Peroxidases Enhance Macrophage-Mediated Cytotoxicity Via Induction of Tumor Necrosis Factor (42841)

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Abstract. **Tumor necrosis factor (TNF) is a monokine which is involved in macrophage-mediated cytotoxicity (MMC). We have previously reported that peroxidases can activate thioglycollate-induced macrophages to the tumoricidal state *in vitro*. The present study was undertaken in an attempt to correlate peroxidase-induced MMC with production of TNF. Horseradish peroxidase (HRP) was used as the principal model for these studies. Resident and thioglycollate-induced macrophages exposed to peroxidases were examined for both MMC against 3T12 cells and production of TNF. Thioglycollate-induced macrophages exposed to HRP, bovine lactoperoxidase, or human myeloperoxidase demonstrated enhanced secretion of TNF. When exposed to HRP, both resident and thioglycollate-induced macrophages secreted significant amounts of TNF and acquired the ability to lyse 3T12 cells. However, resident macrophages were considerably less efficient in both their cytotoxic activity and TNF secretion. Macrophage-mediated cytotoxicity was eliminated by the addition of specific antisera to TNF. In addition, replacement of culture supernatants within 24 hr after exposure of the macrophages to HRP increased tumor cell killing in the absence of additional detectable TNF production, suggesting that other factors may be involved in peroxidase-induced MMC. These results indicate that TNF is intimately associated with peroxidase-induced MMC and suggest a possible role for peroxidases as immunomodulators via augmentation of macrophage capacities and functions.** [P.S.E.B.M. 1989, Vol 190]

Macrophages (Mϕ) play a pivotal role in the initiation and regulation of immunologic responses. Much evidence indicates that these cells also participate in the host's defense against neoplastic cells (1–3). Many Mϕ functions are mediated by proteins secreted by these cells in response to various stimuli. One such protein is tumor necrosis factor (TNF). Tumor necrosis factor was first described by Carswell *et al.* (4) as a tumor-specific cytolytic factor found in the serum of BCG-primed mice after exposure to endotoxin. In addition to its cytolytic activity, TNF also is capable of enhancing phagocytosis by neutrophils, increasing eosinophil cytotoxicity, and is pyrogenic (5). The role of TNF in macrophage-mediated cytotoxicity (MMC) appears to be well established (6, 7).

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Peroxidases are a group of heme-containing oxidative enzymes which catalyze the oxidation of certain substrates by H_2O_2. These enzymes have been shown to function as cytotoxic molecules in combination with H_2O_2 and halide ions (8). A recent study by Wei *et al.* (9) indicated that peroxidases are capable of activating thioglycollate-induced (TG) Mϕ to the cytotoxic state *in vitro*. Other studies have suggested that certain exogenous peroxidases possess antitumor activity *in vivo* (10). This study presents evidence that exposure of Mϕ to peroxidases stimulates the production of TNF from both resident and TG Mϕ *in vitro*. This explains, in part, the ability of peroxidases to enhance MMC. In addition, the data presented here suggest a possible immunoregulatory role of endogenous peroxidases through the activation of Mϕ *in vivo*.

Materials and Methods

Mice. Age-matched male and female C57BL/6 mice, 6–8 weeks old, were obtained from The Jackson Laboratory (Bar Harbor, ME).

Reagents. Horseradish peroxidase Type VI (HRP) and lactoperoxidase (LPO) were purchased from Sigma

Chemical Co. (St. Louis, MO). Myeloperoxidase (MyPO) was obtained from Calbiochem (La Jolla, CA). Peroxidase solutions were prepared immediately prior to use and filter sterilized using a 0.22-μm Millex-GS filter (Millipore, Bedford, MA). Dulbecco's modification of Eagle's minimal essential medium (DMEM) (GIBCO, Long Island, NY) supplemented with 2% fetal bovine serum (FBS) (Hyclone, Logan, UT), 25 m*M* Hepes (Sigma), and 25 μg/ml gentamicin (Sigma) were used for cultivation of Mϕ and preparation of peroxidase solutions. This will be referred to as complete DMEM. Phosphate-buffered saline (PBS) was prepared as described previously (9).

Assay of Endotoxin Activity. All solutions were tested for endotoxin activity using a Limulus assay (Associates of Cape Cod, Woods Hole, MA). All media and reagents contained less than 1.0 ng of endotoxin/ml. *Escherichia coli* endotoxin (serotype O127:B8) (Sigma) was used as a positive control.

