

TUMOR CELL ANTI-OXIDANT DEFENSES

Inhibition of the Glutathione Redox Cycle Enhances Macrophage-mediated Cytolysis*

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The secretion of reactive oxygen intermediates is one means by which leukocytes can lyse tumor cells without phagocytosis (1-9). We have identified two settings in which macrophages make use of this mechanism: when the respiratory burst is triggered by pharmacologic agents (5, 6) or by anti-tumor antibody (7, 8). In both cases, activated macrophages, which can secrete abundant hydrogen peroxide (10, 11), destroy tumor cells. Resident peritoneal macrophages or those elicited with certain sterile inflammatory agents secrete little H_2O_2 (10, 11) and are not cytolytic (5, 7).

Tumor cells vary widely in their susceptibility to lysis by H_2O_2 (5, 12) or by peroxide-producing leukocytes (5). However, little is known about anti-oxidant defenses of tumor cells, which might affect their susceptibility or resistance to oxidative injury.

In this report, we studied six murine tumors differing >50-fold in their susceptibility to lysis by enzymatically generated H_2O_2 . We measured the activities of two peroxide-catabolizing pathways: catalase and the cyclic oxidation and reduction of glutathione. We inhibited both pathways, and observed the effects on lysis of the tumors by fluxes of H_2O_2 and by macrophages in the presence of phorbol myristate acetate (PMA)¹ or anti-tumor antibody. The results suggest that the tumor cell glutathione cycle can be a rate-limiting factor in cytotoxicity. Inhibition of this pathway makes tumor cells more susceptible to macrophage-mediated oxidative injury.

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¹ *Abbreviations used in this paper:* Ara-C, cytosine-1- β -D-arabinofuranoside hydrochloride; BCG, bacille Calmette-Guérin; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-(cyclohexyl)-1-nitrosourea; FBS, fetal bovine serum heated at 56°C for 30 min; γ -GCS, gamma-glutamylcysteine synthetase; GPO, glutathione peroxidase; GR, glutathione reductase; GS, glutathione synthetase; GSH, reduced glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; HS, horse serum heated at 56°C for 30 min; ID₅₀, dose causing 50% inhibition; LD₅₀, dose lethal to 50% of cells; MEM, Eagle's minimum essential medium, alpha variant; methyl-CCNU, 1-(2-chloroethyl)-3-(4-transmethylcyclohexyl)-1-nitrosourea; PBS, Dulbecco's phosphate-buffered saline; PMA, phorbol myristate acetate; RPMI-10% FBS, RPMI supplemented with 100 μ g/ml streptomycin, 100 U/ml penicillin, 300 μ g/ml glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, and 10% heat-inactivated FBS.

Materials and Methods

Tumors. J774 cells were maintained in spinner culture, and were the kind gift of Ms H. Plutner, Dr. J. Unkeless, and Dr. I. Mellman, The Rockefeller University, New York. The other tumors were used either as ascites or as stationary suspension cultures in Eagle's minimum essential medium (alpha variant) (MEM), supplemented with 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 U/ml penicillin, and 5–10% heat-inactivated horse serum (HS) (Flow Laboratories, Inc., Rockville, Md.). P388 lymphoma cells were passed in (BALB/c \times DBA/2) F_1 (CD2F₁) mice (Simonsen Laboratories, Gilroy, Calif.), TLX9 and NK lymphomas in C57BL/6 mice (Simonsen Laboratories; The Jackson Laboratory, Bar Harbor, Maine; or Trudeau Institute, Saranac Lake, N. Y.), and YAC-1 lymphoma in A strain mice (The Jackson Laboratory, or Trudeau Institute). NK lymphoma, which arose in a C3 strain mouse around 1959 (13), was obtained through the courtesy of Dr. J. Mayo, Division of Cancer Treatment, National Cancer Institute, Frederick Cancer Research Center, Frederick, Md., from a Hungarian stock. The other tumors were obtained as described (5, 7).

Diet. For most studies, mice were fed *ad libitum* with rodent laboratory chow 5001 (Ralston Purina Co., St. Louis, Mo.) and tap water. Where indicated, the mice were fed a selenium-deficient diet according to the formulation of Rotruck et al. (14) supplemented with 100 IU/kg tocopherol acetate (ICN Nutritional Biochemicals, Cleveland, Ohio). As a control, littermates were fed the same diet repleted with 1 ppm sodium selenite, to which was added 0.05% by weight of a mixture of FD & C red dye No. 2, yellow No. 5, and blue No. 1 as an aid in identification. The special diets were stored at -20°C to retard spoilage. Mice receiving the special diets were only given deionized water double-distilled in glass. They gained weight at the same rate as littermates receiving chow 5001. Tumors grew in all three groups of mice at the same rate.

Effector Cells. Peritoneal cells were obtained from untreated CD2F₁ female mice (resident cells); 10–42 d after intraperitoneal injection of viable bacille Calmette-Guérin (BCG) (BCG cells); 4–5 d after intraperitoneal injection of thioglycollate broth (thioglycollate cells); or 12–18 h after thioglycollate broth (granulocytes). Details of the elicitation, collection, hypotonic lysis, and differential counting of these populations have been presented elsewhere (5, 7, 10). Effector:target ratios are reported on the basis of the proportion of macrophages detected among the resident, BCG, or thioglycollate cells, or the granulocytes in the acute thioglycollate-broth exudates, as described elsewhere (10). To prepare cytolytic T cells, C57BL/6 and CD2F₁ mice were injected intraperitoneally with spleen cells of the opposite strain. 25–32 wk later, spleens were pressed through a 40-gauge wire screen with glass stoppers, and washed in RPMI-1640 supplemented with 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 U/ml penicillin, 300 $\mu\text{g}/\text{ml}$ glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, $5 \times 10^{-5}\text{M}$ 2-mercaptoethanol, and 10% heat-inactivated fetal bovine serum (RPMI-10%FBS) (Flow Laboratories, Inc.). Erythrocytes were lysed with 1.6% ammonium chloride. 7×10^6 responder cells were incubated with 4×10^6 irradiated (2,000 rad) stimulator cells in 2 ml RPMI-10%FBS in 16-mm wells (Costar, Data Packaging, Cambridge, Mass.) for 5 d. The cells were washed and replated at 4×10^6 /well with 1×10^6 fresh, irradiated stimulator cells. Effector cells were collected 2–5 d later.

