Biochimica et Biophysica Acta 938 (1988) 434–446
Elsevier

BBA 73866

Local and metastatic tumor growth and membrane properties of LM fibroblasts in athymic (nude) mice

A.B. Kier [a], M.T. Parker [b] and F. Schroeder [c]

[a] *Departments of Pathology and Lab Animal Medicine, College of Medicine, University of Cincinnati, Cincinnati, OH 45267-0529,* [b] *Department of Veterinary Pathology, College of Veterinary Medicine, University of Missouri, Columbia, MO 65212 and* [c] *Department of Pharmacology and Medical Chemistry, College of Pharmacy, Department of Pharmacology and Cell Biophysics, College of Medicine, University of Cincinnati, Cincinnati, OH 45267-0004 (U.S.A.)*

(Received 13 October 1987)

Key words: Phospholipid; Metastasis; Fibroblast; (Mouse)

LM fibroblasts grown in a chemically-defined, serum-free medium readily incorporated choline or one of three analogues of choline, namely *N*,*N*-dimethylethanolamine, *N*-monomethylethanolamine, or ethanolamine into membrane phospholipids. The effect of these phospholipid manipulations in vitro on tumor growth and metastasis was examined in nude mice. Serum and choline-fed cells most frequently metastasized (74% and 68%, respectively), while frequency of lung metastasis was 46%, 42% and 17% in mice injected with cells fed with dimethylethanolamine, monomethylethanolamine, and ethanolamine, respectively. Metastases from cells cultured with serum, choline or dimethylethanolamine, but not from monomethylethanolamine or ethanolamine, were extensive and highly invasive. The specific activity of the ($Na^+ + K^+$)-ATPase but not of 5′-nucleotidase was significantly decreased in local tumor plasma membranes from choline analogue-fed cells as compared to tumor plasma membranes from choline-fed cells. When compared to the choline-fed tumor cells, the specific activities of three mitochondrial enzymes, namely NADH dependent, rotenone insensitive NADH-dependent, and rotenone sensitive NADH-dependent cytochrome-*c* reductase, were significantly increased in the choline analogue-supplemented cells. The arachidonic acid content of phosphatidylcholine in plasma membranes, microsomes, and mitochondria was significantly decreased in tumor membranes from choline analogue-fed cells as compared to tumor membranes from choline-fed cells. As compared to local tumor plasma membranes, the lung metastasis plasma membranes had elevated ($Na^+ + K^+$)-ATPase specific activity, phospholipid oleic and arachidonic acid content, and fluidity. In contrast, the 5′-nucleotidase specific activity, the content of cholesterol, phospholipid, and phosphatidylethanolamine were decreased in lung metastasis plasma membranes. In summary, membrane alterations of LM tumor cells in vitro (1) were not completely reversed in vivo, and (2) affected metastatic ability.

Introduction

The study of tumor metastasis has been hampered by lack of a suitable model system that would produce a high incidence of metastases from local tumors in a short period of time. Most experimental murine tumors do not readily metastasize from their local tumor sites [1]. Athymic (nude) mice have been useful models for heterogeneic tumor study [2], but most animal [3–11] and human [12–15] tumor cell lines produce encapsulated, non-metastatic tumors at the site of injection. Tumor cell lines which have been shown

Correspondence: F. Schroeder, Department of Pharmacology and Medicinal Chemistry, College of Pharmacy, Department of Pharmacology and Cell Biophysics, College of Medicine, University of Cincinnati, Cincinnati OH 45267-0004, U.S.A.

to metastasize include the B16 melanoma [16], Lewis lung carcinoma [11], UV2237 sarcoma [17], CMT64 lung carcinoma [18], and certain human malignant melanomas [19–21]. The LM cell-nude mouse model system [22] utilized in this report allowed study of a naturally-metastasizing cell line which develops local tumors and metastases with high incidence and in a short period of time (28 days).

Lipids have been shown to have a role in neoplastic processes: fatty acids and/or cholesterol can affect cell-mediated and antibody-mediated immune response of the host to tumors [16,23–37], tumorigenicity of tumors [28–33], immunogenicity of tumors [33], cell differentiation [34], and resistance of tumor cells to antineoplastic drugs [35,36]. There have been few studies comparing plasma membrane structure and membrane lipid composition either from local versus metastatic tumors [37] or from high versus low metastatic cell lines [38–40]. In this report, we studied the effect of phospholipid polar head group manipulation in vitro on (1) metastatic ability, and (2) certain membrane properties of the local and metastatic tumors.

A portion of this work was presented as an abstract: F. Schroeder and A.B. Kier, Prospectives of chemotherapy based on membrane dynamics and composition of primary and metastatic tumor cells. 14th International Cancer Congress, Budapest, Hungary, August 21–27, 1986.

Materials and Methods

Cell culture and choline analogue supplementation. LM cells were originally obtained from the American Type Culture Collection (CCL 1.2). Cells were removed from liquid nitrogen, cultured in Corning 75 cm^2 monolayer plastic tissue culture flasks with Higuchi medium containing 10% calf serum for one week. The cells were then transferred to suspension cell culture in Higuchi medium without serum for three weeks, and then used for culture with choline or choline analogue containing Higuchi medium [22,41]. Higuchi medium is a lipid-free, protein-free, serum-free, chemically-defined medium initially containing choline (40 $\mu g/ml$) [22,41] or, in the choline analogue-supplemented cell cultures, one of three choline analogues, *N,N*-dimethylethanolamine, *N*-monomethylethanolamine, or ethanolamine (also at 40 $\mu g/ml$) instead of choline. Choline, dimethylethanolamine, monomethylethanolamine and ethanolamine contain 3, 2, 1 and 0 methyl groups, respectively. Log phase cells were cultured for three days, sedimented, and resuspended in vehicle (choline-containing medium) for immediate injection into mice.

Injection of mice. A dose response curve was established by injection of 10^2 to $2 \cdot 10^7$ cells per mouse in a manner similar to that described below. For the choline analogue experiments, a 0.5 ml aliquot ($1 \cdot 10^7$ LM cells) was inoculated subcutaneously over the left thigh of six- to eight-week old athymic (nude) mice (BALB/C, nu/nu, Charles River Laboratories, Wilmington, NJ). Unless otherwise stated, five mice were inoculated per group. Mice were house in filter-covered suspended polycarbonate cages with wood shavings and automatic watering system. They were fed commercial rodent chow. Tumor volume was determined every two days as described elsewhere [22].

