

ANTITUMOR EFFECTS OF HYDROGEN PEROXIDE IN VIVO*

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Hydrogen peroxide, a secretory product of mononuclear phagocytes (1), accounts for a considerable portion of their nonphagocytic lysis of tumor cells in at least three circumstances: when certain secretagogues are added (2-4), when antitumor antibody is present (5, 6), or when the tumor cells are coated with eosinophil peroxidase.¹ Granulocytes also secrete H₂O₂, which may participate in their cytotoxic effects in a variety of situations (7-13). Finally, preformed or enzymatically generated H₂O₂, with or without a peroxidase, lyses tumor cells (2, 14-19).

In the present study we sought to devise a nontoxic way to deliver hydrogen peroxide to sites of malignancy *in vivo* and to test its antitumor efficacy. Glucose oxidase was chosen for this purpose because its substrates, glucose and oxygen, are abundant in the body fluids, because its sole products are H₂O₂ and gluconic acid (20), and because a flux of H₂O₂ generated enzymatically *in situ* might be less toxic than injection of preformed H₂O₂. To prolong the retention of the H₂O₂-generating system at the site of administration, glucose oxidase was coupled covalently to polystyrene microspheres.

Below are described the distribution of parenterally injected glucose oxidase-latex particles (GOL),² their antitumor effect in the peritoneal cavity and subcutaneous tissues of the mouse, the role of supplemental oxygen, the synergy between GOL and another antitumor agent capable of inhibiting a major peroxide-catabolizing pathway in tumor cells, and the relative lack of toxicity of this novel treatment.

Materials and Methods

Coupling of Enzymes to Polystyrene Microspheres. Carboxylated latex particles were obtained as a 10% suspension from Dow Chemical Co., Indianapolis, Ind. The manufacturer reported their diameter as $0.907 \pm 0.0061 \mu\text{m}$ (mean \pm SD). 13.2 ml of latex suspension was washed three times by centrifugation at 15,000 *g* for 15 min at 4°C in 30 ml 0.2 M sodium acetate buffer, pH 5.4. The pellet was resuspended in 44 ml of the buffer and mixed with 26.1 ml of glucose oxidase (β -D-glucose:oxygen 1-oxidoreductase, E.C. 1.1.3.4, type V, from *Aspergillus niger*), representing ~160 mg of protein with an activity of 1,050-1,400 Sigma units/ml (Sigma Chemical Co., St. Louis, Mo.), or with the same volume of catalase from beef liver, catalog number C100 from Sigma Chemical Co., representing about 886 mg of protein with an activity of 1.17×10^6 Sigma units/ml (one Sigma unit of glucose oxidase catalyzes the oxidation of 1 μmole of β -D-glucose/min at 35°C in air-saturated buffer, pH 5.1; one Sigma unit of catalase

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² *Abbreviations used in this paper:* BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CPK, creatine phosphokinase; GOL, glucose oxidase-latex particles; i.p., intraperitoneal; MEM, Eagle's medium essential medium, alpha variant; s.c., subcutaneous.

degrades 1 μ mole of H_2O_2 /min at 25°C in 0.05 M phosphate buffer, pH 7.0). Immediately before use, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide-metho-*p*-toluenesulfonate (Aldrich Chemical Co., Milwaukee, Wis., or Sigma Chemical Co.) was dissolved in the acetate buffer at a concentration of 254 mg/ml, and 17.6 ml was added to the latex-enzyme suspension, which was then stirred for 30 min at 4°C. After centrifugation, the pellet was resuspended in 44 ml of 1 M glycine (Fisher Scientific Co., Fair Lawn, N. J.), and the suspension was stirred for 30 min at room temperature. The latex was then washed four times by centrifugation in 30 ml of 0.9% NaCl and resuspended in 21 ml of 0.9% NaCl. Where indicated, the beads were boiled for 20 min to inactivate the enzyme.

Tests of Enzyme-coupled Beads. H_2O_2 production by GOL was measured by the loss of fluorescence due to oxidation of scopoletin in the presence of horseradish peroxidase at 37°C, as described (1). The buffer (Krebs-Ringer phosphate) (1) was equilibrated with air, contained 5.5 mM glucose, and had a pH of 7.35–7.40. The K_m of GOL for glucose was determined by measuring the initial rate of H_2O_2 production (V) at various concentrations of glucose (S) and plotting V/S vs. V (K_m for glucose = 16.7 mM; correlation coefficient = 0.95). The K_m of GOL for O_2 was estimated in an oxygen polarograph (Yellow Springs Instrument Co., Yellow Springs, Ohio) using Krebs-Ringer phosphate buffer with 100 mM glucose at 37°C in the presence of 1,744 Sigma units/ml of catalase. Under these conditions, H_2O_2 production was assumed to be twice the observed oxygen consumption. The curve of H_2O_2 produced (y) vs. time (x) fit the function, $y = a + b \ln(x)$, with a correlation coefficient of 0.99. The first derivative of this function was used to determine instantaneous rates of H_2O_2 formation (V) at given O_2 concentrations (S). A plot of V/S vs. V indicated a K_m for O_2 of 0.01 mM (correlation coefficient = 0.94). The activity of catalase-latex was measured by determining the number of beads that reduced by 50% the rate of H_2O_2 generation (1.3 nmol/min) by glucose oxidase and was compared with the volume of native catalase solution necessary to achieve the same result. The specific activity of the catalase-latex was 7.5% of that of the initial enzyme preparation. The number of latex beads per milliliter was determined in a hemocytometer with a $\times 40$ water immersion objective with phase contrast. The protein content of the native and bound enzyme preparations was measured by the method of Lowry et al. (21) with a bovine serum albumin standard. The sterility of the final latex suspensions was assessed by streaking 0.01-ml aliquots on two to four tryptic soy broth agar plates with or without sheep's blood.