Use of Pharmacologic Agents. The following preservative-free compounds were obtained through the courtesy of Dr. V. L. Narayanan, Drug Synthesis and Chemistry Branch, and Dr. J. D. Douros, Natural Products Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.: 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU; NSC 409962); 1-(2-chloroethyl)-3-(4-transmethylcyclohexyl)-1-nitrosourea (methyl-CCNU; NSC 95441); 1-(2-chloroethyl)-3-(cyclohexyl)-1-nitrosourea (CCNU; NSC 79037); cis-diaminedichloroplatinum (NSC 119875); and doxorubicin hydrochloride (NSC 123127). The following were from Sigma Chemical Co., St. Louis, Mo.: mitomycin C, cytosine-1- β -D-arabinofuranoside hydrochloride (Ara-C), 2-amino-6-mercaptapurine (6-thioguanine), 5-fluorouracil, 4-(bis[2-chloroethyl]amino)-L-phenylalanine (melphalan), 3-amino-1,2,4-triazole, chlorambucil, and catalase (thymol-free). Cyclophosphamide monohydrate was a gift from Mead Johnson & Co., Evansville, Ill. Methotrexate was from Lederle Laboratories, Pearl River, N. Y.

Tumor cells were labeled with $\text{Na}_2 [^{51}\text{Cr}]\text{O}_4$ as described before (5), washed by centrifugation, resuspended in MEM-1% HS, and divided into two aliquots of 5×10^6 – 1×10^7 cells in 1 ml or

1×10^7 – 2×10^7 cells in 2 ml. Each tube then received 0.1 or 0.2 ml of vehicle (MEM-1% ethanol) (final ethanol concentration, 0.09%), or an equal volume of vehicle that contained 1.1 mg/ml BCNU (final concentration: 100 μ g/ml or 0.467 mM). After 10 min at 37°C, the tumor cells were washed once in MEM-1% HS and twice in MEM-5% HS. The BCNU was stored at –20°C, weighed out immediately before use, dissolved in cold absolute ethanol, and diluted with cold, serum-free MEM before addition to the tumor cells. During the above procedures, the pH of the medium was maintained no higher than 7.3 by gassing with 5% CO₂/95% air. Careful control of the pH and serum concentration was necessary to avoid toxicity (15–18). Other drugs were also used at 0.467 mM for 10–30 min exposures, except for aminotriazole. The latter was present at 25–50 mM during the 45–60 min period of ⁵¹Cr-labeling, and was also present throughout the cytotoxicity assays at 25–50 mM.

Cytotoxicity Assays. To determine susceptibility to lysis by H₂O₂, 4×10^4 ⁵¹Cr-labeled tumor cells in 0.2 ml MEM-5% HS were added to round-bottomed microtest plate wells (Linbro Division, Flow Laboratories, Inc., Hamden, Conn.) which contained 0.02 ml of glucose oxidase (Type V, Sigma Chemical Co.) diluted in normal saline. The plates were incubated at 37°C in 5% CO₂/95% air for 4.5 h, then centrifuged at 550 g for 5 min. 0.1 ml of the supernate was removed for gamma counting. In some experiments, the wells were made to contain 0.27% trypan blue, and the ability of the tumor cells to exclude the dye was observed in a hemocytometer. For experiments with J774 and NK lymphoma cells, in which high concentrations of glucose oxidase were employed, the glucose concentration of the medium was increased to 44 mM. To test cell-mediated cytotoxicity, various numbers of effector cells and 2×10^4 ⁵¹Cr-labeled tumor cells were added to the wells in a total vol of 0.2 ml of MEM-5%, either alone, or in the presence of 100 ng/ml PMA, 0.0033% dimethylsulfoxide, anti-tumor alloantisera, or nonimmune allosera, as described (7). In some experiments with selenium-deficient tumor cells, Neuman-Tytell medium (Grand Island Biological Co., Grand Island, N. Y.) was used with 0.25 mg/ml bovine serum albumin. The plates were usually centrifuged at 160 g for 1 min, then incubated 4.5 h, before harvest as above. To test antibody-mediated, complement-dependent lysis, 4×10^4 ⁵¹Cr-labeled tumor cells in 0.2 ml MEM-5% HS were added to wells with 0.05 ml of various dilutions of heat-inactivated rabbit anti-mouse lymphocyte serum (Microbiological Associates, Walkersville, Md.) and 0.05 ml absorbed, screened, fresh-frozen rabbit serum (Pel-Freez, Rogers, Ariz.). The supernate was sampled 3.5 h later. The percent specific release was calculated as before, reporting the mean and simple proportional SEM for triplicates (5).

Biochemical Assays. The activity of glucose oxidase was measured with the scopoletin assay for H₂O₂ (10) in Krebs-Ringer phosphate buffer, pH 7.35–7.41, at 37°C in air, with either 5.5 or 40 mM glucose. These conditions were chosen to mimic those in the cytotoxicity assay, even though they are suboptimal for the enzyme in terms of the concentrations of glucose, oxygen, and hydrogen ion. To measure glutathione reductase (GR), 5×10^6 – 2×10^7 nonradiolabeled tumor cells were treated with BCNU or with vehicle as described above, washed three times in ice-cold Dulbecco's phosphate-buffered saline (PBS) (Grand Island Biological Co.) by 30-s centrifugations in a microfuge (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) and resuspended to 1 ml in PBS. They were made 0.2% in Triton X-100 (Sigma Chemical Co.) and centrifuged for 10 min. Aliquots of the supernate were removed for protein determination by the method of Lowry et al. (19) using a bovine serum albumin standard. 0.1-ml aliquots were used to measure GR by the method of Roos et al. (20). Preliminary experiments showed that the activity of GR was not affected by preincubating the tumor cells for 45 min in Na₂CrO₄ (21) at the concentrations used to radiolabel the cells for cytotoxicity assays. Glutathione peroxidase (GPO) was measured in the same lysate supernate by the method of Paglia and Valentine (22). Values were the same using cumene hydroperoxide (Polysciences, Inc., Warrington, Pa.) as substrate rather than H₂O₂. Catalase was measured according to Baudhuin et al. (23). Glutathione was measured in acid-soluble extracts of triton lysates by a modification of the method of Tietze (24) as described in detail elsewhere (B. A. Arrick, C. F. Nathan, Z. A. Cohn, and O. W. Griffith, manuscript in preparation). The total of reduced plus oxidized glutathione is expressed as nanomoles of the tripeptide per milligram cell protein.

Results

Levels of Catalase, GPO GR, and Glutathione in Tumor Cells. Four lymphomas, a mastocytoma, and a histiocytoma were chosen for study, because they spanned a 54-fold range in susceptibility to lysis by the H₂O₂-generating enzyme, glucose oxidase (Table I). At every concentration of glucose oxidase employed, lysis was abolished by catalase (not shown). The six tumors differed 56.7-fold in their specific activity of catalase, 5.3-fold in GPO, and 3.3-fold in GR. However, the levels of these enzymes did not correlate with the resistance of the tumors to H₂O₂. In contrast, the specific cellular content of glutathione correlated with the logarithm of the flux of peroxide causing 50% lysis ($r = 0.91$).

