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Activation of Macrophages by Peroxidases[1] (42374)

RU QI WEI,* STANLEY S. LEFKOWITZ,* DORIS L. LEFKOWITZ,*
JOHANNES EVERSE†

*Departments of *Medical Microbiology and †Medical Biochemistry, Texas Tech University Health Sciences Center, Lubbock, Texas 79430*

Abstract. Peritoneal macrophages from C57BL/6 mice were activated *in vitro* with various peroxidases and their cytotoxic activity toward 3T12 cells was determined. Destruction of 3T12 cells by macrophages stimulated with horseradish peroxidase, lactoperoxidase, and microperoxidase was observed at peroxidase concentrations as low as 9, 1.6, and 200 n*M*, respectively. A 50% cytotoxic effect was obtained at peroxidase concentrations of 0.9, 1.6, and 1.5 μM, respectively. The macrophage-stimulating activity of horseradish peroxidase was not destroyed by boiling. This, together with the high activity of microperoxidase, indicates that the macrophage-stimulating activity of the peroxidases is probably associated with the heme portion of the enzymes. On a molar basis the peroxidases are much less potent macrophage activators than interferon ($\alpha + \beta$) and endotoxin. Nevertheless, our data clearly indicate that peroxidases are a group of enzymes capable of inducing macrophage activation, resulting in cytostatic and/or cytocidal activity.

The functional diversity of macrophages as phagocytic cells is becoming increasingly recognized and has been reviewed recently (1). It is believed that as one of their functions, macrophages play a central role in tumor cell killing (2–4).

In order for the macrophage to function optimally *in vitro,* it must be activated by appropriate stimuli. A number of different substances have been shown to possess the ability to stimulate macrophages into a state in which they exert tumoricidal activity. These substances include interferon and other lymphokines, lipopolysaccharides, muramyl dipeptide, BCG,[2] and many others. Present evidence indicates that macrophage activation proceeds to different levels, dependent on the type of stimulus used, prior to their acquiring cytotoxic properties. This subject has recently been reviewed (4, 5).

Recent experiments (6) indicate that mouse peritoneal macrophages can be activated by horseradish peroxidase (HRP) to destroy intracellular parasites of the genus *Leishmania.* This ability to kill parasites was lost when the macrophages were treated with peroxidase inhibitors. Other investigators have shown that eosinophil peroxidase-coated tumor cells were 32 times more sensitive to lysis than normal tumor cells when incubated with macrophages (7). This tumor cell killing was enhanced by the production of H_2O_2 which was generated during the reaction. Studies from this laboratory have indicated that HRP, either free or immobilized, was effective in causing the regression of Novikoff hepatoma tumors in rats (8).

These observations encouraged us to investigate the ability of various peroxidases to stimulate macrophages in expressing cytotoxic activity. In this communication we demonstrate that HRP and other peroxidases can activate mouse peritoneal macrophages *in vitro,* and enhance their potential to destroy certain target cells.

Materials and Methods. *Materials.* Horseradish peroxidase type VI, lactoperoxidase, microperoxidase type III, lipopolysaccharide from *Escherichia coli* 0127:B8 (phenol extract), 3-amino-L-tyrosine, and 3-amino-1,2,4-triazole were purchased from Sigma Chemical Company (St. Louis, Mo.); murine interferon ($\alpha + \beta$) with a specific activity of 1.7×10^6

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[2] Abbreviations used: BCG, bacillus calmette–guerin; HRP, horseradish peroxidase; MEM, minimum essential medium; PBS, phosphate-buffered saline; FCS, fetal calf serum; IFN, interferon.

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units per mg protein was obtained from Lee Biomolecular (Los Angeles, Calif.); Eagle's MEM and Dulbecco's modified Eagle's MEM were products of GIBCO (Grand Island, N.Y.); and fetal calf serum was purchased from Sterile Systems, Inc. (Logan, Utah). The thioglycollate broth was a product of Baltimore Biological Laboratories (Baltimore, Md.).

Solutions of the peroxidases were made immediately prior to use and were filtered through a Millipore filter type Millex-GS (0.22 μm). All solutions contained <0.4 ng/ml of endotoxin as determined by the limulus amebocyte lysis assay which was obtained from Associates of Cape Cod Inc. (Woods Hole, Mass.).

Cell cultures. 3T12 (Balb/c) spontaneously transformed mouse fibroblasts were obtained from the American Type Culture Collection (Rockville, Md.). All cultures and assays were done in either Eagle's MEM or Dulbecco's modified MEM. The medium was supplemented with 0.025 mg/ml gentamycin.

