

# Effect of Metabolic Inhibitors on the Ability of Tumor Cells to Express Antigen and Bind Complement Components C4 and C3<sup>1</sup>

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## SUMMARY

Metabolic inhibitors commonly used in the treatment of cancer increase the ability of antibody and complement to kill guinea pig tumor cells *in vitro*. No correlation was found between increased killing and changes in cell surface antigen concentration or binding of complement components C4 and C3.

## INTRODUCTION

In preceding papers (14-16) we have shown that 2 diethylnitrosamine-induced guinea pig hepatoma cells, line 1 and line 10, will become sensitive to killing by antibody plus GPC<sup>4</sup> if the cells are pretreated with metabolic inhibitors and drugs commonly used in the treatment of cancer patients. This increased sensitivity could not be correlated with inhibition of surface antigen movement as measured by indirect fluorescent antibody techniques (2).

The experiments reported in this paper were designed to establish whether the increased sensitivity of the inhibitor-treated tumor cells was due to increased expression of antigen or binding of the initial components of GPC (C4 and C3).

## 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. 13.

Two antigenically distinct diethylnitrosamine-induced he-

patic tumors, line 1 and line 10, passed in strain 2 guinea pigs were collected as described in Refs. 18 and 19 and after washing were resuspended in Medium 1640 (Grand Island Biological Co., New York, N. Y.: No. 187G) containing 15% FCS (Grand Island Biological Co.; No. 614) and antibiotics (RPMI 1640-15% FCS).

All buffers were prepared as described in Ref. 13. Sheep erythrocytes with cell-bound GPC4 (EC4<sup>8p</sup>) were prepared as described in Ref. 8. Sheep erythrocytes with cell-bound GPC3 (EC43<sup>8p</sup>) were prepared as described in Ref. 9. Partially purified GPC1 and GPC2 were prepared as described in Ref. 13.

**Sera and Antisera.** Rabbit IgM antisera to sheep Forssman antigen was prepared as described in Ref. 13.

Rabbit anti-GPC3 was supplied by Dr. H. S. Shin (Johns Hopkins University) (17). Guinea pig anti-GPC4 was prepared as described in Ref. 8.

Rabbit anti-line 1 and anti-line 10 antisera were prepared as described in Ref. 1.

All antisera were heated at 56° for 30 min, absorbed with sheep erythrocytes at 4°, and stored at -20°. The antitumor antisera were further absorbed with appropriate tumor cells. Normal guinea pig sera (JEM Research Products, Inc., Kensington, Md.) were stored at -30°.

**Treatment of Tumor Cells with Inhibitors.** Tumor cells were treated with inhibitors as described in the preceding paper (16). Briefly, 500,000 line 1 or line 10 cells were cultured in 10 ml RPMI 1640-15% FCS containing actinomycin D, puromycin, adriamycin, azacytidine, or methotrexate for 17 hr at 37° under air-5% CO<sub>2</sub>. Controls consisted of cells cultured in RPMI 1640-15% FCS free of inhibitors. The concentrations of inhibitors in the medium were the lowest concentrations giving maximum sensitivity to antibody-GPC-mediated killing. After being cultured for 17 hr the control and inhibitor-treated cells were washed with prewarmed RPMI 1640-15% FCS, without inhibitors, and VBS-gel, and they were suspended to 10<sup>6</sup>/ml in VBS-gel.

**Antibody-Complement-mediated Cytotoxicity of Nucleated Cells.** The method for determining antibody-complement-mediated cytotoxicity has been described (11). Briefly, 0.1-ml dilutions of antisera were added to 0.1 ml VBS-gel containing target cells (10<sup>6</sup>/ml). After incubation for 30 min at 30° and washing, 0.1 ml guinea pig serum diluted 1/8 was added and the mixture was incubated at 37° for 60 min. The cells were suspended in 0.1 ml of 0.4%

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<sup>4</sup> The abbreviations used are: GPC, guinea pig complement; C1, C4, etc., components of complement; RPMI, Roswell Park Memorial Institute; FCS, fetal calf serum; VBS-gel, Veronal-buffered NaCl solution containing 0.1% gelatin, 0.001 M Mg<sup>2+</sup>, and 0.00015 M Ca<sup>2+</sup>; CIFT, C1 fixation and transfer test.

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trypan blue, and the percentage of cells taking up the dye was determined. Controls included cells incubated with Veronal-buffered saline, alone or plus complement diluted 1/8.

**CIFT.** This method for estimating cell-bound complement-fixing antigen-antibody complexes on a molecular basis has been described in detail (1, 13).