Treatment of Mice. (BALB/c \times DBA/2) F_1 (CD2 F_1) female mice were obtained from Simonsen Laboratories, Gilroy, Calif., or from Charles River Breeding Laboratories, Wilmington, Mass. and entered into experiments at 7–14 wk of age. In several experiments DBA/2 mice (The Jackson Laboratory, Bar Harbor, Maine) or (DBA/2 \times BALB/c) F_1 (D2CF $_1$) mice from Trudeau Institute, Saranac Lake, N. Y. were used. In a given experiment, all mice were of one strain, chosen randomly from a single shipment from one supplier, and were within 1 wk of each other in age. P388 lymphoma and P815 mastocytoma were maintained by passage of ascites (19). For experiments, the cells were washed by centrifugation in Eagle's minimum essential medium (MEM), alpha variant, containing 100 U/ml penicillin and 100 μ g/ml streptomycin (Flow Laboratories, Inc., Walkersville, Md.) and counted in a hemocytometer. The desired number of cells in 0.5–1.0 ml of MEM was injected intraperitoneally (i.p.). GOL or an equivalent volume of 0.9% NaCl was then given through a separate i.p. injection.

For experiments involving 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (Bristol Laboratories, Syracuse, N. Y.), the mean weight of the mice was used to calculate a dose of 30 mg/kg. Sterile BCNU was dissolved in absolute ethanol and diluted in MEM so that the desired amount was injected i.p. in 0.5 ml, with a final ethanol concentration of 2%. Controls received 0.5 ml of MEM-2% ethanol.

Some of the mice were exposed for 2–3.5 h to 100% oxygen saturated with water at 1 atm pressure by placing them in a gas-tight lucite chamber into which O_2 (Matheson Gas Products, East Rutherford, N. J.) was passed after bubbling through water. Gas exited through a water seal, in which the O_2 tension exceeded 500 mm Hg when the chamber was fully occupied. The chamber temperature was maintained at 24.0°–25.5°C by a water jacket connected to a circulating temperature regulator.

For experiments with subcutaneous (s.c.) tumors, P388 cells were suspended in Krebs-Ringer

phosphate buffer without glucose and injected through a 26-gauge needle under the abdominal skin in a volume of 0.1 ml.

Where indicated, 30% hydrogen peroxide (analytical grade, Mallinckrodt, Inc., Paris, Ky.) was assayed by the scopoletin method, diluted in 0.9% NaCl, and injected i.p. Ethylhydroperoxide and cumenehydroperoxide were from Polysciences, Inc., Warrington, Pa.

Other Tests. 15 min after i.p. injection of BCNU into mice bearing advanced P815 ascites, the tumor cells were collected, washed three times at 4°C in Dulbecco's phosphate-buffered saline (Grand Island Biological Co., Grand Island, N. Y.), and assayed for glutathione reductase as described (19). Serum levels of glucose, urea nitrogen, creatinine, bilirubin, glutamic-pyruvic transaminase, and creatine phosphokinase were measured in the clinical laboratories of The New York Hospital by standard methods. The erythrocyte count was determined with a hemocytometer.

Estimation of the Percent Killing of P388. In three separate experiments, survival time was determined for groups of mice (5-33 per group) injected i.p. with P388 cells ranging in number over three to seven orders of magnitude. Survival in days (x) was plotted against \log_{10} cells injected (y), and the best fit of the data determined to the equation, $y = ax + b$, by linear regression. The following assumptions were made: that all dividing tumor cells gave rise to two daughters, each of which divided; that generation time was constant until death of the host; and that all hosts died when a fixed number of P388 cells was attained. Then, doubling time of the tumor cells in hours = $t_d = 24(\log 2)/(-a)$. Using the mean t_d from the three experiments (14.28 ± 1.94 h), the percent tumor cell kill in a given experiment with GOL was estimated as $100(1 - [2^{24n/t_d}]^{-1})$, where n = days of increased survival compared with controls.

Results

Characteristics of GOL. The properties of nine consecutive preparations of GOL are summarized in Table I. The average GOL suspension contained 5.8×10^{10} beads/ml. In air-saturated buffer at pH 7.4 with 5.5 mM glucose, 1 ml of beads typically produced 172 nmol H_2O_2 /min (range, 99-315). 99-100% of this activity was sedimentable in a microfuge or retained by a filter (0.22 μ m pore size) and thus appeared to be bound to the beads. On the average, 1.4% of the added protein was coupled to the beads; their specific activity was 17% of that of the initial enzyme preparation.

