

Eradication of Interleukin 5-transfected J558L Plasmacytomas in Mice by Hydrogen Peroxide-generating Stealth Liposomes¹

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ABSTRACT

Certain human tumors are extensively infiltrated by eosinophils and contain extracellular deposits of eosinophil peroxidase, which uses hydrogen peroxide as a substrate to produce highly toxic hypohalous acids. We hypothesized that J558L HI, an interleukin 5-transfected murine plasmacytoma that is infiltrated by numerous degranulating eosinophils, would be especially sensitive to killing by hydrogen peroxide generated by glucose oxidase (β -D-glucose: oxygen-oxido reductase; EC 1.13.4). Here we report that 4 i.v. injections of 0.5 ml of hydrogen peroxide-generating, anionic Stealth liposomes containing 50 μ g of glucose oxidase eradicated s.c. implants of 10⁶ J558L HI plasmacytoma cells in 6 of 13 mice. By contrast, the J558L HI tumors grew rapidly in 13 of 13 untreated mice and in 10 of 10 mice treated with daily i.v. injections of 50 μ g of unencapsulated (free) glucose oxidase ($P = 0.002$ by log-rank test of survival curves constructed using the Kaplan-Meier method). Antisense transfected J558L tumors that did not contain eosinophils were not eradicated by the peroxide-generating liposomes in any of the 10 mice that were tested. Treatment with the liposomes was well tolerated for the first three doses (given on days 3, 4, and 5 after tumor inoculation). The fourth dose given on day 10 produced significant allergic toxicity and was, therefore, omitted in a second trial with only minimal reduction in the therapeutic response. We conclude that peroxide-generating, anionic Stealth liposomes can eradicate plasmacytomas infiltrated by eosinophils in mice. Our results, therefore, suggest that peroxide-generating compounds may be a useful experimental approach for treating those human tumors that are naturally infiltrated by eosinophils but resistant to conventional therapies.

INTRODUCTION

Certain human tumors such as lymphomas and a subset of carcinomas are sometimes extensively infiltrated by degranulating eosinophils (1-8). By administering a radiolabeled monoclonal antibody directed against EPO³ to patients whose lymphomas were infiltrated by eosinophils, we have shown that these tumors also contained extensive extracellular deposits of EPO (9, 10).

EPO is a heme-enzyme that catalyzes the production of cytotoxic hypohalous acids from hydrogen peroxide and halides or pseudohalides such as thiocyanate (11-13). These cytotoxic compounds readily kill tumor cells and vascular endothelium (14-17), prompting us to hypothesize that naturally occurring EPO deposition within certain tumors could sensitize the tumors to killing by exogenous hydrogen peroxide. In a previous report (18), however, we unexpectedly showed that EPO within free granules obtained from eosinophil sonicates actually protected tumor cells from killing by hydrogen peroxide *in vitro*.

Because our *in vitro* experiments using free granules might not have adequately duplicated the *in vivo* adherence of EPO to tumor cells or endothelial cells, it remained uncertain if naturally occurring

EPO deposits would sensitize tumors to hydrogen peroxide or protect them. An animal model of eosinophil degranulation within a tumor was clearly needed to resolve this issue.

In the current study, the animal tumor model that we selected for testing the effects of *in vivo* eosinophil degranulation uses the IL-5-secreting, transfected J558L cell line that was developed by Krüger-Krasagakes *et al.* (19). J558L is a heavy-chain-loss variant of the murine plasmacytoma cell line J558. It is of BALB/c origin. A mouse IL-5 cDNA fragment was cloned into the *Bgl*III site of the plasmid pLTR in sense (pLTR.IL-5S) and antisense (pLTR.IL-5AS) orientations. Transfectants were then produced and assayed for IL-5 activity, and two stable cell lines were eventually subcloned: J558LAS, which produces no IL-5; and 5D17 (J558L-HI), which produces approximately 500 units of IL-5 per 10⁶ cells/48 h. When inoculated into mice, the HI IL-5-secreting transfectant develops high numbers of eosinophils within the tumors and continues to grow *in vivo* at the same rapid rate as AS, the non-secreting, antisense transfectant (19). Most importantly, the J558L-HI tumor mimics the human lymphomas with eosinophilia that have been shown by us to express mRNA coding for IL-5 (5, 6, 20).