Macrophage Collection. Thioglycollate-induced peritoneal Mϕ were collected as described previously (9). Briefly, mice were killed by cervical dislocation, followed by peritoneal lavage with approximately 8 ml of PBS. The cells were washed three times with serum-free DMEM, followed by resuspension in complete DMEM at a concentration of 1×10^6 cells/ml. One-hundred microliters of the cell suspension were added to each well of a 96-well tissue culture cluster (Falcon) and incubated for 2 hr at 37°C. Nonadherent cells were removed by washing twice with 100 μl of DMEM. Two-hundred microliters of complete DMEM were added to each well, and the Mϕ were incubated for 48–72 hr prior to use. Resident peritoneal Mϕ were collected in the same manner, but without prior induction with TG.

Macrophage Cytotoxicity Assay. Macrophage-mediated cytotoxicity was assayed as described by Olsson *et al.* (10). Briefly, TG Mϕ were collected and cultured as described above. After 48 hr of incubation, 100 μl of DMEM containing the indicated concentration of peroxidase were added to each well. Control wells received DMEM without peroxidase. NIH 3T12 cells were used as target cells at an effector to target cell ratio of 16:1. After 6 hr of incubation, the culture supernatants were aspirated from the wells and frozen until assayed for TNF activity. Two-hundred microliters of fresh complete DMEM were added to each well and the cells were incubated for another 42 hr. At this time the cells were fixed in 10% phosphate-buffered formalin for 10 min and stained with 0.5% methylene blue in 10 m*M* borate buffer (pH 8.4). The plates were washed extensively with borate buffer to remove unbound stain and allowed to air dry. The dye was extracted from the cells by addition of 0.1 *N* HCl, and absorbance was measured at 630 nm using a Dynatech microtiter plate reader. Although macrophages do take up some methylene blue, this amount does not greatly influence the assay. Activity of resident Mϕ was assayed in the same manner, with the exception of the effector to target cell ratio which was increased to 30:1. The calculation of cytotoxicity was performed as follows: For each treatment, including controls, the optical density of wells containing Mϕ alone were subtracted from the corresponding wells containing Mϕ plus target cells to obtain the optical density corresponding to the target cells alone. The differences between six or more cultures were used to calculate the mean optical density value for each treatment. The value obtained from untreated cultures (controls) was considered as 100% (i.e., 0% target cell killing). The means of treated cultures were expressed as a percentage of the controls using the following formula to obtain the relative target cell killing:

$$\% \text{ cytoxocity} = \left[1 - \left(\frac{\text{mean of treated cultures}}{\text{mean of control cultures}}\right)\right] \times 100$$

Each experiment was repeated a minimum of three times.

Tumor Necrosis Factor Assay. Tumor necrosis factor was assayed as described by Ruff and Gifford (12) using murine L929 cells. One-hundred microliters of DMEM (2% FBS) containing 1.5×10^4 cells were added to each well of a 96-well tissue culture cluster (Falcon) and incubated for 24 hr at 37°C. The media were aspirated, and 100 μl of the samples to be tested were added to the first well of a row and serially diluted 2-fold across the plate. Control wells received 100 μl of DMEM. In addition, all wells received 100 μl of medium containing 2 μg/ml actinomycin D. The cells were incubated for 20 hr at 37°C, followed by the addition of 50 μl of 0.033% neutral red (w/v) in PBS. The cells were incubated for 1 hr more at 37°C, followed by washing twice with 200 μl of PBS. The neutral red taken up by the cells was then extracted by the addition of 200 μl of 50% ethanol in 100 m*M* NaH_2PO_4. Absorbance of the solutions from each well was measured at 550 nm using a microtiter plate reader. The TNF titers were calculated as follows: The percentage of cytotoxicity was determined using the formula:

$$\% \text{ cytotoxicity} = \left[1 - \left(\frac{\text{OD of cells exposed to supernatants}}{\text{OD of control cells}}\right)\right] \times 100$$

Two simultaneous equations of the form $y = ax + b$ were solved, where y = % cytotoxicity above and below the theoretical 50% point and x = the reciprocal of the corresponding dilutions. Then 0.50 was substituted for y and the TNF titer was calculated and expressed as units/100 μl.