**Quantitation of GPC4 and GPC3 Bound to Tumor Cells.** The methods for the quantitation of these 2 components have been described in detail (8-10). They are based on the inhibition of specific anti-C4 and anti-C3 antibody by the respective antigens. The component to be measured is added to a standard amount of anti-complement antibody, and the residual antibody activity is determined by its capacity to sensitize appropriate indicator cells for lysis by GPC. By comparing the observed lysis with inhibition-of-lysis curves obtained with known amounts of specific components, the amount of test antigen can be estimated. The tests were carried out in 1.0-ml polystyrene Fisher tubes (Fisher Scientific 4-975-145) for use with the Fisher Model 59 centrifuge. The cells were collected after centrifugation at  $1500 \times g$  for 1 min at room temperature.

## RESULTS

**Measurements of Antigen Expression on Tumor Cells by CIFT.** In the preceding paper (16) we reported that both line 1 and line 10 tumor cells become sensitive to killing by antibody and GPC after the cells were incubated in culture medium containing selected metabolic inhibitors. For determination of whether the increase in sensitivity was due to an increase in antigen expression, the number of complement-fixing antibody-antigen complexes was determined with CIFT.

Following treatment with the inhibitors for 17 hr at  $37^\circ$ , the tumor cells were washed, resuspended to  $10^6$ /ml in VBS-gel, and examined for the sensitivity to antibody-complement-mediated killing and antigen expression. Table 1 gives the results of 4 experiments obtained with 4 different inhibitors of line 1 cells. Each experiment was performed on a different day with freshly prepared line 1 cells. Following inhibitor treatment, the cells of each preparation were more susceptible to killing by either antibody plus GPC. Control values for cells alone or cells plus GPC (not shown) were near 10% whether or not the cells were treated with inhibitors. Increased killing could not be correlated with changes in expression of antigen. Antigen expression as measured by CIFT could be either higher (azacytidine), lower (puromycin, adriamycin), or the same (methotrexate, adriamycin) as the concurrent controls.

Similar experiments were performed with line 10 tumor cells treated with inhibitors and their untreated controls. The results of 4 experiments in which different preparations of line 10 cells treated with inhibitors are shown in Table 2. Following treatment with the inhibitors, increased susceptibility to killing by antibody and GPC was noted. As expected, 6-mercaptapurine and hydroxyurea were not as effective as the other inhibitors tested. Control values for

Table 1  
Measurement of antigen expression and sensitivity to killing by antibody plus complement of line 1 hepatoma cells following treatment with inhibitors

Dilution of anti-Forsman antibody, 1/10; dilution of anti-line 1 antibody, 1/10.

$$\% \text{ trypan blue-stained cells} = \left( 1 - \frac{\% \text{ nonstained cells in experiment}}{\% \text{ nonstained cells in cell control}} \right) \times 100$$

	Anti-Forsman antibody		Anti-line 1 antibody	
	% trypan blue-stained cells	C1-fixing sites/cell ( $\times 10^{-4}$ )	% trypan blue-stained cells	C1-fixing sites/cell ( $\times 10^{-4}$ )
Experiment 1				
No treatment	24	4.9	52	3.3
Adriamycin (50 $\mu$ g/ml)	100	1.8	100	3.9
Experiment 2				
No treatment	50	4.0	24	6.8
Azacytidine (20 $\mu$ g/ml)	87	4.4	97	8.6
Experiment 3				
No treatment	51	3.2	33	4.6
Methotrexate (200 $\mu$ g/ml)	85	3.1	97	4.0
Experiment 4				
No treatment	37	10.4	12	8.1
Puromycin (25 $\mu$ g/ml)	82	4.2	82	2.9

cells alone or cells plus GPC (not shown) were near 10% whether or not the cells were treated with inhibitors. As noted with line 1 cells, increased killing of line 10 cells could not be ascribed to increased antigen expression. Decreased amounts of both Forsman and tumor-specific antigen was noted following treatment of cells with adriamycin and puromycin, while the other inhibitors showed either increase, decrease, or no change of one or the other antigen.

The results with line 1 and line 10 cells taken together suggest that antigen expression (as measured by CIFT) does not play a major role in determining the sensitivity of these tumor cells to killing by antibody and complement.

We next applied techniques designed to measure the amount of GPC components bound to tumor cells treated with inhibitors and control cells.

