

Enhancing Effect by Metabolic Inhibitors on the Killing of Tumor Cells by Antibody and Complement¹

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SUMMARY

Two chemically induced, antigenically distinct guinea pig hepatoma cell lines, line 1 and line 10, which are resistant to killing by rabbit anti-Forsman or specific antitumor antibody and complement, can be rendered susceptible when the cells are pretreated with metabolic inhibitors and drugs commonly used for the treatment of cancer patients. The effect appears within 7 hr after initial contact with the inhibitors and is dependent on temperature and on inhibitor concentration; the effect is reversible within 7 hr, and the process of reversion is also temperature dependent.

Not all preparations of tumor cells were rendered susceptible following treatment with inhibitors. In some cases, susceptibility to killing by complement was observed with anti-Forsman antibody but not antitumor antibody.

No clear correlation between known metabolic inhibitory activity of the inhibitors and conversion to the sensitive state could be made.

The results suggest that properties of nucleated cells, which are under metabolic control, play an important role in the killing efficiency of antibody and complement.

INTRODUCTION

It is generally known that different types of nucleated cells are not equally susceptible to the action of antibody and complement. In analyzing the reasons for this variability, we have concluded that resistance to cytotoxicity is in part due to an intrinsic property of the cell; *i.e.*, cells can either prevent damage to their surface or repair damaged sites. We obtained support for the idea of damage prevention or repair from preliminary studies on the effect of metabolic inhibitors (22) and chemotherapeutic drugs (21) on immune cytotoxicity.

The purpose of this paper is to document in detail various aspects of inhibitor-cell interaction to elucidate further the

mechanism whereby nucleated cells resist cytotoxic attack by antibody and complement.

MATERIALS AND METHODS

Animals. Inbred Sewall-Wright strain 2 guinea pigs were obtained from the Laboratory Aids Branch, Division of Research Services, NIH. The animals weighed approximately 250 g.

Cells and Reagents. Sheep erythrocytes were collected and washed as described in Ref. 20.

Two antigenically distinct diethylnitrosamine-induced hepatic tumors (ascitic form), line 1 and line 10 passed in strain 2 guinea pigs, were collected as described in Refs. 16, 26, and 27 and suspended in Medium 1640 (GIBCO⁴ No. 187G) containing 15% FCS (GIBCO No. 614) and 1% antibiotic-antimycotic mixture (GIBCO No. 524) (RPMI 1640-15% FCS).

The inhibitors used in these studies were: hydroxyurea (Schwarz/Mann, Orangeburg, N.Y.; Lot X1326); puromycin dihydrochloride (Sigma Chemical Co., St. Louis, Mo.; Lot 43C-0403); 5FU (Roche Laboratories, Nutley, N.J.; Lot 0776-08213); Mitomycin C (Schwarz/Mann; Lot 771138); actinomycin D (Schwarz/Mann; Lot 41036); cytosine arabinoside (NCI, Cancer Therapy Evaluation Branch, Bethesda, Md.; NSC 63878); 6MP (NCI; NSC 755); vincristine sulfate (NCI; NSC 67574); methotrexate (NCI; NSC 740); cyclophosphamide (Meade Johnson & Co., Evansville, Ind.); adriamycin (NCI; NSC 123127); azacytidine (NCI; NSC 102816).

Sera and Antisera. Rabbit IgM antiserum to sheep Forsman antigen was prepared as described previously (20). Ten ml were heated at 56° for 30 min and passed through a column of Sephadex G-200. The proteins in the void volume were collected, pooled, and stored at -30° until used.

Rabbit anti-line 1 and anti-line 10 antisera were prepared as described in Ref. 1. The antisera were heated at 56° for

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⁴ The abbreviations used are: GIBCO, Grand Island Biological Co. (Grand Island, N.Y.); RPMI, Roswell Park Memorial Institute; FCS, fetal calf serum; 5FU, 5-fluorouracil; NCI, National Cancer Institute; 6MP, 6-mercaptopurine; HUC, human complement; GPC, guinea pig complement.

30 min and absorbed with sheep erythrocytes; the appropriate tumor cells at 4°, filtered, and stored at -30°.

Normal HUC, absorbed with sheep erythrocytes and line 1 and line 10 cells at 4°, and normal GPC (JEM Research Products, Inc., Kensington, Md.) were stored at -35°.

Antibody-Complement-mediated Cytotoxicity of Nucleated Cells. This method for determining antibody-complement-mediated killing has been described (16). Briefly, 0.1 ml of diluted antiserum was added to 0.1 ml of tumor cells (10⁶/ml). After incubation for 30 min at 30°, the cells were washed and incubated with 0.1 ml GPC or HUC (diluted 1/8) for 60 min at 37°. One-tenth ml 0.4% trypan blue was added, and those cells taking up the dye were counted visually. Controls included cells plus culture medium and cells plus complement alone. The error of the test is less than ±10%; a drug was considered effective if the sensitivity of the drug-treated cells to killing by antibody plus complement was 2-fold or more higher than that of the untreated cells.

