These two procedures were carried out on the whole homogenate, the low-speed (600 × *g*) centrifugal sediment and its supernatant (starting material for the membrane isolation), the plasma membrane pellet and its supernatant at 105,000 × *g*, and, finally, the pellet and its supernatant at 200,000 × *g*. Five to 17 determinations were made on each tumor strain, each sample consisting of 4.4–39 g of wet tumor tissue. There was no appreciable protein content difference in the whole cell homogenate among the six mammary carcinomas, but the membranes of metastasizing mammary carcinoma contained slightly more protein than those of nonmetastasizing ones. Homogenate 5′-AMPase activity was seven times greater in the highly immunogenic,

TABLE 1. *5′-Nucleotidase activity* in metastasizing and nonmetastasizing mammary tumors*

Tumor strain	No. of samples	Whole cell	Membrane	Supernatant
SMT-2A (M)†	17	0.89 ± 0.16 (0.75–1.25)‡	0.16 ± 0.06 (0.06–0.31)	0.31 ± 0.09 (0.22–0.37)
TMT-50 (M)	15	2.75 ± 0.46 (0.19–3.37)	4.87 ± 1.44 (2.37–8.00)	1.95 ± 0.32 (1.25–2.37)
BCG-MT (M)	5	2.82 ± 0.56 (2.00–3.37)	4.95 ± 1.37 (3.00–6.37)	1.82 ± 0.56 (1.12–2.62)
MT-91	5	2.70 ± 0.65 (1.62–3.25)	5.72 ± 1.15 (3.87–6.87)	1.48 ± 0.39 (1.00–1.87)
MT-W9A	7	2.12 ± 0.32 (1.75–2.62)	5.20 ± 2.77 (1.51–10.62)	1.30 ± 0.24 (1.00–1.75)
MT-W9B	12	6.54 ± 0.78 (5.50–8.12)	19.16 ± 5.09 (12.00–22.75)	2.28 ± 0.63 (1.50–3.87)

* μmol of inorganic phosphorous released/mg of protein per hr.
† M, metastasizing.
‡ Mean ± SD (range).

nonmetastasizing MT-W9B than in the widely metastasizing SMT-2A. However, there was no difference in homogenate enzyme activity between two weakly metastasizing (BCG-MT and TMT-50) and two nonmetastasizing control (MT-W9A and MT-91) tumors. The 5′-AMPase activity of plasma membrane was nearly 120 times greater in MT-W9B than in SMT-2A, whereas with the other tumors (MT-W9A compared with TMT-50 and MT-91 compared with BCG-MT) the difference was very slight. In the 105,000 × *g* supernatants, MT-W9B had about seven times the enzyme activity of SMT-2A, while the weakly metastasizing MT cells had 20–50% more enzyme than their nonmetastasizing controls (Table 1). When the ratio of 5′-AMPase activity in the plasma membrane to its supernatant fraction was compared, the nonmetastasizing MT-W9B had most of the enzyme bound to the membranes (75.2%), while in the widely metastasizing SMT-2A the bulk of the enzyme was solubilized in the supernatant (78.9%) (Table 2).

The solubilization of plasma membrane or cell surface coat in SMT-2A seemed to be complete, for when the 200,000 × *g* supernatant fraction was assayed for 5′-AMPase activity, some 90% of the activity originally present in the 105,000 × *g* supernatant was recovered. This observation further verified that the 105,000 × *g* supernatant did not contain plasma membrane fragments. There was little difference in 5′-AMPase activity between weakly metastasizing, nonimmunogenic mammary carcinomas (TMT-50 and BCG-MT) and their nonmetastasizing, immunogenic controls (MT-W9A and MT-91). Fluctuation of this enzyme activity was observed within the strain of weakly metastasizing mammary carcinomas from one transplantation generation to another, indicating a possible heterogeneity of these tumor cell clones. When different generations of the weakly metastasizing tumor TMT-50 were further compared for enzyme activity, the speed and extent of metastasis corresponded roughly to the amount of this enzyme in the supernatant fraction (Fig. 3). Thus, 5′-AMPase activity in the plasma membrane seems to relate quantitatively to their metastasizing capacity. Dissociation and solubilization of immunogenic determinants from the cell surface, as indicated by the activity of this enzyme, may increase their metastasizing capacity. Therefore, our general conclusion is that in the mammary carcinomas studied thus far, the greater the membrane-bound 5′-AMPase activity, the greater the immunogenicity, and consequently the weaker the metastasizing capacity. This conclusion has been further confirmed by the analysis of this and other plasma membrane marker enzymes on seven additional mammary carcinomas, two non-metastasizing and five metastasizing (17).