**Quantitation of GPC4 and GPC3 Bound to Inhibitor-treated Tumor Cells.** We wished to determine whether the increased sensitivity of inhibitor-treated tumor cells is accompanied by a change in the number of C4 and C3 fixed to the cell surfaces. Line 10 tumor cells were incubated in RPMI 1640-20% FCS with or without metabolic inhibitors for 17 hr at  $37^\circ$ . The cells were washed in medium free of inhibitor and resuspended to  $10^6$ /ml VBS-gel.

For determination of the amount of C4 and C3 bound to

Table 2

Measurement of antigen expression and sensitivity to killing by antibody plus complement of line 10 hepatoma cells following treatment with inhibitors

Dilution of anti-Forsman antibody, 1/2; dilution of anti-line 10 antibody, 1/15. See Table 3 for method of calculation of percentage of trypan blue-stained cells.

	Anti-Forsman antibody		Anti-line 10 antibody	
	% trypan blue-stained cells	C1-fixing sites/cell ( $\times 10^{-4}$ )	% trypan blue-stained cells	C1-fixing sites/cell ( $\times 10^{-4}$ )
Experiment 1				
No treatment	11	4.3	10	4.4
6-Mercaptopurine (500 $\mu$ g/ml)	25	3.6	32	4.8
Azacytidine (20 $\mu$ g/ml)	50	2.5	78	6.4
Experiment 2				
No treatment	5	3.0	6	15.4
Adriamycin (40 $\mu$ g/ml)	57	1.3	53	3.7
Experiment 3				
No treatment	16	8.4	20	36.4
Actinomycin D (25 $\mu$ g/ml)	40	4.5	59	34.0
Experiment 4				
No treatment	4	5.4	4	10.2
Puromycin (5 $\mu$ g/ml)	57	3.2	71	3.4
Hydroxyurea (500 $\mu$ g/ml)	11	5.0	25	6.0

the cells, each of 12 tubes received  $10^5$  inhibitor-treated cells in 100  $\mu$ l of medium and each of another 12 tubes received  $10^5$  untreated tumor cells in 100  $\mu$ l of medium. Equal volumes of anti-Forsman antibody, antitumor antibody, or VBS-gel were added to the appropriate tubes, and the mixtures were incubated for 30 min at 30°. After 1 washing with 1.0 ml VBS-gel, all tubes received 100  $\mu$ l GPC diluted 1/8. Two additional tubes were added at this time and received 100  $\mu$ l GPC diluted 1/8. All tubes were incubated for 60 min at 37° and then washed 6 times with 1.0 ml VBS-gel to remove excess GPC. One group of 13 tubes (6 inhibitor-treated, plus 6 untreated, plus 1 GPC alone) was assayed for C4, another group of 13 tubes was assayed for C3 as described in "Materials and Methods," and every tube containing cells was assayed for cytotoxicity. The results of 4 experiments are shown in Table 3.

All preparations of cells treated with inhibitors were susceptible to killing by either antibody plus GPC (Table 3; compare Columns 1 and 2) and that the amount of GPC4 bound to these cells was the same (azacytidine) or less than that bound to control cells (Table 3; compare Columns 3 and 4). Similar results were obtained when the cells were examined for the amounts of C3 bound to their surface.

Similar results were obtained with line 1 tumor cells; that is, increased sensitivity to killing by antibody and GPC was not accompanied by an increase in the quantity of C4 and C3 bound to the tumor cells.

## DISCUSSION

In this and the preceding paper we have shown that 2 guinea pig hepatoma cell lines normally resistant to killing by antibody and GPC can be rendered susceptible after treatment with selected metabolic inhibitors and drugs commonly used to treat cancer patients. In the present paper we have shown that the increased susceptibility is not due to a measurable increase in antigen expression (as measured by CIFT) or binding of the early-acting components of GPC, C4 and C3.

In most cases the amounts of antigen (Forsman or tumor-specific) detected on tumor cells pretreated with inhibitor were less than or equal to that found in the control cells. The only exceptions were line 1 and line 10 tumor cells pretreated with azacytidine. They expressed more tumor-specific antigen than did the control cells. These results confirm and extend our previous observations and those of other investigators that antigen expression and amount of antibody and complement components bound to cells do not correlate in a simple way with the efficiency of cell killing by antibody and complement (4, 5, 7, 10, 12).

Other investigators have also shown that preincubation of cells with metabolic inhibitors may or may not affect expression of a surface antigen. Ferrone *et al.* (6) have shown that the expression of *HL-A* antigens on human lymphoid cells may be decreased on treatment of the cells with puromycin but not with actinomycin D or cycloheximide. Using the Moloney leukemia virus-induced mouse lymphoma cell, Cikes and Klein (3) found that actinomycin D added to logarithmically growing cells enhanced the expression of *H-2* and Moloney leukemia virus-determined antigens. Other metabolic inhibitors, such as puromycin or cycloheximide, could block this effect of actinomycin D.

