Macrophage Activation and Immunomodulation by Myeloperoxidase

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Abstract. **Myeloperoxidase (MyPo) is an enzyme found in neutrophils and monocytes that plays an important role in the microbicidal and cytocidal activities of these cells. The present studies show that this enzyme can also affect both capacities and functions of macrophages. When resident peritoneal macrophages from C57BL/6 mice were exposed to preparations of either human or canine enzyme *in vitro*, tumor necrosis factor (TNF) was released. The amount of TNF produced was dose dependent and could be neutralized with polyclonal anti-TNF. Low levels of interferon were also produced by these cells. In addition, exposure of murine macrophages *in vitro* to this enzyme resulted in increased ability to destroy 3T12 target cells. Intravenous injection of mice with myeloperoxidase induced the production of both TNF and interferon, which could be detected in the sera. Possible mechanisms of TNF induction include radical production by myeloperoxidase or ligand-receptor interaction by the binding of this enzyme to the mannosyl-fucosyl receptor. These results, when taken in their entirety, suggest that this enzyme can modulate the immune response through effects on macrophage function.** [P.S.E.B.M. 1992, Vol 199]

Myeloperoxidase (MyPo) is an enzyme found in the azurophilic granules of neutrophils that may comprise up to 5% of the dry weight of that cell (1). There is considerable evidence that this enzyme is involved in microbicidal activity of the neutrophil via the "cytotoxic triad" (2). The latter represents an oxygen-dependent pathway involving MyPo, a halide ion, and H_2O_2 which functions in the destruction of bacteria, fungi, tumor cells, etc. Evidence for its existence, as well as its importance, has recently been summarized by Klebanoff (3). In addition to neutrophils, monocytes also contain MyPo, whereas mature macrophages (Mø) are devoid of this enzyme (4). It has been reported, however, that Mø can acquire MyPo via phagocytosis of neutrophils or endocytosed granules (5–6). In addition to their phagocytic function, Mø process and present antigen, secrete various immunoregulatory molecules, and become activated to kill certain target cells (7).

Previous reports from these laboratories have shown that horseradish peroxidase, lactoperoxidase, and microperoxidase were able to induce Mø to secrete tumor necrosis factor (TNF), β-interferon (IFN), and to induce macrophage-mediated cytotoxicity (MMC) (8, 9; J. Mone, S. S. Lefkowitz, submitted for publication). The present study was undertaken to determine whether the naturally occurring peroxidase MyPo was capable of stimulating Mø capacities and functions. The present studies show that MyPo can also stimulate the production of TNF and IFN and induce MMC. Therefore, MyPo can function as an immunoregulatory molecule in addition to its previously reported functions.

Materials and Methods

Mice. Age-matched male and female C57BL/6, C3H/HeN, and C3H/HeJ mice, 8–12 weeks old, were obtained from SASCO Laboratories, Omaha, NE. Random-bred, Swiss-Webster mice were obtained from Charles River, Wilmington, MA.

Reagents. Human MyPo purchased from Calbiochem, La Jolla, CA (h-MyPo-C), had a protein to heme ratio (RZ) of 0.73. In addition, human MyPo (h-MyPo)

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purified in this laboratory (h-MyPo-L) had an RZ of 0.63. This material was purified from human neutrophils using Sephadex G-150 (Pharmacia, Piscataway, NJ) according to the methods of Andrews and Krinsky (10). A small amount of this material was repurified and had an RZ of 0.71. Preparations of canine MyPo (c-MyPo) with RZ of 0.74–0.76 were a gift from the late Julius Schultz. Activator solutions were prepared immediately prior to use and filter sterilized using a 0.22-μm Millex-GS filter (Millipore, Bedford, MA). Dulbecco's modified Eagle's medium (DMEM) (Gibco, Long Island, NY) supplemented with 2% fetal bovine serum (Hyclone, Logan, UT), 25 m*M* HEPES (Sigma, St. Louis, MO), and 25 μg/ml of gentamycin sulfate (United States Biochemical, Cleveland, OH) was used for cultivation of Mø. This will be referred to as complete DMEM. Phosphate-buffered saline was prepared as described previously (11). Antibodies to interferon were purchased from Lee BioMolecular, San Diego, CA. Solutions were tested for endotoxin activity using a Limulus amoebocyte lysate assay (Associates of Cape Cod, Woods Hole, MA). All media and reagents used contained ≤0.5 ng of lipopolysaccharide per milliliter.