Inhibition of Tumor Cell GR by BCNU. To investigate the contribution of glutathione to tumor cell anti-oxidant defense, we sought means of inhibiting the enzymes that catalyze its oxidation and reduction. The alkylating and carbamoylating agent BCNU was recently reported to inhibit GR in human erythrocytes without influencing the

TABLE I
Levels of Anti-Oxidant Defenses and Susceptibility to Lysis by H₂O₂

Tumor	I.D. ₅₀ [*]	Protein [‡]	Catalase [§]	GPO	GR [¶]	Glutathione ^{¶¶}
TLX9	0.28 ± 0.02 (4)**	56.6 ± 6.1 (8)	14.6 ± 1.7 (7)	87.5 ± 12.9 (7)	30.4 ± 3.8 (6)	17.2 ± 3.7 (5)
P388	0.31 ± 0.06 (7)	61.1 ± 10.6 (6)	68.7 ± 13.5 (5)	243.8 ± 52.1 (3)	22.7 ± 4.6 (6)	11.8 ± 1.2 (6)
YAC	0.54 ± 0.12 (3)	72.0 ± 6.7 (3)	47.2 ± 14.3 (4)	75.0 ± 14.9 (3)	36.3 ± 2.0 (4)	22.4 ± 5.1 (5)
P815	1.31 ± 0.15 (19)	102.2 ± 9.8 (9)	31.5 ± 6.6 (4)	174.4 ± 29.6 (7)	18.4 ± 1.4 (27)	25.3 ± 2.7 (4)
J774	3.39 ± 2.11 (2)	117.3 ± 6.5 (3)	238.0 ± 14.0 (4)	54.0 ± 4.7 (3)	60.4 ± 3.3 (4)	27.7 ± 6.8 (4)
NK	15.04 ± 0.52 (2)	87.0 ± 11.3 (6)	4.2 ± 2.4 (3)	288.3 ± 54.1 (3)	21.1 ± 0.5 (3)	31.3 ± 2.1 (3)
Ratio, highest: lowest	53.7	2.1	56.7	5.3	3.3	2.7

* Activity of glucose oxidase (nmol H₂O₂/min) required to cause 50% specific release of ⁵¹Cr under the standard conditions (Materials and Methods).

[‡] μg/10⁶ cells.

[§] Baudhuin units × 10⁴/mg protein.

^{||} nmol NADPH/min per mg protein at room temperature.

[¶] nmol glutathione tripeptide (oxidized plus reduced)/mg protein.

** Mean ± SEM. Number of experiments is in parentheses.

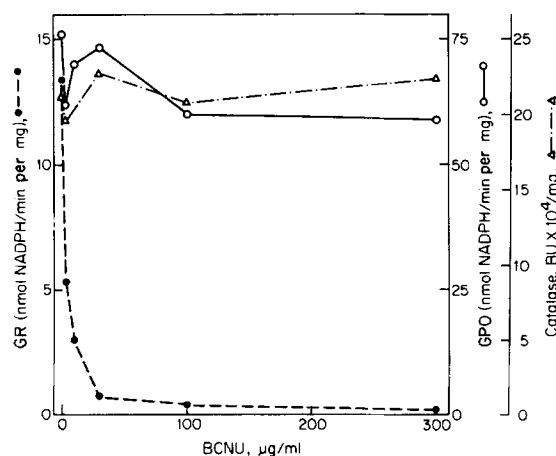


FIG. 1. The effect of BCNU on the activity of GR, GPO, and catalase in P815 cells. The intact tumor cells were incubated with the indicated doses of BCNU for 10 min at 37°C, washed, lysed, and assayed. BU, Baudhuin units.

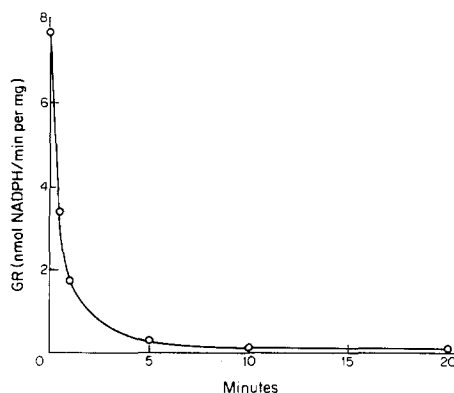


FIG. 2. Time-course of inhibition of GR in P815 cells by BCNU. The intact cells were exposed to 100 $\mu\text{g/ml}$ BCNU for the indicated times at 37°C before being rapidly cooled, washed, lysed, and assayed.

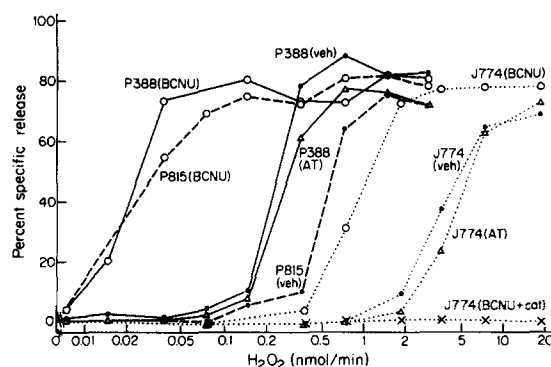


FIG. 3. Effect of BCNU and aminotriazole on sensitivity of P388 (—), P815 (---) and J774 cells (· · ·) to lysis by fluxes of H_2O_2 . ^{51}Cr -release was measured from the tumor cells after 4.5-h incubation with dilutions of glucose oxidase. Before assay, the cells were treated with vehicle alone (veh, ●) or 100 $\mu\text{g/ml}$ BCNU for 10 min (○), or incubated with 25 or 50 mM aminotriazole (AT, Δ) for 45–60 min before and then throughout the assay. Catalase (cat) (3,000 U/ml) abolished lysis in all cases, as shown for J774 (x). Points are means of triplicates. SEM averaged 2.0%.

activity of any other enzyme tested (25). Accordingly, we measured GR in lysates of P815 mastocytoma after exposing the intact cells to the drug for 10 min and then washing it out (Fig. 1). GR was 50% inhibited by 2.3 $\mu\text{g/ml}$ (11 μM) BCNU and 95% inhibited by 100 $\mu\text{g/ml}$. Doses up to 300 $\mu\text{g/ml}$ did not affect the activity of GPO or catalase in the same cells (Fig. 1). Inactivation of GR by BCNU was rapid, being 50% complete in <30 s and essentially complete in 5 min (Fig. 2). Similar results were obtained with P388 and TLX9 lymphomas (data not shown).