Fate of GOL after i.p. Injection. Between 4 and 8 h after injection, most of the fluid of the GOL suspension was cleared from the peritoneal cavity, leaving from 10 to 100 tightly packed macroscopic aggregates of latex particles along the anterior peritoneal lining and the mesentery, in the peripancreatic fat, against the serosal surfaces of the stomach and spleen, between the lobes of the liver, and along the inferior surfaces of the diaphragms. By 13 h, peritoneal lavage recovered only 0.045% of the injected GOL as free particles, with an enzymatic activity ~50% of that of an equivalent number of uninjected particles. Also recovered were 4×10^6 leukocytes, 75% neutrophilic, each containing ~20 GOL particles with activity ~10% of that of an equivalent number of uninjected beads. The GOL aggregates contained rare mononuclear cells at 4 h and were then gradually infiltrated with small numbers of polymorphonuclear

TABLE I
Characteristics of Nine Consecutive Lots of GOL

Characteristic	Mean \pm SEM	Range
Particles per milliliter	$5.8 \times 10^{10} \pm 1.1 \times 10^{10}$	1.3×10^{10} - 1.1×10^{11}
H_2O_2 , nmol/min $\cdot 10^6$ GOL	3.6 ± 0.9	1.0-9.6
Soluble enzymatic activity, % of total	0.3 ± 0.2	0.0-1.3
Specific activity relative to native enzyme, %	17.0 ± 3.8	2.2-37.1
Microbial cultures		negative

leukocytes through the second day. At that time, the vast majority of particles remained extracellular. However, by day 7, the aggregates were heavily infiltrated with mononuclear phagocytes, which engulfed almost all the particles. These inflammatory changes were limited to the aggregates of GOL themselves. There was no gross or histologic evidence of GOL particles or inflammation elsewhere in the peritoneal cavity or in the following organs: brain, eyes, salivary glands, submandibular lymph nodes, lungs, thymus, heart, diaphragms, stomach, duodenum, small bowel, colon, liver, spleen, mesenteric nodes, pancreas, kidneys, adrenals, uterus, ovaries, skeletal muscles, bones, or marrow. At 5 and 10 mo after injection of GOL, the histologic picture was essentially the same as on day 7, except for the appearance of GOL in rare Kupffer's cells by 10 mo.

Prolongation of Survival after i.p. Injection of GOL and 10^6 P388 Lymphoma Cells. To quantify antitumor activity of GOL, we used the P388 lymphoma, because of the highly regular relation between the number of tumor cells injected i.p. and the time to death of the mice. For example, in the experiment shown in Fig. 1, survival time was a linear function ($r = 0.98$) of the logarithm of the number of cells in the inoculum, over all seven orders of magnitude tested. Injection of 100 or more P388 cells killed 100% of the mice. Putative injection of 10 cells killed 40% of the mice. Putative injection of 1 cell killed 20% of the mice (five mice per group).

When 10^6 P388 cells were injected i.p., followed by 1 ml of GOL i.p., the mice lived an average of 3.2 d (27%) longer than those receiving tumor cells and saline. These results were observed in eight experiments, each with a different preparation of GOL. This prolongation of survival was consistent with the destruction by GOL of an average of 97.6% of the tumor cell inoculum (Table II).

Enzymatic activity of the beads appeared to be necessary, because the injection of boiled beads did not prolong survival (Table II). Such beads produced H_2O_2 at a rate averaging 0.3% of that seen with unboiled GOL.

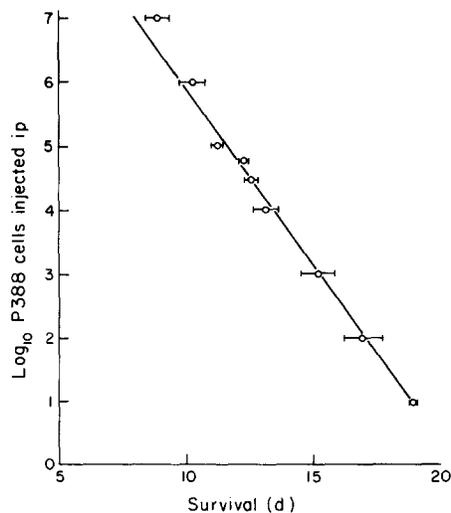


FIG. 1. Survival of CD2F₁ mice injected i.p. with various numbers of P388 lymphoma cells. Means \pm SEM for groups of five, except at doses of 6×10^4 cells (33 mice) and 10 cells (data shown for two mice that died out of five injected). Correlation coefficient = 0.98.

TABLE II
Survival of Mice Injected with 10^6 P388 Cells i.p. with or without GOL

Experiment	Number of mice/group	Days survival (mean \pm SEM)				Estimated percent tumor cell kill¶
		Saline*	SGO‡	BGOL§	GOL	
1	4	12.5 \pm 0.9		11.3 \pm 0.3	15.5 \pm 0.3	97.0
2	7	11.9 \pm 0.7		10.9 \pm 0.7	14.0 \pm 0.3	91.3
3	7	11.4 \pm 0.7			13.3 \pm 0.6	89.1
4	6	11.5 \pm 0.2	12.0 \pm 0.0		15.7 \pm 0.6	99.3
5	1	12			16	99.1
6	1	12			17	99.7
7	4	10.5 \pm 0.7			12.5 \pm 0.7	90.3
8	5	13.0 \pm 0.6			17.0 \pm 3.4	99.1
Overall mean \pm SEM		11.9 \pm 0.3	12.0	11.1 \pm 0.2	15.1 \pm 0.6	97.6

* 1 ml 0.9% NaCl i.p.

‡ 1 ml saline i.p., containing 5 Sigma units of soluble glucose oxidase.

§ 1 ml boiled GOL i.p.

|| 1 ml GOL i.p.

¶ See Materials and Methods.