Macrophage Collection. Thioglycollate-induced peritoneal Mø were collected as described previously (11). Briefly, mice were sacrificed by cervical dislocation, followed by peritoneal lavage with phosphate-buffered saline. The cells were washed three times and resuspended in complete DMEM at a concentration of 1×10^6 cells/ml. One hundred microliters of cell suspension were added to each well of a 96-well tissue culture cluster (Costar, Cambridge, MA) and incubated for 2 hr at 37°C. Nonadherent cells were removed by washing and 200 μl of complete DMEM were added to each well. The Mø monolayers were incubated 48 hr prior to use.

Tumor Necrosis Factor Assay. Peritoneal Mø from C57BL/6 mice at a concentration of 1×10^5/well were exposed to MyPo preparations for either 1 or 6 hr. After this incubation, supernatants were harvested and saved for assay. Tumor necrosis factor was assayed using either murine L929 cells, as described previously (8), or WEHI cells using a neutral red dye uptake assay (12). The TNF titers were calculated with the percentage of cytopathology, as determined using the following formula: Two simultaneous equations of the form $y = ax + b$ were solved where y equals the percentage of cytotoxicity above and below the theoretical 50% point and x equals the reciprocal of the corresponding dilutions. Then 0.50 was substituted for y and the TNF titer was calculated and expressed as units per 100 μl.

Interferon Assay. Sera or supernatants from cell cultures containing IFN were assayed using vesicular stomatitis virus in a plaque reduction assay on L929 cells (13). Interferon titers were expressed as a reciprocal of the dilution producing a 50% reduction in plaque number. Antibodies to α/β-IFN and β-IFN were utilized to neutralize IFN activity.

Macrophage Cytotoxicity Assay. Cytotoxicity (MMC) was assayed as described previously (8, 9, 11). Briefly, Mø were collected and cultured as described above. After 48 hr of incubation, 100 μl of DMEM containing the peroxidase were added to each well. Control wells received DMEM without peroxidase. NIH 3T12 cells were used as target cells at an effector cell to target cell ratio of 16:1. After either 1 hr or 6 hr of incubation, the culture supernatants were aspirated from the well and assayed immediately 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 buffered formalin and stained with 0.5% methylene blue in buffer at pH 8.4. The plates were washed to remove unbound stain and allowed to air dry. The dye was extracted with 0.1*N* HCl, and absorbance was measured at 660 nm using a microtiter plate reader. Cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \left[1 - \left(\frac{O.D.\text{ of cells exposed to peroxidases}}{O.D.\text{ of control cells}}\right)\right] \times 100 \qquad \text{[Eq. 1]}$$

Statistical Analysis of Data. A 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 p transformed prior to analysis. A Student-Newman-Keuls 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.

In Vivo Studies. Random-bred Swiss-Webster mice weighing 25–30 g were given a single intravenous injection of 0.1 ml of MyPo through the tail vein. After 90 min, these animals were bled by cardiac puncture and their sera were saved for both TNF and IFN assays.

