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Correlation Between Lipid Synthesis in Tumor Cells and Their Sensitivity to Humoral Immune Attack

Abstract. *Prolonged incubation of two antigenically distinct, chemically induced guinea pig hepatomas with relatively high concentrations of chemotherapeutic drugs or metabolic inhibitors increases their susceptibility to killing by antibody and complement. This effect is reversible when the cells are cultured in the absence of the drugs. The drug-induced sensitivity and the ability of the cells to recover their resistance to killing are directly correlated to their ability to synthesize complex lipids.*

Most nucleated cells are more resistant than nonnucleated cells to killing by antibody and complement (*1*). Different nucleated cells also differ in their susceptibility to immune killing. Susceptibility or resistance of nucleated cells to killing could not be explained by differences in antigen concentration on the cell surface (*2, 3*), by the mobility of antigen on the cell surface (*4*), by the immunoglobulin class used to sensitize the cells (*1*), or by the amount of early acting complement components fixed to the cell (*1, 2, 5*). Some resistant nucleated cells, however, become susceptible to killing at certain stages in their cell cycle (*6*), or can be rendered susceptible to immune attack by treatment with certain metabolic inhibitors and chemotherapeutic drugs used in the treatment of cancer (*7*). Resistance of nucleated cells to killing by immune attack mechanisms may be an intrinsic property of the target cell under metabolic control. Since the cytotoxic action of antibody and complement occurs on or in the cell membrane (*8*), we were prompted to investigate cellular metabolic pathways associated with membrane structure and function which might be affected by treatments which increase the sensitivity of tumor cells to humoral immune attack. In this report, we present evidence that the ability of tumor cells to resist killing by antibody and complement is related to their ability to synthesize lipids.

The ascitic forms of two antigenically distinct guinea pig hepatomas (line 1 and line 10) induced by diethylnitrosamine were collected as previously described (*9*). These cells are resistant to killing by specific rabbit antitumor antibodies plus guinea pig complement (GPC) (*3*). In addition, cells of line 10, but not of line 1, are resistant to killing by rabbit IgM antibody to Forssman antigen plus GPC (*3*). However, cells of both line 1 and line 10 sensitized either with antibody to specific tumor antigens or with IgM antibody to Forssman antigen are susceptible to killing by human complement (*3*).

Tumor cells of line 1 and line 10 were incubated for various lengths of time with selected metabolic inhibitors and chemotherapeutic agents (see Fig. 1). Those drugs effective in increasing sensitivity to killing were used at the lowest concentrations giving a maximum effect, whereas the drugs ineffective in increasing sensitivity were used at the highest concentrations which were not toxic to the cells (*7*). Control suspensions were prepared concurrently consisting of cells incubated in tissue culture medium alone. At several time intervals during the incubation, drug-treated and control cells were tested for their sensitivity to killing by antibody and GPC and for their ability to synthesize DNA, RNA, protein, and complex carbohydrates (*10*); in addition, the cells were tested for their ability to synthesize complex lipids as measured by following their incorporation of fatty acids into extractable cellular lipids (*11*).

The data presented in Fig. 1 are the summary of results obtained with line 1 tumor cells; similar results were obtained with line 10 cells. Increased susceptibility of the drug-treated tumor cells to killing by IgM antibody to Forssman antigen or antibody to specific tumor antigens plus GPC as compared to nondrug-treated cells similarly tested was noted only after 17 hours of incubation with actinomycin D, adriamycin, mitomycin C, or puromycin (see Fig. 1A). These drugs will be referred to as "effective." Cells incubated with 5-fluorouracil, cytosine arabinoside, cyclophosphamide, vincristine sulfate, 6-mercaptopurine, or hydroxyurea remained resistant to killing at all time intervals tested (see Fig. 1B). These drugs will be referred to as "ineffective." Tumor cells which had been incubated 17 hours with the effective drugs, washed free of drug, and reincubated in drug-free medium recovered their resistance to killing by antibody plus GPC within 4 hours in culture (see Fig. 1A). Those

Table 1. Effect of Atromid-S on the macromolecular synthesis and the susceptibility to antibody-complement mediated killing of line 1 tumor cells.