Statistical Analysis of Data. Student's t test was used to determine the significance of the effects of peroxidases on cytotoxicity. A one-way analysis of variance was used to examine the dose-response effects of peroxidases on MMC. The data were arcsin $\sqrt{p}$ transformed prior to analysis. A Student-Newman-Kull a posteriori test was performed on the transformed means to determine significant treatment level effects among the different groups. Nontransformed means are illustrated in the appropriate figures.

Results

Exposure of TG Mϕ to various peroxidases resulted in the release of a soluble cytotoxic factor into the culture supernatant. This factor was cytotoxic to actinomycin D-treated L929 cells in a manner similar to that reported for TNF. To confirm that L cell cytotoxicity was caused by TNF, Mϕ supernatants which contained cytotoxic activity were preincubated with a 1/25 dilution of polyclonal rabbit antiserum to TNF, which completely abolished the cytotoxic activity of the supernatants (data not shown).

Exposure of TG Mϕ to 8.8 μM HRP resulted in the rapid appearance of TNF activity as illustrated in Figure 1. Tumor necrosis factor activity was detectable within 2 hr after exposure to the enzyme. This activity peaked at 6 hr, then declined for the duration of the experiment. Exposure of peritoneal Mϕ to bovine LPO also resulted in TNF production with essentially similar kinetics (data not shown).

Thioglycollate-induced peritoneal Mϕ produced TNF after exposure to every peroxidase tested as presented in Table I. The peroxidase concentrations shown were the lowest and the highest concentrations employed which induced TNF activity. On a molar basis, HRP was equivalent to LPO with respect to TNF production. Myeloperoxidase was the most efficient inducer. At a concentration of 0.16 μM, MyPO induced as much TNF as 0.88 μM HRP (8 units/100 μl).

Previous results obtained in our laboratory demonstrated that exposure of TG Mϕ to peroxidases resulted in the acquisition of cytotoxicity to 3T12 cells (9). An attempt was made to correlate the production of TNF activity with MMC. When TG Mϕ were exposed to HRP *in vitro*, the Mϕ acquired the ability to lyse 3T12 target cells as shown in Figure 2. Each value represents the log of the mean of three replicate experiments ± SEM. Activation was dose dependent, with as little as 90 nM HRP inducing significant cytolytic activity. However, TNF activity was not detectable in supernatants from Mϕ exposed to 90 nM HRP. The exposure of target cells directly to HRP (without Mϕ) did not result in detectable cytotoxicity, ruling out Mϕ-independent cytolysis of target cells by HRP. Macrophage-mediated cytotoxicity was enhanced by complete replacement of the media 6–24 hr after exposure of the cells to 8.8 μM HRP as shown in Figure 3. Tumor necrosis factor was not detectable in supernatants col-

Table I. Induction of TNF by Peroxidases[a]

Inducer	Concentration	TNF[b] (units/100 μl)
HRP	0.88 μM	8 ± 1.77
	17.70 μM	185 ± 3.18
LPO	1.77 μM	12 ± 3.70
	17.70 μM	174 ± 3.09
MyPO	0.16 μM	8 ± 1.07
LPS	10 ng/ml	2 ± 0.40

[a] TNF activity obtained from TG-induced peritoneal Mϕ exposed to the indicated concentration of peroxidases.
[b] Supernatants were removed 6 hr after exposure of Mϕ to inducers and frozen until assayed.

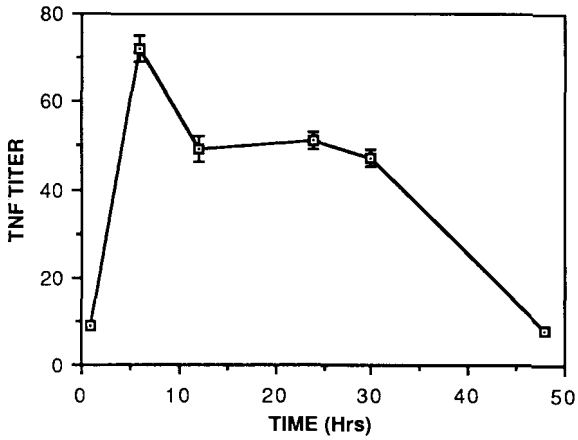

Figure 1. Kinetics of TNF production by thioglycollate-induced macrophages after exposure to 8.8 μM HRP. At the indicated times, the culture supernatants were removed and frozen until assayed for TNF activity. Each value represents the mean TNF titer from three replicate pools ± SEM, expressed as units/100 μl.