Based on our preclinical toxicology and biodistribution studies (21, 22), we then selected GO to test our hypothesis that J558L-HI tumors would be sensitive to killing by exogenous hydrogen peroxide. This enzyme generates abundant hydrogen peroxide *in vivo* under physiological conditions. Acidic liposomes were specifically selected to deliver the glucose oxidase to tumors with eosinophilia because highly cationic, eosinophil major basic protein causes disintegration of acidic liposomes (23).

Stealth liposomes were chosen for this study because they have been shown to accumulate in perivascular clusters within tumors and to have only minimal intramural accumulation in blood vessels within normal tissues (24). In addition, they remain in the blood up to 100 times longer than conventional liposomes and show dose-independent kinetics of blood clearance (25). Consequently, fewer Stealth liposomes will be cleared by the liver, and more will accumulate in implanted tumors (25). These properties are especially important when using GO because the free enzyme is normally cleared very rapidly from the blood by the liver and spleen (21, 22). This report, therefore, describes the results of our experiments using free GO and GO in anionic Stealth liposomes to treat plasmacytomas in mice.

MATERIALS AND METHODS

Tumor Cell Lines. The J558L-HI and antisense (AS) cell lines were generously provided by Dr. Tibor Diamanstein of the Free University of Berlin (Berlin, Germany). They were maintained at 37°C in a humidified cell culture incubator with 5% carbon dioxide in RPMI 1640 supplemented with 10% FCS (Sigma Chemical Co., St. Louis, MO). The human H9 T-cell line (American Type Culture Collection, Rockville, MD) was used in the clonogenic cell-killing assays because human T-cell lymphomas are infiltrated frequently by eosinophils.

For the *in vivo* studies, 10⁶ washed plasmacytoma cells were inoculated s.c. into the shaved and depilated flanks of BALB/c mice (Simonson Labs, Gilroy, CA). At this dosage, virtually all mice develop tumors greater than 10 mm in diameter within 14-16 days. To confirm the presence of eosinophilia in the HI transfectants, representative tumors at 14 days after inoculation were removed,

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³ The abbreviations used are: EPO, eosinophil peroxidase; IL-5, interleukin 5; GO, glucose oxidase.

and routine cryostat sections were prepared. The cryostat sections were then incubated in a chromogenic substrate of EPO (18), counterstained with hematoxylin, cover-slipped, and examined with routine light microscopy for the presence or red-staining eosinophils and EPO deposits.

Clonogenic Assay for EPO-coated Cells. To quantify the sensitivity of EPO-coated, human lymphoma cells to GO, we performed a variation of our previously described clonogenic cell killing assay (18). Instead of using EPO in free granules obtained from sonicated eosinophils, however, we precoated the H9 target cells with an acetate-buffered extract containing EPO and eosinophil major basic protein (17). Successful coating of the tumor cells with EPO was confirmed by incubating cytopreparations of aliquots of the coated cells in the chromogenic substrate for EPO (18).

The EPO-coated, H9 cells were then incubated with 0.025 or 0.005 $\mu\text{g/ml}$ of GO (Boehringer-Mannheim, Indianapolis, IN) in RPMI 1640 supplemented with 10% FCS for 0.5, 1.0, and 2.0 h. Following the incubation, serial dilutions of the cells were prepared for the clonogenic assay as described previously (18). The baseline control consisted of H9 cells incubated in acetate buffer only, without EPO coating, followed by exposure to GO. The negative control consisted of EPO-coated and uncoated cells exposed for 2 h to cell culture medium alone, without any GO. A final control consisted of EPO-coated cells incubated with GO in medium that also contained 10 $\mu\text{g/ml}$ catalase (Sigma) to neutralize hydrogen peroxide. All clonogenic assays were performed in duplicate, and the results were then averaged.