Results

All MyPo preparations were electrophoresed using a conventional sodium dodecyl sulfate-polyacrylamide gel electrophoresis system. The gels were stained with either Coomassie blue or silver stain. It can be seen in Figure 1 that two bands were present in the c-MyPo with mol wt of approximately 60,000 and 14,000. The h-MyPo-C paralleled the canine components in size; however, there was a minor component with an apparent mol wt of 68,000. The h-MyPo-L prepared in this laboratory displayed a 60,000-mol wt band, several higher molecular weight bands, with the primary com-

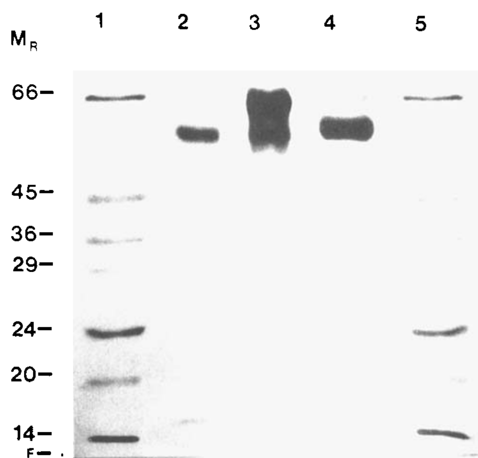


Figure 1. Separation of MyPo preparations by polyacrylamide gel electrophoresis. Lanes 1 and 5 contain molecular weight standards. Lane 2 contains c-MyPo. Lane 3 contains h-MyPo-L and Lane 4 contains h-MyPo-C.

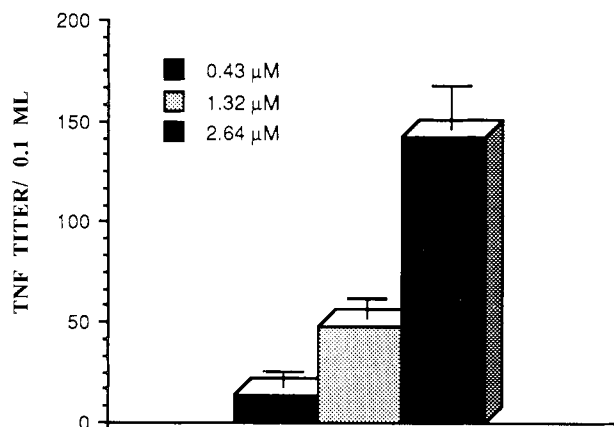


Figure 2. Induction of TNF by h-MyPo-L. Thioglycollate-induced peritoneal macrophages from C57BL/6 mice were cultured 48 hr prior to use. Macrophages were exposed to various concentrations of h-MyPo-L for 6 hr. After incubation, the culture supernatants were collected and assayed for TNF using WEHI 164 cells. Each value represents the mean of triplicate cultures ± SE.

ponent having an apparent mol wt of approximately 68,000.

Myeloperoxidase preparations were tested for their ability to induce thioglycollate-induced murine Mø to secrete TNF *in vitro*. All three preparations induced TNF in a dose-dependent manner (Figs. 2 and 3). Figure 2 shows that 0.43 μM h-MyPo-C induced approximately 11–15 units of TNF/0.1 ml. At 2.64 μM h-MyPo-L, almost 150 units were obtained. Canine MyPo at 1.32 μM induced approximately 45 units of TNF/0.1 ml (Fig. 3). The specificity of TNF induced by preparations of h-MyPo was confirmed by neutralizing activity with specific polyclonal antisera. It can be seen in Table I that anti-TNF (rabbit) completely neutralized cytotoxicity to WEHI cells, whereas normal rabbit serum had no effect.

Kinetic studies exposing thioglycollate-induced Mø to either h-MyPo-L or c-MyPo showed that peak titers