Necropsy. All mice were euthanized by CO_2 or ether chamber on day 28 post-inoculation. External lesions, mouse weight, local tumor size, and gross necropsy observations were recorded. Local tumors were dissected, weighed, and divided: (1) a small portion of the tissue was fixed in formalin for histopathological evaluation; and (2) the rest of the tumor (except for necrotic areas) was placed on ice for immediate membrane isolations. The entire lung of each mouse was perfused intratracheally and fixed and embedded in paraffin as a single whole organ. In later trials, visible lung metastases from three to five animals were trimmed away under a dissecting microscope with small iris scizzors and combined for membrane analysis. Visible metastases appeared as raised, slightly opaque, whitish areas 2–3 mm in diameter. The remaining lung tissue was then perfused with formalin and treated as previously described. Lung tissue from a nude mouse not injected with tumor cells was processed for membrane analysis to serve as a background control. Tissue samples for histopathologic examination were also collected from other potential metastatic sites, including portions of tissue from liver, spleen, kid-

ney, cervical and mesenteric lymph nodes, brain, heart, adrenal glands, Harderian gland, intestine, and pancreas. Tissue samples were placed into 10% neutral buffered formalin and processed by standard histopathologic techniques. Sections of all tissues were cut 4 μm in thickness and stained with hematoxylin and eosin by standard histopathologic methods. Serial step sections were cut on each lung such that the entire lung from each mouse was examined for presence and size of metastases. Lung metastasis size and severity (invasion) were scored by our previously published method [22]. The metastatic score for each mouse in each group was multiplied by the number of mice with that score. These values were then added to arrive at a total group score (metastatic index) and then normalized to the metastatic index obtained for the ethanolamine group. This procedure provided a relative index of metastatic severity comprised of incidence, invasiveness, number of foci, mitotic index, and size of foci.

Tumor membrane fractionation. Tumors were excised from each mouse as described above, rinsed with ice-cold phosphate-buffered saline (pH 7.4), immersed in the same buffer, and immediately placed on ice. All subsequent procedures were performed at 4°C. The tumor was minced under phosphate-buffered saline into 1 mm pieces with a single edge razor blade and then sedimented at $150 \times g$ for 5 min. This procedure was repeated, and the tumor pellet was resuspended in 15 ml of 0.25 M sucrose/1 mM triethanolamine-HCl (pH 7.4) per g (wet weight) of tumor tissue. The tumor was then finely dispersed with a Model SDT Ultra Turrax polytron (Tekmar Co., Cincinnati, OH) followed by homogenization (15–20 up and down strokes) with a tight-fitting Dounce homogenizer. A portion of this crude homogenate was retained for enzymatic analyses, while the remainder was used for isolation of plasma membranes, microsomes, and mitochondria essentially as described previously [41], except that the dextran gradient step was omitted, and the sucrose gradient was centrifuged for only 1.5 h.

Determination of enzyme markers. The specific activities of ouabain-sensitive $(Na^+ + K^+)$-ATPase and 5′-nucleotidase were determined as described previously [40]. NADH-dependent, rotenone-insensitive NADH-dependent, NADPH-dependent and succinate-dependent cytochrome-*c* reductase activities were assayed as described by Sottocasa et al. [42]. Protein was determined by the method of Lowry et al. [43].

Lipid analyses. All organic solvents were glass-distilled, and all glassware was washed with sulfuric acid/dichromate before use. Lipids were extracted and separated into neutral and phospholipid fractions by silicic acid chromatography as described earlier [40]. The neutral lipid fraction was resolved into components by thin-layer chromatography [44]. Individual components were eluted with chloroform and quantitated by the method of Marzo et al. [45]. Total lipid was also determined by the method of Marzo et al. [45]. Cholesterol was determined by gas chromatography [40]. Phospholipids were quantitated by a total lipid phosphate assay [46] and also resolved by two-dimensional thin-layer chromatography. Individual components were visualized with Rhodamine 6G, scraped, eluted, and divided into two parts: one part was for phosphate analysis while the other part was transesterified with BF_3 in methanol [40]. Fatty acid methyl esters were analyzed by gas chromatography also as described earlier [40]. Fatty acid methyl esters were analyzed by gas chromatography also as described earlier [40].

Fluorescence probe incorporation and biophysical characterization. All procedures were performed exactly as described earlier [39,47–50].

Statistics. All values represent the mean $\pm$ S.E. with n, the number of experiments, given in parenthesis. Statistical comparisons were made by Student's t-test.

Results

Initiation of local tumor growth

The number of injected LM tumor cells affected the incidence, time of appearance, rate of growth, and size of local tumors as well as mortality rate of the mice. The threshold number of tumor cells, cultured in choline containing medium, required to initiate to a tumor within 90 days was 100 cells. Lag time, the time from injection to palpable appearance of a tumor, decreased as number of tumor cells injected increased, ranging from four days postinoculation with injection

of 10^7 cells, to 90 days with injection of 10^2 cells. The tumor volume increased rapidly as the number of tumor cells injected was increased, and with increasing time post-injection. Doubling times were only slightly over two days when more than $0.2 \cdot 10^6$ cells were injected, while fewer cells than this amount increased the doubling time by up to 7-fold. From these studies, 10^7 cells were chosen as the standard dose for all choline analogue experiments. The optimal time for allowing tumor development and growth was chosen to be 28 days post-inoculation. Thereafter, the survival rate of the mice rapidly decreased.

Effect of choline analogue supplementation on local tumor development and metastasis

The growth of cultured LM cells in the chemically defined (choline-containing) culture medium was rapid: tumor cell number doubling time was 1.5 days. Supplementation of the cell culture medium with choline analogues in the place of choline for more than three days increased the cell number and cell protein/ml doubling times, especially for cells supplemented with *N*-monomethylethanolamine or ethanolamine. In contrast, if cells cultured with choline analogues for three days were then sedimented and resuspended in choline-containing medium, subsequent growth was the same as for choline-supplemented cells which had never been grown in analogue-containing medium. Thus for the inoculation studies, LM cells were cultured for three days in a choline analogue-containing medium, sedimented, and resuspended in choline-containing medium (vehicle) prior to injection.

Greater than ninety-nine percent of mice in all groups developed local tumors at the site of the subcutaneous injection of LM cells. When cells has been cultured in the presence of 10% calf serum, tumor volume, doubling time, and weight, but not lag time, were significantly increased as compared to cells cultured with choline or choline analogues. At the end of 28 days, local tumor volume, local tumor volume doubling time, and local tumor weight were approximately 7 cm^3, 2.3 days, and 11 g, respectively, in all choline and choline analogue groups. Mouse body weight (minus the weight of the tumor) was relatively constant among groups at an average of approximately 17 g. At 28 days after inoculation, the local tumor accounted for approximately 40% of the total mouse weight in each group. Consistent with earlier results [22], necrosis was evident in the center of some of the local tumors. However, for all biochemical and biophysical determinations, necrotic areas were not used.

All major organs were examined histopathologically from all mice in all groups. Tumor metastasis was observed only in lung tissue. Lung metastasis was evident in 68% of the mice injected with LM cells cultured in the presence of choline (Table I). In contrast, the incidence of lung metastasis was significantly decreased to 46, 42, and 17% for mice injected with LM cells that had been cultured with *N*,*N*-dimethylethanolamine, *N*-monomethylethanolamine, or ethanolamine, respectively. Interestingly, the histopathology of the tumor metastases was markedly different at this time point (day 28): LM cells cultured with monomethylethanolamine or ethanolamine formed lung

TABLE I

INCIDENCE OF LUNG METASTASIS WITH LM FIBROBLASTS INJECTED IN NUDE MICE

Values represent the mean ± S.E. of three experiments using 5, 15 and 30 mice per group, respectively. *P* refers to statistical significance in % lung metastasis as compared to choline by Student's *t*-test.