RESULTS

Antibody-Complement-mediated Killing of Inhibitor-treated Tumor Cells. Two and one-half million line 1 or line 10 tumor cells in 5.0 ml RPMI 1640-15% FCS con-

taining appropriate concentrations of inhibitors (determined from dose-response experiments described below) were incubated at 37° under air-5% CO₂. Control suspensions were prepared concurrently with medium alone. After 17 hr the cells were washed 3 times with 10 ml prewarmed fresh RPMI 1640-15% FCS, resuspended to 10⁶/ml of the culture medium, and assayed for their susceptibility to the killing by antibody and GPC or HUC. Charts 1A, 2A, and 3A (line 10 cells) and Charts 1B, 2B, and 3B (line 1 cells) illustrate the results of experiments determining the effect of inhibitors on tumor cells collected at different times. Only complement control values are given, since they are not significantly different from the cell controls (22).

The data in Chart 1A show that, of the purine and pyrimidine analogs tested, only azacytidine was effective in increasing the sensitivity of line 10 cells to killing by GPC with either of the antibodies. Different preparations of line 10 cells treated with azacytidine differed from one another in their sensitivity to immune attack. Tumor cells treated with 6MP, 5FU, or cytosine arabinoside were as resistant to killing as were control cells.

Line 1 tumor cells could be killed by anti-Forsman antibody and GPC following treatment with any of the 4 inhibitors (Chart 1B). However, only tumor cells treated

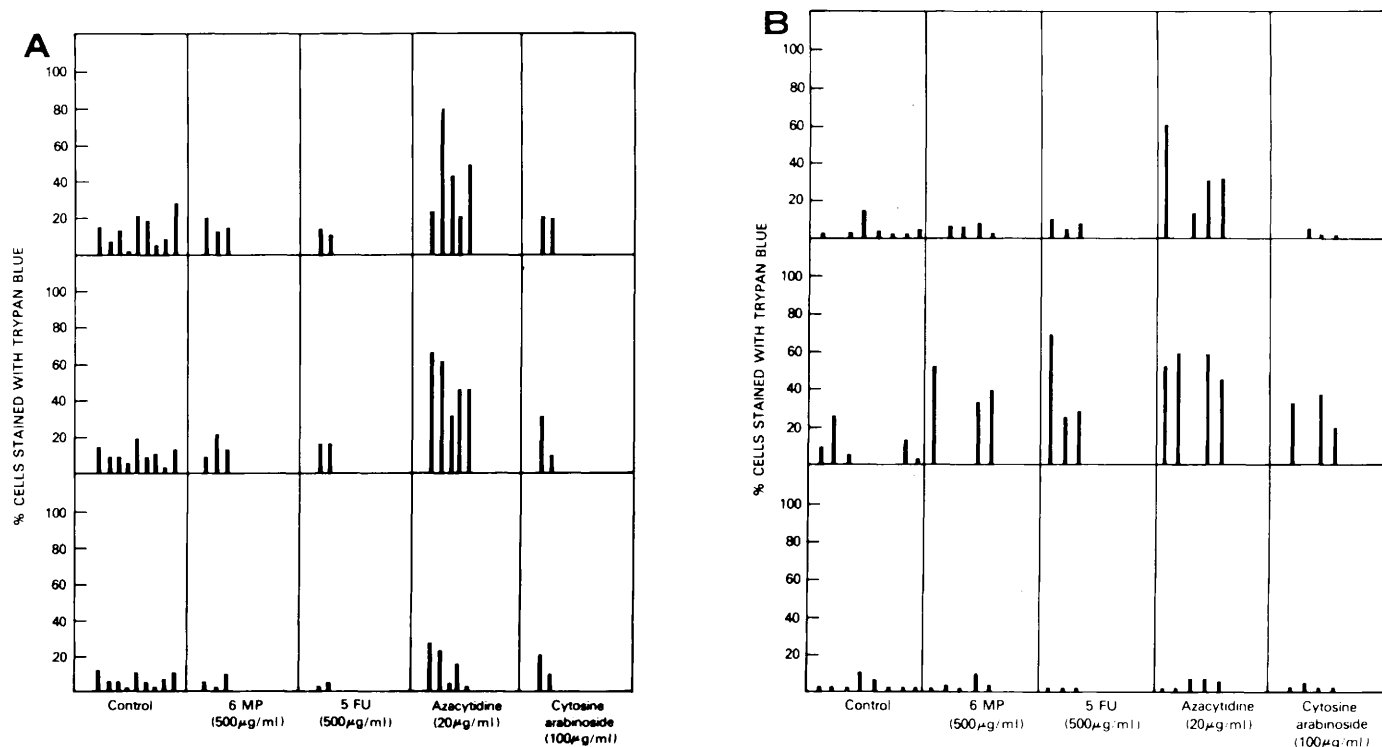


Chart 1. A, effect of purine and pyrimidine analogs on sensitivity of line 10 tumor cells to killing by antibody and GPC. Tumor cells were incubated in RPMI 1640-15% FCS containing the indicated concentration of inhibitor for 17 hr at 37° in air-5% CO₂ prior to testing for sensitivity to antibody-complement-mediated killing. Top, specific rabbit anti-line 10 antibody diluted 1/20 plus GPC diluted 1/8; middle, rabbit anti-Forsman antibody diluted 1/2 plus GPC diluted 1/8; bottom, tissue culture medium plus GPC diluted 1/8. B, effect of purine and pyrimidine analogs on sensitivity of line 1 tumor cells to killing by antibody and GPC. Tumor cells were incubated in RPMI 1640-15% FCS containing the indicated concentration of inhibitor for 17 hr at 37° in air-5% CO₂ prior to testing for sensitivity to antibody-complement-mediated killing. Top, specific rabbit anti-line 1 antibody diluted 1/30 plus GPC diluted 1/8; middle, rabbit anti-Forsman antibody diluted 1/100 plus GPC diluted 1/8; lower, tissue culture medium plus GPC diluted 1/8.

with azacytidine were sensitive to killing by specific antitumor antibody and GPC. As illustrated with line 10 cells, different preparations of line 1 cells also showed variance in their susceptibility to killing after treatment with inhibitors.