Concentration of Atromid-S*	Percentage inhibition of incorporation of				Cells stained with trypan blue (%)	
	[^{3}H]Thymidine	[^{3}H]Uridine	^{14}C-Labeled amino acids	[^{14}C] Palmitic acid	IgM antibody to Forssman antigen (1:80) plus GPC (1:8)	Antibody to line 1 antigens (1:10) plus GPC (1:8)
None†	0	0	0	0	6	1
$1 \times 10^{-4}M$	14	3	34	37	8	3
$5 \times 10^{-4}M$	24	19	35	70	34	27
$1 \times 10^{-3}M$	56	62	52	68	37	29

*Line 1 cells (5×10^5/ml) were incubated with Atromid-S for 60 minutes at 37°C. †Untreated control cells incorporated, in terms of counts per minute, approximately 10,000 [^{3}H]thymidine, 7500 [^{3}H]uridine, 6000 ^{14}C-labeled amino acids, and 25,000 [^{14}C]palmitic acid.

cells incubated with the ineffective drugs remained resistant to killing at all time intervals tested after they were recultured in the absence of drug (see Fig. 1B).

Tumor cells incubated for only 1 hour with either effective or ineffective drugs were maximally inhibited (70 to 100 percent) in their ability to synthesize DNA, RNA, and protein, and in their ability to incorporate glucose, glucosamine, and galactosamine (Fig. 1, A and B). The cells remained maximally inhibited in their incorporation of isotopically labeled precursors into DNA, RNA, protein, or complex carbohydrate throughout the entire time course of the experiments, even after the cells incubated with the effective drugs had regained their resistance to killing by antibody and complement (Fig. 1A). This indicated that resistance of these cells to killing by antibody and complement was not directly dependent upon their ability to synthesize DNA, RNA, or protein, or on their ability to incorporate glucose or amino sugars.

Tumor cells incubated with the effective drugs were not maximally inhibited (70 percent) in their ability to incorporate [^{3}H]fucose and [^{14}C]*N*-acetyl neuraminic acid (sialic acid) until after 17 hours of incubation; this corresponded to the time the cells were increased in their sensitivity to killing (Fig. 1A). However, at no time did the cells regain their ability to incorporate these labeled compounds even after the cells had regained their resistance to antibody-complement mediated killing (Fig. 1A). In addition, cells incubated with the ineffective drugs were also inhibited in their incorporation of these compounds even though the cells were never rendered susceptible to killing at any of the time intervals (Fig. 1B). These results indicate that resistance of the tumor cells to killing was not directly correlated to the incorporation of fucose or sialic acid; these compounds are known to label preferentially membrane glycoproteins and glycolipids (*12*).