TABLE 2. *Ratio of 5′-nucleotidase concentration in unsolubilized and solubilized plasma membranes of mammary tumors*

Tumor strain	600 × *g* (%)		105,000 × *g* (%)	
	Sediment	Supernatant*	Membrane	Supernatant
SMT-2A	4.3	95.5	20.1	78.9
TMT-50	14.8	82.1	51.6	48.0
BCG-MT	10.3	89.1	55.6	44.5
MT-91	15.1	82.4	56.7	41.0
MT-W9A	12.2	88.5	58.5	40.1
MT-W9B	20.2	79.2	75.2	22.8

* Starting material for the 105,000 × *g* centrifugation.

Identification of Tumor Cell Surface Antigens in the Sera of Mammary Carcinoma Hosts. Two major questions emerge from the observation of tumor cell surface properties described above. Do the absence of glycocalyx and the low plasma membrane specific 5′-AMPase activities in spontaneously metastasizing, non-immunogenic MT cells represent a loss of glycocalyx production or easy dissociability of the glycocalyx from the cell surface? Conversely, do the thick glycoprotein coat and the high levels of plasma membrane-associated 5′-AMPase activity in nonmetastasizing, immunogenic cells signify an increased production of glycocalyx or the ability of these cells to retain this substance on the cell surface? To determine whether the solubilized cell surface antigens or glycocalyx are released by the tumor cells, we prepared antisera in rabbits against the plasma membrane and 105,000 × *g* supernatant fractions of the various MT strains for use in immunodiffusion by Ouchterlony procedure. Thus, the sera of rats bearing mammary carcinomas were tested for free tumor cell surface antigens.

Four groups of New Zealand white rabbits were immunized with plasma membrane and 3-fold concentrated supernatant fractions from the nonmetastasizing MT-W9B and from the metastasizing SMT-2A tumors. Initially the rabbits were injected intramuscularly with 1 ml of 1:1 homogenized mixture of either membrane in 0.85% NaCl, or supernatant, with Freund's complete adjuvant, each ml containing 3 mg of protein. On the second and third day, each rabbit was injected intradermally into five different sites (0.2 ml per site) with 1 ml of either membrane homogenate in 0.85% NaCl or supernatant without adjuvant, each ml also containing 3 mg of protein. In the second and third week, on three successive days, the multiple intradermal injections were repeated with homogenate of membrane or supernatant, without adjuvant,

containing 3 mg of protein per ml. In the seventh week, they were exsanguinated, and the antisera were absorbed with lyophilized normal rat liver powder to remove nonspecific immunoprecipitation.

Immunodiffusion experiments were carried out as follows: 30 μl of antisera, containing antibodies against the supernatant concentrate of the two representative mammary carcinomas, were placed in the center well; 20 μl and 30 μl of 3-fold concentrate of tumor host sera were placed in wells 1 and 3; and an equivalent amount and concentration of pooled normal syngeneic rat sera in wells 2 and 4. A thick immunoprecipitation band between the center well and wells 1 and 3 (Fig. 4) indicated solubilized tumor cell surface antigen in the sera of rats bearing the metastasizing tumor SMT-2A, but no such precipitation line was noted against the nonmetastasizing MT-W9B host sera. Therefore, one may conclude at this point that the absence of glycocalyx in the spontaneously metastasizing, non-immunogenic tumor cells is not necessarily due to the loss of its synthetic capacity, but more to its easy dissociability from the cell surface.