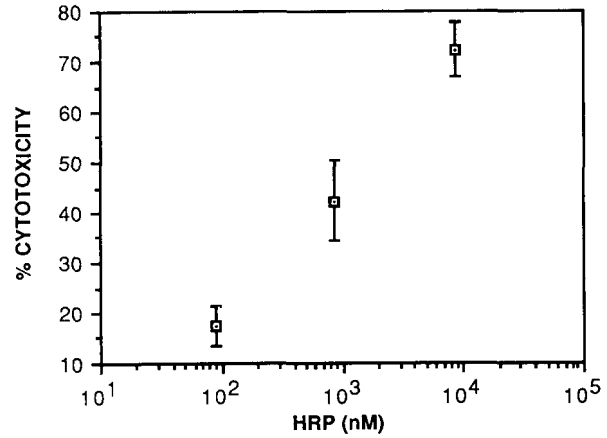

Figure 2. Macrophage-mediated cytotoxicity after exposure to HRP. Thioglycollate-induced macrophages were exposed to the indicated concentrations of HRP and cultured for 48 hr with 3T12 cells (16:1 effector to target cell ratio). The cells were fixed and stained with methylene blue and the percentage of cytotoxicity was determined by comparing the optical density of control cultures with peroxidase-treated cultures. Each value represents the mean from three replicate experiments ± SEM.

lected at any time following media replacement, demonstrating that additional TNF activity was not released by the Mϕ. The simultaneous exposure of TG Mϕ to HRP and anti-TNF serum significantly reduced MMC, indicating that TNF activity was necessary for cytotoxicity (Figure 4). It can be seen that a 1/50 dilution of anti-TNF reduced cytotoxicity by approximately 80%. In addition, the use of heat-inactivated FBS (1 hr at 56°C) reduced cytotoxicity (data not shown). All results were compared with controls which did not receive peroxidases. This minimized the contribution of spontaneous MMC in the assay.

Resident peritoneal Mϕ were also exposed to HRP and assayed for cytolytic activity and TNF production. The results of two representative experiments are shown in Table II. Exposure of resident Mϕ to 8.8 μM HRP resulted in a 15-20% cytotoxicity to 3T12 cells when compared with controls without HRP. Significant TNF activity was also detected 6 hr after exposure to the enzyme. Tumor necrosis factor activity was not detectable in supernatants from controls which were not exposed to HRP.

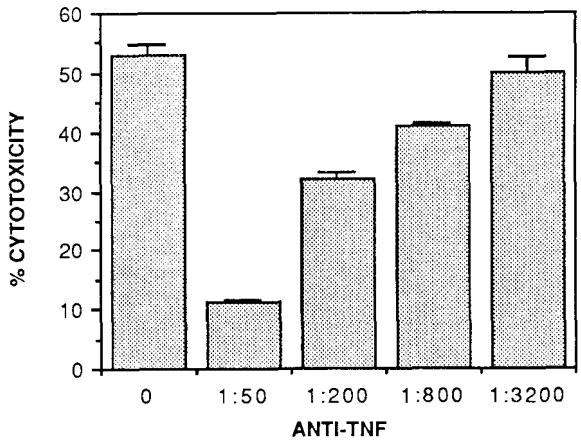

Figure 4. The effect of antitumor necrosis factor on macrophage-mediated cytotoxicity. Macrophages were incubated with 8.8 μM HRP and dilutions of antibody to TNF, followed by the addition of 3T12 target cells (16:1 effector to target cell ratio). The cells were incubated for 48 hr, fixed, and stained with methylene blue. Optical density was read spectrophotometrically. The reduction in cytotoxicity is expressed as a percentage of controls which did not receive antibody. Each value represents the mean from three replicate experiments ± SEM.

Discussion

Much experimental evidence has accumulated which suggests that Mϕ play a role in the host's defense against malignancy (2, 3, 13). The activation of Mϕ to the cytotoxic state *in vitro* has been demonstrated to occur in a stepwise fashion requiring both a priming signal derived from T cells (γ-interferon (γ-IFN)) and exposure to small amounts of endotoxin (1, 14). Previous studies in this laboratory have demonstrated that peroxidases can activate TG Mϕ to become cytotoxic (9). The present study was intended to further explore the mechanism of peroxidase-induced Mϕ activation. We have found that exposure of either resident or TG Mϕ to HRP resulted in the release of significant amounts of TNF. The kinetics of TNF production in the culture supernatants agree with data from other investigators regarding the appearance of TNF-specific mRNA in the cytoplasm of stimulated cells (15, 16).