Mice. Age-matched, female C-57BL/6 mice, 8–12 weeks old, were purchased from the Jackson Laboratories (Bar Harbor, Maine).

Macrophage monolayers. Mice were injected with 1 ml of thioglycollate broth 4 days prior to macrophage collection. Peritoneal macrophages were collected using phosphate-buffered saline (PBS) containing 10 units Heparin/ml. The animals were killed by cervical dislocation and approximately 8 ml PBS was injected into the peritoneal cavity which was gently massaged for 1 min followed by a withdrawal of the fluid. The collected cells were washed three times in PBS and adjusted to 1×10^6 cells/ml in Dulbecco's MEM without serum. The cell suspension (100 μl) was added to each well of a 96-well microtiter plate. The cells were incubated under 5% CO_2 at 37°C for 2 hr. After attachment, the peritoneal cells were washed twice with media without serum and supplied with 250 μl of Dulbecco's MEM containing 2% FCS. The cells were then cultured for a minimum of 3 days prior to the addition of target cells. The adherent cells routinely consisted of >99% macrophages. (A differential count of the exudate cells consistently indicated a macrophage content of 75–78%.)

Cytotoxic quantitations. Cytotoxic activity of the macrophages was measured using methods similar to those of Weinberg *et al.* (9). These methods have been modified (10) and are described below. After cultivation of mouse peritoneal macrophage monolayers for 3 days, the medium was removed and 50 μl of fresh medium containing 2% FCS was added. Macrophages were exposed to the activating factor, which either remained present during the entire experiment or was removed by washing the cell monolayers prior to the addition of the target cells. MEM (100 μl) containing from 3.3 to 6.6×10^3 3T12 cells was added to each well. Approximately 15 to 30 macrophages were present per target cell. After 48 hr of incubation, the medium was removed and the macrophages were washed with PBS.

The number of target cells present was determined by at least two of the following three methods. First, the cells were fixed in methanol and stained with giemsa. Results were obtained by counting the number of 3T12 cells per microscopic field in four different areas of each well, as described by Weinberg *et al.* (9). Second, a modification of the procedure of Olsson *et al.* (11) was used in which the cells were fixed with 10% phosphate-buffered Formalin for 10 min and then washed with 0.01 *M* borate buffer, pH 8.4. Cultures were then stained with 1% methylene blue for 30 min, followed by at least four washings with buffer. After drying at room temperature, 200 μl of 0.1 *N* HCl was added to each well. The solution was quantitatively collected from each well with the use of 1 ml of deionized water, transferred to a cuvette, and the optical density at 664 nm was determined spectrophotometrically. Third, the absorption at 660 nm of the HCl solutions in each well was determined directly with the use of a Dynatech minireader. Consistent results were obtained with the three methods.

Each experiment consisted of a series of experimental points, each done at least in triplicate, as well as the following controls: macrophages only; macrophages with 3T12 cells, but no other additions; 3T12 cells with activator, but without macrophages; and 3T12 cells only. Control points were routinely determined in triplicate and the results were averaged. The percentage cytotoxicity was calculated from the average value obtained from each experimental point and the average value obtained from the wells containing macrophages and 3T12 cells without other additions, using the formula

$$100\% - \frac{\text{experimental}}{\text{control}} \times 100 = \%\ \text{cytotoxicity}.$$

Results. *Activation of mouse macrophages by peroxidases.* Thioglycollate-induced mouse peritoneal macrophages exposed to small amounts of HRP exhibited increasing levels of cytotoxic activity toward 3T12 cells with increasing levels of the peroxidase. This is illustrated in Figure 1A. Effects were noted with