Enhanced Susceptibility of Tumor Cells to Lysis by H_2O_2 After Exposure to BCNU. Exposure of P815 mastocytoma cells to 100 $\mu\text{g/ml}$ BCNU for 10 min was nontoxic, as judged by exclusion of trypan blue and release of ^{51}Cr measured over a period of 18 h. However, BCNU treatment made the tumor cells much more susceptible to lysis by small fluxes of H_2O_2 . For example, in the experiment illustrated in Fig. 3, 18.7 times less glucose oxidase was required to cause 50% specific release of ^{51}Cr from P815 cells after pulsing them with BCNU. Similar results were seen with all six of the

TABLE II
Effects of BCNU and Aminotriazole on Sensitivity of Tumor Cells to Lysis by Glucose Oxidase

	Tumor					
	TLX9	P388	YAC	P815	J774	NK
Ratio of LD ₅₀ for glucose oxidase						
Control*:BCNU‡	6.4 ± 4.1 (2)¶	21.3 ± 11.6 (4)	7.7 ± 4.0 (3)	5.5 ± 1.7 (9)	6.0 ± 2.6 (6)	4.3 ± 0.9 (2)
Control:AT§	1.0 (1)	1.0 ± 0.2 (2)	ND**	1.0 ± 0.2 (2)	2.6 ± 0.8 (4)	2.6 (1)
Control:BCNU + AT	ND	4.5 (1)	ND	5.3 (1)	6.5 ± 1.6 (4)	7.4 (1)
Spontaneous release						
Control	8.1 ± 1.3 (9)	8.8 ± 0.4 (7)	5.2 ± 0.2 (3)	8.4 ± 0.6 (14)	14.5 ± 1.6 (7)	13.9 ± 3.3 (4)
BCNU	30.0 ± 8.0 (2)	23.8 ± 5.5 (4)	16.2 ± 4.7 (3)	12.2 ± 0.7 (9)	16.7 ± 0.7 (6)	11.8 ± 1.8 (2)
AT	9.9 (1)	8.0 ± 2.7 (2)	ND	8.7 ± 1.4 (2)	14.6 ± 1.7 (4)	9.9 ± (1)
BCNU + AT	ND	28.7 (1)	ND	14.5 (1)	17.0 ± 1.3 (4)	13.8 (1)

* Tumor cells were treated for 10 min with 0.09% ethanol in MEM-1% HS and then washed.

‡ Tumor cells were treated for 10 min with 100 µg/ml BCNU in 0.09% ethanol in MEM-1% HS.

§ Tumor cells treated for 45-60 min with 25 or 50 mM aminotriazole (AT); with the same concentration of aminotriazole present during the assay.

|| Percent release of ⁵¹Cr from 4 × 10⁴ tumor cells after 4.5 h in 0.2 ml MEM-5% HS without glucose oxidase.

¶ Mean ± SEM. Number of experiments is in parentheses.

** Not done.

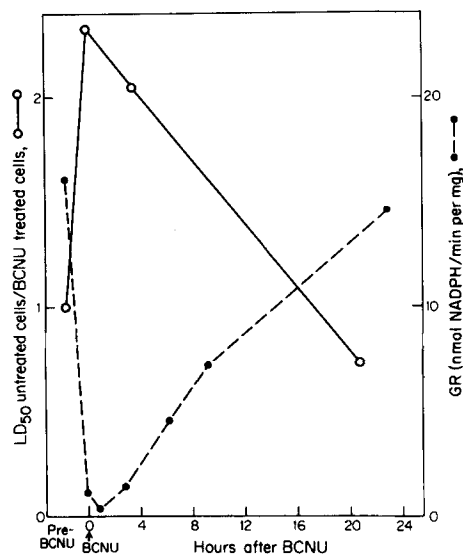


FIG. 4. Time-course of recovery of GR activity and resistance to H₂O₂, after exposure of P815 cells to 100 µg/ml BCNU for 10 min. The LD₅₀ of glucose oxidase for treated and control cells was determined at each time point from curves like those in Fig. 3.

tumors, whether they were initially more sensitive or more resistant to H₂O₂ than was P815 (Fig. 3), and whether or not BCNU elevated their spontaneous release of ⁵¹Cr (Table II). Lysis of the BCNU-treated tumors by glucose oxidase was abolished by catalase (Fig. 3). Results of all 26 such experiments are summarized in Table II.

The temporal association between inhibition of GR by BCNU and enhanced sensitivity to H₂O₂ is seen in Fig. 4. Both functions recovered in a linear fashion, reaching pretreatment values by 21-23 h.

The effect of the concentration of BCNU on H₂O₂ sensitivity was evaluated by

both ^{51}Cr release and dye exclusion (Fig. 5). Over the range from 3 to 100 $\mu\text{g}/\text{ml}$ BCNU, the effect of the drug on GR (Fig. 1) paralleled its effect on peroxide sensitivity (Fig. 5A). At 300 $\mu\text{g}/\text{ml}$ BCNU, an artifact was introduced into the ^{51}Cr assay such

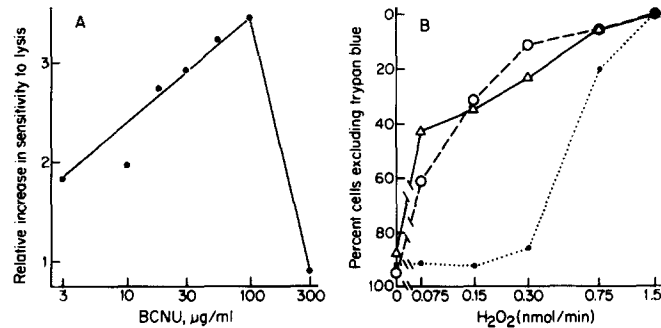


FIG. 5. Effect of BCNU concentration on lysis of P815 by glucose oxidase. (A) Tumor cells were exposed to the indicated concentrations of BCNU for 10 min. The LD_{50} of glucose oxidase for untreated and control cells were determined as in Fig. 3 (B) Trypan blue exclusion by P815 cells was tested after 10 min of exposure of the cells to vehicle alone (●---●), 100 $\mu\text{g}/\text{ml}$ BCNU (○---○), or 300 $\mu\text{g}/\text{ml}$ BCNU (Δ — Δ), followed by 5 h of incubation with different concentrations of glucose oxidase under the same conditions as in (A). 100 $\mu\text{g}/\text{ml}$ BCNU decreased the LD_{50} for glucose oxidase by a factor of 4.3, as measured by trypan blue exclusion, and by a factor of 4.7 as measured by ^{51}Cr release in the same experiment.