The sensitivities of line 10 and line 1 cells to killing following treatment with a folate antagonist (methotrexate), 2 alkylating agents (cyclophosphamide and Mitomycin C), an inhibitor of protein synthesis (puromycin), or an inhibitor that affects mitosis (vincristine sulfate) are shown in Chart 2. The control untreated cells for comparison are shown in Chart 1. Treatment of line 10 cells with puromycin or Mitomycin C rendered them very susceptible to killing by either antibody and GPC (Chart 2A). One of 4 and 3 of 5 preparations of line 10 cells were susceptible following treatment with methotrexate or vincristine sulfate, respectively. Cyclophosphamide treatment was of doubtful effectiveness.

Line 1 tumor cells showed similar results; *i.e.*, Mitomycin C or puromycin treatment was very effective with all cell preparations tested, while vincristine sulfate and methotrexate were active for some of the cell preparations. Two of 4 cyclophosphamide-treated preparations of line 1 cells were sensitive to killing by anti-Forsman antibody, and none of the 4 preparations were sensitive to killing by specific anti-line 1 antibody and GPC.

Chart 3 shows the results obtained with line 10 and line 1 cells treated with inhibitors that interact with DNA. The control untreated cells for comparison are shown in Chart 1.

Line 10 cells were susceptible to killing by GPC with either of the antibodies following treatment with actinomycin D, adriamycin, or hydroxyurea (Chart 3A). Again, as shown above, not all preparations of the tumor cells were susceptible to killing following drug treatment and hydroxyurea did not appear to be as effective as actinomycin D or adriamycin. Similar results were observed following treatment of line 1 cells except that hydroxyurea-treated cells were resistant to killing by tumor-specific antibody but not by anti-Forsman antibody (Chart 3B).

The results of the next experiment showed that increased susceptibility to killing could be demonstrated with human serum as a source of complement. However, cells of both tumor lines are sensitive to killing by either antibody and HUC without pretreatment with inhibitors (15). Therefore the experiments demonstrating increased killing were performed with dilutions of antibody at which no killing or minimal killing by HUC occurred. As shown in Table 1, line 10 cells were more sensitive than the untreated control cells to killing by either antibody and HUC following treatment with puromycin, Mitomycin C, methotrexate, 5FU, or 6MP. Increased killing by specific antitumor antibody and HUC was observed with cells pretreated with the remaining inhibitors except hydroxyurea. Line 1 tumor cells showed increased susceptibility to both antibody and HUC following pretreatment with actinomycin D, puromycin, adriamycin, cytosine arabinoside, vincristine sulfate, azacytidine, or 5FU. Increased susceptibility to killing by anti-

