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EOSINOPHIL-MEDIATED MAMMALIAN TUMOR CELL CYTOTOXICITY: ROLE OF THE PEROXIDASE SYSTEM[1]

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Guinea pig eosinophil peroxidase (EPO) had a potent cytotoxic effect on a line of mouse ascites lymphoma cells designated LSTRA when combined with hydrogen peroxide and a halide. Cytotoxic activity, as measured by ^{51}Cr release, was dependent on each component of the EPO-H_2O_2-halide system, was completely inhibited by catalase, azide, and aminotriazole, and was partially inhibited by cyanide and gelatin under our experimental conditions. The EPO system was more effective at pH 6.0 than at pH 7.0. At pH 7.0 and with added gelatin (0.005%) to reduce background ^{51}Cr release, iodide was the only effective halide with 10^{-5} M required. Chloride (0.1 M), although ineffective alone under these conditions, significantly increased cytotoxic activity at otherwise ineffective iodide concentrations (10^{-6} and 10^{-7} M). At pH 6.0, iodide, bromide, chloride, or thiocyanate ions could be employed as the halide cofactor.

Intact guinea pig eosinophils (96% purity), when stimulated by preopsonized zymosan or phorbol myristate acetate (PMA), were cytotoxic to the LSTRA cells. A chloride-containing medium at pH 7.4 was employed; the further addition of iodide was required for optimum cytotoxic activity with both stimuli and was an absolute requirement when zymosan was used. The cytotoxic effect was inhibited by catalase, azide, and aminotriazole but not by cyanide under our experimental conditions. When PMA-stimulated human neutrophils were employed as the effector cells, the effect on the LSTRA cells was comparable to that of eosinophils except that the addition of iodide to the chloride-containing medium did not significantly increase cytotoxicity; and cyanide, like azide, aminotriazole, and catalase, was completely inhibitory. These findings indicate that eosinophils, like neutrophils, are cytotoxic to mammalian tumor cells when appropriately stimulated and suggest that this cytotoxicity can be mediated by the EPO-H_2O_2-halide system.

Eosinophils, like neutrophils and monocytes, contain a peroxidase in cytoplasmic granules. Eosinophil peroxidase (EPO),[3] however, differs from the neutrophil and monocyte peroxidase (myeloperoxidase, MPO) in primary structure, heme prosthetic group, and in the reactions that they catalyze (1-3). Like MPO, EPO can participate in a peroxidase-H_2O_2-halide antimicrobial system that is effective against several species of microorganisms (4-6). Intact eosinophils are bactericidal, but they appear to be less efficient in this regard than are neutrophils (7-11), possibly due to decreased phagocytic activity (7-10; however, see 11). Eosinophils also can function as effector cells in an antibody-dependent cytotoxic reaction against erythrocytes (12), Chang liver cells (12), Girardi human heart cells (12), and the schistosomulum stage of *Schistosoma mansoni* (13-18). There is histochemical evidence that damage to schistosomula is associated with the release of peroxidase from the eosinophils and its deposition on the surface of the organisms (19, 20). Eosinophils, like neutrophils, respond to perturbation of the plasma membrane with a respiratory burst, in which oxygen is converted to hydrogen peroxide (7, 8, 21, 22). This raises the possibility that eosinophils can release the components of the peroxidase system into the extracellular fluid where they can react with a halide to damage adjacent cells.

In this study, the cytotoxic activity of the cell-free EPO-H_2O_2-halide system against a line of mouse ascites lymphoma cells was investigated and compared to the cytotoxic activity of intact eosinophils activated by either phagocytosis or phorbol myristate acetate (PMA). Evidence was obtained that suggested that eosinophil toxicity to the tumor cells was mediated in part by the extracellular release of components of the peroxidase system.

MATERIALS AND METHODS

Target cells. A Maloney virus-induced ascites lymphoma cell line designated LSTRA was maintained by serial i.p. passage in BALB/c mice. The cells were harvested by peritoneal lavage, processed, and labeled with ^{51}Cr ($Na_2{}^{51}CrO_4$, 100 to 300 μCi/μg Cr, Amersham/Searle Corp., Arlington Heights, Ill.) as previously described (23). The stock cell suspensions contained 2×10^6 viable ^{51}Cr-labeled LSTRA cells per milliliter in 0.1 M sodium sulfate for experiments with isolated EPO and in 0.154 M sodium chloride for experiments with intact eosinophils.

Effector cells. Eosinophils were obtained from guinea pig peritoneal exudate cells after weekly i.p. injections of polymyxin B (24). The peritoneal cells were washed once in modified Hanks' solution without calcium, magnesium, and phenol red (MHS, Microbiological Associates, Walkersville, Md.) with 0.1% gelatin (Difco Lab., Detroit, Mich.) added (MHS/gel). Contaminating erythrocytes were removed by hypotonic lysis and the eosinophils were purified by sedimentation through Percoll

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[3] Abbreviations used in this paper: EDTA, ethylenediamine tetracetic acid; EPO, eosinophil peroxidase; MHS, modified Hanks' solution; MHS-gel, modified Hanks' solution with 0.1% gelatin; MPO, myeloperoxidase; NaCl/gel, 0.154 M sodium chloride with 0.1% gelatin; PBS, phosphate-buffered saline, pH 7.0; PMA, phorbol myristate acetate.