Furthermore, coupling of glucose oxidase to the beads was required, because administration of soluble glucose oxidase with an enzymatic activity equivalent to 3 ml of GOL afforded no increase in survival (Table II). The dose of soluble glucose oxidase tested in this experiment corresponded to the dose lethal to 10% of the mice, as determined in preliminary experiments, and was 65% of the dose lethal to 50% of the mice. Thus, it was the highest dose of soluble glucose oxidase that could be tested for therapeutic benefit.

In contrast, the use of no more than 1 ml of GOL per mouse was dictated by its cost, not by toxicity (see below). In two experiments, 0.3 or 0.1 ml of GOL did not prolong survival after injection of 10^6 P388 cells (not shown).

Effect of Interval between Injection of GOL and P388. The tight packing of GOL in aggregates \sim 3 h after its injection presumably restricted diffusion of substrates and products. To estimate the duration of effective enzymatic activity of GOL and to evaluate the role of inflammatory cells, GOL was injected 20, 6, 3, 1, and 0 h before 10^6 P388 cells. GOL was effective if injected 0 or 1 h before P388, but not if injected 3–20 h before. In contrast, the modest number of inflammatory cells detected in the peritoneal washings peaked at the latter times, when an antitumor effect was no longer evident (Fig. 2). This suggested that GOL exerted its effect over the first few hours by a mechanism independent of newly arriving inflammatory cells.

Effect of Supplemental Substrates. In vivo, the concentrations of oxygen and glucose are probably suboptimal for glucose oxidase (20, 22–26). Table III shows the effect of exposing mice to 100% O_2 at 1 atm for 2–3.5 h after injection of 10^6 P388 cells and 1 ml GOL i.p. In five experiments, survival was prolonged an average of 4.7 d (42%), consistent with destruction of 99.6% of the tumor cell inoculum. Supplemental O_2 had no effect on the survival of mice given P388 without GOL (cf. Tables II and III).

Supplemental glucose in various amounts up to 0.36 mmol per mouse (presumably enough to triple the blood glucose concentration) did not prolong survival from P388 lymphoma, when injected alone, with GOL, or with GOL plus supplemental O_2 (not shown).

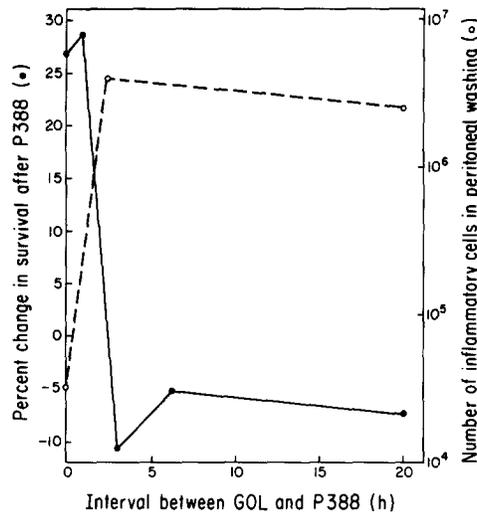


FIG. 2. Rate of loss of protective effect of GOL after i.p. injection, compared with accumulation of inflammatory cells. Five mice per group received 1 ml GOL i.p., and after the indicated intervals, a subsequent injection of 10^6 P388 cells. Their survival was compared to that of mice receiving 1 ml 0.9% NaCl i.p. followed immediately by P388 cells (11.2 ± 0.4 d). The peritoneal cavity of an additional mouse was washed out with MEM before injection of GOL or 3 or 20 h after injection, and the total number of cells, differential count, and percent peroxidase-positive cells were determined. The total number per mouse of polymorphonuclear leukocytes or peroxidase-positive cells recovered (whichever was larger) is indicated on a logarithmic scale.

Dependence of the Antitumor Effect on H_2O_2 . Reversal of the antitumor effect of GOL by boiling the beads indicated a need for enzymatic activity (Table II) but did not identify which of the three enzymatic effects of glucose oxidase was most important: production of H_2O_2 , production of gluconic acid, or consumption of glucose. To make this distinction, mice were injected simultaneously with P388, with GOL, and with latex beads to which catalase had been covalently coupled, and then given supplemental O_2 for 2 h. As shown in Table IV, catalase-latex alone had no effect on the survival of the mice. GOL prolonged their survival by 4.8 d, consistent with killing 99.6% of the P388 cells. Catalase-latex abolished the effect of GOL. Since catalase

TABLE III
Survival of Mice Injected with 10^6 P388 Cells i.p. with or without GOL followed by 2-3.5-h Exposure to 100% O_2

Experiment	Number of mice/group	Days survival (mean \pm SEM)		Estimated percent tumor cell kill§
		Saline*	GOL‡	
1	3	11.3 \pm 0.3	17.0 \pm 0.6	99.9
2	2	10.5 \pm 0.5	15.0 \pm 0.0	99.5
3	4	10.5 \pm 0.6	16.0 \pm 1.1	99.8
4	5	13.2 \pm 1.0	16.2 \pm 0.5	97.0
5	5	10.6 \pm 0.5	15.4 \pm 2.0	99.6
Overall mean \pm SEM		11.2 \pm 0.5	15.9 \pm 0.3	99.6

* 1 ml 0.9% NaCl i.p.

‡ 1 ml GOL i.p.

§ See Materials and Methods.