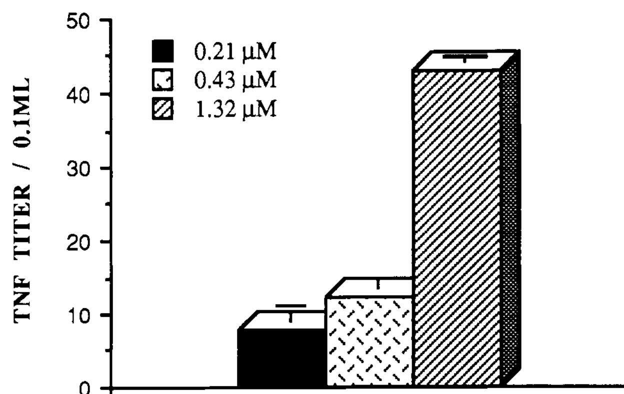


Figure 3. Induction of TNF by canine myeloperoxidase. Thioglycollate-induced peritoneal macrophages from C57BL/6 mice were cultured in DMEM 48 hr prior to use. Macrophages were exposed to various concentrations of peroxidase for 6 hr. Supernatants were harvested and separated into three pools and assayed for TNF using WEHI 164 cells. Each value represents the mean of triplicate cultures ± SE.

Table I. Neutralization of Myeloperoxidase-Induced Tumor Necrosis Factor[a]

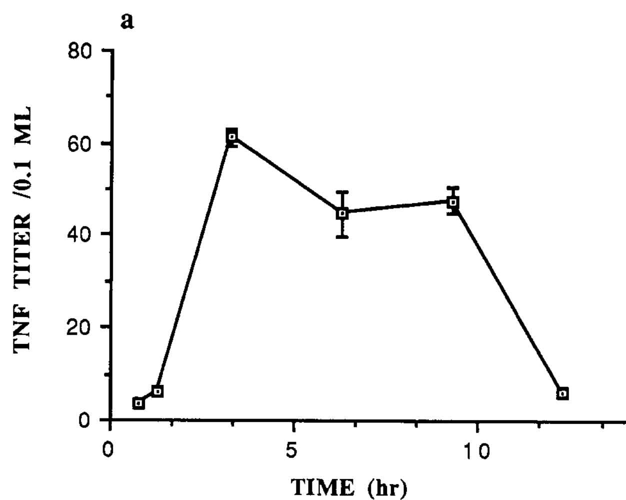
Myeloperoxidase	Dose (μM)	Control	+AB[b]
h-MyPo-C	0.33	16 ± 4.16	<2
h-MyPo-L	2.51	41 ± 3.22	<2

[a] Thioglycollate-induced peritoneal macrophages from C57BL/6 mice were exposed to two different preparations of human myeloperoxidase in culture. After 6 hr of incubation, the culture supernatants were assayed for TNF using a WEHI-164 cell assay. Each value represents the mean of triplicate cultures ± SE.
[b] Anti-TNF (rabbit).

of TNF occurred by approximately 3 hr after induction (Fig. 4). These titers remained at these levels for about 9 hr and then declined. By 12 hr, TNF was barely detectable.

Studies were undertaken to determine whether MyPo could induce thioglycollate-induced Mø to become cytotoxic. Two different strains of mice were employed, the endotoxin-resistant C3H/HeJ mice and the endotoxin-sensitive C57BL/6 mice. When Mø from C57BL/6 mice were exposed to 0.43 μM h-MyPo-C, approximately 40% cytotoxicity to 3T12 target cells was obtained (Table II). A similar level of toxicity was noted using Mø from C3H/HeJ mice. When Mø were exposed for 1 hr to 2.51 μM h-MyPo-L *in vitro*, MMC was also demonstrated. It can be seen in Table II that 20% cytotoxicity was obtained with this preparation.

Studies were undertaken to determine what effects MyPo would have on the induction of IFN. Peritoneal Mø were exposed to high levels of either c-MyPo or highly purified h-MyPo-L. Three to six hours later, the supernatants were harvested and assayed for IFN. Low levels of IFN (2–4 units)/50μl were obtained (data not shown). When mice were injected intravenously and