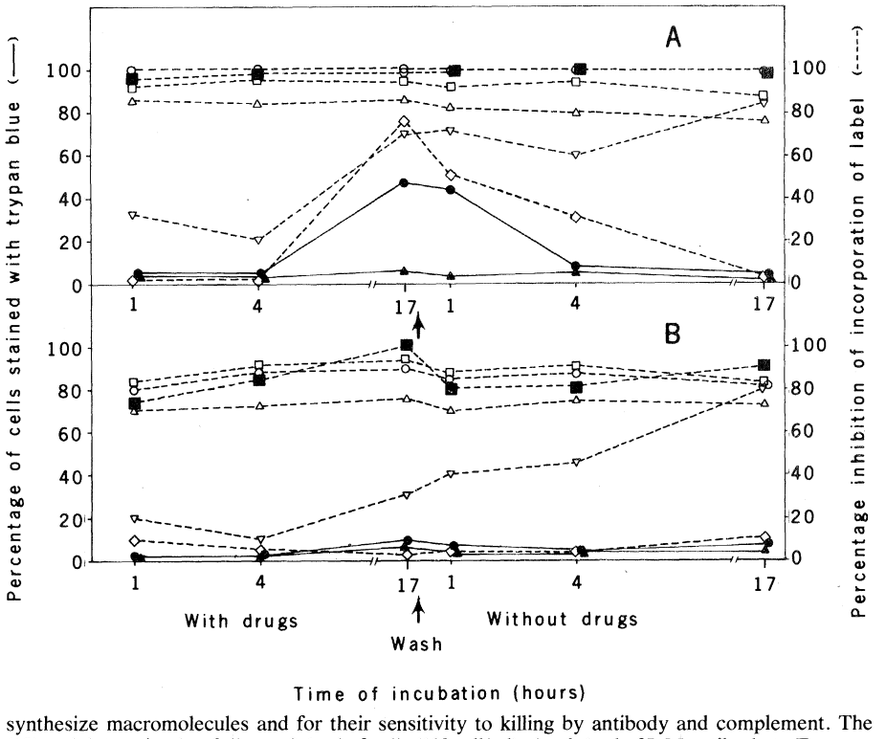

Fig. 1. Effect of treatment of line 1 tumor cells with metabolic inhibitors on the ability of the cells to synthesize macromolecules and on their sensitivity to killing by antibody and GPC, and the reversibility of these effects. The cells were incubated at 37°C in an atmosphere of air plus 5 percent CO_2 at a density of 5×10^5 cell/ml in RPMI 1640 tissue culture medium plus 20 percent fetal calf serum containing one of the following compounds: actinomycin D [National Cancer Institute (NCI), Cancer Therapy Evaluation Branch, NSC-3053, 25 μg/ml]; adriamycin (NCI, NSC-123127, 40 μg/ml); mitomycin C (NCI, NSC-26980, 25 μg/ml); cyclophosphamide (NCI, NSC-26271, 100 μg/ml); 5-fluorouracil (NCI, NSC-19893, 500 μg/ml); cytosine arabinoside (NCI, NSC-63878, 100 μg/ml); vincristine sulfate (NCI, NSC-67574, 20 μg/ml); 6-mercaptopurine (NCI, NSC-755, 500 μg/ml); puromycin dihydrochloride (Sigma, 5 μg/ml); or hydroxyurea (Schwarz/Mann, 500 μg/ml). After 1, 4, and 17 hours in culture with the drugs, the cells were washed and tested for their ability to synthesize macromolecules and for their sensitivity to killing by antibody and complement. In addition, after 17 hours in culture with the drugs, the cells were washed and reincubated at 37°C in drug-free RPMI 1640 plus 20 per cent FCS. One, 4, and 17 hours later, the cells were tested for their ability to synthesize macromolecules and for their sensitivity to killing by antibody and complement. The cytotoxicity assays were performed in VBS-gel (*18*) and determined as follows: 0.1 ml of cells (10^6 cell/ml) plus 0.1 ml of IgM antibody to Forssman antigen diluted 1:80 or antibody to line 1 tumor antigen diluted 1:15 were incubated for 30 minutes at 30°C. The cells were washed two times with VBS-gel; 0.1 ml of GPC diluted 1:8 was added to the cells, and the mixture was incubated for 60 minutes at 37°C. At this time, 0.1 ml of 0.4 percent trypan blue was added, and the percentage of cells stained with trypan blue was determined. Controls consisted of cells plus GPC alone and cells plus VBS-gel alone; the viability of control cells was > 90 percent throughout the experiments. The tumor cells were tested for their ability to synthesize macromolecules as follows: 0.5 ml of drug treated or untreated cells (5×10^5 cell/ml) were incubated for 60 minutes at 37°C in RPMI 1640 plus 20 percent FCS containing [^{3}H]thymidine (20 μc/ml; Amersham/Searle, 24.6 mc/mmole), [^{3}H]uridine (20 μc/ml; Amersham/Searle, 46 c/mmole), [^{3}H]glucosamine hydrochloride (1 μc/ml; Amersham/Searle, 3 c/mmole), [^{14}C]galactosamine hydrochloride (10 μc/ml; Amersham/Searle, 3 mc/mmole), [^{3}H]glucose (12.5 μc/ml; Amersham/Searle, 20 c/mmole), [^{3}H]fucose (20 μc/ml; New England Nuclear, 2780 c/mmole), [^{14}C]*N*-acetyl neuraminic acid (1.25 μc/ml; Amersham/Searle, 214 mc/mmole), [^{14}C]palmitic acid (5 μc/ml; Amersham/Searle, 57.9 mc/mmole), [^{14}C]linoleic acid (3 μc/ml; Amersham/Searle, 61 mc/mmole), or [^{14}C]oleic acid (0.5 μc/ml; New England Nuclear, 810 mc/mmole), or in media deficient in essential amino acids containing ^{14}C-labeled amino acids (20 μc/ml; Schwarz/Mann, approximately 320 mc/mmole, L-arginine, L-lysine, L-valine, L-leucine). The TCA precipitable activity was measured as described in (*13*). All determinations were performed in duplicate. Blanks consisting of medium plus labeled compounds alone were prepared; these values remained at < 50 count/min throughout. The incorporation of the labeled fatty acids into extractable complex cellular lipids was tested as described in (*11*). (A) The cells were treated with actinomycin D (similar results were obtained with adriamycin, puromycin, and mitomycin C). (B) The cells were treated with 5-fluorouracil (similar results were obtained with cyclophosphamide, cytosine arabinoside, vincristine sulfate, 6-mercaptopurine, and hydroxyurea). Symbols: ●, drug-treated cells tested with IgM antibody to Forssman antigen plus GPC; ▲, untreated control cells tested with IgM antibody to Forssman antigen plus GPC (a similar pattern of sensitivity to killing was observed when drug-treated and untreated cells were tested with antibody to line 1 tumor cells plus GPC); ○, [^{3}H]thymidine (control cells incorporated approximately 10,000 count/min); ■, [^{3}H]uridine (control cells incorporated approximately 10,000 count/min); □, ^{14}C-labeled amino acids (control cells incorporated approximately 5000 count/min); △, [^{3}H]glucosamine hydrochloride (control cells incorporated approximately 5000 count/min; similar results were obtained with [^{14}C]galactosamine and [^{3}H]glucose); ▽, [^{3}H]fucose (control cells incorporated approximately 25,000 count/min; similar results were obtained with [^{14}C]*N*-acetyl neuraminic acid); ◇, [^{14}C]palmitic acid (control cells incorporated approximately 50,000 count/min; similar results were obtained with [^{14}C]linoleic acid and [^{14}C]oleic acid).

