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Effective tumor immunization induced by cells of elevated membrane-lipid microviscosity

(tumor antigens/immunotherapy/lipid fluidity/cholesterol/cholesteryl hemisuccinate)

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ABSTRACT **The immunogenicity of a series of mouse tumor lines propagated *in vivo* (T and B lymphomas and mammary adenocarcinoma) was tested after alteration of the cell membrane-lipid microviscosity. Tumor cells used for immunization were first treated to alter the lipid content, then irradiated and injected intraperitoneally into syngeneic mice. A second identical immunization was performed 14 days later. The degree of immunization in the treated mice was assessed by survival time after challenge with untreated viable tumor cells of the same origin as the immunizing cells. For all tumors tested, enrichment of the immunizing cells with cholesterol or cholesteryl hemisuccinate, which increased the membrane-lipid microviscosity significantly, afforded a marked increase in immunization, compared to that obtained with cells that were only irradiated. Furthermore, in over 90% of the mice that were pretreated with cholesteryl hemisuccinate-enriched cells, tumor growth after the challenge was not detectable. Because the lipid-modifying treatments of the immunizing cells involve no toxic substances, these results may provide the basis for a potent approach to immunotherapy of human cancer.**

The active immunotherapy approach to treatment of cancer is based on the notion that malignant cells bear tumor-associated antigens (TAA) that can elicit immune rejection (1–4). Although TAA have not been identified unequivocally, indirect evidence strongly supports the view that such antigens are present on malignant cells. The most cogent experimental evidence for the existence of TAA is the positive immunization response obtained in mice (1–3) or in humans (4–6) after treatment with irradiated tumor cells. The antigenicity of tumor cells, however, is too weak to provoke an effective immune opposition to tumor growth.

During the past 3 years we have presented experimental evidence implying that alteration of the membrane-lipid fluidity can displace the equilibrium position of membrane proteins vertically to the plane of the membrane (7–12). This process was termed "vertical displacement" (8), and the associated modulation of membrane function was termed "passive modulation" (11, 12), to distinguish it from "active modulation," which requires metabolic energy (13). Recently, an increase in accessibility of the Rh(D) antigen (14) and the θ antigen (unpublished observations) was shown to accompany the increase in membrane-lipid microviscosity, consistent with the vertical displacement hypothesis. Similarly, others have reported that synthetic antigens incorporated into liposomes are more immunogenic *in vivo* when the surrounding lipid matrix is in a rigid state (15, 16), presumably due to a greater antigen exposure. These observations led us to determine whether the immunogenicity of tumor cells could be augmented by increasing the lipid microviscosity of the cell membranes. As demonstrated in the present study, effective prophylactic immunization against homologous tumor cells in mice can be achieved by treating the animals with irradiated tumor cells of increased membrane-lipid microviscosity.

MATERIALS AND METHODS

Mice. Females of the C57BL/6 and C3H/eB strains, 5–7 weeks old, were obtained from the breeding colony of the Weizmann Institute. The mice were kept in groups of 10 per cage, fed on Purina Chow and water ad lib.

Tumors. The five *in vivo* propagated tumors used in the present study were induced in two strains of mice. Three of these tumors were induced in female C3H/eB mice by the chemical carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA) dissolved in polyethylene glycol (PEG-400) administered by stomach tube, four times at weekly intervals (1 mg of DMBA per feeding). The tumor designated 38-C-54, induced in an intact mouse at a 140-day latency, was characterized as a T lymphoma (17); the one designated 38-C-13, induced in an adult thymectomized T-depleted mouse at a latency of 150 days, was shown to be a B lymphoma (18), and 38-C-38, induced in an adult thymectomized mouse at a 300-day latency, was diagnosed as a mammary adenocarcinoma.

The two other tumors used in the present study originated in C57BL/6 mice treated with the radiation leukemia virus variant D-RadLV, with additional x-ray coleukemogenic treatment (19). The ascitic lymphoblastic tumor, designated ALC, originated from a solid thymoma propagated *in vivo* as an ascitic tumor for about 40 generations. The other tumor, designated T-lymphoma D-RadLV, originating from a primary thymoma, has been propagated *in vivo* for several generations.