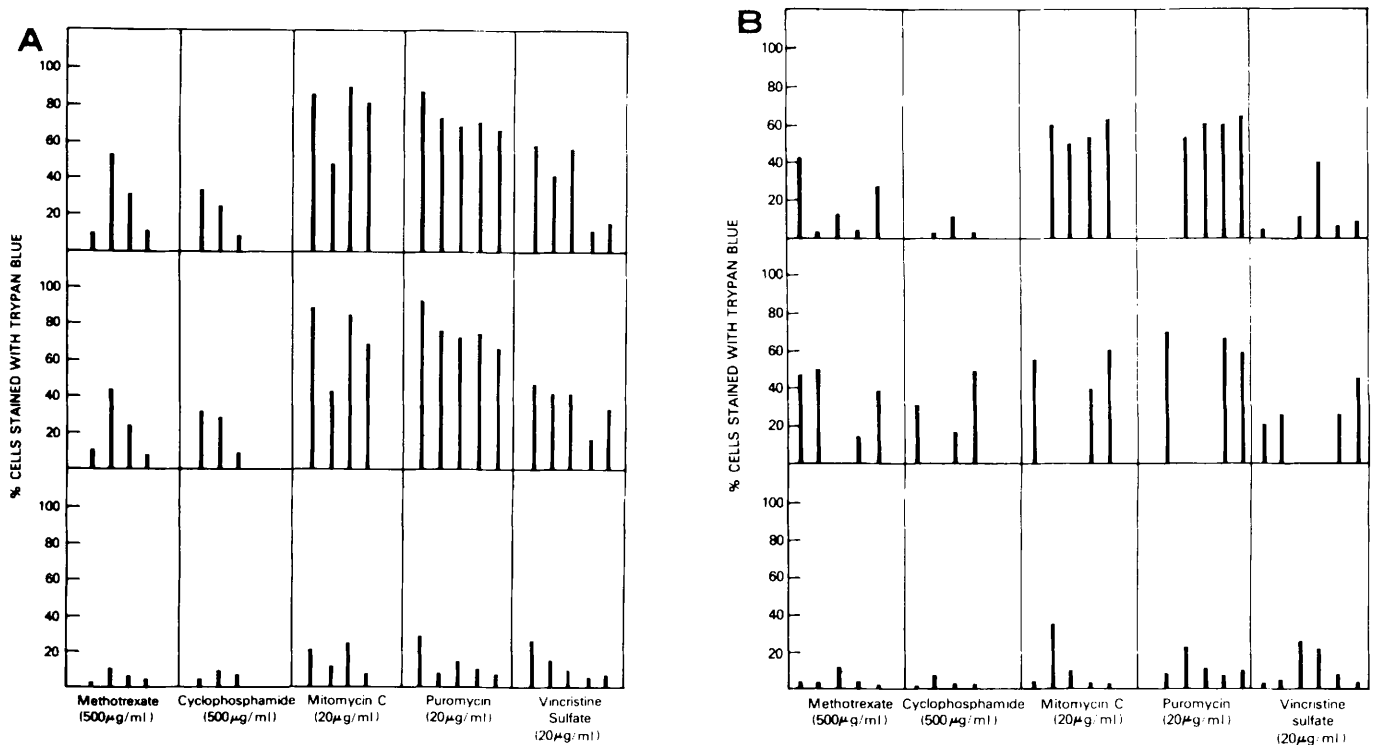


Chart 2. *A*, effect of an antifolate antagonist, alkylating agents, inhibitor of protein synthesis, and inhibitor of mitosis on sensitivity of line 10 tumor cells to killing by antibody and GPC. See Chart 1A for treatment and reagents used in the *upper*, *middle*, and *lower* panels. *B*, effect of an antifolic antagonist, alkylating agents, an inhibitor of protein synthesis, and an inhibitor of mitosis on sensitivity of line 1 tumor cells to killing by antibody and GPC. See Chart 1B for treatment and reagents used in *upper*, *middle*, and *lower* panels.

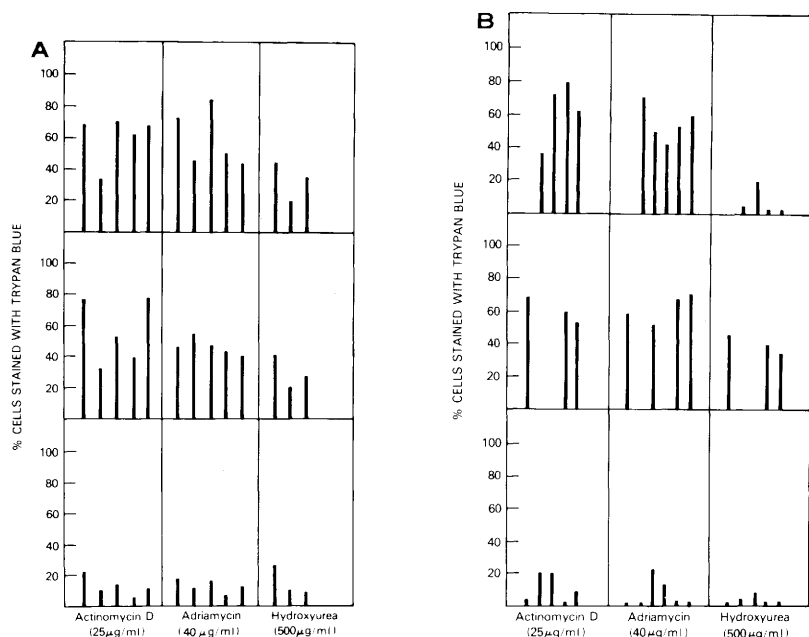


Chart 3. *A*, effect of inhibitors that interact with DNA and sensitivity of line 10 tumor cells to killing by antibody and GPC. See Chart 1*A* for treatment and reagents used in the upper, middle, and lower panels. *B*, effect of drugs that interact with DNA and sensitivity of line 1 tumor cells to killing by antibody and GPC. See Chart 1*B* for treatment and reagents used in upper, middle, and lower panels.

Table 1
Sensitivity of inhibitor-treated line 10 and line 1 cells to killing by antibody and HUC

Both tumor cell lines were cultured for 17 hr at 37° in air-5% CO₂ in RPMI 1640-20% FCS containing the inhibitors listed. They were then washed and tested for their sensitivity to killing by antibody and human complement diluted 1/8.