TABLE IV
Abrogation of Effect of GOL by Catalase-Latex

Injection*	Days survival (mean ± SEM)
Saline‡	10.6 ± 0.5
Catalase-latex§	10.4 ± 0.6
GOL	15.4 ± 2.0
GOL + catalase-latex	9.6 ± 0.2

* Five mice per group were injected with 10⁶ P388 cells i.p. plus the substances indicated. All mice received a total of 1 ml MEM and 1 ml saline. They were then placed in 100% O₂ for 1 h and 49% O₂ for 1 h.

‡ 0.9% NaCl.

§ 5 × 10⁹ particles with a catalase activity equivalent to 7,430 Sigma units (see Materials and Methods).

|| 1 × 10¹¹ particles generating 103 nmol H₂O₂/min under assay conditions (see Materials and Methods).

would be expected to break down H₂O₂, without affecting the consumption of glucose or production of acid by GOL, it appeared that H₂O₂ was necessary for the antitumor effect of GOL.

Effect of GOL against Lower Doses of P388 i.p. No mice were cured by GOL after being injected with 10⁶ P388 cells i.p. However, injection of 1 ml GOL led to long-term survival of increasing proportions of mice injected with fewer P388 cells. Two such experiments, with observation periods in excess of 441 and 303 d, are illustrated in Fig. 3. GOL appeared to cure 23% of the mice after injection of 10⁵ P388 cells, 77% after 10⁴ cells, and 92% after 10³ cells. Boiled GOL cured no mice, even when only 10³ P388 cells were injected (Fig. 3). In addition, soluble glucose oxidase, at a dose

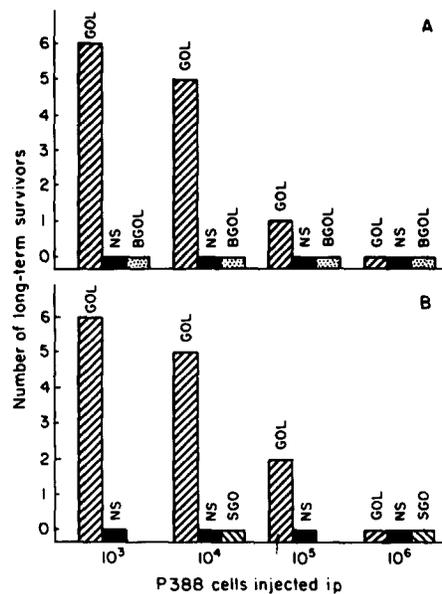


FIG. 3. Survival of mice injected with the indicated numbers of P388 cells i.p. followed by 1 ml of the following: GOL (glucose oxidase-latex), BGOL (boiled GOL), NS (normal saline), or SGO (soluble glucose oxidase, 5 Sigma units). There were seven mice per group followed for 441 d in the experiment shown in A, and 6 mice per group followed for 303 d in B.

equivalent to 3 ml of GOL, cured none of the mice injected with as few as 10^4 P388 cells (Fig. 3), nor even prolonged their survival (15.5 ± 0.4 d, compared with 15.3 ± 0.8 d for mice given saline).

Effect of GOL against s.c. P388. When injected s.c., P388 grows as a nodule and metastasizes (unpublished observations). As few as 10^3 P388 cells s.c. killed three of three mice, 10^2 cells s.c. killed two of three mice, and 10 cells s.c. killed one of three mice. As shown in Fig. 4, when 10^5 P388 cells were admixed with 0.03 ml of saline and injected s.c., the tumors grew slowly for 4 d and then expanded rapidly to diameters as great as 2.5 cm, killing six of six mice at 15.7 ± 0.5 d. Admixture of 0.03 ml of boiled GOL (3.9×10^6 particles) delayed the onset of the rapid growth phase by 8 d, but thereafter the nodules grew as fast as in the saline controls, killing all the mice at 23.3 ± 2.8 d. In contrast, injection of the same number of GOL particles together with P388 cells completely suppressed nodule formation in five of six mice. Glucose was not present in the syringe containing GOL and tumor cells, so the antitumor effect seen with GOL and not with boiled GOL presumably commenced after injection. Nonetheless, three of the mice without local nodules died from metastases. When the number of beads was tripled, boiled GOL also began to suppress nodule formation (data not shown).

Effect of GOL against a Peroxide-resistant Tumor: Synergy with BCNU. P815 mastocytoma cells require ~ 4.2 times more glucose oxidase to lyse 50% of the cells than do P388 lymphoma cells (19). 1 ml of GOL did not prolong the survival of mice injected with 10^6 P815 cells i.p., whether or not the mice received supplemental O_2 (not shown). However, in vitro, it is possible to increase the susceptibility of P815 cells to lysis by glucose oxidase by an average of 5.5-fold by exposing the cells to BCNU (19). This effect of BCNU appears to be due to its inhibition of tumor cell glutathione

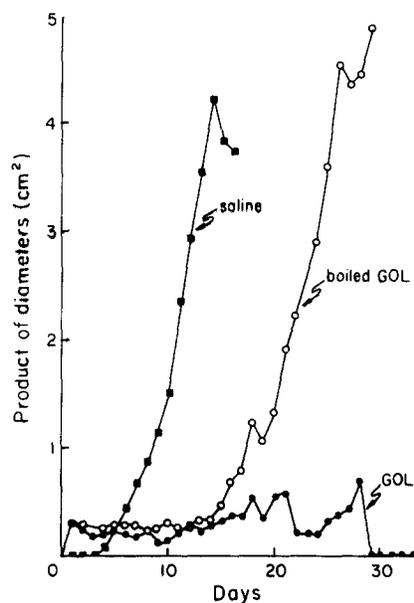


FIG. 4. Growth of nodules of P388 lymphoma after s.c. injection of 10^5 cells mixed with normal saline, GOL, or boiled GOL. Means for six mice per group. In the GOL group, only one mouse developed a nodule; the other five had stable papules due to the GOL itself.