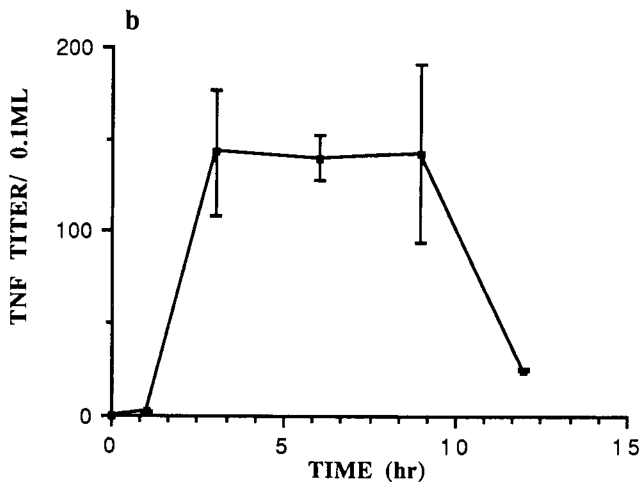


Figure 4. Kinetics of TNF production by macrophages exposed to myeloperoxidase. Thioglycollate-induced peritoneal macrophages from C57BL/6 mice were cultured in DMEM 48 hr prior to use. Macrophages were exposed to either (*a*) 1.32 μM c-MyPo or (*b*) h-MyPo-L for various time periods. Supernatants were harvested and assayed for TNF using WEHI 164 cells. Each value represents the mean ± SE of triplicate cultures assayed.

bled 90 min later, higher levels of circulating TNF were found in their sera. It can be seen in Table III that >512 units were found in certain animals. In addition, lower levels of IFN were made in these animals. It can also be seen that from 5 to 35 units/50 μl were present. From 80 to 95% of IFN activities were neutralized with antibodies to either α + β-IFN or β-IFN (data not shown).

Discussion

It should be noted that the system studied here utilized murine cells and a human enzyme. Clearly, the use of a murine MyPo with murine Mø would be more desirable; however, because of the low yield of MyPo from murine cells (14), the present system was employed. It should also be pointed out that canine enzyme functioned in a similar manner, suggesting that species differences may not be applicable here.

Table II. Macrophage-Mediated Cytotoxicity Induced by Myeloperoxidase[a]

Animal strain	Treatment dose (μM)	Percentage of cytotoxicity	O.D.	*P*<
C57Bl/6				
Control		0	0.379 ± 0.025	
h-MyPo-C	0.33	44	0.211 ± 0.011	0.001
Control		0	0.546 ± 0.013	
h-MyPo-L	2.51	20	0.438 ± 0.019	0.001
Control		0	0.404 ± 0.022	
C-MyPo	6.00	14	0.237 ± 0.019	0.002
C3H/HeJ				
Control		0	1.708 ± 0.252	
h-MyPo-C	0.33	39	1.557 ± 0.240	0.001

[a] Thioglycollate-induced macrophages were cultured for 48 hr, followed by exposure to human MyPo. After incubation, the cultures were washed and 3T12 target cells were added. Forty-two hours later, the monolayers were fixed and stained with methylene blue and the O.D. was determined. Each value represents the mean of nine cultures. Each experiment was repeated at least twice. h-MyPo-C, 6 hr of exposure; h-MyPo-L, 1 hr of exposure; C-MyPo, 6 hr of exposure.

Three different sources of MyPo were used for this study. The MyPo purified in this laboratory was not as pure as the preparations from other sources, as evidenced by several additional minor light bands on gel electrophoresis. This MyPo also had a lower RZ (0.63) compared with the other preparations. In addition, the major component had a slightly higher molecular weight (68,000). Several reports in the literature have also indicated differences in the molecular weight of this enzyme (15–18). The commercial preparation of h-MyPo-C and the c-MyPo were highly purified, as evidenced by both RZ (≥0.73) and electrophoresis.

Lipopolysacharride levels in these preparations were too low (<1 ng/mg of protein) to induce the biological effects noted. Furthermore, the effects of MyPo on the endotoxin-resistant mouse strain C3H/HeJ were similar to those obtained with C57BL/6 mice, confirming the lack of participation of lipopolysaccharide in these studies. Previous studies by these authors using other peroxidases also showed minimal effects, if any, of trace levels of lipopolysaccharide in the peroxidase preparations on either TNF production or MMC (19).