Lipid Alteration of Cell Membranes. Cells were incubated in serum-free medium in which 3.5% wt/vol polyvinylpyrrolidone (PVP, M_r 40,000, Sigma), the well-known plasma expander, was substituted for the serum. To decrease the membrane fluidity, cells were treated with cholesterol (>99%, Sigma), cholesteryl hemisuccinate (Sigma), or stearic acid (>99%, Sigma), which were kept in ethanolic stock solutions, each at 5 mg/ml. To render the membranes more fluid, cells were treated with egg lecithin (>99%, Makor Chemicals Ltd., Jerusalem), which was stored in ethanolic stock solution at 10 mg/ml. The lipid ethanolic solution was diluted 1:20 in the case of cholesterol, lecithin, or cholesteryl hemisuccinate, and 1:200 in the case of stearic acid, into a solution of 3.5% PVP in phosphate-buffered saline containing 0.2% glucose and 1% bovine serum albumin (Sigma). In the control mixture, only ethanol was introduced. The resultant dispersions were centrifuged at 3000 × *g* for 5 min, and the remnants of undispersed material

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Abbreviations: ALC, ascitic lymphoblastic cells; DMBA, 7,12-dimethylbenz[*a*]anthracene; PVP, polyvinylpyrrolidone; RIP, relative immunity parameter; TAA, tumor-associated antigens.

Table 1. Properties of ascitic lymphoblastic cells (ALC) and mammary adenocarcinoma cells (MAC) used for immunization

Treatment*	$\bar{\eta}$ (25°C),† poise		Molecules incorporated per cell‡	
	ALC	MAC	ALC	MAC
None	2.6	3.2		
Cholesterol depletion	2.4	2.4		
Cholesterol enrichment	4.0	5.4	2×10^9	1×10^9
Cholesteryl hemisuccinate enrichment	6.8	6.4	8×10^9	3×10^9
Stearic acid enrichment	3.8			

* Cells were incubated in PVP/bovine serum albumin/lipid mixtures for 3 hr at room temperature.

† Apparent membrane microviscosity $\bar{\eta}$ determined by fluorescence depolarization of 1,6-diphenyl-1,3,5-hexatriene. Because this compound is incorporated to some extent into intracellular organelles, the values of $\bar{\eta}$ correspond only partially to the cell plasma membrane (21). One poise = 0.1 Pa·sec.

‡ Determined with [^{3}H]cholesterol and [^{3}H]cholesteryl hemisuccinate.

in the pellet were discarded. Cells were incubated in the PVP/lipid dispersion (10^6 cells per ml) at room temperature with gentle shaking for 2–3 hr. The cells were then washed three times with phosphate-buffered saline to minimize any antigenic reaction of PVP (20).

The effectiveness of the lipid treatment was assessed by the incorporation of ^{3}H-labeled cholesterol or cholesteryl hemisuccinate and by fluorescence polarization analysis of the apparent membrane microviscosity, with 1,6-diphenyl-1,3,5-hexatriene as a probe (21). The viability of the treated cells was monitored by trypan blue exclusion.

Immunization and Tumor Challenge. The treated cells were dispersed in phosphate-buffered saline and exposed to γ irradiation at a dosage of 10,000 rad (1 rad = 0.01 gray). Treated, irradiated cells (10^7 cells in 1 ml of phosphate-buffered saline) were injected intraperitoneally into each mouse. A second identical immunization was performed 14 days later. Subcutaneous or intraperitoneal challenge with 5×10^4 viable cells per mouse was performed 14 days after the second immunization. The degree of immunization was assessed by survival and tumor size after challenge.