In contrast to these data, tumor cells incubated with the effective drugs were not maximally inhibited (75 percent) in their incorporation of fatty acids into complex cellular lipids until after 17 hours of culture, corresponding to the time the cells were increased in their sensitivity to killing by antibody and complement (Fig. 1A). Four hours after washing and reculture in drug-free medium, the cells recovered their ability to incorporate fatty acids into complex lipids, concomitant with the recovery of their resistance to killing (Fig. 1A). None of the ineffective drugs inhibited fatty acid incorporation into cellular lipid at any of the time intervals (Fig. 1B). In addition, the inhibition of incorporation of fatty acids into complex lipids caused by the effective drugs could not be attributed to interference in transport of the molecules into the cell (data not presented). These results indicated a direct correlation between the resistance of the cells to humoral immune attack and their ability for complex lipid synthesis.

Physical means of inhibiting macromolecular synthesis were also studied. Line 1 cells heated for 30 minutes at 43°, 44°, or 45°C were inhibited by 80 to 90 percent in their ability to synthesize DNA, RNA, and protein; these cells, however, remained resistant to killing by antibody plus GPC and were not inhibited in their ability to incorporate fatty acids into complex lipid. Furthermore, line 1 cells x-irradiated with 6000 or 9000 roentgen were inhibited in DNA, RNA, and protein synthesis when tested immediately, 6 and 16 hours after irradiation (*13*). However, these cells were rendered susceptible to antibody-complement mediated killing and were inhibited in fatty acid incorporation only 6 and 16 hours after irradiation.

Attempts were made to inhibit lipid synthesis in line 1 cells without affecting DNA, RNA, or protein synthesis in order to determine whether these cells were sensitive to killing by antibody and GPC. Line 1 cells (5×10^5/ml) were incubated for 60 minutes at 37°C with the antilipidemic drug, Atromid-S (*14*). As shown in Table 1, DNA, RNA, and protein synthesis of line 1 cells incubated with $5 \times 10^{-4}M$ Atromid-S were inhibited by only 24, 19, and 35 percent, respectively; incorporation of palmitic acid into complex cellular lipids, however, was inhibited by 70 percent. These cells were increased in comparison to untreated cells in their sensitivity to killing by IgM antibody to Forssman antigen or antibody to specific tumor antigens plus GPC (Table 1). At a lower concentration ($1 \times 10^{-4}M$), the drug inhibited lipid synthesis by only 37 percent and was not effective in rendering the cells sensitive to killing; at a higher concentration ($1 \times 10^{-3}M$), the drug inhibited DNA, RNA, and protein synthesis to as great an extent as lipid synthesis (Table 1).