Medium supplement	% tumor incidence	% lung metastasis	*P*	Histopathology
Choline	99 ± 1	68 ± 4	–	invasion
N,*N*-Dimethylethanolamine	99 ± 1	46 ± 3	0.025	invasion
N-Monomethylethanolamine	98 ± 1	42 ± 4	0.025	emboli
Ethanolamine	99 ± 1	17 ± 3	0.001	emboli
10% calf serum	99 ± 1	74 ± 4	0.400	invasion
No supplementation	99 ± 1	30 ± 3	0.050	invasion

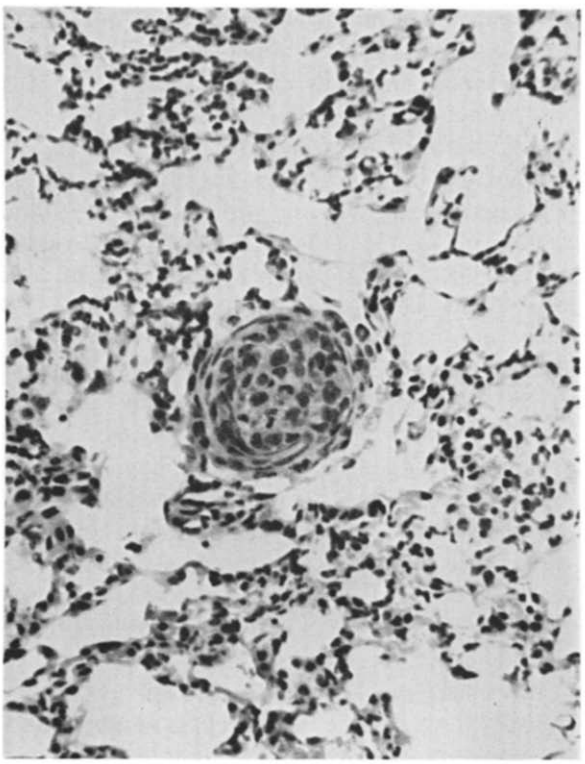

Fig. 1. Tumor embolus from the lung of a mouse injected subcutaneously with LM cells grown in medium containing ethanolamine. Tumor cells are packed within the vessel in center of the photo. Hematoxylin and eosin stain, ×244.

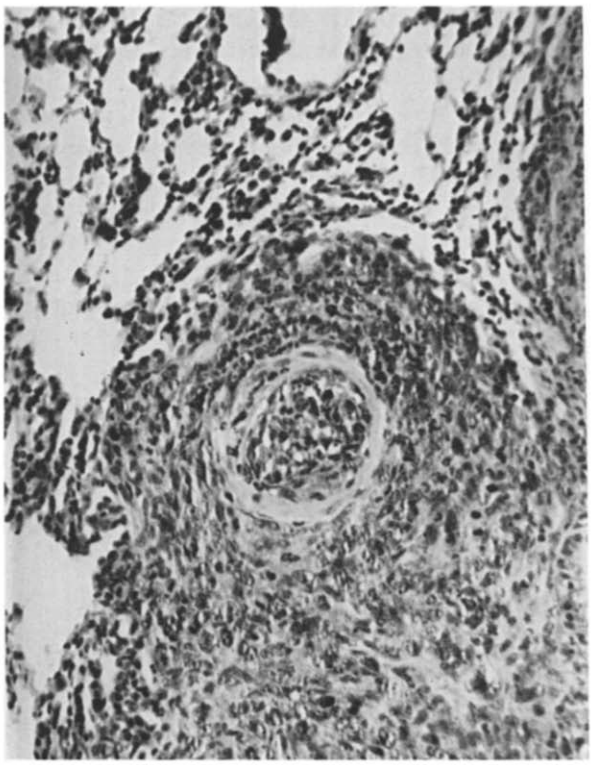

Fig. 2. Tumor embolus with extensive invasion into the lung interstitium. Metastasis taken from the lung of a mouse injected with LM cells grown in medium containing *N,N*-dimethylethanolamine. Tumor cells have destroyed the morphology of the vessel and invaded the surrounding lung parenchyma. Hematoxylin and eosin stain, ×166.

emboli with little evidence of invasion (Fig. 1), while tumor metastases from serum, choline, or *N,N*-dimethylethanolamine-grown cells showed extensive invasion from the vasculature into the surrounding lung interstitium (Fig. 2). When LM cells were cultured for three days in the complete absence of choline or choline analogues, cell growth stopped, although the cells remained viable. These cells produced local tumors with the same high frequency as other groups, but frequency of metastasis was low (30%, Table I). However, these metastases appeared highly invasive and had a high mitotic index. The metastatic indices were 4.7, 3.7, 3.0, and 1.0 for mice injected with choline, *N,N*-dimethylethanolamine, monomethylethanolamine, and ethanolamine supplemented cells, respectively. The size of the metastatic lesions in the groups showing invasion were more numerous, larger, and in addition had mitotic indices approximately three times those of the emboli-only groups. Thus, two effects were noted: (1) choline substitution with *N*-monomethylethanolamine or ethanolamine significantly ($P < 0.01$) decreased both the incidence and extent of metastasis, while (2) choline or choline analogue deprivation significantly ($P < 0.01$) decreased metastatic incidence but not extent of metastasis.

Effect of choline analogues on membrane characteristics of local tumors

Considerable data in the literature exists as to a role of primary tumor cell membranes in the neoplastic process. The altered metastatic incidence of choline analogue supplemented LM cells could indicate an alteration of some membrane property. Therefore, plasma membranes, microsomes, and mitochondria were isolated from local tumors (Table II). The specific activity of (Na^+ +

TABLE II

ACTIVITIES OF PLASMA MEMBRANE, MICROSOMAL, AND MITOCHONDRIAL ENZYMES IN PURIFIED MEMBRANE FRACTIONS ISOLATED FROM TUMORS OF ATHYMIC (Nu/Nu) MICE INJECTED WITH LM CELLS CULTURED IN VITRO WITH CHOLINE ANALOGUES

Plasma membrane fractions were purified approximately 5-fold with respect to crude homogenate (specific activity of $(Na^+ + K^+)$-ATPase); microsomes were purified 3.5-fold with respect to crude homogenate (NADPH–cytochrome-*c* reductase specific activity); mitochondria were purified 3-fold with respect to crude homogenate (NADH-dependent cytochrome-*c* reductase specific activity). Values represent the mean ± S.E. ($n = 5$). [a], [b] and [c] refer to $P < 0.005$, 0.025 and 0.05, respectively, as compared to choline by Student's *t*-test.