Treatment	Concentration of inhibitors (µg/ml)	% cells stained with trypan blue			
		Anti-Forsman + C (1/2)	Anti-line 10 + C (1/30)	C	Medium
Line 10	Control	34	6	4	1
	Actinomycin D 25	48	33	13	5
	Puromycin 25	95	84	43	24
	Mitomycin C 25	79	65	20	16
	Hydroxyurea 500	43	24	10	14
	Adriamycin 40	61	68	23	21
	Vincristine sulfate 20	38	53	21	13
	Cytosine arabinoside 100	43	36	12	12
	Azacytidine 20	61	56	24	20
	Methotrexate 500	79	43	11	6
	Cyclophosphamide 100	45	28	3	7
	5FU 500	67	50	4	12
	6MP 500	58	47	16	10
Line 1	Tissue culture medium	10	0	2	2
	Actinomycin D 25	45	34	21	8
	Puromycin 20	82	75	29	36
	Mitomycin C 20	36	69	22	5
	Hydroxyurea 500	30	9	1	4
	Adriamycin 40	60	48	20	12
	Vincristine sulfate 20	38	23	9	6
	Cytosine arabinoside 100	22	15	4	2
	Azacytidine 20	50	55	11	7
	Cyclophosphamide 500	16	12	10	2
	5FU 500	37	24	3	2
	6MP 500	41	9	3	6

line 1 antibody but not anti-Forssman antibody occurred following pretreatment with Mitomycin C. The opposite was observed following pretreatment with hydroxyurea or 6MP. No increase in susceptibility to either antibody and HUC occurred following pretreatment with cyclophosphamide.

Effect of Inhibitor Concentration in Increasing the Sensitivity of Tumor Cells to Antibody-GCP-mediated Killing. Dose-response experiments were carried out with several of the inhibitors to determine the concentration of inhibitor that was least toxic but that gave maximal sensitivity to antibody-GPC-mediated killing. Two and one-half million line 10 tumor cells in 5.0 ml RPMI 1640-15% FCS containing different concentrations of inhibitors were incubated at 37° under air-5% CO₂. Controls, treatment, washing, and testing for susceptibility were as described above. Chart 4 shows the results obtained with 5 of the 12 inhibitors used in these studies. Sensitivity to killing by either antibody and complement was dependent on the concentration of inhibitors used to treat the cells. Adriamycin was an exception; a 40- $\mu\text{g/ml}$ dose was more effective than 200 $\mu\text{g/ml}$. In repeat experiments with this inhibitor on different preparations of line 10 cells, a 200- $\mu\text{g/ml}$ dose was either toxic for the cells or was as effective as 40 $\mu\text{g/ml}$.

Similar experiments carried out with line 1 tumor cells also showed that increased sensitivity was dependent on the concentration of inhibitors used to treat the cell.

Effect of Antibody and Complement Concentration on Killing of Inhibitor-treated Tumor Cells. Line 10 tumor cells were cultured for 17 hr with actinomycin D (25 $\mu\text{g/ml}$), azacytidine (20 $\mu\text{g/ml}$), Mitomycin C (25 $\mu\text{g/ml}$), or vincristine sulfate (20 $\mu\text{g/ml}$); washed, and resuspended to 10⁶/ml RPMI 1640-15% FCS. The cytotoxicity test was carried out as described in "Materials and Methods." Chart 5 shows the results obtained with varying antibody concentration and constant GPC. In all cases killing was dependent on antibody concentration, and complement control values were not significantly different from cell control values (*i.e.*, cells plus medium alone).

To determine whether the increased susceptibility to killing was dependent on complement concentration, untreated control and inhibitor-treated tumor cells were sensitized with near-saturating levels of antitumor antibody and then varying GPC concentration was added. In all cases studied the percentage of cells killed was dependent on complement concentration. Chart 6 gives representative curves obtained with Mitomycin C- or actinomycin D-treated cells. Similar results were obtained with cells treated with other active inhibitors or with anti-Forssman antibody-sensitized cells. In other experiments antibody alone or complement plus antibody specific for the opposite tumor did not kill the inhibitor-treated cells. Antibody dose-response experiments were also performed with line 1 cells treated with puromycin (25 $\mu\text{g/ml}$), methotrexate (500 $\mu\text{g/ml}$), azacytidine (20 $\mu\text{g/ml}$), 5FU (500 $\mu\text{g/ml}$), and 6MP (500 $\mu\text{g/ml}$). All inhibitor-treated cells showed increased sensitivity to killing by anti-Forssman antibody and GPC compared to the control untreated cells (Chart 7A).