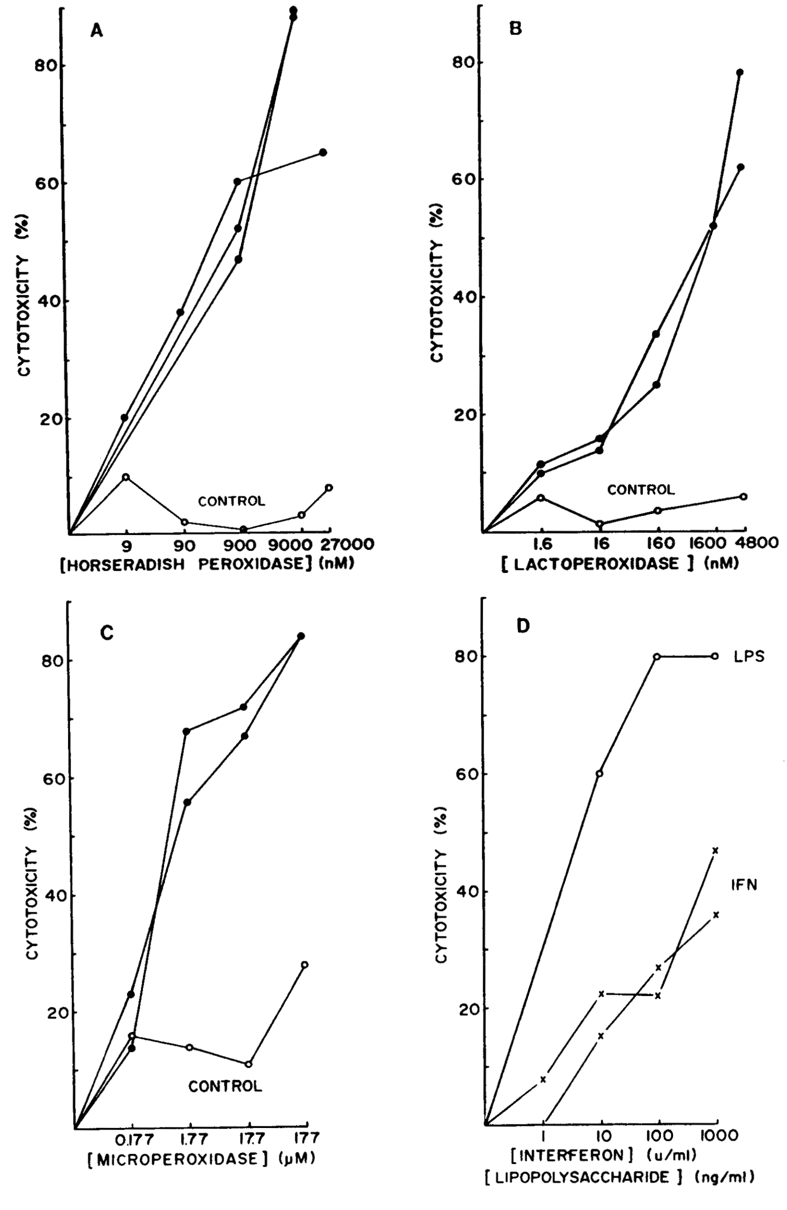

FIG. 1. Cytotoxic activity of macrophages to 3T12 cells following activation by the substances indicated in the figures. Peroxidases: (●), results of one or more typical experiments; (○), cytotoxicity in the absence of macrophages. Lipopolysaccharide and interferon: graphs indicate results of one or two representative experiments; cytotoxicity in the absence of macrophages was not detectable.

concentrations as low as 9 nM (0.1 unit) of HRP. The highest concentrations of HRP (9 μM) resulted in a 80 to 90% inhibition of tumor cell growth. When resident macrophages were used under the same conditions, minimal inhibition of tumor cell growth was obtained. Exposure of 3T12 cells to similar levels of HRP in the absence of macrophages yielded a cytotoxicity of 8 ± 3%.

In other experiments the HRP was incubated with the macrophages for 20 hr after which the cells were washed and then incubated with the target cells for 48 hr. The results obtained were not significantly different from those obtained when the HRP was allowed to remain present during the 48-hr incubation with the 3T12 cells.

Figures 1B and 1C show the effects of lactoperoxidase and microperoxidase on tumor cell inhibition. With both of these substances, cytotoxicity of peritoneal macrophages to 3T12 cells increased significantly with increasing levels of the peroxidases. It was found, however, that higher levels of microperoxidase exerted significant toxicity to 3T12 cells in the absence of macrophages; this was not observed with the other peroxidases (note control values in Figure 1C).

Since the concentrations of the peroxidases used are comparable, a comparison of their relative potency as macrophage activators can be made. To do this we calculated the concentrations of peroxidase needed to produce a 50% cytotoxic effect under our assay conditions. These concentrations were found to be 0.9, 1.6, and 1.5 μM for horseradish peroxidase, lactoperoxidase, and microperoxidase, respectively. Thus, under our assay conditions the peroxidase enzymes used were essentially equal in potency.