		Specific activity (nmol/min per mg)			
	Supplement:	choline	*N*,*N*-dimethyl-ethanolamine	*N*-monomethyl-ethanolamine	ethanolamine
Plasma	$(Na^+ + K^+)$-ATPase	56.4 ± 3.9	40.8 ± 3.8 [a]	42.9 ± 3.6 [c]	41.3 ± 3.6 [b]
	5′-Nucleotidase	25.0 ± 2.2	20.0 ± 2.0	22.0 ± 1.7	21.0 ± 1.5
Microsomes	NADPH-dependent cytochrome-*c* reductase	30.3 ± 3.1	27.7 ± 1.5	28.4 ± 2.6	32.1 ± 2.9
Mitochondria	NADH-dependent cytochrome-*c* reductase	188 ± 5	142 ± 25	234 ± 15 [b]	299 ± 27 [a]
	Rotenone-insensitive NADH-dependent cytochrome-*c* reductase	167 ± 5	130 ± 18	218 ± 17 [b]	276 ± 24 [b]
	Rotenone-sensitive NADH-dependent cytochrome-*c* reductase	37 ± 4	25 ± 6	53 ± 5 [c]	59 ± 6 [c]

K^+)-ATPase but not 5′-nucleotidase was significantly decreased ($P < 0.025$) in plasma membranes of local tumors derived from LM cells cultured with *N*,*N*-dimethylethanolamine, *N*-monomethylethanolamine, and ethanolamine as compared to local tumors from choline supplemented cells. These differences were not present in plasma membranes isolated from each group of cultured cells prior to injection into the nude mice. The mitochondrial enzymes NADH-dependent cytochrome-*c* reductase, rotenone insensitive NADH-dependent cytochrome *c* reductase, and rotenone sensitive NADH-dependent cytochrome-*c* reductase were significantly increased ($P < 0.025$) in local tumor mitochondria from animals injected with *N*-monomethylethanolamine and ethanolamine supplemented LM cells. Differences in mitochondrial protein/g tumor were not observed. Microsomal NADPH-dependent cytochrome-*c* reductase activity was unaltered.

Lipid composition of local tumor cell membranes

All lipid classes were elevated in the plasma membranes from local tumors of mice injected with LM cells cultured with 10% calf serum. The cholesterol/phospholipid ratio exhibited a slight but not statistically significant, decreasing trend in the local tumor plasma membranes of tumors from mice injected with choline analogue supplemented cells (0.77 ± 0.18, 0.66 ± 0.21, 0.68 ± 0.19, and 0.51 ± 0.13, respectively). In contrast, the total lipid, phospholipid, cholesterol, triacylglycerol and free fatty acid content of local tumor cell membranes from mice injected with LM cells cultured with choline analogues did not differ significantly ($P < 0.05$) from that of tumor membranes from mice injected with LM cells cultured with choline.

The phosphatidylcholine/phosphatidylethanolamine ratio in membranes from the cultured cells supplemented with choline analogues prior to injection into the mice was significantly different from that of choline-supplemented cells ($P < 0.05$) [41]. The phospholipid species composition of the cultured cell membranes (in vitro [41]) was dramatically different from that of tumor (in vivo) membranes, especially in plasma membrane and microsomes. In contrast, no statistically significant differences in the phospholipid composition of plasma membranes, microsomes, and

TABLE III

FATTY ACID COMPOSITION OF PHOSPHATIDYLCHOLINE OF LM TUMOR MEMBRANES FROM LM CELLS GROWN IN SUSPENSION WITH MEDIUM CONTAINING DIFFERENT ANALOGUES OF CHOLINE

LM cells were grown in media containing choline (TME), *N,N*-dimethylethanolamine (DME), *N*-monomethylethanolamine (ME), or ethanolamine (E). Numbers refer to fatty acid nomenclature: the figure in front of the colon represents the number of carbon atoms while the figure after the colon refers to the number of double bonds. Long chain refers to 18 carbons or longer while short chain refers to less than 18 carbon fatty acids. The t indicates trace (detection limit 0.2%). Values represent the mean ± S.E. ($n = 5$). [a] refers to $P < 0.025$ with respect to choline by Student's *t*-test.

Supplement	Fatty acid composition (%)								Unsaturated/ saturated	Long chain/ short chain
	14:0	16:0	16:1	18:0	18:1	18:2	20:4	other		
Plasma membranes										
TME	0.4±0.1	34.0±3.4	2.2±0.4	24.2±1.6	21.7±2.0	10.1±1.6	3.0±0.3	4.5	0.7	1.7
DME	1.6±0.2	20.2±2.7	3.1±1.1	25.1±2.8	19.8±3.0	8.2±1.8	1.4±0.4 [a]	0.6	0.5	1.2
ME	t	39.5±4.0	2.9±0.5	24.8±3.4	17.1±2.0	8.5±1.4	1.3±0.5 [a]	5.9	0.6	1.4
E	3.6±1.8	33.1±2.4	2.5±0.6	23.8±1.3	21.9±0.9	10.1±1.0	1.3±0.5 [a]	3.7	0.7	1.6
Microsomes										
TME	0.5±0.1	33.6±2.1	1.9±0.4	20.7±1.0	30.5±2.3	10.7±1.4	2.2±0.3	t	0.8	1.8
DME	t	37.5±0.5 [a]	t	29.5±1.8 [a]	21.3±3.2 [a]	9.9±1.3	3.8±0.7	t	0.5 [a]	1.8
ME	2.3±0.8	40.2±1.9 [a]	1.3±0.3	26.1±0.8 [a]	20.3±2.3 [a]	5.3±1.0 [a]	0.8±0.2 [a]	4.2	0.5 [a]	1.3 [a]
E	0.5±0.2	39.1±1.1 [a]	t	25.6±1.2 [a]	19.2±3.1 [a]	3.4±0.7 [a]	t [a]	12.2	0.5 [a]	1.5
Mitochondria										
TME	t	16.0±2.0	t	18.9±1.5	26.5±2.0	t	24.9±2.8	13.7	1.9	5.3
DME	t	43.4±1.9 [a]	t	38.3±2.8 [a]	10.0±2.1 [a]	t	4.0±1.0 [a]		0.2 [a]	1.3
ME	t	43.9±3.1 [a]	t	33.0±1.2 [a]	t [a]	t	4.4±1.4 [a]	12.3	0.2 [a]	1.0
E	6.4±1.8	38.5±2.4 [a]	t	35.7±3.1 [a]	14.5±1.8 [a]	t	11.3±2.0 [a]	t	0.3 [a]	1.6

mitochondria in tumors from nude mice injected with choline analogue supplemented as opposed to choline-supplemented cells were observed. The ratio of phosphatidylcholine/phosphatidylethanolamine and the ratio of anionic to neutral zwitterionic phospholipids was unaltered.

The fatty acid composition of phosphatidylcholine, one of the two major phospholipid components, for microsomes and mitochondria of choline analogue tumors was significantly altered ($P < 0.025$) (Table III). The complete fatty acid profile was altered and the ratio of unsaturated/saturated fatty acids was significantly decreased ($P < 0.025$) in phosphatidylcholine for microsomes and mitochondria of choline analogue as compared to tumors arising from choline-grown cells. The arachidonic acid content (20:4) of phosphatidylcholine from plasma membranes, microsomes, and mitochondria of tumors from choline analogue supplemented cells was significantly decreased ($P < 0.025$) as compared to choline supplemented cells (Table 3). In contrast, there were no consistent differences in the fatty acyl chain composition of either the total phospholipids, phosphatidylethanolamine (the other major phospholipid component) triacylglycerols, or free fatty acids.