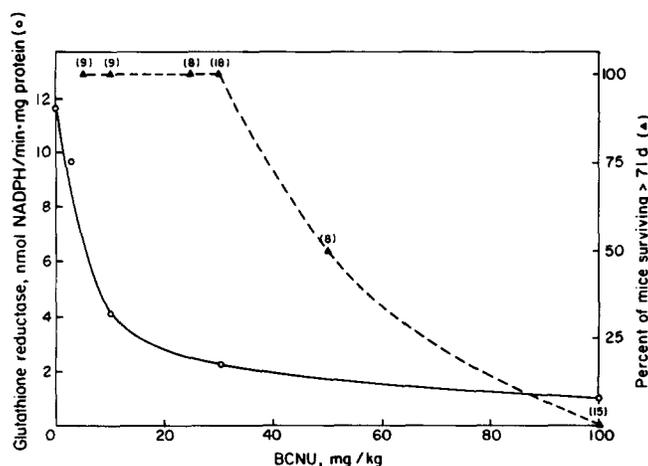


FIG. 5. Effect of dose of BCNU injected i.p. on the survival (▲) of normal mice (numbers of mice indicated in parentheses) or the activity of glutathione reductase (O) in P815 ascites cells.

reductase (19). Accordingly, we determined the dose of BCNU necessary to inhibit glutathione reductase in P815 ascites cells in vivo (Fig. 5). 30 mg BCNU/kg, the highest nonlethal dose tested (Fig. 5), inhibited glutathione reductase >80%. In the experiment shown in Table V, this dose of BCNU alone led to long-term survival in only 15% (2 of 14) mice given 10^6 P815 cells i.p. GOL alone cured none of the mice. However, the combination of BCNU and GOL produced 86% (6 of 7) long-term survivors (>422 d). No such synergistic effect was seen with boiled GOL (Table V). The combination of BCNU and GOL was not tested against P388 lymphoma, because BCNU alone produced 100% long-term survivors when given after as many as 10^7 P388 cells (not shown).

Toxicity of GOL. Mice injected with 1 ml GOL i.p. appeared healthy throughout the period of observation (up to 441 d). Among >300 mice injected with GOL, no deaths could be attributed to this agent. Erythrocyte counts and serum levels of glucose, urea, creatinine, bilirubin, glutamic-pyruvic transaminase, and creatine phosphokinase (CPK) were measured before GOL and at intervals up to 1 wk after its injection into mice exposed to 100% O_2 for 3.5 h. The results are given in Table

TABLE V
Synergy between BCNU and GOL against P815 Mastocytoma

Latex injection*	BCNU‡	Survivors/total number of mice§
None	-	0/7
	+	2/7
Boiled GOL	-	0/7
	+	0/7
GOL	-	0/7
	+	6/7

* 1 ml i.p. Mice not getting latex got 0.9% NaCl.

‡ 30 mg/kg i.p. in 0.5 ml MEM-2% ethanol. Mice not getting BCNU got the vehicle alone.

§ Survivors observed for 422 d.

VI. The blood tests remained normal, except for a single low erythrocyte count and elevated CPK values after the 8th hour. The results of complete autopsies performed up to 10 mo after injection of GOL were described above.

Effect of Preformed H₂O₂ and Organic Hydroperoxides. 30 mmol H₂O₂/kg i.p. was lethal within ~30 s, probably because of the formation of O₂ emboli after contact of H₂O₂ with erythrocyte catalase (27, 28). After 10 mmol H₂O₂/kg i.p., mice looked severely ill, but recovered. The latter dose of H₂O₂ extended the survival of mice given 10⁶ P388 cells immediately beforehand from 12.4 ± 0.7 to 15.2 ± 0.5 d. Mice given 10⁶ P815 cells i.p., followed by 10 mmol H₂O₂/kg i.p., lived no longer than controls given 0.9% NaCl alone. Ethylhydroperoxide and cumenehydroperoxide were injected i.p. in the expectation of avoiding gas formation. Both, however, were highly toxic, even at doses as low as 1 mmol/kg.

Discussion

It is of considerable interest to learn whether any proposed mediator of cytotoxicity can exert an antitumor effect in vivo. Possible obstacles to in vivo efficacy include the size of the anatomic compartments, the chemically reactive nature of the extracellular fluids, washout by the blood and lymph, metabolism by adjacent and distant tissues, and the intermingling of normal and neoplastic cells.

Hydrogen peroxide contributes to the lysis of tumor cells by macrophages and granulocytes in a variety of experimental settings in vitro (2-13).¹ The studies reported here showed that H₂O₂ generated by a solid-phase enzyme can also kill tumor cells in vivo, in the peritoneal cavity, without harm to the host.