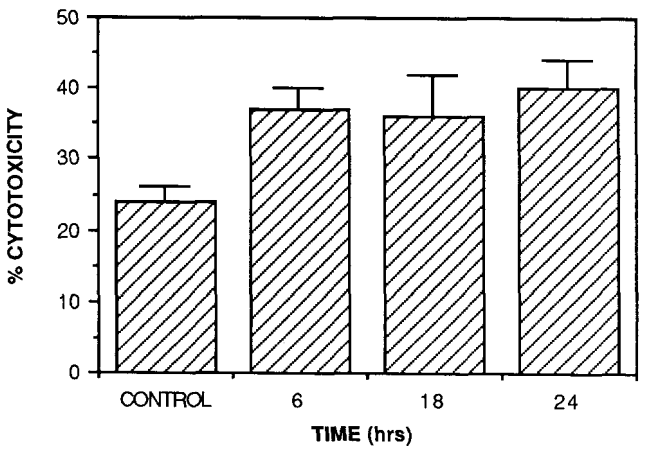

Figure 3. Effect of medium replacement on macrophage-mediated cytotoxicity. Thioglycollate-induced macrophages were exposed to 8.8 μM HRP and cultured with 3T12 cells (16:1 effector to target cell ratio). With the exception of the control, the media were removed at the indicated times and replaced with fresh DMEM. The cells were incubated for 48 hr, fixed, and stained with methylene blue. The percentage of cytotoxicity was determined by comparing the optical density of cultures which were not exposed to HRP with peroxidase-treated cultures. Each value represents the mean from three replicate experiments ± SEM.

Three peroxidases from widely diverse sources were examined for their ability to stimulate TNF production from TG Mϕ. Human MyPO is isolated from azurophilic granules of peripheral blood granulocytes (17, 18). Myeloperoxidase is released in both free and granule-enclosed forms following degranulation (19, 20). In addition, it has been reported that tissue Mϕ actively scavenge MyPO in tissues from sites of inflammation (21, 22). Bovine lactoperoxidase is isolated from cows' milk and plays a role in the protection of newborn calves from infection (23). Horseradish peroxidase is a plant peroxidase which was used as a model peroxidase due to its availability. Interestingly, all three peroxidases were capable of activating TG-induced Mϕ to the cytotoxic state *in vitro*, albeit, with widely differing efficiencies. The effectiveness of the peroxidases in inducing TNF production (on a molar basis) was: MyPO (mol wt 105 kD) > LPO (mol wt 77 kD) or HRP (mol wt 40 kD). It is intriguing to note that the most effective stimulator of TNF production was the peroxidase isolated from mammalian neutrophils. This is not too surprising if one considers the possibility that peroxidases may function as Mϕ activators *in vivo*.

The likelihood that a common contaminant was present in all of the peroxidase solutions tested, which could account for the observed activities, appears to be minimal. Analysis of the peroxidases used in this study

Table II. Enhancement of Resident Macrophage-Mediated Cytotoxicity after Exposure to HRP

Experiment	HRP (μM)	TNF (units/100 μl)	% Cytotoxicity	Mean[a] OD	P^b
1	0	<2	0	0.370 ± 0.018	
	8.8	3.93 ± 0.14	15	0.314 ± 0.015	0.02
2	0	<2	0	1.010 ± 0.045	
	8.8	6.17 ± 0.58	20	0.809 ± 0.038	0.01

[a] Mean OD of target cells ± SEM.
[b] Significance level $P \leq 0.05$.

by polyacrylamide gel electrophoresis did not reveal any common protein bands which could account for the observed activities. Bacterial lipopolysaccharide (LPS) is the most likely possible contaminant, considering the widely varying sources and methods of isolation and purification of the peroxidases tested. To rule out the possibility of endotoxin contamination, all solutions were tested and found to contain < 1.0 ng/ml of endotoxin activity by Limulus assay. In addition, commercially obtained LPS was examined for its ability to induce TNF production and to activate Mϕ to the cytotoxic state in our assay system. It was found that at least 10 ng/ml of LPS were required to induce detectable levels of TNF or detectable MMC. However, the possibility of extremely low levels of LPS acting synergistically with peroxidases to activate Mϕ cannot be ruled out at this time.