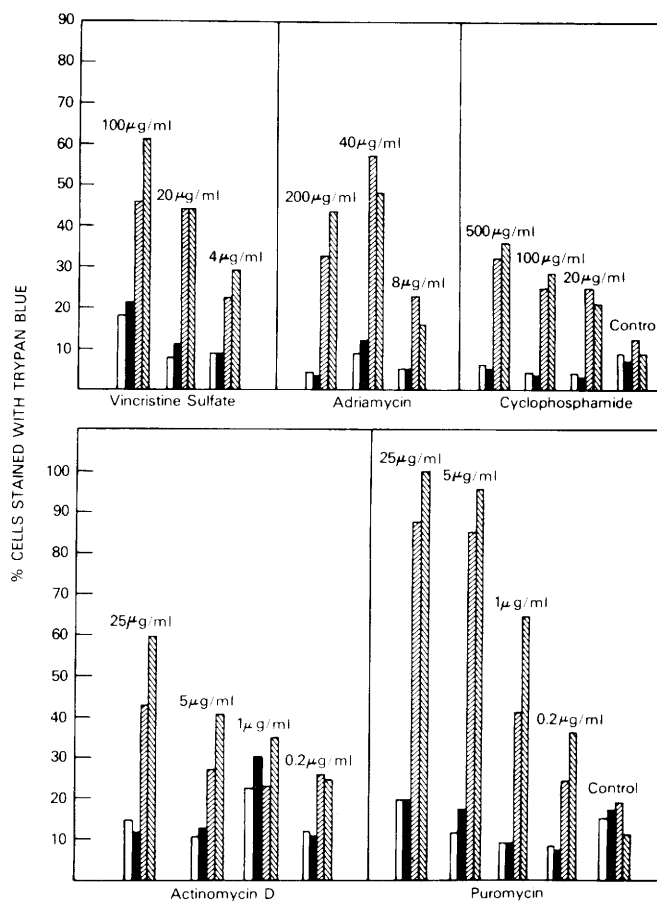


Chart 4. Effect of inhibitor concentration on sensitivity of line 10 tumor cells to killing by anti-antibody and GPC. Tumor cells were incubated for 17 hr at 37° in air-5% CO₂ in RPMI 1640-20% FCS containing the concentrations of inhibitor indicated above each group of 4 bars. Controls are results with tumor cells cultured in medium free of inhibitors. □, cells plus buffer control; ■, cells plus GPC diluted 1/8 control; ▨, cells plus anti-Forssman antibody diluted 1/2 plus GPC diluted 1/8; ▩, cells plus specific antitumor antibody diluted 1/20 plus GPC diluted 1/8.

Increased sensitivity to killing by anti-line 1 antibody and GPC was observed following treatment with puromycin, methotrexate, and azacytidine (Chart 7B). Tumor cells treated with 5FU were killed to approximately 55% following sensitization with saturating levels of anti-line 1 antibody, while 6MP-treated cells were as resistant as the untreated cells to all antibody concentrations tested.

Effect of Temperature on Inhibitor Treatment of Tumor Cells. In these experiments line 10 tumor cells were cultured at 4° and 37° in RPMI 1640-15% FCS containing actinomycin D (25 $\mu\text{g/ml}$), puromycin (5 $\mu\text{g/ml}$), adriamycin (50 $\mu\text{g/ml}$), azacytidine (20 $\mu\text{g/ml}$), or medium free of inhibitors. After incubation for 17 hr the cells were washed, resuspended to 10⁶/ml, and examined for the sensitivity to killing by anti-Forssman or specific anti-line 10 and GPC. In all cases studied the cells cultured with inhibitors at 4° were as resistant to killing by either antibody and GPC as were cells cultured in inhibitor-free medium (Table 2). On

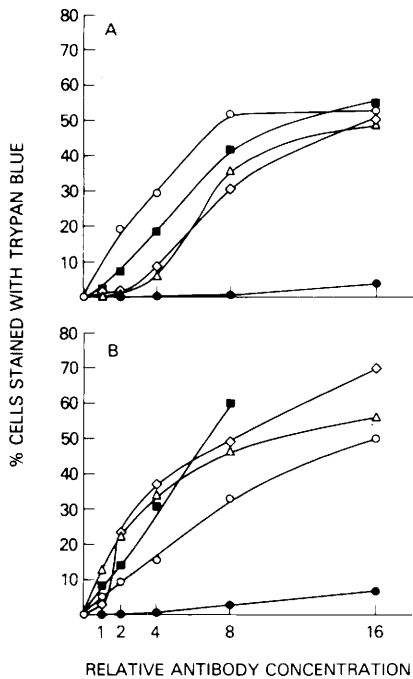


Chart 5. Effect of antibody concentration on killing of inhibitor-treated line 10 tumor cells. Tumor cells cultured with actinomycin D (O), Mitomycin C (■), vincristine sulfate (◇), azacytidine (Δ), or medium free of inhibitors (●) for 17 hr at 37°. See text for concentration of inhibitors used and cytotoxicity test. *A*, anti-Forsman (1 = 1/32); *B*, specific anti-line 10 antibody (1 = 1/160). GPC used at a dilution of 1/8.

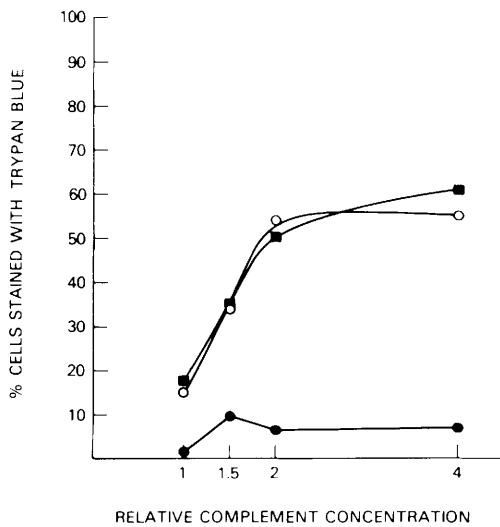


Chart 6. Effect of GPC concentration on killing of antibody-coated, inhibitor-treated line 10 cells. Tumor cells treated with actinomycin D (O), Mitomycin C (■), or medium free of inhibitors (●) for 17 hr at 37°. Cells coated with specific anti-line 10 diluted 1/10. 1 = 1/32 dilution of GPC.

the other hand, cells cultured at 37° with the inhibitors were sensitive to killing.