Both preparations of h-MyPo and the preparation of c-MyPo induced TNF and MMC. Since both the sources of the enzyme and the methods of purification were different, it is unlikely that induction of these activities could be due to a common trace contaminant. On a weight basis, the commercial preparation (h-

Table III. Induction of Tumor Necrosis Factor and Interferon by Myeloperoxidase *In Vivo*[a]

Inducer	Conc.	Animal pool	TNF Titer/ 0.1 ml	IFN Titer/ 0.05 ml
Canine	13.2 μM			
		1	214 ± 10[b]	8 ± 1
		2	205 ± 6	7 ± 1
		3	>512	18 ± 2
		4	>512	23 ± 10
Human	4.3 μM			
		5	53 ± 3	9 ± 1
		6	116 ± 6	15 ± 1
		7	174 ± 8	35 ± 10
Lipopolysaccharide	100 ng	8[c]	245 ± 7	ND
Poly(I):poly(C)[d]	2 mg/kg	9[c]	ND	95 ± 11
Bovine serum albumin	200 μg	10[c]	<2	<2

[a] Swiss-Webster mice were given a single intravenous injection of MyPo in 0.1 ml of saline and bled 90 min later. The sera collected were assayed for TNF and IFN, as described in Materials and Methods.
[b] Each value represents the mean of triplicate cultures ± SE.
[c] Pool of three animals.
[d] Poly(I):poly(C) was injected intraperitoneally.

MyPo-C) had the highest level of specific activity. The preparation h-MyPo-L was less pure and was less effective in its ability to induce the capacities and functions measured in this study. Interestingly, although the canine preparation induced moderate amounts of TNF, this peroxidase was less effective in MMC (data not shown). It is conceivable that these differences could relate to induction of different forms of TNF, i.e., membrane bound versus secreted (20). The latter investigators showed that the membrane-bound form was responsible for MMC.

In another study, investigators have reported the production of β-IFN by murine Mø exposed to either peroxidative enzymes or certain glycosylated proteins (J. Mone and S. S. Lefkowitz, submitted for publication). These studies prompted an investigation of the ability of MyPo to induce IFN both *in vitro* and *in vivo*. Only very low levels of IFN were produced *in vitro*. The intravenous injection of MyPo resulted in the induction of high levels of TNF by 90 min. When these same sera were assayed for IFN, low levels were obtained. It was noted that higher levels of IFN were correlated with the higher levels of TNF. It should be emphasized that the MyPo preparations used for *in vivo* studies were highly concentrated (70–200 μg/animal).

The mechanisms involved in the induction of TNF, IFN, and MMC by MyPo are not known. Neither is the relationship of these activities to enzymatic activity understood. Recent studies (J. Mone and S. S. Lefkowitz, submitted for publication; 19) have suggested that enzymatic activity of horseradish peroxidase was not necessary for induction of TNF and IFN or activation of Mø to the cytocidal state. These studies indicated that enzymatically inactive proteins from this enzyme can also induce these activities. It is known that horseradish peroxidase binds to the mannosyl-fucosyl receptor (MFR). The MFR, which has been extensively studied by Stahl and coworkers (21, 22), binds primarily mannosylated proteins. Other glycosylated proteins also bind to the MFR, but with lesser affinity. It should be emphasized that MyPo is also a glycoprotein with several mannose residues that bind to the MFR on the surface of Mø (5). It is conceivable that simple binding to this receptor may be sufficient to induce the activities reported in this study. The importance of carbohydrates for recognition by specific receptors is becoming increasingly recognized (23). A recent review refers to the likelihood that the MFR may play an important role in host defenses because it mediates the recognition and presumed killing of microorganisms (24). The MFR could also be part of a primitive "immune" recognition system based on carbohydrate-specific interactions (24). A recent paper describes the induction of TNF by a receptor-ligand interaction that mediates cell-cell adhesion (25). Studies underway in this laboratory with a more heavily glycosylated h-MyPo suggest a greater potency in these types of studies. Radicals have also been reported to be involved in TNF induction (26, 27). The production of radicals either directly or indirectly by MyPo via the cytotoxic triad is well documented (3). Therefore, the effects of MyPo, as measured in this study, could be through the production of radicals and/or binding to the MFR.