All modern models of cell membranes include a central role for lipids as necessary structural and functional components (*15*). In addition, there is evidence that complement can induce physical or chemical changes, or both, in the cell membrane (*8, 16*). It has been suggested that these changes may be associated with the lipid component of the membrane (*17*). The biochemical mechanisms of action of the drugs used in our studies have been studied extensively, but little is known regarding the mechanism whereby they increase the sensitivity of tumor cells to immune attack. Our present observations indicate that the metabolic inhibitors and chemotherapeutic agents used in our experiments may enhance tumor cell sensitivity to humoral immune attack by inhibiting cellular lipid synthesis, but not the synthesis of DNA, RNA, protein, or complex carbohydrates. This suggests that the resistance of cells to immune attack is related to their ability to synthesize lipid. There are several possibilities whereby this relationship can be explained: (i) lipid synthesis may serve as a mechanism to repair damage to the membrane resulting from complement action, (ii) synthesis of cellular lipids may be necessary to maintain areas on the cell surface that can serve as substrates for complement without disturbing membrane integrity, (iii) the synthesized lipids could be part of anticomplementary moieties on the cell membrane affecting the binding or activation of complement components, or (iv) the synthesized lipids may be exported from the cell and become anticomplementary to fluid phase complement.

By identifying the particular lipid moieties that may be essential to the ability of these tumor cells to resist killing by antibody and complement, it may be possible to determine what role lipid synthesis plays in enabling tumor cells to resist immune attack. This may not only further our understanding of the biology of tumor cells but could result in more informed methods of cancer treatment.

SEYMOUR I. SCHLAGER
SARKIS H. OHANIAN
Laboratory of Immunobiology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

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19. We thank T. Borsos for his invaluable support and advice.

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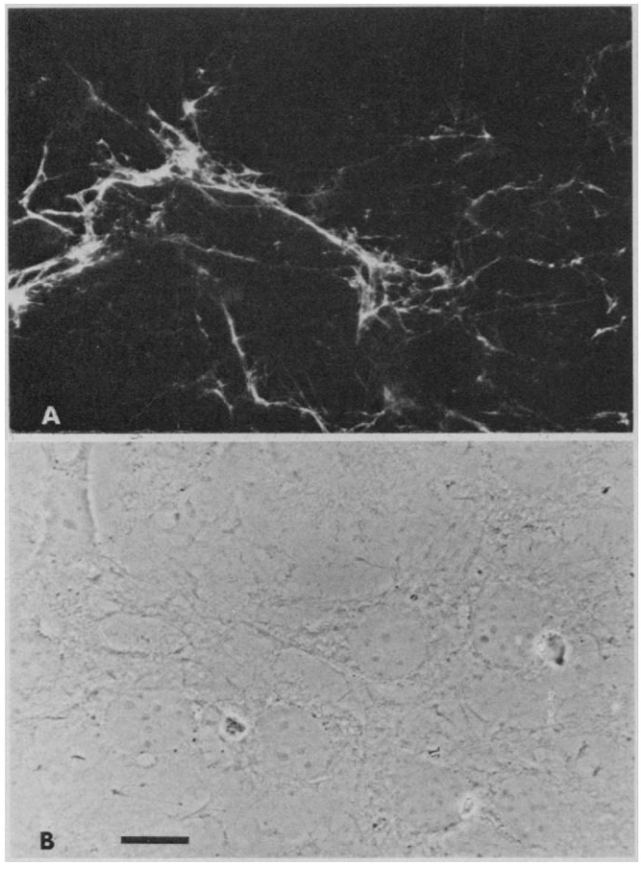
Control of a Cell Surface Major Glycoprotein by Epidermal Growth Factor