Preliminary studies suggested that the dissociated glycocalyx or cell surface antigen(s) are glycoproteins and are probably related to human carcinoembryonic antigen (CEA). Furthermore, the levels of this perchloric acid-extractable seromucoid substance in the blood rose with growth of metastasizing mammary carcinoma (18). Radioimmunoassay of sera from the rats bearing metastasizing mammary carcinomas with ^{125}I-labeled goat anti-human CEA sera indicated crossreactivity, although they were not antigenically identical (Kim, Chu, and Tunis, to be published). It was also found that the dissociated tumor cell surface antigen(s) in the blood of metastasizing mammary carcinoma hosts interfered with blastogenic capacity of circulating lymphocytes to mitogenic stimulation with lectins (Kim and Han, unpublished data). Blocking of the specific cell-mediated immune response by circulating tumor cell surface antigen(s) observed in the metastasizing mammary carcinoma system may be analogous to the "blocking serum factors" in the sera of cancer patients reported by the Hellströms (19). The blocking effect in our tumor system appears to have been caused by the excess of circulating tumor cell surface antigen(s) rather than by cytotoxic antibodies or by antigen–antibody complexes. Baldwin *et al.* (20) have demonstrated the cytotoxic lymphocyte blocking of tumor antigens *in vitro*. The reason for the "blocking serum factors" being found only in a few animal tumor systems (21–23), while they are frequently found in the sera of many human cancer patients (19), may be that the conventional animal tumors rarely metastasize spontaneously and seldom shed their tumor cell surface antigen(s), while human cancers usually metastasize and also seem to frequently release their surface antigen(s) into the systemic circulation in the form of CEA and/or related neoantigens (19, 24, 25). Another indirect clue for the identity of the "blocking serum factors" is that blocking factors and humoral cytotoxic antibody are mutually antagonistic, and the latter has an "unblocking effect" on the former (19). In cancer patients, extirpation of tumor masses reduces or eliminates serum blocking factors and, in certain tumor hosts, CEA as well (26–29), while it evokes or stimulates the production of specific cytotoxic antibodies (29–32). Therefore, we postulate that the level of free tumor cell surface antigen(s) or the blocking serum factors depends primarily on the antigen-shedding capacity of tumor cells and on the levels of cytotoxic antibodies in the systemic circulation. However, these suppositions remain to be tested, for the evidence in man is at best fragmentary.

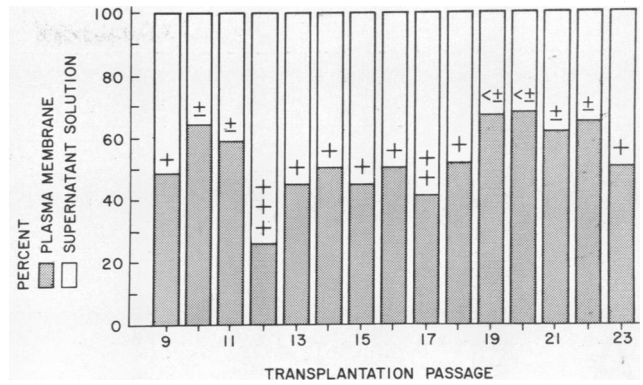

FIG. 3. Fluctuation of metastasizing capacity with 5′-nucleotidase activity in weakly metastasizing mammary carcinoma TMT-50. +++ = widely, rapidly metastasizing; ++ = moderately; + = slightly, slowly; ± = sparsely, occasionally; <± = microscopically, only at autopsy.

Interrelationship Between Soluble and Insoluble Tumor Cell Surface Antigens. To investigate the immunological relationship between plasma membrane bound and unbound antigens from metastasizing and nonmetastasizing mammary carcinomas, immunodiffusion experiments were carried out with 20 μl of rabbit antisera (3-fold concentrate) containing antibodies against the plasma membrane fractions and their supernatants from the two mammary carcinomas in the center well of four separate slides, and 100 μg (by protein content) of the antigens in wells 1–4, as follows: no. 1, SMT-2A membranes; no, 2, SMT-2A supernatant; no. 3, MT-W9B supernatant; and no. 4, MT-W9B membranes. Fig. 5 illustrates the interrelationship between soluble and insoluble antigens of plasma membranes of the two mammary carcinomas. The antisera against the insoluble plasma membrane antigen of the nonmetastasizing MT-W9B were antigenically unique in that they produced a single heavy precipitation band against MT-W9B membranes only (Fig. 5A). However, the solubilized fraction in the supernatant of this tumor was antigenically identical to the supernatant of SMT-2A, as shown by crossreactivity (Fig. 5B). Solubilization of MT-W9B membrane-

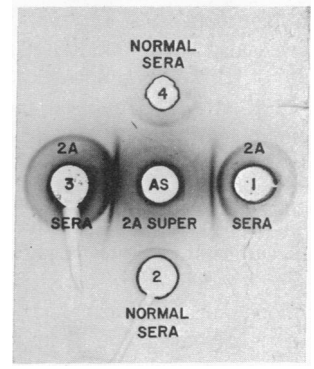

FIG. 4. Demonstration of solubilized tumor cell surface antigen in the blood of metastasizing mammary carcinoma SMT-2A bearing rats. Well no. 1, 20 μl of 3-fold concentrate of SMT-2A host sera; well no. 3, 30 μl of SMT-2A host sera; wells no. 2 and 4, 20 and 30 μl of 3-fold concentrate of normal syngeneic female rat sera, respectively; center well (AS), rabbit antisera against SMT-2A supernatant (3-fold concentrate).