Myeloperoxidase is involved in a number of reactions. Probably one of the most important functions of this enzyme is its role in the cytotoxic triad. This enzyme plus H_2O_2 and a halide ion play a major role in the destruction of a number of microorganisms as well as mammalian cells (3). Immunosuppression by MyPo through oxidative injury to lymphocytes has also been reported (28). It has been reported recently that in an inflammatory reaction, intercellular levels of this

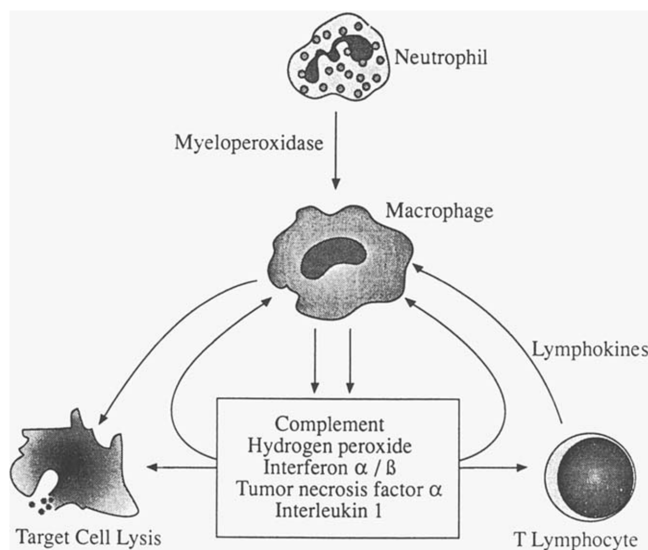


Figure 5. Paradigm for immunomodulation by myeloperoxidase.

enzyme may vary from 50 to 150 nM (29). The lower levels of MyPo utilized in the present study that induced both TNF and MMC approached these amounts; however, it should be kept in mind that the levels of MyPo in a natural environment would be difficult to relate to the amounts and type of exposure utilized in the present study. Another recent report (6) demonstrated that MyPo, either in granulocytes or in the form of granules, is readily taken up by Mø. The acquired MyPo, in contrast to endogenous peroxidase, has a half-life of 6–9 hr. A number of investigators have reported that MyPo taken up by Mø could "arm" these cells, resulting in an increase in a number of biochemical activities, including the production of oxidants (5, 6). Others (30) have described a stimulatory effect of certain iron-containing compounds (ferro-mitogens), including horseradish peroxidase, on peripheral blood mononuclear cells. They reported that this mitogenic effect could be blocked using either antioxidants or peroxidase inhibitors. These results support a possible role of MyPo and free radicals in immune regulation.

These data taken in their entirety suggest a new role for MyPo, that of an immunomodulator. We propose the following scenario: Granulocytes and mononuclear cells accumulate at the site of inflammation. Granulocytes release both soluble and granule-associated MyPo. Macrophages acquire MyPo, which, in turn, induces the production of TNF and β-IFN. By autocrine and paracrine pathways, these monokines induce the production of other cytokines, such as interleukin-1. These cytokines, in turn, activate other cells of the immune system. Figure 5 illustrates a scheme in which MyPo could function as an immunoregulator.

Currently, studies are being conducted to determine the effects of MyPo on the induction of other monokines, such as interleukin 1 and interleukin 6. Future studies will address the immunoregulatory effect of this enzyme *in vivo*.

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