Production of GO in Stealth Liposomes. Stealth acidic liposomes (0.2- μm diameter) were synthesized using the protocol of Wu *et al.* (26) and a 30:30:33:5 composition of phosphatidyl choline:phosphatidic acid:cholesterol:1,2 distearoylglycerol-3-phosphoethanolamine-*N*-polyethylene glycol-2000 (Avanti Polar Lipids, Inc., and Liposome Technology, Menlo Park, CA). The lipids were first dissolved in chloroform and then combined in the appropriate ratios. Vacuum drying was used to evaporate the organic solvent, and the lipids were then rehydrated in HBSS containing GO (30 mg/ml).

After overnight hydration, the lipids and GO were freeze-thawed in liquid nitrogen 10 times and then passed 31 times through a double 200-nm polycarbonate filter (Millipore) in a Lipofast (Lipex Biomembranes, Vancouver, British Columbia, Canada) extruder. The acidic liposomes were then resuspended in HBSS at a concentration of 10 mM total lipid. Prior to use, the liposomes were separated from free GO by gel chromatography on a Sephadex minicolumn. Spectrophotometric assays demonstrated that approximately 5% of the original GO was incorporated into the liposomes, and the enzyme did not appear to have lost any of its activity from the processing.

Accumulation of GO in Plasmacytomas. The concentration of GO in s.c. plasmacytomas was measured in groups of three mice at 5, 15, and 30 min after i.v. injections of a 50- μg dose of GO in acidic stealth liposomes or free GO. A capture ELISA was then used to measure the GO in the tumor homogenates (21).

Stability of Anionic Stealth Liposomes. This experiment was designed to compare the ability of 10 μl of 0.1% Triton X-100 (positive control), 10^5 sonicated human eosinophils (a test of cationic, eosinophil granule proteins), PBS (negative control), and pH 4.2 acidic acetate buffer (acidic positive control) to liberate GO from 100 μl of acidic Stealth liposomes. After 15 min of incubation at room temperature, the test and control mixtures were fractionated on a Sephadex minicolumn (Pharmacia, Piscataway, NJ) to separate GO-liposomes from free GO in the supernatant, and aliquots of the supernatants were then assayed colorimetrically for the activity of liberated GO (21).

Survival Studies. These experiments were performed to determine if free GO or GO-Stealth liposomes could retard the growth of J558L HI and antisense tumors grown in BALB/c mice. In brief, 36 female BALB/c mice were inoculated s.c. into the right flank with 1×10^6 washed J558L HI tumor cells obtained directly from cell culture. On days 3, 4, 5, and 10 after inoculation, 13 of the mice received i.v. injections of 50 μg of GO in Stealth liposomes. Another 13 mice were the negative control and received sham injections of liposome vehicle only. The remaining 10 mice received i.v. injections of 50 μg of free (unencapsulated) GO. For comparative purposes, another group of 10 mice received s.c. inoculations of 10^6 washed, antisense-transfected tumor cells and was then treated with GO liposomes as described above.

The diameters of the tumors were measured daily for up to 35 days, and the endpoint was designated to be a tumor diameter of 15 mm. This endpoint was selected as a surrogate marker and humane alternative to death of the mice.

Survival curves were constructed using the Kaplan-Meier method, and the effects of GO and GO liposomes on survival were assessed using the log-rank test. Mice that survived for 35 days were then necropsied and examined for the presence of residual tumor.

After the results of the first experiment were obtained, the treatment protocol was repeated in a second group of mice to verify the initial findings. In this repeat experiment, however, the peroxide-generating liposomes were administered only on days 3, 4, and 5 after tumor injection to eliminate the allergic toxicity.

RESULTS

Tumor Cell Lines. The J558L HI tumors consistently contained high numbers of eosinophils, particularly at the edges of the tumors (Fig. 1). In addition, there appeared to be deposition of peroxidase activity on the vascular endothelium. By contrast, the antisense tumors had few or no eosinophils (data not shown).

Clonogenic Assays. The EPO-coated H9 cells had abundant peroxidase activity on the membranes of the cells (Fig. 2). When the EPO-coated cells were incubated with GO for varying periods of time, there were practically no surviving clonogenic units (Table 1). In the absence of EPO precoating, there were many surviving clonogenic units, except after a 2-h exposure to 0.025 $\mu\text{g/ml}$ of GO. The addition of catalase to the reaction mixture neutralized the killing of EPO-coated cells by GO.