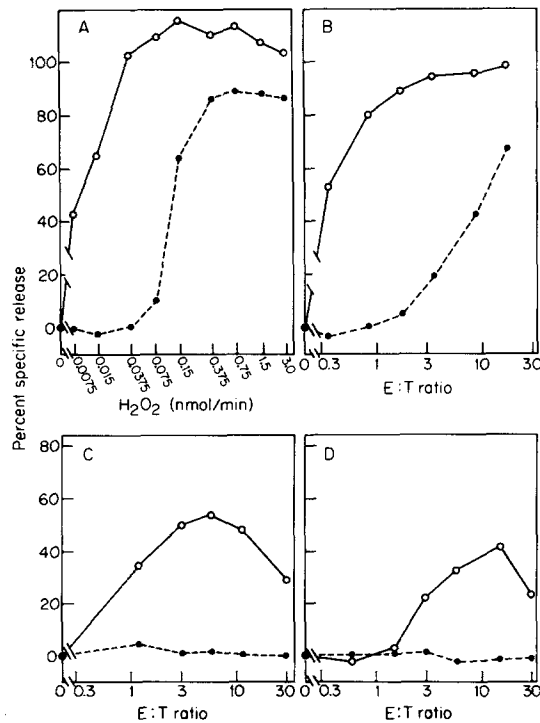


FIG. 6. Effect of BCNU on lysis of P388 cells by PMA-stimulated peritoneal cells. (●) vehicle-treated P388. (○) P388 exposed to 100 $\mu\text{g}/\text{ml}$ BCNU for 10 min. (A) Lysis by glucose oxidase. (B-D) Lysis by peritoneal cells in the presence of 100 ng/ml PMA. (B) BCG-activated macrophages. (C) thioglycollate broth-elicited macrophages. (D) resident macrophages. SEM averaged 2.7%.

that the release of radiolabel underrepresented the degree of cell lysis. It should be noted that at 100 $\mu\text{g}/\text{ml}$ BCNU, the results of the ^{51}Cr -release assay and the dye exclusion test agreed closely (Fig. 5 B). This dose was used in all subsequent experiments.

Enhancement of PMA-dependent, Macrophage-mediated Cytotoxicity by BCNU. We next examined the sensitivity of BCNU-treated tumor cells to injury by macrophage populations. The agent used to induce the release of oxygen intermediates in this case was PMA. Fig. 6 shows the results with BCG-activated (Fig. 6 B), thioglycollate broth-elicited (Fig. 6 C), and resident macrophages (Fig. 6 D). After exposure of P388 lymphoma cells to BCNU, the BCG-activated macrophages became 33-fold more effective in their lytic activity. Of considerable interest was the cytolytic capacity of both resident and thioglycollate broth-elicited macrophages against the BCNU-treated target. The same macrophage populations were incapable of lysing untreated P388 cells. The cytolytic efficiency of each macrophage population can be compared

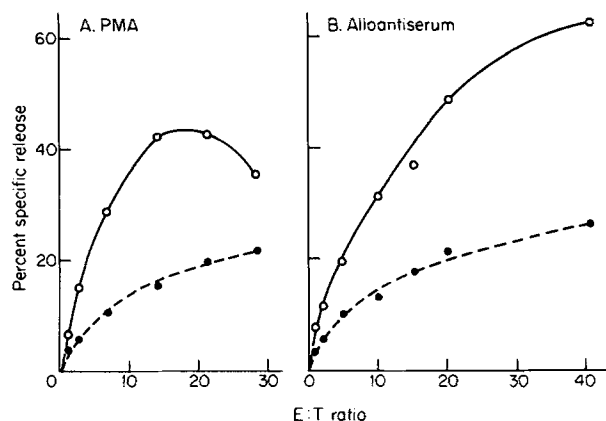


FIG. 7. Effect of BCNU on lysis of P815 cells by BCG-activated macrophages in the presence of (A) PMA, 100 ng/ml or (B) anti-tumor alloantiserum. (●) P815 cells treated with vehicle alone. (○) P815 treated with 100 $\mu\text{g}/\text{ml}$ BCNU for 10 min. SEM averaged 1.3%.

TABLE III
Effect of BCNU on Antibody-dependent, Macrophage-mediated Lysis of P815

Exp.	E:T ratio*	Specific release of ^{51}Cr		Specific release (BCNU/vehicle)
		Vehicle‡	BCNU§	
1	16	20.2 \pm 0.7	49.8 \pm 2.7	2.47
2	15	28.2 \pm 0.7	41.3 \pm 1.1	1.46
3	23	37.9 \pm 1.6	74.7 \pm 1.7	1.97
4	41	26.4 \pm 1.4	62.7 \pm 0.3	2.38
5	20	12.6 \pm 0.5	31.7 \pm 2.1	2.52
6	29	43.1 \pm 2.2	58.5 \pm 4.4	1.36

* Various numbers of macrophages from BCG-treated mice were incubated for 4.5 h with 2×10^4 ^{51}Cr -labeled P815 cells that had been exposed to alloantiserum.

‡ Target cells were treated for 10 min with 0.09% ethanol in MEM-1% HS.

§ Target cells were treated for 10 min with 100 $\mu\text{g}/\text{ml}$ BCNU in 0.09% ethanol in MEM-1% HS.

|| Means \pm SEM for triplicates.

with the lysis by enzymatically generated H_2O_2 in the same experiment (Fig. 6A). The observed degrees of cell-mediated lysis would be expected if the BCG-activated macrophages released $5.9 \text{ nmol } H_2O_2/10^6$ cells per 5 min, the thioglycollate broth-elicited cells released 1.1 nmol, and the resident cells released 0.14 nmol. These rates agree closely with those previously observed (10, 11).

Less striking but still pronounced effects were noted using P815, a tumor with greater intrinsic resistance to peroxide (Fig. 7A). In three experiments, BCG-activated macrophages were 2.9 ± 0.1 times more active against the BCNU-treated tumor cells at any given effector:target ratio.

Enhancement of Antibody-dependent, Macrophage-mediated Cytotoxicity by BCNU. Activated macrophages bind and lyse tumor targets coated with specific antibody (7). At least 62% of this activity was oxygen-dependent (8). The ability of BCNU to modify the lysis of alloantiserum-coated P815 cells is illustrated in Fig. 7B and Table III. In six such experiments, BCNU treatment of the target enhanced cytolysis by BCG-activated macrophages by a factor of 2.0 ± 0.2 . The increased efficiency of the reaction against BCNU-treated tumor cells was also manifest as an eightfold reduction in the amount of antibody required to achieve the same degree of lysis (Fig. 8).