Effect of denatured horseradish peroxidase. The finding that microperoxidase, which is virtually devoid of protein, has about the same activity as horseradish peroxidase and lactoperoxidase on a molar basis, could suggest that the protein portion of the peroxidases may not be essential for the macrophage-stimulating activity. To test this possibility we did several experiments in which we used a horseradish peroxidase solution that had been incubated at 100°C for 10 or 30 min prior to its addition to the macrophage cultures. Although this treatment causes a complete denaturation of the protein, the macrophage-stimulating activity was largely retained (Table I). This result confirms that the protein portion does not play an essential role in the activation of the phagocytes. We must conclude therefore that the macrophage-stimulating activity is mainly or exclusively associated with the heme portion of the peroxidases.

Activation of macrophages by interferon and lipopolysaccharide. Murine interferon ($\alpha + \beta$) was found capable of activating mouse peritoneal macrophages as measured by the inhibition of 3T12 cell proliferation. Figure 1D illustrates the macrophage-stimulating activity of IFN as a function of its concentration. Significant tumoricidal activity was observed with concentrations as low as 100 units IFN/ml, and approximately 40% cytotoxic activity was obtained using 1000 units IFN/ml. The same figure also illustrates the potent effect of LPS, which activates macrophages at concentrations of less than 1 ng/ml. A concentration of 100 ng/ml resulted in an 80% inhibition of tumor cell growth, which did not increase with higher concentrations of LPS.

Although IFN in the amounts used in our experiments appears not to be a very potent activator of macrophages, its potency can be considerably increased when used in conjunc-

TABLE I. EFFECT OF BOILED HORSERADISH PEROXIDASE ON MACROPHAGE ACTIVATION AND TARGET CELL INHIBITION

Expt	Control	Native HRP (nM)			Boiled HRP (nM)		
		90	900	9000	90	900	9000
I	40 ± 3[a]	38 ± 4	20 ± 5	10 ± 4	25 ± 5	18 ± 4	8 ± 2
II	71 ± 2	N.D.[b]	52 ± 2	39 ± 1	N.D.	46 ± 3	38 ± 1

[a] All values represent means of OD readings from 5–7 wells in a microtiter plate ± SEM; $P \leq 0.03$ for both native and boiled HRP compared to controls which were not exposed to the enzyme.

[b] N.D., not determined.

tion with LPS. When macrophages were incubated with IFN for 4 hr and washed prior to addition of target cells, less than 10% cytotoxicity was observed, even at IFN concentrations as high as 1000 units/ml. Similarly, incubation of macrophages with 10 ng LPS for 1 hr produced approximately 27% cytotoxicity. However, when the phagocytes were primed with 100 units/ml IFN for 4 hr, followed by a 1-hr exposure to 10 ng/ml LPS, cytotoxic activities as high as 84% were obtained. The results demonstrate that IFN can act both as a primer and a trigger. Using IFN as a primer and LPS as a trigger was more effective than either alone. Similar results have been reported previously (4, 5).

Discussion. A large number of chemically unrelated compounds are capable of stimulating macrophages to various levels of activation and some of these compounds activate macrophages to a tumoricidal state. In this communication we have demonstrated that another group of compounds, the peroxidases, are able to promote tumoricidal activity in macrophages.

Several reports in the literature have indicated that macrophages can be activated by peroxidases. Horseradish peroxidase has been used in this manner to destroy intracellular parasites of the genus *Leishmania* (6). Eosinophil peroxidase has been used to sensitize *T. cruzi* to mouse peritoneal macrophages (12). In addition, eosinophil peroxidase-coated tumor cells were shown to be more sensitive to lysis by macrophages than noncoated tumor cells (7). In the latter two observations the peroxidase was allowed to interact with the target cell rather than with the macrophage. To the best of our knowledge this is the first time that a peroxidase has been shown to be capable of activating macrophages to the tumoricidal state.

Endotoxin is frequently present as a contaminant in many reagents. It can be seen from these studies, as well as from others (9), that endotoxin can significantly enhance macrophage activation. In order to rule out the possibility that the observed results were due to endotoxin contamination, all reagents including peroxidases were tested using the limulus amebocyte lysis assay. All reagents were found to contain <0.4 ng/ml endotoxin after filtration through a 0.22-μ filter. Therefore the possibility that the results reported in these studies may reflect endotoxin contamination is minimal.

The cytotoxic assays used in the present studies in principle do not distinguish between cytostasis and cytolysis. However, Hibbs and his colleagues have shown that the 3T12 cell is a lytic cell type, when exposed to activated macrophages (9, 13). In concert with their observations we have observed sufficient loss of target cells in selected experiments to clearly indicate cytolysis. Based on this information we assume that the macrophage-mediated reduction in tumor cell number in our experiments is a cytolytic process.