To be effective, the enzyme that generated H₂O₂ had to be coupled to particles. Soluble glucose oxidase, given in maximum sublethal doses, did not kill an appreciable number of tumor cells, perhaps because it left the peritoneal cavity too quickly. A single injection of preformed H₂O₂ killed substantial numbers of tumor cells in the peritoneal cavity, but only at doses nearly lethal to the mice. Organic hydroperoxides were even more toxic than H₂O₂. In contrast, glucose oxidase coupled to latex particles was able to destroy up to 99.6% of an i.p. inoculum of 10⁶ P388 lymphoma cells, and 100% of smaller inocula, without evident toxicity.

TABLE VI
Effect of GOL on Blood Tests

Injection*	Hours after injection	Erythrocytes/ milliliter	Glucose	Urea nitrogen	Creatinine	Bilirubin	SGPT‡	CPK§
			mg/dl	mg/dl	mg/dl	mg/dl	U/liter	U/liter
Saline	3.5	9.1 ± 0.8 × 10 ⁹	173 ± 6	21.8 ± 0.9	0.4 ± 0.2	0.4 ± 0.3	12.5 ± 0.5	270 ± 47
GOL¶	3.5	7.1 × 10 ⁹	161	27.7	0.5	0.6	12.8	133
GOL	8	8.5 × 10 ⁹	175	21.9	0.5	0.1	11.1	801
GOL	23	7.8 × 10 ⁹	183	21.0	0.7	0.0	19.7	3,379
GOL	47	11.1 × 10 ⁹	179	20.5	0.6	0.0	15.2	860
GOL	170	6.6 × 10 ⁹	207	21.2	0.8	0.0	15.8	3,591
Reported normal values**		10.1 × 10 ⁹	174	ND‡‡	1.1	0.4	10-23	262

* Mice were injected with 1 ml of the indicated material i.p. and then exposed to 100% O₂ for 3.5 h.

‡ Serum glutamate-pyruvate transaminase.

§ Creatine phosphokinase.

|| Means ± SEM for three mice.

¶ Values from individual mice.

** From reference 25.

‡‡ Data not given.

That the effects of GOL were mediated by H_2O_2 is supported by three lines of evidence: increased antitumor activity after augmenting the concentration of the substrate, O_2 ; abolition of the antitumor effect by boiling the GOL to inactivate the enzyme; and complete protection of the tumor cells by simultaneous injection of catalase coupled to latex particles. The latter result militates against glucose consumption or acid production playing an important role in the antitumor action of GOL.

The possibility remains that the H_2O_2 produced by GOL elicits inflammatory changes, which in turn suppress tumor growth. Two considerations make this unlikely. First is the minimal extent of inflammation detected histologically. From 3 to 20 h after injection, there was a scant neutrophilic exudate. After ~ 3 h, the vast majority of beads came together in a limited number of tightly packed aggregates. During the first week, these aggregates were infiltrated by macrophages, which ingested the beads. Autopsies at intervals up to 10 mo after the injection of GOL revealed no other inflammatory changes. Second, GOL exerted an antitumor effect for up to 1 h after its injection, but not thereafter. As noted, cellular inflammatory changes only became detectable later.

In contrast to the results with P388 lymphoma, GOL alone was ineffective against P815 mastocytoma, a more peroxide-resistant tumor (19). *In vitro*, BCNU inhibited glutathione reductase in P815 cells and thereby sensitized them to lysis by H_2O_2 generated by glucose oxidase (19). Doses of BCNU well tolerated by the mice also inhibited P815 cell glutathione reductase *in vivo*. As predicted, the combination of GOL and BCNU apparently cured mice given 10^6 P815 cells *i.p.* However, proof is lacking that the synergistic interaction between GOL and BCNU *in vivo* had the same basis as that observed *in vitro*.

Supplemental O_2 , but not glucose, enhanced the antitumor effect of GOL. In mouse arterial blood, the concentrations of O_2 and glucose are normally ~ 0.14 and 9.6 mM, respectively (25). In the peritoneal cavity, the O_2 concentration is lower, probably ~ 0.06 mM (26). With GOL, we observed a K_m for oxygen of 0.01 mM and a K_m for glucose of 16.7 mM. However, these values, or the wide-ranging values reported from experiments in which soluble glucose oxidase was used (20, 22–24), may be misleading under the conditions prevailing in the peritoneal cavity after the injection of GOL. For example, assuming no replenishment, GOL would be calculated to exhaust the content of O_2 in the injected fluid in ~ 3 min. In that time, the glucose content in the same fluid would be expected to have diminished by only 9%. Thus, regardless of the K_{ms} , it is plausible that O_2 rather than glucose would be rate limiting for the production of H_2O_2 by GOL in the peritoneal cavity. The actual rate of H_2O_2 production by GOL *in vivo* was not determined, but presumably was much lower than that measured under standard assay conditions *in vitro* (Table I).

GOL did not appear to be toxic to mice observed for about half their normal lifespan, as judged by their appearance and by complete post-mortem examinations. For up to 1 wk after injection of GOL, normal values were recorded for the erythrocyte count, blood glucose, and tests of function of the kidneys and liver. However, CPK was elevated in most of the samples assayed. It is doubtful that this elevation is significant, inasmuch as histologic changes were not noted in the heart, brain, or skeletal muscle, the usual sources of clinically significant serum concentrations of

CPK. Trauma to muscle may have released CPK into the wound during collection of blood samples from the axillary vessels, giving artifactual elevations.