Specificity of Action of BCNU. BCNU did not simply make tumor cells more susceptible to any form of humoral or cell-mediated injury. Thus, antibody plus complement did not lyse BCNU-treated target cells any more readily than native target cells (Table IV). Likewise, the lysis of tumor cells by allosensitized T cells was not affected by BCNU (Table IV). This was true even when BCNU itself was slightly toxic to certain of the tumor cells, as judged by elevation in their spontaneous release of ^{51}Cr (Table II). Finally, of 11 anti-neoplastic agents, only BCNU sensitized tumor cells to lysis by H_2O_2 under the conditions tested. The following drugs were incubated with TLX9 or P815 cells at 0.47 mM for 10–30 min, and gave the indicated ratio of half lethal doses (LD_{50}) of glucose oxidase for control cells:drug treated cells: CCNU (1.13), methyl-CCNU (0.91), chlorambucil (1.26), melphalan (0.99), 5-fluorouracil (0.87), Ara-C (0.72), methotrexate (0.64), doxorubicin hydrochloride (0.61), mitomycin C (0.88), and cis-diaminedichloroplatinum (0.76).

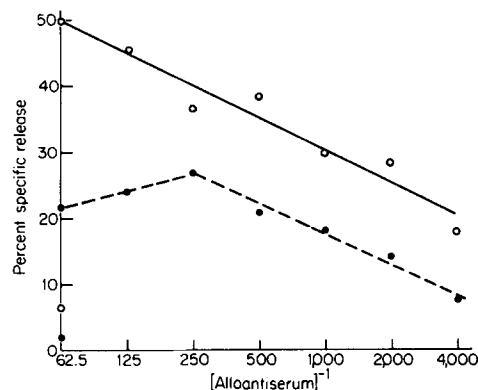


FIG. 8. Effect of BCNU on antibody-dependent lysis of P815 cells by BCG-activated macrophages as a function of the concentration of alloantiserum used to sensitize the targets. (●) vehicle-treated P815. (○) BCNU-treated P815. Results with nonimmune alloserum are indicated by separate symbols on the Y axis. The effector:target ratio was 16. SEM averaged 1.5%.

TABLE IV
Lack of Effect of BCNU on Susceptibility of Tumor Cells to Lysis by Antibody Plus Complement or by Allosensitized Spleen Cells

Tumor	Lytic agents	Specific release of ⁵¹ Cr from tumors treated with	
		Vehicle*	BCNU‡
TLX9	Antibody§		
	1:100 + C'¶	76.0 ± 0.3¶	59.0 ± 4.3
	1:300 + C'	37.3 ± 1.4	27.2 ± 3.9
	1:1,000 + C'	9.8 ± 1.5	5.1 ± 0.7
P815	C57BL/6 anti-CD2F ₁ spleen cells**		
	E:T, 50	70.6 ± 7.8	81.2 ± 2.4
	E:T, 15	45.5 ± 2.5	45.3 ± 0.8
	E:T, 5	20.1 ± 1.1	18.6 ± 1.1
	E:T, 1.5	9.2 ± 1.0	8.1 ± 0.9
	E:T, 0.15	1.2 ± 0.6	1.2 ± 0.9
TLX9	CD2F ₁ anti-C57BL/6 spleen cells**		
	E:T, 50	93.8 ± 2.6	88.1 ± 9.8
	E:T, 15	83.0 ± 6.3	69.2 ± 4.9
	E:T, 5	57.3 ± 2.1	40.5 ± 4.9
	E:T, 1.5	18.2 ± 2.2	16.4 ± 2.7
	E:T, 0.15	2.8 ± 0.6	-1.2 ± 2.0

* Tumor cells were exposed to 0.09% ethanol in MEM-1% HS for 30 min (1st experiment) or 10 min (2nd and 3rd experiments).

‡ Tumor cells exposed to 100 µg/ml BCNU in vehicle as in footnote * above.

§ Rabbit anti-mouse lymphocyte antiserum, heat-inactivated.

¶ Fresh normal rabbit serum, 1:20. Specific release with complement (C') but without antibody was 6.0% for vehicle-treated cells and 1.9% after BCNU.

¶ Means ± SEM for triplicates.

** Mice were immunized in vivo and their spleen cells boosted twice in vitro as described in Materials and Methods. P815 shares the major histocompatibility antigens of CD2F₁ mice. TLX9 arose in a C57BL/6 mouse. Effector cells were also tested at each effector:target (E:T) ratio against the homologous target, both vehicle-treated and BCNU-treated; specific release ranged from -15% to 3.2%.

Inhibition of GPO by Dietary Deprivation of Selenium. Another method of interrupting the glutathione redox cycle, in the absence of drugs, was to take advantage of the selenium dependence of GPO (26). Mice were fed a selenium-deficient diet for 4 wk and were then employed as hosts for the ascites form of TLX9 lymphoma. After 10 wk of passage in selenium-depleted mice, the GPO activity of TLX9 cells was reduced 85% (Fig. 9B), with no effect on tumor cell GR (Fig. 9C), catalase (Fig. 9D), or glutathione (data not shown). The selenium-deficient tumor cells were threefold more sensitive to lysis by H₂O₂ than the controls (Fig. 9A). In three such experiments, sensitivity of TLX9 cells from selenium-deficient mice increased 3.4 ± 0.6 times compared with TLX9 cells from selenium-repleted mice. Similar results were seen with P815 cells.

PMA-triggered cytolysis by BCG-activated macrophages and by granulocytes was markedly augmented against selenium-deficient TLX9 cells, compared with tumor cells from selenium-repleted mice (Fig. 10).

Inhibition of Catalase. The catalase activity of J774 was inhibited 100% by 60 min

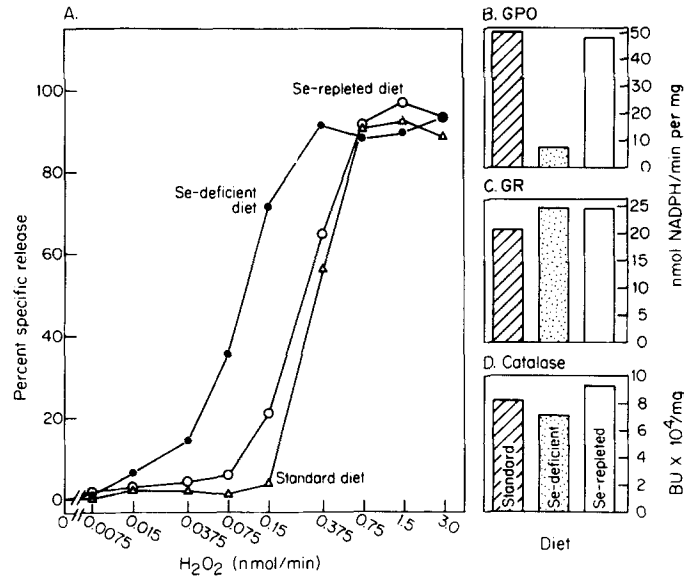


FIG. 9. Effect of dietary selenium (Se) deprivation on (A) peroxide-dependent lysis and (B-D) anti-oxidant enzyme levels of TLX9 cells. BU, Baudhuin units.