Since macrophages produced H_2O_2 the possibility exists that the cytotoxic activity is in fact exerted by the peroxidases, which in the presence of H_2O_2 and a halide ion form a toxic system. In this case the cytotoxic activity would involve a direct attack by the peroxidases on the tumor cells, and the macrophages would merely contribute the required H_2O_2. This possible mechanism is unlikely to take place in our experiments, based on the following findings. First, in none of our experiments did we observe a decrease in the number of macrophages present, indicating that the toxic activity was specifically directed against the 3T12 cells. Since the peroxidase–H_2O_2–halide system is toxic to all types of cells one would expect that macrophages are also subject to this toxic activity. This was not observed. Second, when the macrophages were exposed to HRP for 20 hr followed by extensive washing prior to the addition of the 3T12 cells no difference in the cytotoxic effects was observed. Thus, removal of the remaining soluble HRP did not affect the results, suggesting that a direct contact between HRP and the tumor cells is not essential for the tumoricidal activity to occur. Third, in a single experiment in which we used a mixed population of macrophages (obtained from two different strains of mice) we observed clear evidence of macrophages destroying macrophages. This result appears to rule out the possibility that macrophages may be more resistant to the toxic effects of the peroxidase–H_2O_2–halide system than the 3T12 cells. Based on these three observations we conclude that the macrophages are directly involved in the killing of the tumor cells rather than in playing an indirect role.

The experiment with the mixed-macrophage populations further suggests the interesting possibility that peroxidases activate macrophages to a stage in which they, rather than being just tumoricidal, recognize and kill any cells that are non-"self," including tumor cells. We intend to further explore this possibility in a forthcoming communication.

The similarity in potency between the lactoperoxidase and horseradish peroxidase was noted. We therefore tested microperoxidase, which is a degradation product of cytochrome C, consisting of a heme moiety with 12 amino acids still attached. In other words, it is a peroxidase that is practically devoid of protein structure. We found that its potency was similar to the horseradish and lactoperoxidase. This indicates that the protein portion of the peroxidases does not play an essential role in the mechanism of macrophage activation; apparently the macrophage-activating activity resides predominantly, if not solely, in the heme portion of the molecule. This conclusion is further substantiated by the finding that denaturation of the protein portion of the horseradish peroxidase by boiling did not abolish its macrophage-stimulating activity. It is noteworthy that boiling also did not completely abolish the peroxidase activity of the enzyme; using guaiacol as a substrate approximately 40% of the enzymatic activity was retained.

For comparative purposes we also determined the potency of lipopolysaccharide and murine interferon ($\alpha + \beta$) to activate macrophages under identical conditions. In order to compare the potencies of all activators tested, we calculated the concentrations required to promote a 50% inhibition of tumor cell growth under our assay conditions. The results are presented in Table II. In order to do this for our interferon preparation, we assumed that pure murine interferon ($\alpha + \beta$) is about 10^9 units/mg and has a MW of 20,000 (14). We further assumed that the impurities in our interferon preparations are inert. It is clear from the results that on an equimolar basis interferon and LPS are orders of magnitude more potent macrophage activators than the peroxidases.

TABLE II. POTENCY OF VARIOUS COMPOUNDS AS MACROPHAGE ACTIVATORS

Activator	Concentration producing 50% toxicity (μM)
Interferon (α and β)[a]	4.5×10^{-5}
Lipopolysaccharide[b]	1×10^{-5}
Interferon, followed by LPS	3×10^{-7}
Horseradish peroxidase	0.9
Lactoperoxidase	1.6
Microperoxidase	1.5

[a] Based on the assumptions that pure interferon contains 10^9 units/mg protein and has an average MW of 20,000 (14).

[b] Based on an average MW of 300,000 (17).

Buchmueller and Mauel (6) found that the macrophage-activating activity of horseradish peroxidase was inhibited in the presence of 50 m*M* 3-aminotriazole. This observation suggests that the enzymatic integrity of the peroxidases must be maintained in order to obtain macrophage activation and that the actual macrophage-activating factor could be a product generated by the enzymatic activity of the peroxidases rather than the peroxidases themselves. Unfortunately, in our laboratory 3-aminotriazole at 50 m*M* concentration proved to be too toxic to our macrophages, preventing us from determining its effect in our system. Nevertheless, other observations also indicate that enzymatically active peroxidases may be involved in the activation of certain cells. Several recent studies have shown that mast cell degranulation can be induced by eosinophil peroxidase (15, 16). These studies further showed that the degranulation is in fact promoted by the peroxidase–H_2O_2–halide system, which at high concentrations is cytotoxic but at low concentrations induces specific physiological events such as degranulation and histamine release. Whether such a system is also operative in macrophage activation remains to be established. Nevertheless, these data suggest the exciting possibility that endogenous peroxidases may have important regulatory functions involving various cells of the reticuloendothelial system.