RESULTS

Treatment of tumor cells for 2–3 hr with lipid dispersions in PVP was found to be more efficient for cholesterol enrichment or depletion than treatment by established methods with liposomes (22) or with lipid-modified sera (23). Thus, during this relatively short treatment, significant lipid alteration was achieved without the adverse effects of liposomes or serum components. In addition, the cell viability was practically unaffected and remained above 85% after all treatments. The effects of the various treatments of ALC and mammary adenocarcinoma cells, assessed both by incorporation of [^{3}H]cholesterol or [^{3}H]cholesteryl hemisuccinate and by the apparent membrane-lipid microviscosity, are shown in Table 1. Similar effects of the corresponding treatments were obtained with the other tumor cells used in this study (data not shown).

Mice were immunized twice with 10^7 irradiated tumor cells with modified membrane lipid content, and challenged with 5×10^4 viable homologous cells. The mean durations of survival after challenge in the different test groups are summarized in Table 2. The detailed survival profiles of three tumor lines are illustrated in Fig. 1. The tumor size, which was most readily assessed after subcutaneous challenge in the mammary adenocarcinoma, qualitatively paralleled the survival pattern (see Fig. 1*C* and Table 2). Each of the experiments presented in Table 2, except the experiment with the mammary adenocarcinoma, was repeated three times with no significant variations in the results.

A relative immunity parameter (RIP) was operationally defined as:

$$\text{RIP} = \frac{\text{D-50 with immunization}}{\text{D-50 without immunization}} - 1, \qquad [1]$$

in which D-50 is the day of 50% survival after challenge. The RIP value would be negative in the case of immunosuppression, would approach zero for nonimmunogenic cells, would vary from 0 to 1 for weakly immunogenic cells, and would exceed 1 for highly immunogenic cells. Values of this parameter obtained after various immunizations are listed in Table 2. As shown, the RIP values for cells that were only irradiated indicate that the inherent immunogenicity of the untreated tumor cells is weak but not negligible, except for the ALC cells, which display moderate immunogenicity. The latter characteristic probably relates to the blastogenic-immature nature of the ALC cells. In all tumors tested, enrichment of membrane cholesterol

Table 2. Resistance to tumor growth in immunized mice after challenge with viable tumor cells, reflected by the day of 50% survival (D-50) and the relative immunity parameter (RIP*)

Treatment	T lymphoma ALC, D-RadLV-induced		T lymphoma 127, D-RadLV-induced		T lymphoma 38-C-54, DMBA-induced		B lymphoma 38-C-13, DMBA-induced		Mammary adenocarcinoma 38-C-38, DMBA-induced	
	D-50	RIP	D-50	RIP	D-50	RIP	D-50	RIP	D-50	RIP
No cells	17	0	14	0	27	0	27	0	20	0
Untreated cells	41	1.4 ± 0.2	20	0.4 ± 0.1	32	0.2 ± 0.1	33	0.2 ± 0.1	29	0.5
Cholesterol depleted	41	1.4 ± 0.2	ND		29	0.1 ± 0.1	30	0.1 ± 0.1	40	1.0
Cholesterol enriched	55	2.2 ± 0.3	31	1.2 ± 0.2	48	0.8 ± 0.1	>100	>5	80	3.0
Cholesteryl hemisuccinate enriched	>100	>5	ND		ND		ND		>100	>5
Stearic acid enriched	38	1.2 ± 0.2	ND		55	1.0 ± 0.2	28	0.1 ± 0.1	ND	

Mice were immunized with the first four tumors by two intraperitoneal inoculations of 10^7 irradiated cells per mouse at an interval of 14 days. The treated mice were challenged 14 days later, with the same tumor cells used for immunization, by intraperitoneal inoculation of 5×10^4 cells per mouse. For mammary adenocarcinoma, the same immunization was used, except that the challenge was inoculated subcutaneously. ND, not determined.

* The degree of immunogenicity of the tumor, as defined in Eq. 1. Values are ± SD, obtained from three separate experiments (10 mice each), except for mammary adenocarcinoma.