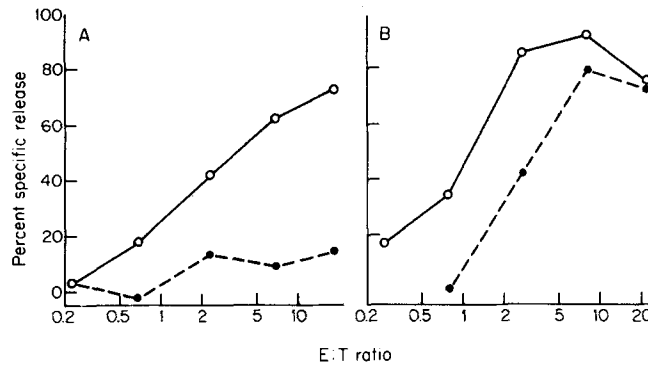


FIG. 10. PMA-dependent lysis of TLX9 cells from selenium-deficient (○) and selenium-repleted mice (●) by (A) BCG-activated macrophages and (B) peritoneal granulocytes. SEM averaged 4.5%.

incubation in 50 mM aminotriazole at 37°C. Catalase in P388 and P815 cells was inhibited 90% by incubation in 25 mM aminotriazole for 45 min. Inhibition of catalase in the latter tumors was probably increased even more by the further inclusion of aminotriazole in the cytolysis assay itself, because aminotriazole inhibits catalase more effectively in the presence of H_2O_2 (27). Used as described, aminotriazole did not augment the lysis of TLX9, P388, or P815 cells by enzymatically generated H_2O_2 (Table II and Fig. 3), by reagent H_2O_2 added as a bolus (data not shown), or by BCG-activated macrophages in the presence of PMA or anti-tumor antibody (data not shown). The sensitivity of J774 histiocytoma and NK lymphoma to H_2O_2 was enhanced to a modest degree by aminotriazole in some but not all experiments (Table II and Fig. 3).

Discussion

If the biochemical basis of cytotoxicity is known in a given experimental setting, it becomes feasible to hypothesize the pathways the target cell might employ to reduce or escape injury. If such pathways are identified, means can be sought to block them.

Reactive oxygen intermediates, including H_2O_2 , appear to be prominently involved in the cytotoxicity of activated macrophages in the presence of PMA (6) or anti-tumor antibody (8). Tumor cells are likely to possess several mechanisms, enzymatic and nonenzymatic, for disposal of H_2O_2 . We focused on three enzymes within tumor cells—catalase, GR, and GPO—and the substrate, glutathione.

Of these four variables, only the content of glutathione correlated with the susceptibility of six different tumors to lysis by fluxes of H_2O_2 . More important, inhibition of the cyclic oxidoreduction of glutathione markedly sensitized each of the tumor lines to oxidant injury.

Novel approaches were necessary to establish this point. Specific inhibitors of GR or GPO are not available. Familial, congenital, or acquired deficiencies of these enzymes have been identified in human leukocytes or erythrocytes (28), but not, to our knowledge, in tumor cells from man or mouse. Agents are available that rapidly oxidize glutathione to its disulfide in intact cells (29), but their effect is transient, because the cells reduce the disulfide back to glutathione (29, 30). In our hands, one such agent, diazenedicarboxylic acid bis-(*N,N*-dimethylamide), or diamide, had no effect on peroxide-mediated lysis of tumor cells (data not shown). Therefore, we explored four separate ways to interrupt the glutathione redox cycle over a period of hours (Fig. 11).

First, we inactivated GR by pulsing the tumor cells with BCNU. This approach was based on the work of Frischer and Ahmad (25) and Babson and Reed (31), who demonstrated inhibition of GR in nonmalignant human and murine cells by BCNU. Frischer and Ahmad observed no effect of BCNU on 17 other enzymes in erythrocytes, including those of the hexose monophosphate shunt and the Embden-Meyerhof path (25). We found that BCNU inhibition of GR in intact tumor cells was potent (dose causing 50% inhibition [ID_{50}]: $11 \mu M$) and rapid (50% inhibition in 30 s). Moreover, the action of BCNU was relatively specific, in that catalase and GPO were unaffected; the tumor cells were not made more susceptible to lysis by antibody and complement, nor by allosensitized T cells; and 10 other anti-neoplastic agents, including alkylating

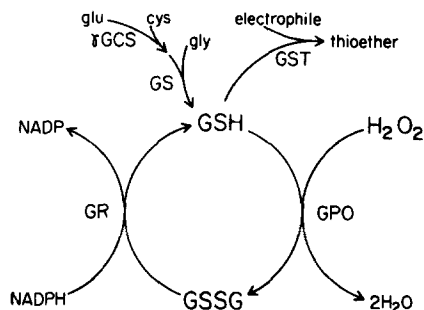


FIG. 11. The oxidation-reduction cycle of glutathione and several related reactions. The enzymes shown are: GPO; GR; γ -GCS, γ -glutamyl-cysteine synthetase; GS, glutathione synthetase; GST, glutathione *S*-transferase. The substrates include: GSH, reduced glutathione; GSSG, glutathione disulfide; glu, glutamate; cys, cysteine; gly, glycine.

compounds, were without similar effects. BCNU-induced inhibition of GR and sensitization of tumor cells to oxidative injury shared the same dose-response curve, and were reversible with the same time-course.

When GR was inactivated within tumor cells, the tumor cells could be injured by fluxes of H_2O_2 which were otherwise inconsequential; they could be killed by numbers of activated macrophages or granulocytes which were otherwise insufficient; and they could be lysed by nonactivated macrophages, which were otherwise ineffective. They could be killed by activated macrophages in the presence of anti-tumor antibody in concentrations which were otherwise too dilute.

As a second, independent way of interfering with the redox cycle of glutathione, we inhibited GPO, a selenoenzyme (26), by passage of the tumor cells in mice fed a selenium-deficient diet. This approach was based on the work of a number of laboratories, in which leukocyte (32-34) or cardiac (35) GPO was inhibited through selenium deprivation. Previous attempts to deplete tumor cells of selenium have been disappointing, perhaps because of the ability of the tumor to command the host's selenium reserves (36). Nonetheless, with time, we observed a selective decrease in activity of GPO in lymphoma and mastocytoma cells, with no effect on GR, catalase, or glutathione. The GPO-deficient tumor cells were much more sensitive to lysis by enzymatically generated H_2O_2 and by PMA-triggered granulocytes and macrophages. This effect could be prevented by making the diet replete with selenium, and it could be reversed by incubating the cells in serum-containing medium (data not shown).