Accumulation of GO in Plasmacytomas. Injection of GO-Stealth liposomes into tumor-bearing mice yielded tumor concentrations of GO that were approximately 10-fold higher than tumor concentrations achieved by injections of free GO (Table 2). The mice tolerated the GO-liposome injections well, and there was no evidence of any acute toxicity such as acral cyanosis, lethargy, or ruffled fur.

Stability Assay. The results of the stability assay are presented in Fig. 3. The eosinophil granules liberated almost as much GO from the liposomes as the detergent Triton X-100. As expected, acidic buffer also destabilized the acidic liposomes.

Survival Studies. The effect of GO-liposome treatment on the survival of mice bearing J558L HI plasmacytomas was pronounced ($P = 0.002$ by log-rank test; Fig. 4). Specifically, injections of peroxide-generating liposomes into 13 mice bearing J558L HI plasmacytomas eradicated the tumors in 6 of the mice that were observed

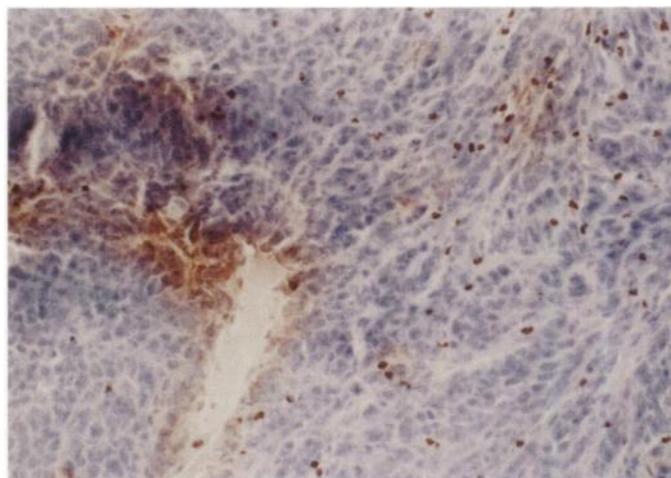


Fig. 1. Cryostat section of J558L HI tumor incubated in chromogenic substrate for EPO. Note the numerous red eosinophils scattered throughout the tumor and the extensive deposition of peroxidase activity in a blood vessel. In the antisense transfected tumor, eosinophils and EPO were much less prominent. Hematoxylin counterstain and aminothylcarbazole substrate with KCN inhibition of pseudoperoxidase and myeloperoxidase. $\times 250$.

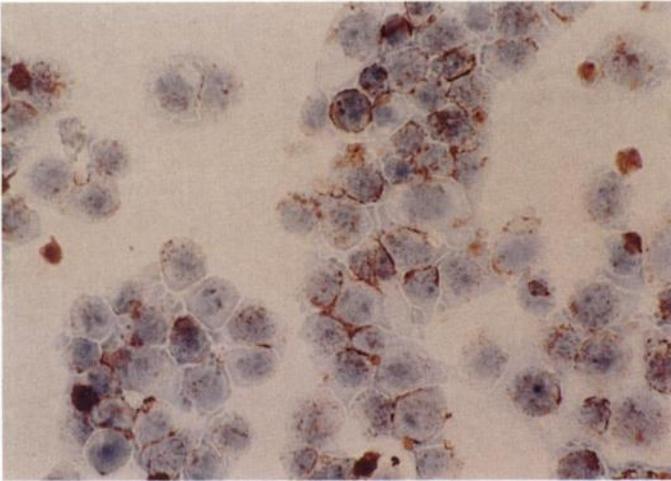


Fig. 2. Demonstration of EPO-coated H9 cells. The EPO deposits are visualized as multiple, punctate, orange granules on the membranes of most of the cells in this cytopreparation. The conditions are the same as in Fig. 1. $\times 400$.