It is possible that the tumor membrane lipid composition of LM cells supplemented with choline resulted from a permanent alteration of the LM cell population phenotype in the primary tumor of the nude mouse. This possibility was tested by examining the phospholipid species composition and the sterol content both before choline supplemented cells were injected into mice and after the cells were grown as local tumors, excised and returned to tissue culture in choline supplemented medium. The sterol content and phospholipid species composition of cultured LM cells were not influenced by passage as local tumors in the nude mouse.

Comparison of local tumor versus lung metastasis membrane properties

Plasma membranes, microsomes and mitochondria were isolated from local tumors, lung metastases, and lung tissue of nude mice injected with LM fibroblasts cultured with choline. Values for lung membranes are included because dissection of small lung metastases (2–3 mm dia) may result in a small amount of contamination with surrounding normal lung tissue. Membrane properties between local tumor, metastasis, and lung tissue differed markedly in enzyme specific activty, lipid composition and structure. The specific activities of $(Na^+ + K^+)$-ATPase and 5′-

TABLE IV

ACTIVITIES OF PLASMA MEMBRANE, MICROSOMAL, MITOCHONDRIAL ENZYMES IN PURIFIED MEMBRANE FRACTIONS ISOLATED FROM LUNG, LUNG METASTASES, AND LOCAL TUMORS OF ATHYMIC (Nu/Nu) MICE INJECTED WITH LM FIBROBLASTS

LM cells were cultured for three days in suspension with medium containing choline. The cells were then centrifuged, resuspended in choline medium at $20 \cdot 10^6$ cells/ml, and injected s.c. into the left thighs of athymic (Nu/Nu) mice. After 28 days, the lungs, lung metastases, and local tumors were biopsied, membrane fractions were prepared, and enzyme activities were determined as described in Methods. Plasma membrane fractions were purified approximately 6-fold with respect to crude homogenate (activity of $(Na^+ + K^+)$-ATPase); microsomes were purified 3.5-fold with respect to crude homogenate (NADH–cytochrome-*c* reductase activity). Mitochondria were purified 4-fold with respect to crude homogenate (succinate–cytochrome-*c* reductase activity). Values represent the mean ± S.E. ($n = 5$). [a] refers to $P < 0.01$ as compared to lung and [b] refers to $P < 0.01$ as compared to local tumor.

Membrane	Enzyme	Specific activity (nmol/min per mg)		
		local tumor	lung metastases	lung
Plasma membrane	$(Na^+ + K^+)$-ATPase	$56.4 \pm 3.9^{a,b}$	$89.0 \pm 4.1^{a,b}$	28.9 ± 2.9^{b}
	5′-Nucleotidase	25.0 ± 2.2^{a}	12.9 ± 2.4^{b}	13.3 ± 1.5^{b}
Microsomes	5′-Nucleotidase	21.2 ± 2.8^{a}	13.8 ± 2.3^{b}	14.8 ± 0.3^{b}
	NADPH-dependent cytochrome-*c* reductase	46.8 ± 5.0^{a}	48.7 ± 7.4^{b}	160.8 ± 8.3^{b}
Mitochondria	Succinate-dependent cytochrome-*c* reductase	188.0 ± 5.0^{a}	$87.3 \pm 12.3^{a,b}$	10.7 ± 0.8^{b}

TABLE V

PHOSPHOLIPID COMPOSITION OF LOCAL TUMOR, LUNG METASTASES, AND LUNG PLASMA MEMBRANES FROM NUDE MICE INJECTED WITH LM FIBROBLASTS

All procedures were as described for the preceding Table IV. Values represent the mean ± S.E. ($n = 3$–5). [a] and [b] refer to $P < 0.05$ for lung metastasis versus local tumor and for lung metastasis versus lung, respectively, by Student's t-test.

Tissue	% Composition					Phosphatidylcholine/ phosphatidylethanolamine
	phosphatidylcholine	phosphatidylethanolamine	phosphatidylinositol + phosphatidylserine	sphingomyelin + lysophosphatidylcholine	other	
Local tumor	35.0 ± 3.1	43.1 ± 2.3	14.4 ± 1.2	6.3 ± 2.0	2.0	0.81
Lung metastases	34.5 ± 3.4 [b]	31.9 ± 1.4 [a,b]	12.5 ± 2.3	18.2 ± 1.2 [a,b]	3.8	1.11 [a,b]
Lung	45.8 ± 1.2	8.6 ± 2.5	11.9 ± 4.4	26.8 ± 2.4	6.9	5.32

nucleotidase were higher and lower ($P < 0.05$), respectively, in plasma membranes from lung metastases as compared to local tumors (Table IV). The specific activity of succinate-dependent cytochrome-*c* reductase was lower in mitochondria from lung metastases than in mitochondria from local tumors (Table IV). Neither metastases nor local tumor membrane enzymatic activity profiles resembled those of lung tissues. It is also possible, however, that host inflammatory cells recruited into the tumor could have altered the biochemical characteristics of the metastases and not been represented by the values found for the local tumor material where a biopsy may not be representative of other areas of tumor growth. Therefore, local tumors and lung metastases were returned to culture in chemically defined, serum-free medium (see Methods) for at least six weeks. Untransformed cells as well as inflammatory cells, unlike the LM tumor cells, were unable to survive in this medium. Plasma membranes, microsomes, and mitochondria were isolated from the cultured local tumor and cultured metastatic cells. Plasma membrane ($Na^+ + K^+$)-ATPase and 5′-nucleotidase specific activity from the cultured metastatic cells was 2.2-fold greater and 7-fold less, respectively, than in plasma membranes from cultured local tumor cells. Thus, it seems unlikely that the differences in membrane properties between local and metastatic tumors were due to either inclusion of adjacent lung tissue, or to recruitment of host inflammatory cells.

The lipid composition of membranes from local tumor, lung tissue, and lung metastases was also determined. The phosphatidylethanolamine content of lung metastasis and sphingomyelin + lysophosphatidylcholine content of lung metastasis plasma membranes were significantly ($P < 0.05$) decreased and increased, respectively, as compared to those of local tumor plasma membranes (Table V). The lung metastasis phospholi-

TABLE VI

FATTY ACID COMPOSITION OF PHOSPHOLIPIDS OF LOCAL TUMOR, LUNG METASTASES, AND LUNG PLASMA MEMBRANE PHOSPHOLIPIDS FROM NUDE MICE INJECTED WITH LM FIBROBLASTS

LM cells were grown in medium containing choline, the cell in vehicle were injected into nude mice, tissues were isolated and plasma membranes were isolated as described in Materials and Methods. Fatty acid nomenclature was as described in the legend to Table III. The t indicates trace (detection limit 0.2%). Values represent the mean ± S.E. ($n = 3$–5). [a] and [b] refer to $P < 0.05$ for local tumor versus lung metastasis and lung metastasis versus lung, respectively, by Student's t-test.