Oxidative injury of tumor cells was augmented less by selenium deprivation than by treatment with BCNU. Two factors may have contributed to this difference. BCNU inhibited GR almost completely, whereas selenium deprivation resulted in only an 85% reduction in the activity of GPO. Furthermore, H_2O_2 can oxidize glutathione nonenzymatically, but the nonenzymatic reduction of glutathione disulfide is extremely slow. Thus, the glutathione redox cycle should be blocked more effectively by inhibition of GR than by inhibition of GPO.

As a third way to interrupt the glutathione cycle, we used an inhibitor of γ -glutamyl-cysteine synthetase to block glutathione synthesis (37) (Fig. 11). As a fourth method, we depleted tumor cell glutathione through thioether formation with a halogenated hydrocarbon, as catalyzed by endogenous glutathione *S*-transferase (38) (Fig. 11). These last 2 approaches will be described elsewhere (B. A. Arrick, C. F. Nathan, Z. A. Cohn, and O. W. Griffith, manuscript in preparation). The diversity of these four pharmacologic and dietary manipulations, all giving the same end result, strongly supports the interpretation that they each sensitize tumor cells to oxidant injury by interfering with the redox cycle of glutathione.

Catalase activity varied 56-fold in the six tumors studied. However, there was no correlation between the specific activity of catalase and the native resistance of tumor cells to peroxide-mediated lysis. Inhibition of catalase with aminotriazole did not augment lysis of TLX9, P388 or P815 cells by activated macrophages or by enzymatically generated H_2O_2 . With two other tumors, inhibition of catalase enhanced peroxide-mediated lysis to a modest degree. It is of interest that the latter two tumors were those with the highest and lowest levels of catalase. In another macrophage effector function, catalase appears to be of more consequence: the ability of macrophages to kill the intracellular parasite *Toxoplasma gondii* is regulated in part by the catalase activity of both the macrophage and the parasite (39).

Additional anti-oxidant defenses undoubtedly exist in tumor cells, besides those identified here. This is illustrated by the finding that inhibition of GR, GPO, and/or catalase in tumors of differing sensitivities to H_2O_2 did not render them equally sensitive. Other substances which might serve as anti-oxidants in tumor cells include other peroxidases, superoxide dismutase, vitamins C and E, free and protein cysteine, and unsaturated fatty acids.

The work described here may shed some light on the pulmonary toxicity which afflicts up to 20% of patients who receive large cumulative doses of BCNU (40, 41). The high oxygen tension in the pulmonary alveolus, compared with the rest of the body, may predispose the lung to oxygen toxicity when anti-oxidant defenses are impaired. Alveolar macrophages, in particular, rely heavily on the glutathione redox cycle for disposal of H_2O_2 (42). It is known that familial GR deficiency leads to oxidative autotoxicity in monocytes and granulocytes (20). Therefore, the pulmonary toxicity of BCNU may stem in part from its inactivation of GR in alveolar macrophages and other pneumocytes, with oxidative injury initiating an inflammatory response. We are currently studying the effect of BCNU on macrophages.

BCNU treatment of target cells had no effect on cytotoxicity mediated by T cells. This observation argues against an oxidative mechanism in that process, but does not exclude one. Tumor cell defenses might not be rate-limiting in T cell-mediated lysis. An injury might be delivered at a site not readily repaired, or by an oxidant not efficiently scavenged, by the cytosolic glutathione redox cycle.

It should also be noted that the procedures used here for decreasing the levels or the oxidoreduction of glutathione are all reversible. As such, they are not well suited to the study of spontaneous macrophage-mediated cytotoxicity, which is a relatively slow process *in vitro*.

However, the findings reported here do argue strongly for the involvement of reactive oxygen intermediates, especially H_2O_2 , in the cytotoxicity of activated macrophages in the presence of PMA (6) or anti-tumor antibody (8). The ability of the tumor cell to catabolize H_2O_2 appears to be a limiting factor in its susceptibility to lysis in both settings. It is possible that interference with glutathione reduction could also affect antibody-dependent, cell-mediated cytotoxicity in other ways, such as by promoting the capping of antigen-antibody complexes on the tumor cell surface (30).

These studies raise the possibility that therapeutic strategies involving macrophage-mediated, antibody-dependent cytolysis, or other forms of oxidant injury, might be augmented by the appropriate use of specific chemotherapeutic agents acting to inhibit tumor cell anti-oxidant defense.

Summary

The basis of resistance to oxidative injury was studied in six murine tumor cell lines that differed 54-fold in their resistance to enzymatically generated H_2O_2 . The tumors varied 56.7-fold in their specific activity of catalase, 5.3-fold in glutathione peroxidase (GPO), 3.3-fold in glutathione reductase (GR), and 2.7-fold in glutathione. There was no correlation among the levels of the three enzymes, and tumor cell resistance to lysis by H_2O_2 . However, the logarithm of the flux of H_2O_2 necessary to cause 50% lysis of the tumor cells correlated with their content of glutathione ($r = 0.91$). The protective role of glutathione was analyzed by blocking GR and GPO, the catalysts

of the glutathione redox cycle. This was facilitated by the demonstration that the anti-neoplastic agent 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) was a potent inhibitor of GR in intact tumor cells. BCNU inactivated tumor cell GR with a 50% inhibitory dose of 11 μ M and a $t_{1/2}$ of inhibition of 30 s. Complete inhibition of GR was attained with no effect on GPO or catalase. Tumor cells whose GR was inactivated by BCNU could be lysed by fluxes of H_2O_2 to which they were otherwise completely resistant. They could be killed by phorbol myristate acetate (PMA)-stimulated, bacille Calmette-Guérin-activated macrophages in numbers which were otherwise insufficient, and by nonactivated macrophages, which otherwise were ineffective. BCNU-treated target cells were also much more sensitive to antibody-dependent, macrophage-mediated cytotoxicity. However, such tumor cells were no more sensitive than controls to lysis by alloreactive T cells or by antibody plus complement.

Next, we deprived tumor cells of selenium by passage in selenium-deficient mice. GPO was inhibited 85% in such cells, with no effect on GR or catalase. Tumor cells with reduced GPO activity were markedly sensitized to lysis by small fluxes of H_2O_2 or by PMA-stimulated macrophages or granulocytes. In contrast, inhibition of catalase with aminotriazole had no effect on the sensitivity of three tumors to peroxide-mediated lysis, and had modest effects with two others.

Thus, the oxidation-reduction cycle of glutathione serves as one of the major defense mechanisms of tumor cells against three related forms of oxidant injury: lysis by fluxes of H_2O_2 , by PMA-triggered macrophages, and by macrophages in the presence of anti-tumor antibody.

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