The basis for the apparently selective toxicity of GOL toward tumor cells rather than normal host tissues is not known. Various cells differ markedly in their susceptibility or resistance to H_2O_2 (2, 17, 19). It has not been determined whether the cells comprising the intraabdominal tissues are more resistant to H_2O_2 than the tumor cells used in these experiments. It is possible that normal cells are protected against H_2O_2 not only by intrinsic antioxidant defenses but also by washout and catabolism of H_2O_2 in the circulation by which they are served.

In the National Cancer Institute's prescreening program for new drugs, CD2F₁ mice are given 10^6 P388 cells i.p., followed by five daily injections of the agent under study. A trial is considered positive if survival is prolonged 20%.³ In this report, we administered GOL or H_2O_2 only once per mouse. It would be of interest to test multiple injections of these agents, and to investigate possible enhancement of their effects by peroxidases and by inhibitors of H_2O_2 catabolism other than BCNU.

Over the past 30 yr, there have been numerous attempts to treat tumor-bearing patients or animals with H_2O_2 . As a single agent, preformed H_2O_2 has usually been ineffective when injected into a solid tumor or into the circulation (29-35). On the other hand, animals with malignant ascites were said to survive longer than controls after injection of H_2O_2 into the peritoneal cavity (33, 36, 37). Unfortunately, none of the latter studies presented full data on this point.

In 1920, Oliver and Murphy (27) gave H_2O_2 by vein to patients who appeared to be dying of pneumonia in an attempt to generate oxygen through the catalytic decomposition of H_2O_2 in the bloodstream. The dramatic recovery of some of their patients encouraged other investigators to use parenteral H_2O_2 to increase the oxygen tension in tumors during radiotherapy (29, 32, 38-41) or chemotherapy (34, 35, 37, 38). Enhancement of the effect of radiotherapy or chemotherapy was usually claimed, although none of the clinical trials and few of the studies in animals were adequately controlled. In one well-designed study, radiation therapy was more effective when H_2O_2 was given intravenously 20 s before but not when given 5 min before or immediately after irradiation (29). Such results strongly suggested that H_2O_2 acted by enhancing the oxygen effect of radiation rather than by injuring tumor cells directly (29). Enhanced oxygenation has also been invoked to explain the enhancement of chemotherapy by H_2O_2 (35).

The antitumor effects of H_2O_2 generated by glucose oxidase cannot be attributed to enhanced tumor oxygenation because glucose oxidase consumes more oxygen than catalase releases.⁴ To our knowledge, the only previous report on the administration of glucose oxidase to tumor-bearing animals involved a single, incompletely controlled experiment in which the glucose oxidase was mixed with catalase (42). Prolongation of survival of the treated animals was attributed to glucose consumption (42).

Thus, we believe that ours is the first report to establish convincingly that H_2O_2 can exert a direct antitumor effect in vivo and thereby prolong the survival of the host. Injection of solid-phase glucose oxidase into the tumor bed was not associated with significant toxicity by the criteria employed. Finally, we extended earlier

³ Abbott, B., Division of Cancer Treatment, National Cancer Institute. Personal communication.

⁴ $Glucose + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$; $H_2O_2 \xrightarrow{\text{catalase}} H_2O + \frac{1}{2} O_2$.

observations (35, 37) that H_2O_2 can synergize in vivo with certain antitumor drugs already in clinical use. These findings help lay the groundwork for experimental therapies based on oxidative injury of tumor cells.

Summary

Glucose oxidase, covalently coupled to polystyrene microspheres (GOL), produced H_2O_2 at an average rate of 3.6 nmol/min per 10^9 beads under standard assay conditions. Injection of 1.3×10^{10} to 1.1×10^{11} GOL i.p. prolonged the survival of mice by 27% after injection of 10^6 P388 lymphoma cells in the same site, consistent with destruction of 97.6% of the tumor cells. Placing mice for several hours in 100% O_2 , the probable rate-limiting substrate for GOL, afforded a 42% prolongation of survival from P388 lymphoma, consistent with destruction of 99.6% of the tumor cells. When the P388 inoculum was 10^5 , 10^4 , or 10^3 cells, GOL led to long-term survival (presumed cure) of 23%, 77%, and 92% of the mice, respectively, consistent with reduction of the injected tumor dose to <10 cells. Subcutaneous growth of 10^5 P388 cells (~ 300 lethal dose to 50% of mice) was suppressed in 83% of mice by admixture of GOL with the tumor cell inoculum. GOL alone had no effect against a more peroxide-resistant tumor, P815 mastocytoma. However, P815 cell glutathione reductase could be inhibited in vivo by well-tolerated doses of the antitumor agent, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU). BCNU alone cured few mice with P815. Together, BCNU and GOL apparently cured 86% of mice injected with 10^6 P815 cells i.p. The protective effect of GOL was abolished by boiling it to inactivate the enzyme, by co-injection of catalase coupled to latex beads, or by delaying the injection of tumor cells for 3 h, by which time the beads had formed aggregates. Soluble glucose oxidase, in doses threefold higher than that bound to GOL, had no detectable antitumor effect. A single injection of preformed H_2O_2 readily killed P388 cells in the peritoneal cavity, but only at doses nearly lethal to the mice. In contrast, GOL had very little toxicity, as judged by the normal appearance of the mice for over 400 d, gross and microscopic findings at autopsy, and various blood tests. GOL injected i.p. remained in the peritoneal cavity, where it was gradually organized into granulomata by macrophages, without generalized inflammation. Thus, an H_2O_2 -generating system confined to the tumor bed exerted clear-cut antitumor effects with little toxicity to the host.

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