Tissue	Local tumor	Lung metastases	Lung
Fatty acid composition (%)			
14:0	1.0 ± 0.4	t	t
16:0	16.3 ± 0.8	17.3 ± 1.0 [b]	24.4 ± 2.2
16:1	2.5 ± 0.2	0.9 ± 0.8 [a,b]	2.9 ± 1.1
18:0	20.9 ± 0.4	31.1 ± 0.4 [a,b]	24.4 ± 0.7
18:1	23.9 ± 1.1	29.6 ± 0.8 [a,b]	25.1 ± 1.5
18:2	11.4 ± 0.4	7.3 ± 1.7 [a]	6.8 ± 1.5
20:4	7.8 ± 0.2	10.9 ± 2.1 [a]	14.3 ± 1.4
Other	15.6	2.9 [a]	2.1
Ratios			
Unsatd. / Satd.	1.6	1.1 [a]	2.0
Long chain / Short chain	3.9	4.4 [b]	2.7

pid composition differed significantly ($P < 0.05$) from that of lung plasma membranes. The phospholipid fatty acid composition of lung metastasis plasma membranes was significantly ($P < 0.05$) enriched in certain unsaturated fatty acids such as arachidonic and oleic acids, as compared to that of the local tumor plasma membranes (Table VI). The lung metastasis phospholipid fatty acid composition also differed significantly ($P < 0.05$) from that of normal lung tissue. In contrast, the ratio of cholesterol/phospholipid was not significantly different in plasma membranes from lung metastases (0.83 ± 0.09) as compared to those of local tumors (0.77 ± 0.08). However, both differed markedly from the cholesterol/phospholipid ratio of lung tissue plasma membranes (0.35 ± 0.09).

The plasma membrane structural properties of metastases differed markedly from those of local tumors. Characteristic breakpoints in Arrhenius plots of *trans*- or *cis*-parinaric acid fluorescence intensity (indicative of lipid phase, clustering, or microdomain alteration) indicated breakpoints near 22° and 36°C in plasma membranes of lung metastases but no breakpoints in primary tumor membranes (Table VII). *trans*-Parinaric acid and diphenylhexatriene fluorescence indicated that the plasma membranes from lung metastases had slightly more bulk fluidity than those from the local tumor membranes; limiting anisotropy and order parameter were significantly ($P < 0.05$) lower in plasma membranes of metastases as compared to those of local tumors (Table VIII).

TABLE VII

CHARACTERISTIC TEMPERATURES OF PLASMA MEMBRANES FROM LUNG, LUNG METASTASES, AND LOCAL TUMORS OF NUDE MICE INJECTED WITH LM FIBROBLASTS

Characteristic temperatures were determined from breakpoints in Arrhenius plots as described in Methods. Values represent the mean ± S.E. ($n = 6$).

Tissue	Characteristic temperature (°C)
trans-Parinaric acid	
Local tumor	none
Lung metastases	22 ± 1, 36 ± 1
Lung	37 ± 1
cis-Parinaric acid	
Local tumor	none
Lung metastases	22 ± 2, 37 ± 1
Lung	36 ± 1
1,6-Diphenyl-1,3,5-hexatriene	
Local tumor	none
Lung metastases	none
Lung	none

TABLE VIII

DYNAMIC AND STRUCTURAL PROPERTIES OF PLASMA MEMBRANES FROM LUNG METASTASES AND LOCAL TUMORS FROM NUDE MICE INJECTED WITH LM CELLS

All fluorescence parameters were determined as described in Methods. Numbers in parentheses refer to the fractional fluorescence intensity. Values represent the mean ± S.E. ($n = 6$). [a] refers to $P < 0.05$ between lung metastases and local tumor by Student's *t*-test.

Parameter	Local tumor	Lung metastases
trans-Parinaric acid		
Polarization	0.330 ± 0.002 [a]	0.315 ± 0.002 [a]
Lifetime τ_1 (ns)	3.5 ±0.3 (0.46)	4.1 ±0.6 (0.47)
Lifetime τ_2 (ns)	15.2 ±1.8 (0.54)	17.5 ±2.6 (0.53)
Limiting anisotropy	0.219 ± 0.002 [a]	0.203 ± 0.001 [a]
Rotational rate (ns)	1.9 ±0.1 [a]	2.3 ±0.1 [a]
Order parameter	0.750 ± 0.006 [a]	0.721 ± 0.004 [a]
1,6-Diphenyl-1,3,5-hexatriene		
Polarization	0.325 ± 0.004 [a]	0.309 ± 0.003 [a]
Lifetime τ_1 (ns)	3.6 ±0.7 (0.14)	6.7 ±0.5 [a] (0.47) [a]
Lifetime τ_2 (ns)	9.3 ±0.5 (0.86)	11.4 ±1.1 [a] (0.63) [a]
Limiting anisotropy	0.211 ± 0.002 [a]	0.193 ± 0.002 [a]
Rotational rate (ns)	1.8 ±0.1 [a]	2.3 ±0.1 [a]
Order parameter	0.733 ± 0.008 [a]	0.702 ± 0.006 [a]

Discussion

Two objectives of this study were to determine if in vitro phospholipid manipulation of LM cells affected (1) their ability to form metastatic tumors, and/or (2) membrane properties of the local and/or metastatic tumors when choline or choline analogue-grown LM cells were injected into nude mice. We were particularly interested if certain membrane properties of metastatic cells differed from those of local tumor cells. The model system chosen for this study was LM fibroblasts, a derivative of L-929 cells, because of (1) their ability to grow in totally defined medium, allowing the

singular manipulation of one component of their culture medium (choline), without the variation inherent in serum-requiring cells, (2) their extensive growth and cell membrane characterization [12,41,51], and (3) unlike A-9 cells [2,5], another derivative of L-929 cells, their ability to form both local and metastasizing tumors in nude mice in a short time (28 days) with high frequency (> 99%) (Ref. 22 and this report).

The first objective was satisfied in that in vitro manipulation of the LM cell phospholipid composition markedly reduced the metastatic ability of LM cells. Moreover, when metastasis was present from cells cultured with *N*-monomethylethanolamine or ethanolamine, only embolus formation but not invasion was observed at the time of necropsy (28 days). The results also showed that at the dose of cells injected (10^7 cells/mouse) in vitro manipulation of LM cell phospholipid composition did not affect their ability to form local tumors in nude mice. One may not, however, conclude on the basis of this single assay that modifying LM membrane lipid did not change LM tumorigenic properties. Differences might have been observed at a lower dose of tumor cells.

The cause and reason for the apparent decreased metastatic rate and invasiveness of choline supplemented cells with altered phospholipid composition is not known. It could be due to changes in host effector recognition in nude mice (which have good NK- and macrophage-mediated responses but lack effective T-cell-dependent responses). Indeed, increased exposure of anionic phospholipids in the membranes of red blood cells enhances their recognition and removal by macrophages [52,53]. One anionic phospholipid, phosphatidylserine, has been proposed to be important in macrophage recognition and cytolysis [54]. Supplementation of LM cells with ethanolamine rather than choline did not, however, increase the plasma membrane content of anionic phospholipids. Both in vitro (cultured cells) and in vivo (local tumors), the isolated plasma membranes from choline and ethanolamine supplemented cells had anionic/neutral zwitterionic phospholipid ratios of 0.22 ± 0.01 versus 0.23 ± 0.01 and 0.16 ± 0.03 versus 0.15 ± 0.02, respectively. The content of phosphatidylserine (10.1 ± 0.59 versus $11.6 \pm 0.7\%$) was not significantly different.