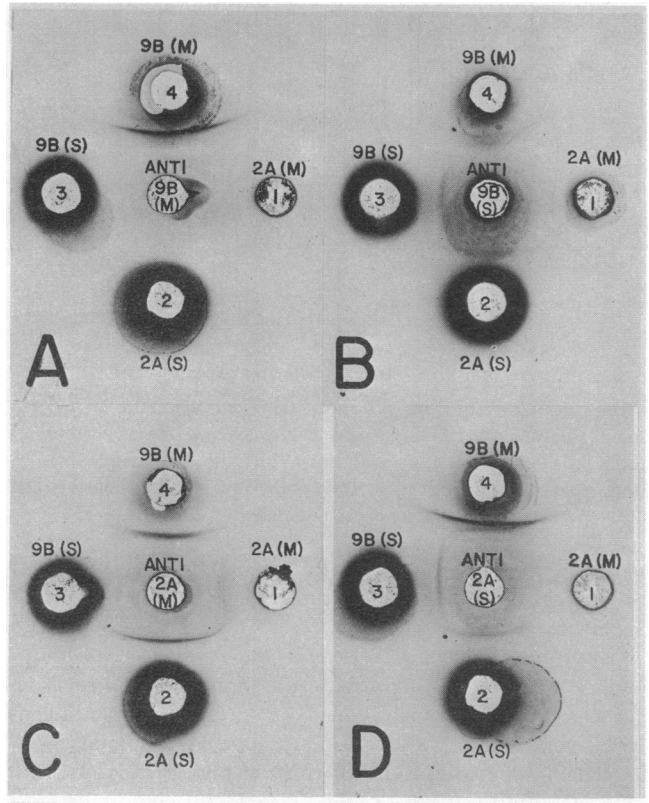

FIG. 5. Interrelationship between soluble and insoluble antigens of plasma membranes of metastasizing and nonmetastasizing mammary tumors. 9B (M), plasma membranes of nonmetastasizing MT-W9B; 9B (S), supernatant of MT-W9B; 2A (M), plasma membranes of metastasizing SMT-2A; 2A (S), supernatant of SMT-2A; ANTI, rabbit antisera against plasma membranes and supernatant fractions.

bound antigen(s) may have been caused by vigorous mechanical fractionation. No precipitation band was noted against SMT-2A membranes, indicating that little or no residual membrane-bound cell surface antigen remained in metastasizing tumor cells (Fig. 5B, C, and D).

The solubilized surface coat of the metastasizing, nonimmunogenic SMT-2A cells was again found to be antigenically identical with the plasma membrane (Fig. 5C). These immunodiffusion plates also reflected the relative concentration of surface antigen(s) in these fractions. The solubilized coat of SMT-2A was also related, but not identical, to the plasma membrane of the immunogenic, nonmetastasizing MT-W9B cells (Fig. 5C and D). Thomson and Alexander (33) described multiple neoantigens with different molecular weights on tumor cell surface. In addition, Ran and Witz (34) and Vanky *et al.* (35) reported that immunoglobulin and/or antibodies were chemically eluted from immunogenic tumor cell surface. In our studies, it is not clear whether this partially overlapping antigenicity demonstrated on xenogenic antisera is caused by immunochemical similarity or by an insoluble surface antigen being tightly locked in as an antigen–antibody complex. The membrane-bound antigen of MT-W9B seems to represent the tumor specific transplantation antigen of this tumor, for when normal syngeneic rats were immunized against this tumor with radiation-killed MT-W9B cells, the rats rejected subsequent live cell challenges, but accepted all other syngeneic mammary carcinomas, whether they were immunogenic or non-immunogenic. Further immunological and chemical analyses of soluble and insoluble antigens and the effect of non-immunogenic and immunogenic tumor cell surface coats on the host immune responses are needed.

This work was supported in part by the American Cancer Society Grants E-636D and IC-71E, and National Cancer Institute Contract NO1-CB-23864.

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