Effect of Time of Inhibitor Treatment of Tumor Cells. Portions of line 10 tumor cells were incubated in RPMI 1640-15% FCS with and without inhibitors for 17 hr as described earlier. The contents of each incubation mixture were washed and divided into 2 parts. One part of each

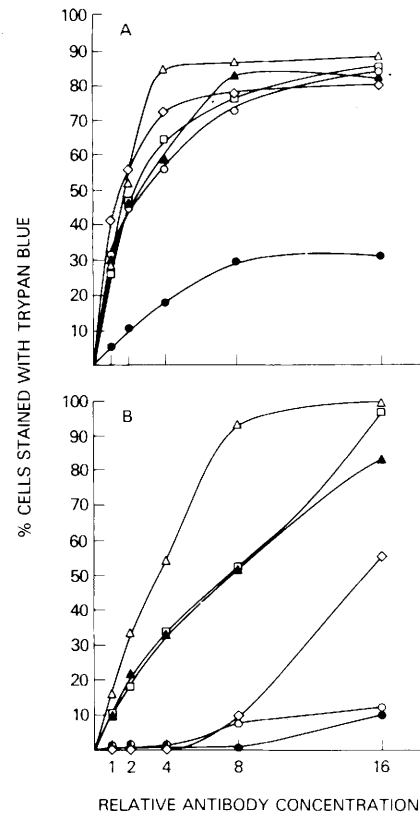


Chart 7. Effect of antibody concentration on killing of inhibitor-treated line 1 tumor cells. Tumor cells were cultured with puromycin (▲), azacytidine (Δ), methotrexate (□), SFU (◇), and 6MP (O) or medium free of inhibitors (●), for 17 hr at 37°. See text for concentration of inhibitors used and cytotoxicity test. *A*, anti-Forsman antibody (1 = 160); *B*, specific anti-line 1 antibody (1 = 1/100) GPC used at a dilution of 1/8.

mixture received medium with inhibitor, and the other part received medium without the inhibitor. Samples were removed at this period of time (0 time) and after incubation for an additional 4, 7, and 24 hr; washed; and checked for sensitivity to antibody- and GPC-mediated cytotoxicity as described in "Materials and Methods." Anti-Forsman antibody diluted 1/2 or anti-line 10 antibody diluted 1/20 were used to sensitize the cells for killing by GPC. Chart 8 shows results obtained with puromycin (5 μg/ml). It will be seen that the inhibitor-treated cells begin to show resistance to killing by antitumor antibody (Chart 8*A*) or anti-Forsman antibody (Chart 8*B*) after culturing for 4 to 7 hr in medium free of inhibitor and that maximal resistance to both antibodies and GPC is reached within 24 hr. The reversion of inhibitor-treated cells from susceptible to resistant occurred at 37° but not at 4° (data not shown). This chart also shows that untreated cells transferred to medium with puromycin became sensitive to killing within 4 to 7 hr and reach maximum sensitivity to both antibodies and GPC within 24 hr. Similar results were obtained with other active inhibitors.

DISCUSSION

We have extended our preliminary observations (21, 22) that antibody-sensitized line 1 and line 10 tumor cells that

Table 2
Antibody-complement-mediated killing of line 10 cells cultured for 17 hr at 4° and 37° in the presence of inhibitors

Treatment ^a	% cells stained with trypan blue ^b							
	4°				37°			
	Anti-Forssman + C	Anti-line 10 + C	C alone	Medium alone	Anti-Forssman + C	Anti-line 10 + C	C alone	Medium alone
Tissue culture medium	11	4	1	2	5	0	2	2
Actinomycin	6	5	2	1	38	37	10	9
Puromycin	7	9	1	2	79	75	7	11
Azacytidine	7	6	3	3	37	44	4	6
Adriamycin	6	4	2	1	40	45	7	4

^a See text for concentration of inhibitors used and treatment of cells.

^b Anti-Forssman antibody diluted 1/2; anti-line 10 antibody diluted 1/20.

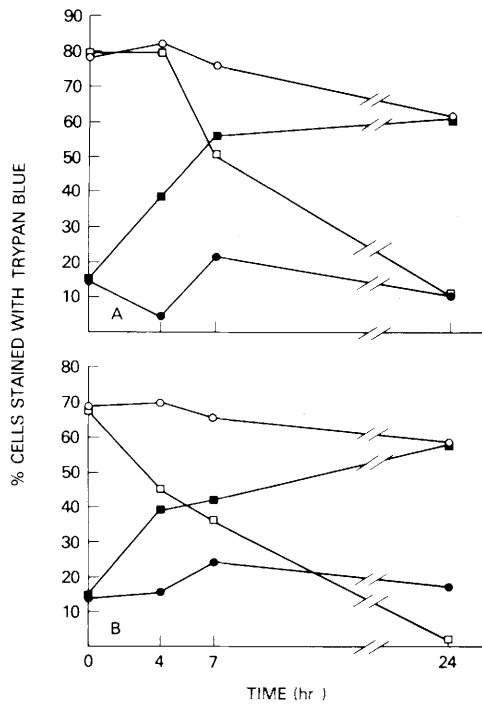


Chart 8. Effect of time of inhibitor treatment on sensitivity of line 10 tumor cells to antibody-complement-mediated cytotoxicity. See text for procedures. Tumor cells cultured in medium without puromycin (●); cultured for 17 hr without puromycin, washed, and cultured in medium with puromycin (■); cultured with puromycin (○); cultured for 17 hr with puromycin, washed, and cultured in medium without puromycin (□). A, specific anti-line 10 diluted 1/20; B, anti-Forssman antibody diluted 1/2. GPC diluted 1/8.