Table 1 Clonogenic assays of EPO-coated H9 cells exposed to glucose oxidase

Glucose oxidase concentration	Exposure time (h)	Clonogenic units ^a	
		+EPO	No EPO
0.025 $\mu\text{g/ml}$	0.5	0	958 \pm 710
	1.0	2 \pm 1	895 \pm 680
	2.0	0	9 \pm 6
0.005 $\mu\text{g/ml}$	0.5	0	1760
	1.0	0	1760
None	2.0	0	805 \pm 750
	2	1760	1760

^a Results are expressed as the mean number of surviving clonogenic units/well \pm 1 SD ($n = 2$) in the original, undiluted inoculum, with or without EPO precoating. Under the conditions of this assay, the maximum number of surviving clonogenic units detectable was 1760/well (equivalent to no killing). When catalase was included in the assay mixture, the killing of EPO-coated cells by glucose oxidase was completely inhibited (clonogenic units/well $>$ 1650).

Table 2 Concentration of glucose oxidase in s.c. plasmacytomas

Time after i.v. injection	Concentration of GO ($\mu\text{g/g}$ tumor) ^a	
	Free GO	GO-Stealth Liposomes
5 min	0.22 \pm 0.2 ($n = 3$)	2.04 \pm 0.46 ($n = 3$)
15 min	0.78 \pm 0.06 ($n = 3$)	1.08 \pm 0.07 ($n = 3$)
30 min	0.046 \pm 0.03 ($n = 3$)	0.68 \pm 0.09 ($n = 3$)

^a Concentration was measured by capture ELISA in homogenates of wet tumor tissue.

for 35 days after injection, and the liposomes delayed tumor growth in the remainder of the mice. In treated mice that reached the 35-day endpoint, there was no gross or microscopic evidence of residual tumor.

By contrast, the J558L HI tumors reached a diameter of 15 mm in 14 days or less in all 13 untreated mice. Similarly, 10 of 10 mice treated with free GO developed tumors $>$ 15 mm diameter within 15 days of inoculation (not plotted). GO-liposome treatment of mice bearing J558L antisense tumors that did not contain eosinophils failed to eradicate the tumor in any of the 10 mice that were tested, and all of these mice also reached the endpoint within 15 days of tumor inoculation.

The first three injections of GO-liposomes were well tolerated in all of the mice. The fourth dose given on day 10, however, was accompanied by immediate lethargy, huddling, and ruffled fur in most of the mice. Sera from three of these animals were tested for the presence of antibodies to GO by the ELISA that we have described previously

(27). All three mice that were tested had detectable levels ($>$ 0.1 $\mu\text{g/ml}$) in their blood of antibodies directed against GO.

In the second experiment using only three doses of GO-liposomes, 4 of 13 treated mice bearing the J558L HI plasmacytoma did not develop any tumors during the entire observation period, and the effect of treatment on the survival of this group compared to the other groups remained significant ($P = 0.01$ by log-rank test). The other results of the second experiment were essentially the same as those described for the first experiment, except that there was no allergic toxicity because the fourth dose of peroxide-generating liposomes was not administered.

DISCUSSION

We have demonstrated that i.v. injections of peroxide-generating, anionic Stealth liposomes eradicated in 46% of mice a genetically engineered plasmacytoma that was infiltrated by numerous degranulating eosinophils. The observed inhibition of tumor growth seemed to depend on the presence of eosinophilia (and presumably, EPO) within the tumor because a similar effect was not observed in mice that were inoculated with the IL-5 antisense-transfected tumor that was not infiltrated by eosinophils. Furthermore, only anionic Stealth lipo-

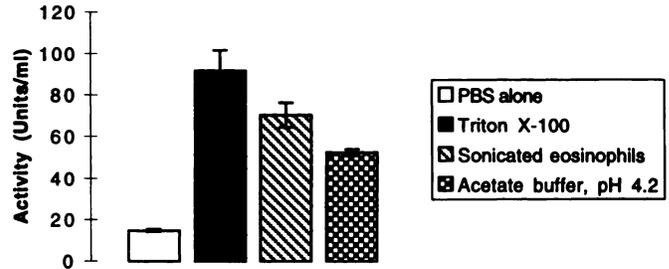


Fig. 3. Liberation of GO from acidic Stealth liposomes. When GO-Stealth liposomes were incubated for only 15 min with sonicated eosinophils, practically all of the GO was liberated from the liposomes into the supernatant. The detergent Triton X-100 and acidic acetate buffer also destabilized the acidic liposomes and liberated GO into the supernatant. Comparatively little GO was liberated after incubation in PBS alone. Bars, SD.