The inhibitors therefore may modify the susceptibility of cells to antibody and complement without affecting Forsman or tumor-specific antigen concentration or binding of complement components to the membrane.

It would appear that the drug induced increased sensitivity of cells to killing by antibody and complement could be due to (a) a more efficient binding and/or activation of the terminal components of complement or (b) a reduction in the defense and repair mechanism of the cells under immunological attack. It is not now possible to conclude which of these mechanisms is involved. There is evidence that alterations in susceptibility to lysis during the cell reproduction cycles are not due to alterations in the binding of the terminal components of complement (7). It would appear that a more efficient activation of complement components and reduction of the efficiency of the cell membrane defenses are the most likely explanations for the effects of the drugs.

Table 3

Measurement of GPC4 and GPC3 on inhibitor-treated line 10 tumor cells sensitized with anti-Forsman or specific antitumor antibody

	% trypan blue-stained cells		Molecules C4/cell ( $\times 10^{-5}$ ) <sup>a</sup>		Molecules C3/cell ( $\times 10^{-5}$ ) <sup>a</sup>	
	No treatment	Inhibitor treated	No treatment	Inhibitor treated	No treatment	Inhibitor treated
Experiment 1 (5 $\mu$ g puromycin)						
Line 10 + anti-Forsman (1/2) + GPC (1/8)	0	63	18.0 $\pm$ 0.8	6.8 $\pm$ 0.5	10.1 $\pm$ 0.45	6.9 $\pm$ 0.35
Line 10 + anti-line 10 (1/15) + GPC (1/8)	0	63	69.3 $\pm$ 2.2	45.0 $\pm$ 0.2	12.8 $\pm$ 0	9.8 $\pm$ 0.15
Line 10 + buffer + GPC (1/8)	11	9	5.2	1.0	ND <sup>b</sup>	0.7
Line 10 + buffer + buffer	2	7	ND	ND	ND	1.6
Experiment 2 (50 $\mu$ g adriamycin)						
Line 10 + anti-Forsman (1/2) + GPC (1/8)	6	52	6.8 $\pm$ 0.95	4.0 $\pm$ 0.8	4.95 $\pm$ 0.45	4.7 $\pm$ 0.1
Line 10 + anti-line 10 (1/15) + GPC (1/8)	6	51	45.5 $\pm$ 0.5	21.8 $\pm$ 0.8	15.3 $\pm$ 0.5	7.0 $\pm$ 1.0
Line 10 + buffer + GPC (1/8)	0	5	ND	ND	1.2	1.6
Line 10 + buffer + buffer	1	5	ND	ND	0.3	3.6
Experiment 3 (25 $\mu$ g actinomycin D)						
Line 10 + anti-Forsman (1/2) + GPC (1/8)	7	26	22.6 $\pm$ 0.95	17.6 $\pm$ 0.6	14.8 $\pm$ 0	11.8 $\pm$ 0.04
Line 10 + anti-line 10 (1/15) + GPC (1/8)	11	43	90.0 $\pm$ 0.75	76.0 $\pm$ 1.0	17.3 $\pm$ 0	17.9 $\pm$ 0.7
Line 10 + buffer + GPC (1/8)	9	14	3.1	1.2	3.2	1.5
Line 10 + buffer + buffer	13	11	1.3	1.2	3.2	1.3
Experiment 4 (20 $\mu$ g azacytidine)						
Line 10 + anti-Forsman (1/2) + GPC (1/8)	11	58	10.5 $\pm$ 0.5	9.95 $\pm$ 0.6	7.0 $\pm$ 0	9.6 $\pm$ 0.16
Line 10 + anti-line 10 (1/15) + GPC (1/8)	12	63	30.5 $\pm$ 0.1	28.3 $\pm$ 2.0	10.4 $\pm$ 0	14.1 $\pm$ 0.45
Line 10 + buffer + GPC (1/8)	0	1	0.91	1.3	2.8	7.0
Line 10 + buffer + buffer	0	2	1.55	1.9	0.7	0.42

<sup>a</sup> Number of molecules per cell calculated from an estimated molecular weight of 230,000 for GPC4 and 185,000 for GPC3. Calculations made after subtracting in each experiment the amount of C4 and C3 in control tubes receiving only GPC, diluted 1/8.

<sup>b</sup> ND, none detected after correcting for control tube receiving only GPC, diluted 1/8.

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