are normally resistant to killing by GPC can be made sensitive by incubating the cells with actinomycin D, puromycin, Mitomycin C, and some inhibitors commonly used in the treatment of cancer patients. This effect was dependent on inhibitor concentration, temperature, and time and was reversible. The sensitivity of these cells to antibody and HUC, to which they are normally susceptible,

can be further increased by inhibitor treatment. The observation with HUC is similar to the findings of other investigators who used cultured human cell lines normally sensitive to *HL-A* alloantibody and rabbit C (12). They found that cycloheximide and puromycin, but not actinomycin D treatment, increased the sensitivity of the cells.

One observation during these experiments was that occasionally 1 preparation of tumor cells may not be rendered susceptible to killing following treatment with a particular inhibitor, although most other preparations could be rendered susceptible by the same inhibitor. The reason for this variability is not clear at the moment; however, since the tumor cells used in these studies were obtained after growth *in vivo*, such variation may represent the effect of some host factors.

The mechanism whereby these inhibitors increase susceptibility to killing is not understood at present. The fact that agents that inhibit the synthesis of macromolecules also increase cell susceptibility to immune injury would suggest that at least part of the reason for the observed resistance of nucleated cells to killing by antibody and complement may be due to a property of the cell that is under metabolic control. This property of the cell may be the ability for preventing damage to cell surface areas under attack by complement and/or the ability for repairing surface areas damaged by complement. While we have no evidence to support the existence of such a property, our experiments on reversibility of inhibitor effects and the effect of temperature on inhibitor treatment support the hypothesis that macromolecular synthesis is necessary. These observations indicated that the effect of inhibitor was reversible; *i.e.*, cells treated with drugs for 17 hr and were therefore susceptible to killing reverted within 4 to 7 hr to resistant state. The reversion could not be ascribed to cell division, because the absolute number of cells did not change during the experiment. This was expected because the doubling time of line 10 cells is about 48 hr (8). The effectiveness of inhibitors in increasing cell susceptibility to killing depends on temperature; cells become sensitive to killing at 37° but not at 4°.

Furthermore reversion of inhibitor-treated cells from susceptible to resistant was also temperature dependent; the cells reverted at 37° but not at 4°.

Studies concerning the variability in the sensitivity of cells taken from different phases of their growth cycles suggest that cellular changes may be responsible for differences in resistance to killing by antibody and complement (2-4, 6, 13, 14, 18, 19, 24). Although the cells used by the various investigators were from different species and of different morphological types, some were more sensitive at either G₁ or S, and others were more sensitive at G₀ phases of their growth cycle. In some of these reports changes in membrane properties were indicated as the cause of increase in susceptibility to killing, usually without change in antigen concentration.

Other investigators have shown that cells treated with some of the inhibitors also used in our study either increased, decreased, or had no effect on antigen expression (5, 11). In the following paper (23) we report that increased sensitivity of pretreated cells to antibody-complement-mediated killing cannot be ascribed to changes in antigen expression or binding of GPC4 and GPC3 to the tumor cell membranes. This would confirm and extend our previous observations and those of other investigators that antigen expression, quantity of cell-bound antibody, or complement components cannot be correlated with cell killing (6, 10, 14-16, 19).

It has been shown that differences in membrane fragility to osmotic shock can be associated with cells taken from various phases of their growth cycle (9, 25). In preliminary experiments inhibitor-treated cells were found to be slightly more fragile than untreated control cells to sucrose solutions of low osmolarity (S. H. Ohanian, M. Segerling, and T. Borsos, unpublished observations).

Regardless of the mechanism of action of the various inhibitors, our observations may furnish a rational approach to studying cellular defense mechanisms against immune injury. It has been known for many years that the susceptibility of cells to the action of complement can be altered by treating the cell surface with various agents, e.g., enzymes. To explain variability in cell susceptibility in the absence of extraneous agents and to explain the need for thousands of complement-fixing antibody-antigen complexes on nucleated cells to effect killing, the existence of cellular repair (or defense) mechanisms has been postulated (15, 19). There is little or no evidence in the literature for the existence of such mechanisms.

Our studies indicate that intracellular events under metabolic and/or synthetic control modulate the susceptibility of the cell to killing by complement. Since the action of complement takes place on or in the cell membrane, we postulate that the intracellular events affected by the inhibition most probably control membrane integrity. Further experiments are needed to identify the end point(s) and pathways effecting control of cell membrane integrity.

The experiments reported here may imply that at least some part of the *in vivo* effectiveness of chemotherapeutic drugs may be due to their ability to increase the susceptibility of the tumor cell to immune attack by complement.

O'Neill and Davies (17) and Davies (7) reported that mice were protected against EL4 lymphoma when rabbit serum containing anti-EL4 antibody was injected (into EL4-bearing mice) shortly after the injection of nitrogen mustards of cytosine arabinoside. Our studies may provide a rational basis for combining chemotherapy and immunotherapy in the treatment of neoplastic diseases.

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