Another possibility for the decreased metastatic ability of ethanolamine supplemented LM cells is changes in cell adhesion properties (platelet binding, endothelial cell binding, homotypic aggregation, etc.). Elsewhere [51] we demonstrated in vitro that ethanolamine supplementation decreased the lag time for homotypic concanavalin A mediated agglutination nearly 4-fold. Concomitantly, heterotypic (sheep red blood cell) agglutination (%) increased nearly 3-fold. These differences were not due to alterations in sialic acid content but correlated with alteration in aminophospholipid content or exposure [51,55]. Consistent with this, lung metastatic plasma membranes had higher phosphatidylethanolamine content than did local tumor plasma membranes. In addition, ethanolamine-enriched cells in culture had elevated phosphatidylethanolamine, perhaps decreasing their ability to produce metastases. The less metastatic choline-analogue supplemented cells had decreased ratios of phosphatidylcholine/phosphatidylethanolamine in culture, while lung metastases had increased ratios. This ratio affects cell-substrate adherence [56,57]. The abnormal adhesiveness of tumor cells is thought to contribute to the metastatic ability of tumor cells in a complex manner [58]. Supplementation of normal RSV transformed chick embryo fibroblasts with ethanolamine or *N*-monomethylethanolamine decreased the substrate adhesiveness of these cells. As described above, cultured LM cells supplemented with choline analogues were more adhesive to one another and more adhesive to non-tumor cells [51]. Similarly, low metastatic B-16 melanoma cell variants showed less adherence to other tumor cells and greater adherence to normal cells than did high metastatic variants [59]. Membrane adhesion sites to substrate in SV transformed 3T3 cells are enriched in phosphatidylethanolamine and decreased in phosphatidylcholine as compared to untransformed 3T3 cells [60]. These observations would lead us to predict decreased metastatic ability of choline analogue supplemented LM cells that we observed.

It should be noted, however, that modification of phospholipid composition also resulted in changes in arachidonic acid content, a lipid believed to play important roles in malignancy. The enzyme systems for methylating phosphatidyl-

ethanolamine to phosphatidylcholine are largely lacking in tumorigenic cells, such as mouse LM fibroblasts [61], mouse ascites [62], rat hepatomas [62], and murine leukemia cells [63]. Production of phosphatidylcholine by *N*-methylation of phosphatidylethanolamine results in enhanced incorporation of arachidonic acid into phosphatidylcholine as compared to that occurring through the classical pathway utilizing choline directly [64]. Arachidonic acid is the well known precursor of prostaglandins, which are thought to have a role in the metastatic process. Phosphatidylcholine of LM local tumor membranes of mice injected with choline analogue supplemented cells had significantly less arachidonic acid (Table III), perhaps indicating less ability of these tumor cells to form prostaglandins. In general, increased prostaglandin content has been found in the blood, urine, and tumor cells of cancer patients [65]. Thus, a decrease in arachidonic acid esterified to phosphatidylcholine of local tumor cells may decrease the malignancy of these cells as manifested by decreased metastasis. An interesting corollary to this possibility may be that metastatic cells (which may be more malignant than encapsulated local tumor cells) should therefore have more arachidonic acid. Indeed, the data in Table VI showing increased arachidonic acid in lung metastasis plasma membranes as compared to local tumor plasma membranes support this interpretation.

The second objective of this study was to determine if there were differences in membrane properties between tumors growing at the local site of inoculation and their lung metastatic counterparts. The plasma, microsomal, and mitochondrial membranes of lung metastases differed markedly from those of the local tumors with respect to membrane enzymes, phospholipid content, cholesterol content, phosphatidylethanolamine content, and unsaturated fatty acid (especially arachidonic and oleic acid) content. These properties were also distinct from those of lung tissue. The physical properties of the metastasis versus local tumor plasma membranes displayed significant differences in Arrhenius plots of fluorescence probe characteristic breakpoints (two versus one breakpoint) and in lipid order indicating that the metastatic cell plasma membranes were more fluid than those of the local tumor cells. The membranes of lung metastasis of Lewis Lung Carcinoma cells have also been reported to be more fluid than in the corresponding local tumor in C57B1/6 mice injected s.c. with the tumor cells [37]. At this time it is premature to relate these differences in membrane properties of metastatic versus local tumor cells to the known biological properties of metastatic cells. This difficulty is largely due to paucity of data on purified plasma membranes and other membranes from metastases obtained either in vitro or in vivo.

In summary, the LM cell/nude mouse system can be used to investigate the effect of in vitro manipulation of tumor cell nutrition on metastatic ability of the cells. The frequency and extent of formation of metastatic tumors from LM fibroblasts in nude mice was dependent on whether the cells were cultured in the presence of choline or of choline analogues. Membrane properties of local versus lung metastatic tumor cells differed significantly.

Acknowledgements

This investigation was supported primarily by USPHS CA 24339 and in part by USPHS GM 31651. A.B.K. is the recipient of a Special Emphasis Research Career Development Award USPHS RR 00013.

The authors wish to thank Messrs. J. Waters, J. Gardiner, E. Hubert, and Mrs. K. Marklewitz for their expert technical assistance.

References

1 Kim, U. (1970) Science 167, 72–74.
2 Freedman, V.H. and Shin, S. (1978) in The Nude Mouse in Experimental and Clinical Research (Fogh, J. and Giovanella, B.C., eds.), pp. 353–384, Academic Press, New York, NY.
3 Bilello, J.A., Freedman, V.H. and Shin, S. (1977) J. Natl. Cancer Inst. 58, 1691–1694.
4 Freedman, V.H. and Shin, S. (1974) Cell 3, 355–359.
5 Freedman, V.H., Brown, A.L., Klinger, H.P. and Shin, S. (1976) Exp. Cell Res. 98, 143–151.
6 Poulsen, C.O., Fialkow, P.J., Klein, E., Klein, G., Rygaard, J. and Weiner, F. (1973) Int. J. Cancer 11, 30–39.
7 Schmidt, M. and Good, R.A. (1975) J. Natl. Cancer Inst. 55, 81–87.
8 Shin, S., Baum, S.G., Fleischer, N. and Rosen, O.M. (1975) J. Cell. Science 18, 199–206.
9 Shin, S., Freedman, V.H., Risser, R. and Pollack, R. (1975) Proc. Natl. Acad. Sci. USA 72, 4435–4439.