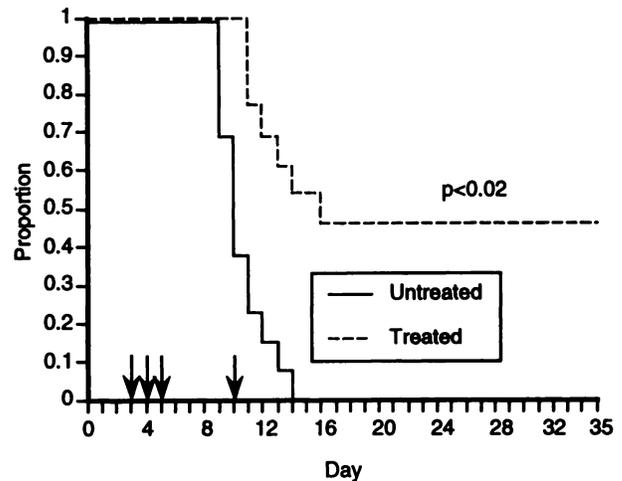


Fig. 4. Kaplan-Meier survival curves of mice bearing J558L HI plasmacytomas. Among the animals treated with GO-liposomes on the days indicated with arrows, 6 of 13 had no evidence of tumor at the end of the 35-day observation period. Untreated mice uniformly attained the endpoint (tumor diameter, 15 mm) within 15 days of inoculation of tumor. Treatment with free (unencapsulated) GO failed to delay or eradicate the tumors (not plotted). In addition, J558L antisense tumors that were treated with GO-liposomes had survival curves that were essentially similar to the untreated J558L HI tumors (endpoints within 15 days). A subsequent repeat of this experiment using only three treatments yielded similar results.

somes were capable of delivering inhibitory quantities of GO to the tumor. Thus, we propose that peroxide-generating drugs encapsulated in anionic Stealth liposomes may be a novel approach for experimental treatment of those human tumors that are naturally (1–8) or artificially (28, 29) infiltrated by eosinophils and resistant to conventional treatment.

The binary killing system that we have described in this report relies upon the deposition of EPO within tumors to provide the tumor specificity and upon the anionic Stealth liposomes to deliver the cytotoxic substrate, hydrogen peroxide, to the EPO in the tumor. Our *in vitro* clonogenic assays and *in vivo* survival studies have demonstrated clearly that this binary killing system is extraordinarily powerful, even in the presence of naturally occurring, potential inhibitors such as plasma thiocyanate, which can be converted by EPO and hydrogen peroxide to the weak oxidizer, hypothiocyanite (13). The most likely explanation for the effective cell killing in the presence of plasma thiocyanate is that the excess hydrogen peroxide that was produced continuously by GO then spontaneously reacted with the hypothiocyanite to form cyanosulfurous acid and cyanosulfuric acid (30). The latter two compounds are highly cytotoxic and bactericidal (30) and probably accounted for the *in vitro* and *in vivo* killing that we observed. Most importantly, this explanation also accounts for our finding that catalase completely neutralized killing of EPO-coated cells by GO in the clonogenic assays.

Another novel aspect of our study was our observation that sonicated eosinophil granules destabilized anionic Stealth liposomes and liberated GO from the liposomes. This finding was not particularly surprising in view of the highly cationic nature of most eosinophil granule proteins. It is significant, however, because it suggests that anionic Stealth liposomes may be an ideal vehicle for delivering drugs to inflamed sites that are infiltrated by degranulating eosinophils and presumably characterized by hyperpermeable blood vessels.

Our studies of GO concentrations in mouse plasmacytomas confirmed the reports by others that Stealth liposomes deliver much higher quantities of a drug to a tumor than administration of free drug alone (24, 25). The only apparent limitation to this approach turned out to be the intrinsic immunogenicity of the GO, which became apparent after the fourth dose that was administered 10 days after tumor injection. In our second experiment where only three doses of the liposomes were given, we observed no allergic reactions and only a slightly reduced therapeutic efficacy compared to four doses. Obviously, additional detailed studies will need to be performed to determine the dosages and timing needed to attain optimum therapeutic results from GO-liposomes. In future reports, therefore, we will describe such optimization studies.

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