A recent communication from this laboratory (8) reported that the injection of horseradish peroxidase and lactoperoxidase into hepatoma-bearing animals results in significant tumor regression. The mechanism by which these peroxidases promote tumor regression *in vivo* is uncertain, but the toxic activity appeared to be specifically directed against the tumor tissues. Our present findings indicate that peroxidases are able to activate macrophages to a tumoricidal state *in vitro,* and the possibility therefore exists that they may also be able to do so *in vivo.* If this were the case, the observed specificity of the peroxidase-mediated antitumor activity can easily be explained.

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1. Hume DA, Gordon S. Macrophage Biochemistry. In: Michelson AM, Bannister, JV, eds. Life Chemistry Reports. London, Harwood Academic, Vol 1:pp1–47, 1982.
2. Fidler IJ. Recognition and destruction of target cells by tumoricidal macrophages. Isr J Med Sci **14**:177–191, 1978.
3. Cohn ZA. The activation of mononuclear phagocytes: Fact, fancy and future. J Immunol **121**:813–816, 1978.
4. Johnson WJ, Somers SD, Adams DO. Expression and development of macrophage activation for tumor cytoxicity. In: Adams DO, Hanna MG, eds. Contemporary Topics in Immunobiology. New York, Plenum, Vol 13:pp127–146, 1984.
5. Meltzer MS, Occhionero M, Ruco LP. Macrophage activation for tumor cytotoxicity: Regulatory mechanisms for induction and control of cytotoxic activity. Fed Proc **41**:2198–2205, 1982.
6. Buchmueller Y, Mauel J. Studies on the mechanisms of macrophage activation: Possible involvement of oxygen metabolites in killing of Leishmania Enriettii by activated mouse macrophages. J Reticuloendothel Soc **29**:181–191, 1981.
7. Nathan CF, Klebanoff SJ. Augmentation of spontaneous macrophage-mediated cytolysis by eosinophil peroxidase. J Exp Med **155**:1291–1308, 1982.
8. Everse KE, Lin H, Stuyt EL, Brady JC, Buddingh F, Everse J. Antitumor activity of peroxidases. Brit J Cancer **51**:743–746, 1985.
9. Weinberg JB, Chapman HA, Hibbs JB Jr. Characterization of the effects of endotoxin on macrophage tumor cell killing. J Immunol **121**:72–80, 1978.
10. Lefkowitz SS, Lefkowitz DL, Wei RQ, Everse J. Activation of macrophages with oxidative enzymes. In: DiSabato G, Everse J, eds. Methods in Enzymology. New York, Academic Press, Vol 132, in press.
11. Olsson NO, Leclerc A, Jeannin JF, Martin F. A simple photometric microassay for the quantitative evaluation of macrophage mediated cytotoxicity on adherent cancer cells. Ann Immunol **133**:245–254, 1982.
12. Nogueira NM, Klebanoff SJ, Cohn ZA. *T. cruzi:* Sensitization to macrophage killing by eosinophil peroxidase. J Immunol **128**:1705–1708, 1982.
13. Weinberg JB, Hibbs JB Jr. Morphological observation of cytotoxicity for tumor cells. In: Herscowitz HB *et al.,* eds. Manual of Macrophage Methodology. New York, Dekker, pp345–355, 1981.
14. Stewart WE II. The Interferon System. New York, Springer-Verlag, 2nd ed, p172, 1981.
15. Henderson WR, Chi EY, Klebanoff SJ. Eosinophil peroxidase-induced mast cell secretion. J Exp Med **152**:265–279, 1980.
16. Chi EY, Henderson WR. Ultrastructure of mast cell degranulation induced by eosinophil peroxidase. J Histochem Cytochem **32**:332–341, 1984.
17. Leive L, Morrison DC. Isolation of lipopolysaccharides from bacteria. In: Ginsburg V, ed. Methods in Enzymology. New York, Academic Press, Vol 28:p254, 1972.

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