The data presented here suggest that peroxidases may play an immunoregulatory role in tumor surveillance by activating tissue Mϕ to the cytotoxic state *in vivo*. Particularly encouraging in this regard are the results obtained with resident Mϕ. We have found that exposure of resident Mϕ to HRP not only stimulated TNF production, but also renders the Mϕ cytotoxic to target cells. In other words, HRP seems to act as both a primer and a trigger for activation of resident Mϕ in lieu of the normal activating signals. However, resident Mϕ were much less sensitive to activation by peroxidases. Exposure of resident Mϕ to 8.8 μM HRP, at twice the effector to target cell ratio (30:1), resulted in less than half the cytotoxicity seen with TG Mϕ exposed to 0.88 μM HRP.

When TG Mϕ were exposed to HRP, significant amounts of TNF were released into the culture supernatants and the Mϕ acquired the ability to lyse target cells. The mechanism by which peroxidases exert their effects on Mϕ is under investigation. The primary mediator of Mϕ cytotoxicity appears to be TNF, since exposure of Mϕ to HRP in the presence of anti-TNF eliminates cytotoxicity. Furthermore, we have found that 3T12 cells are highly sensitive to soluble TNF. A recent report suggested that interleukin 1, γ-IFN, and TNF were all required for MMC (24). It is possible that interleukin 1 and γ-IFN are also produced by HRP-induced Mϕ, although the presence of anti-γ-IFN failed to affect either TNF production or MMC. Although HRP has no direct effect on 3T12 cells at the concentrations employed, possible synergy between peroxidases and TNF cannot be ruled out. However, synergy between soluble TNF and HRP seems unlikely since MMC was observed using low concentrations of HRP which did not induce detectable TNF in the culture supernatants.

It is interesting to note that in our system, free TNF in the culture supernatant did not seem to be important in MMC. If the culture media was completely replaced 6 hr after exposure to HRP, cytotoxicity was enhanced when compared with controls where the media were not replaced. Assay of culture supernatants up to 48 hr following media replacement consistently failed to detect TNF activity, indicating that additional TNF was not produced by the Mϕ. These findings suggest that membrane-bound TNF activity may be the essential component in our assay system which is in agreement with others (7). In addition, Espevik and Nissen-Meyer (25) reported that TNF activity associated with paraformaldehyde-fixed Mϕ was capable of lysing neoplastic target cells. What is particularly interesting is the enhancement of cytotoxicity when the culture supernatants were replaced. Since no additional TNF was produced by the Mϕ following media replacement, it seems likely that an additional media-derived component may be playing a role in cytotoxicity of the target cells. Preliminary results obtained with heat-inactivated FBS suggest that one or more complement components might be required for cytotoxicity. This possibility is currently under investigation.

Histologic analysis of malignant tumors often demonstrates the presence of granulocytes and mononuclear cells associated with the tumor mass (26, 27). Many investigators have demonstrated antitumor effects of Mϕ and have postulated that these cells are important in protection against neoplasia (1, 2, 28). In addition, neopterin is often present in the serum of patients with malignant tumors. Neopterin appears to be a product of Mϕ stimulated with γ-IFN and is considered to be a marker of activated Mϕ (29, 30). To explain the relationship of these results to defense against malignancies *in vivo*, we propose the following hypothesis: The growth of malignant cells results in the accumulation of granulocytes and mononuclear cells at the tumor site. Interferon produced by lymphocytes in

response to the tumor primes the Mϕ at the tumor site for cytotoxic activation. The signal for triggering cytotoxic activity may be supplied by granulocytes in the form of soluble and granule-associated MyPO released by degranulation. It has been shown that Mϕ are able to acquire MyPO activity from granulocytes during inflammatory processes (21, 22). However, oftentimes there is a failure to clear the tumor load, indicating either a block in the triggering of the Mϕ or an absence of adequate amounts of the trigger signal. We propose that peroxidases exogenously supplied could, under certain conditions, be capable of activating Mϕ to become tumoricidal and might be a useful rationale for the treatment of certain malignancies.

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