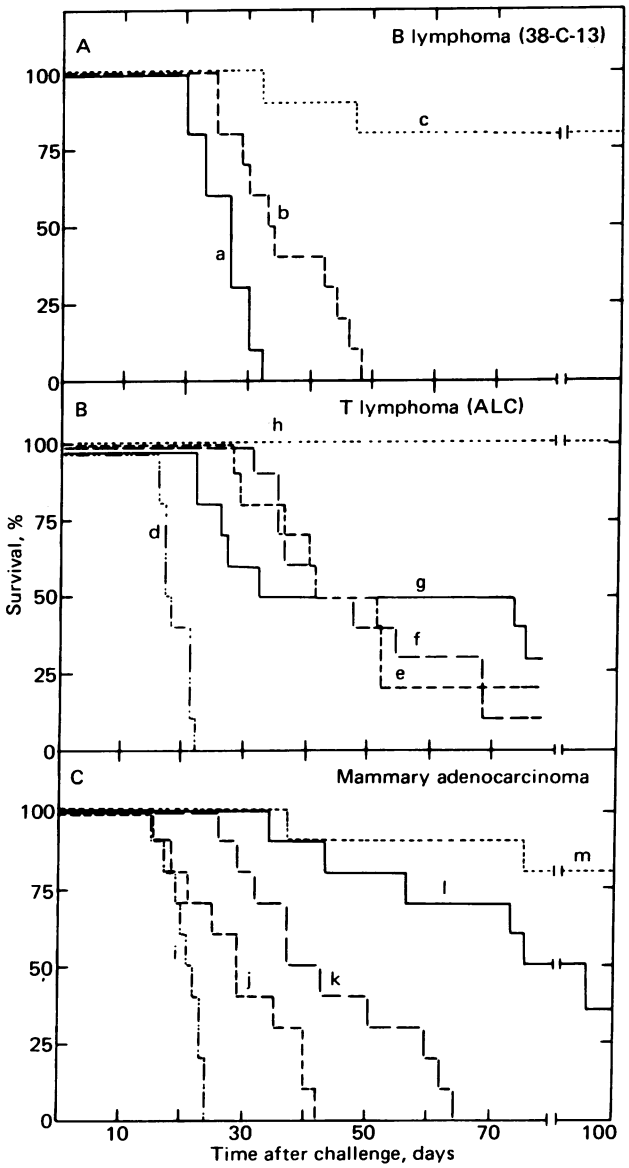

FIG. 1. Effects of modifying membrane lipids of tumor cells used for immunization on survival profiles after challenge with 5×10^4 viable tumor cells per mouse. (*A*) C3H/eB mice were challenged with B lymphoma cells after no treatment (curve a) or after two immunizations with 10^7 irradiated B lymphoma cells (b) or 10^7 irradiated cholesterol-enriched cells (c). (*B*) C57BL/6 mice were challenged with T lymphoma cells after no treatment (d) or after two immunizations with 10^7 T lymphoma cells that were irradiated only (e) or irradiated after cholesterol depletion (f), cholesterol enrichment (g), or cholesteryl hemisuccinate enrichment (h). (*C*) C3H/eB mice were challenged with mammary adenocarcinoma cells after no treatment (i) or after two immunizations with mammary adenocarcinoma cells that were irradiated only (j) or irradiated after cholesterol depletion (k), cholesterol enrichment (l) or cholesteryl hemisuccinate enrichment (m).

consistently increased the apparent immunogenicity of the cells. A remarkable increase in immunogenicity was achieved by incorporation of cholesteryl hemisuccinate, which, as a hydrophilic cholesterol ester, presumably rigidifies the membrane-lipids, as does cholesterol (24).

The specificity of the acquired immunity is now being examined by immunizing mice with lipid-modified syngeneic tumor cells of one kind, followed by a challenge with untreated viable syngeneic tumor cells of a different kind. Preliminary results indicate that the immunity elicited in the treated mice is specific for the tumor cell type used for immunization. It is also important to examine the possibility of immune rejection of a preimplanted tumor, by immunization with lipid-treated and irradiated cells, as a guideline for possible immunotherapeutic application of this method.