Abstract. *When the serum concentration of the culture medium is below 0.7 percent, 3T3 mouse cells lose most of their large external transformation sensitive (LETS) protein at the cell surface. Subsequent addition of epidermal growth factor results in the reappearance of massive fibrillar LETS protein networks on the surface of confluent 3T3 cells. Thirteen other hormones tested do not have this effect. It appears that epidermal growth factor can control the expression of LETS protein.*

There is considerable interest in the large external transformation sensitive (LETS) protein, which is also known as cell surface protein (CSP), fibronectin, or galactoprotein a (*1*). These proteins are the only class of cell surface glycoprotein consistently reduced or absent in numerous transformed cell lines (*2*). There seems to be an inverse correlation between increase in tumorigenicity and decrease in the percentage of cells expressing LETS protein (*3*), and among 50 tumorigenic lines tested, 45 show significant reductions in LETS protein as detected by indirect immunofluorescence (*4*). Furthermore, the addition of LETS protein can partially restore to transformed cells normal morphology, adhesion, and contact inhibition of movement (*5*). Finally, the observation that LETS protein on the cell surface is altered during myogenesis led to the recognition of the involvement of LETS protein in myoblast fusion (*6*).

In attempts to understand the control of the expression of LETS protein at the cell surface, previous investigators showed that cell density (*7*) and cell-cell contact (*3*) are the prime events mediating its expression. It is only at the stage of extensive cell contact that massive fibrillar networks of LETS protein are formed (*3*, *8*). In most of these experiments, 5 to 10 percent serum was present in the culture medium, and it was not generally realized that the serum might have affected the expression of LETS protein. While studying the effect of



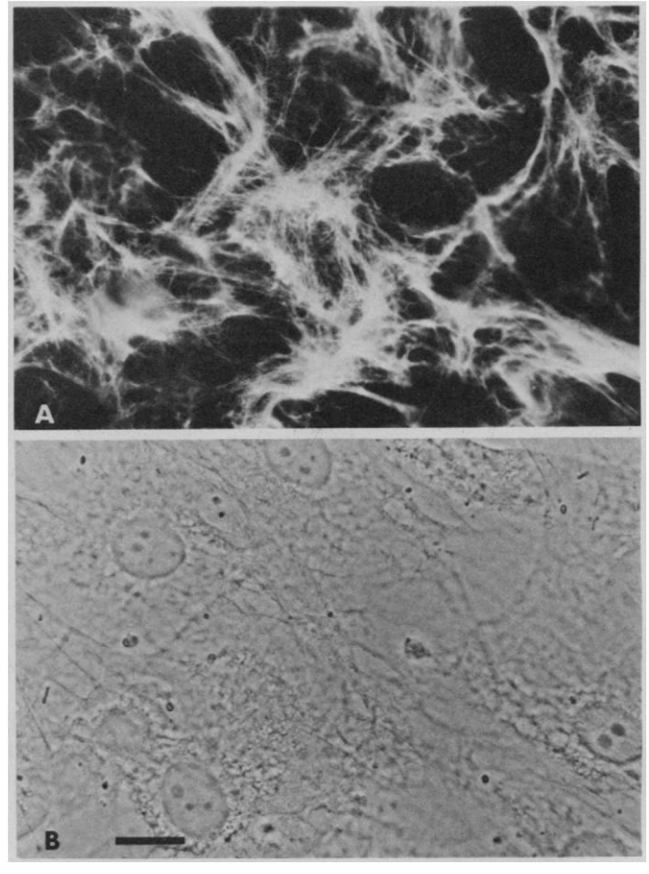


Fig. 1 (left). (A) Indirect immunofluorescence of 3T3 cells with LETS protein antiserum. The procedures for immunofluorescence staining and the specificity of the antiserum were described previously (*3*, *6*). 3T3 cells at confluency were incubated in Dulbecco's modified Eagle's medium containing 0.7 percent calf serum for 2 days. Most of the cells in culture lost LETS protein on the cell surface under these conditions. The photograph is of a field where LETS protein is most prominent. (B) Phase contrast micrograph of the same field. (Scale bar, 15 μm) Fig. 2 (right). (A) A culture similar to that in Fig. 1, but photographed 30 hours after the addition of EGF (1 ng/ml). (B) Phase contrast micrograph of the same field. (Scale bar, 15 μm)