10 Sorda, B., Merenda, C. and Carrel, S. (1977) in Proc. of the 2nd Int. Workshop on Nude Mice (Nomura, T., Ohsawa, N., Tamaoki, N. and Fujiwara, K., eds.), pp. 313–326, G. Fischer Verlag, Stuttgart.
11 Stiles, C.D., Desmond, W., Chuman, L.M., Sato, G. and Safer, M.H., Jr. (1996) Cancer Res. 36, 1353–1360.
12 Fogh, J., Tiso, J., Orfeo, T., Fogh, J.M., Daniels, W.P. and Sharkey, F.E. (1982) in Proc. of the 3rd Int. Workshop on Nude Mice (N.D. Reed, ed.), pp. 447–456, G. Fischer Verlag, New York.
13 Kim, U., Han, T., Ghosh, S., Freedman, V.H., Shin, S. and Pressman, D. (1982) in Proc. of 3rd Int. Workshop on Nude Mice (Reed, N.D., ed.), pp. 367–378, G. Fischer Verlag, New York, NY.
14 Rygaard, J. (1973) Thymus and Self: Immunobiology of mouse mutant nude, J. Wiley, New York, NY.
15 Rygaard, J. and Pulsen, C.O. (1969) Acta Pathol. Microbiol. Scand. 77, 758–760.
16 Fidler, I.J. (1978) in Pulmonary Metastasis (Weiss, L. and Gilbert, H.A., eds.), pp. 43–61, G.K. Hall, Boston, MA.
17 Kripke, M.L., Gruys, E. and Fidler, I.J. (1978) Cancer Res. 38, 2962–2967.
18 Layton, M.G. and Franks, L.M. (1984) Br. J. Cancer 49, 415–421.
19 Giovanella, B.C., Yim, S.O., Morgan, A.C., Stehlin, J.S. and Williams, L.J., Jr. (1973) J. Natl. Canc. Inst. 50, 1051–1054.
20 Giovanella, B.C., Stehlin, J.S. and Williams, L.J., Jr. (1974) J. Natl. Cancer Inst. 52, 921–927.
21 Sordat, B., Fritsche, R., Mach, J.-P., Carrel, S., Ozzelo, L. and Cerottini, J.-C. (1974) in Proc. Int. Workshop on Nude Mice, 1st (J. Rygaard and C.O. Poulsen, eds.), pp. 269–278, Gustav Fischer Verlag, Stuttgart.
22 Kier, A.B. and Schroeder, F. (1982) Transplantation 33, 274–279.
23 Erickson, K.L., Adams, D.A. and McNeill, C.J. (1983) Lipids 18, 468–474.
24 Gurr, M.I. (1983) Prog. Lipid Res. 22, 257–287.
25 Schlager, S.I. and Ohanian, S.H. (1983) Lipids 18, 475–482.
26 Schlager, S.I., Meltzer, M.S. and Madden, L.D. (1983) Lipids 18, 483–488.
27 Shinitzky, M., Skornick, Y., Gorelik, E. and Sindelar, W. (1983) in 13th Int. Cancer Congress, Part B, Biology of Cancer (1) (Mirand, E.A., Hutchinson, W.B. and Mihick, E., eds.), pp. 425–433, A.R. Liss, Inc., New York, NY.
28 Alderson, J.C.E. and Green, C. (1975) Biochem. Soc. Trans. 3, 1009–1011.
29 Corwin, L.M., Varshavsky-Rose, F. and Broitman, S.A. (1979) Cancer Res. 39, 4350–4355.
30 Inbar, M. and Shinitzky, M. (1974) Proc. Natl. Acad. Sci. USA 71, 2128–2130.
31 Inbar, M. and Shinitzky, M. (1974) Proc. Natl. Acad. Sci. USA 71, 4229–4231.
32 Shinitzky, M. and Inbar, M. (1974) J. Mol. Biol. 85, 603–615.
33 Shinitzky, M., Skornick, Y. and Haran-Ghera, N. (1979) Proc. Natl. Acad. Sci. USA 76, 5313–5316.
34 Bhat, N.R. and Volpe, J.J. (1984) J. Neurochem. 42, 1457–1463.
35 Burns, C.P., Luttenegger, D.B., Dudley, D.T., Buettner, G.R. and Spector, A.A. (1979) Cancer Res. 39, 1726–1732.
36 Guffy, M.M., North, J.A. and Burns, C.P. (1984) Cancer Res. 44, 1863–1866.
37 Rivnay, B., Gorelik, E., Segal, S. and Shinitzky, M. (1981) Invasion Metastasis 1, 99–10.
38 Mountford, C.E., Wright, L.C., Holmes, K.T., Mackinnon, W.B., Gregory, P. and Fox, R.M. (1984) Science 226, 1415–1418.
39 Schroeder, F. (1984) Biochim. Biophys. Acta 776, 299–312.
40 Schroeder, F. and Gardiner, J.M. (1984) Cancer Res. 44, 3262–3269.
41 Schroeder, F., Perlmutter, J.F., Glaser, M. and Vagelos, P.R. (1976) J. Biol. Chem. 251, 5015–5026.
42 Sottocasa, G.L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) J. Cell. Biol. 32, 415–438.
43 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
44 Schroeder, F., Goh, E.H. and Heimberg, M. (1979) J. Biol. Chem. 254, 2456–2463.
45 Marzo, A., Ghirardi, P., Sardini, D. and Meroni, G. (1971) Clin. Chem. 17, 145–147.
46 Ames, B.N. (1986) Methods Enzymol. 8, 115–118.
47 Schroeder, F. (1985) in Subcellular Biochemistry, Ch. 29, Vol. 11 (Roodyn, D.B., ed.), pp. 51–101, Plenum Pub. Corp., New York, NY.
48 Schroeder, F. (1988) in Advances in Membrane Fluidity, Vol. 1 (Aloia, R.C., Curtain, C.C. and Gordon, L.M., eds.), Alan R. Liss, Inc., in press.
49 Sweet, W.D. and Schroeder, F. (1986) Biochim. Biophys. Acta 861, 53–61.
50 Sweet, W.D., Wood, W.G. and Schroeder, F. (1987) Biochemistry 26, 2828–2835.
51 Schroeder, F. (1982) Biochemistry 21, 6782–6790.
52 Tanaka, Y. and Schroit, A.J. (1983) J. Biol. Chem. 258, 11335–11343.
53 Schroit, A.J., Madsen, J.W. and Tanaka, Y. (1985) J. Biol. Chem. 260, 5131–5138.
54 Fidler, I.J. (1985) Cancer Res. 45, 4714–4725.
55 Hampton, R.Y., Holz, R.W. and Goldstein, J.J. (1980) J. Biol. Chem. 255, 6766–6771.
56 Hale, A.H., Pessin, J.E., Palmer, F., Weber, M.J. and Glaser, M. (1977) J. Biol. Chem. 252, 6190–6200.
57 Portoukalin, J., Bugand, M., Zwingelstein, G. and Precausta, P. (1977) Biochim. Biophys. Acta 489, 106–118.
58 Weiss, L. (1977) in Cancer Metastasis, Gann monograph on cancer research (Stansly, P.G. and Sato, H., eds.), 20, 25–36. University Park Press, Baltimore, MD.
59 Fidler, I.J. and Nicholson, G.L. (1978) Isr. J. Med. Sci. 14, 38–50.
60 Cathcart, M.K. and Culp, L.A. (1979) Biochemistry 18, 1167–1176.
61 Feller, D.J., Schroeder, J. and Bylund, D.B. (1983) Biochem. Pharmacol. 32, 2217–2223.
62 Figard, P.H. and Greenberg, D.M. (1961) Cancer Res. 22, 361–367.
63 Ramu, A., Glaubiger, D. and Weintraub, H. (1984) Cancer Treatment Rep. 68, 637–641.
64 Kanangi, R., Koizumi, K., Hata-Tanoue, S. and Masuda, T. (1980) Biochem. Biophys. Res. Commun. 96, 711–718.
65 Levine, L. (1981) Adv. Cancer Res. 35, 49–79.