DISCUSSION

It is now widely accepted that tumor cells bear antigenic determinants that can be recognized as nonself and elicit an immune response (1–6). Accordingly, the overt rate of tumor growth would be a function of both the rate of tumor cell division and the opposing rate of tumor rejection. Once the rate of tumor rejection exceeded the rate of multiplication, tumor regression or growth inhibition would be achieved. Hence, augmentation of tumor immunogenicity by exposing cryptic tumor-associated antigens (TAA) could, in principle, potentiate immune rejection of the tumor.

We have recently demonstrated that when the membrane-lipid microviscosity is increased—e.g., by incorporation of cholesterol—the exposure of membrane proteins on both sides of the membrane may be increased. Depletion of cholesterol has the converse effect (8–10). Thus, upon reduction of lipid fluidity, the projection of antigens can be increased (14–16) and the associated immunogenic characteristics can be augmented. It has been shown previously that untreated irradiated tumor cells have some active immunotherapeutic potency (5, 6). In the present study, a moderate degree of immunogenicity was inferred for untreated ALC cells from the value of the relative immunity parameter (RIP) in Table 2. The other tumors that were tested displayed only weak immunogenicity when untreated by lipids.

It has been demonstrated clearly in this study that when the membrane-lipid microviscosity of the immunizing cells was elevated, their immunogenicity was greatly increased, and the latency in tumor growth after challenge with viable tumor cells was prolonged. For virtually all tumors tested, the addition of cholesterol or cholesteryl hemisuccinate to the immunizing cells resulted in a statistically significant increase in survival after challenge. The apparent increase in the RIP value after treatment with cholesteryl hemisuccinate was significantly greater than that after treatment with cholesterol. This difference probably originates from the more efficient incorporation of cholesteryl hemisuccinate, as compared to cholesterol, rather than from a lower potency of the latter (Table 1). Cholesteryl hemisuccinate, which has some characteristics of a fatty acid, appears to be incorporated into the cell membrane more readily than cholesterol, as is also reflected by the more pronounced increase in membrane-lipid microviscosity (Table 1).

The ability of stearic acid to augment the immunogenicity appeared to be much lower than that of cholesterol or cholesteryl hemisuccinate, although it has a similar lipid-rigidifying potency (Table 2). Shortly after incorporation into the plasma membrane, stearic acid penetrates into intracellular organelles, where it participates in phospholipid biosynthesis. It is plausible that the original membrane fluidity, observed before treatment with stearic acid, is rapidly recovered, and the increased exposure of the antigens is reversed. Therefore, it may be inferred from these results that treatment of tumor cells with cholesterol or its derivatives is more effective for increasing immunogenicity than treatment with membrane-rigidifying fatty acids.

In accordance with the observations that exposure of membrane proteins (8–11) and well-characterized antigens (14–16) is directly related to the membrane-lipid microviscosity, it seems likely that the increase in immunogenicity of tumor cells shown in this study is related to a vertical displacement of TAA.

This mechanism implies that TAA are present in the membranes of tumor cells, but that most of them are effectively concealed from the immune system. Indeed, the increase in immunogenicity correlates well with the decrease in membrane fluidity, as demonstrated for both lymphoma and mammary adenocarcinoma cells (Tables 1 and 2). The correlation between membrane-lipid microviscosity and antigenicity is further supported by the observation that cholesterol depletion resulted in only small changes in immunogenicity of tumor cells. It should be stressed, however, that factors other than antigenic modulation could take part in the observed increase in the tumor immunogenicity. These include the duration of the altered membrane fluidity after the cells were introduced into the host, and the effect of lipid fluidity on the association of newly exposed TAA to form effective recognition sites. Therefore, the apparent lipid microviscosity may serve as a reliable, but only qualitative, criterion for estimating the modulation of tumor immunogenicity *in vivo*.

It may be possible to treat tumor-bearing mice by active immunotherapy with lipid-modified irradiated tumor cells. It is hoped that these studies may lead to a potent immunotherapeutic tool for the treatment of human cancer.

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