(Pharmacia Fine Chemicals, Piscataway, N. J.) as previously described (6). The cells were washed three times in MHS/gel, twice in Dulbecco's phosphate-buffered saline, pH 7.0, without calcium and magnesium (PBS) (GIBCO, Grand Island, N. Y.) and suspended in NaCl/gel (0.154 M sodium chloride with 0.1% gelatin) at a concentration of 2×10^7 cells/ml. The final cell suspension contained a mean of 96% eosinophils (range 93 to 98%) with less than 2% heterophils and the remainder mononuclear cells.

Neutrophils were obtained from healthy, normal human volunteers. Blood collected with 0.002% potassium ethylenediamine tetraacetic acid (EDTA) as anticoagulant was mixed with an equal volume of 3% dextran in 0.154 M NaCl and allowed to sediment for 20 min. The cells of the leukocyte-rich supernatant were washed two times in MHS/gel, subjected to hypotonic lysis, and sedimented through Percoll as previously described (6). The cells were washed five times as described above for eosinophils and suspended in NaCl/gel at a final concentration of 2×10^7/ml. The preparation contained a mean of 96% (range 94 to 99%) neutrophils, approximately 1% eosinophils, and the remainder mononuclear cells.

Preparation of EPO. EPO was partially purified from guinea pig peritoneal eosinophils and assayed by guaiacol oxidation as previously described (6).

Cytotoxicity assay. The components of the reaction mixture (see legends to tables and figures) were incubated in 10 x 75 mm glass tubes for 60 min at 37°C. In experiments in which intact eosinophils and preopsonized zymosan were employed, the reaction mixture was centrifuged at 400 × G for 5 min at 22°C to concentrate the cellular components in a pellet before incubation. Cytotoxicity was determined by the ^{51}Cr-release assay as previously described (23). Briefly, 1 ml of ice-cold PBS was added to each tube at the end of the incubation period, the tubes were centrifuged (300 × G, 10 min at 4°C) and 1.0-ml aliquots of the supernatant solution were counted in a well-type scintillation counter. Duplicate tubes were employed for each experimental condition, and the average ^{51}Cr release for each set was used as a single n for statistical analysis. ^{51}Cr release was expressed as a percentage of the maximum releasable activity observed in supernatants of cells treated with 1% Triton X-100 (Sigma Chemical Co., St. Louis, Mo.).

Special reagents. Other chemicals and biologicals were obtained as follows: glucose oxidase (Type V, 200 units/mg), guaiacol, phorbol-12-myristate-13-acetate (PMA), dimethylsulfoxide/(DMSO) from Sigma Chemical Co.; 3-amino-1,2,4-triazole from Schwarz/Mann, Orangeburg, N. Y.; catalase (beef liver, crystalline 60,000 units/mg) from Worthington Biochemical Corp., Freehold, N. J.; zymosan from Nutritional Biochemical Co., Cleveland, Ohio. All other reagents used were of the highest purity available from commercial sources. The catalase was heated at 100°C for 15 min where indicated; zymosan was opsonized with fresh frozen pooled guinea pig serum (Totem Farms, Redmond, Wash.), as previously described (25) and PMA was dissolved in DMSO to the desired concentration.

Statistical analysis. Experimental results are expressed as the mean ± standard error. Differences were compared by using Student's two tailed *t*-test for independent means (not significant NS $p > 0.05$).

RESULTS

Cytotoxic effect of cell-free EPO system. Table I demonstrates the cytotoxic effect of EPO, H_2O_2 and a halide on the LSTRA tumor cell line at pH 7.0 and 6.0. At pH 7.0, incubations were conducted both in the presence and absence of 0.005% gelatin, whereas gelatin was always added at pH 6.0. At pH 7.0 and in the presence of gelatin, incubation with the complete peroxidase system (iodide 10^{-5} M) increased ^{51}Cr release by the LSTRA cells from 19.6% to 72.2% ($p < 0.001$). ^{51}Cr release on deletion of any of the components of this system (EPO, H_2O_2, iodide) was not significantly different from background. The cytotoxic effect also was abolished by decreasing the iodide concentration to 10^{-6} M or by the substitution of 10^{-1} M chloride for iodide. The deletion of gelatin from the reaction mixture resulted in an increase in the background ^{51}Cr release from 19.6 to 27.2% ($p < 0.001$). As in the presence of gelatin, the addition of EPO, H_2O_2 and 10^{-5} M iodide greatly increased ^{51}Cr release, an effect that was dependent on each component of the system. ^{51}Cr release with 10^{-6} M iodide was not significantly greater than background; however, a small but significant increase was observed when iodide was replaced by 10^{-1} M chloride ($p < 0.01$). A cytotoxic effect of the system was observed with either 10^{-6} M iodide or 0.1 M chloride even in the presence of gelatin when the pH was lowered to 6.0 ($p < 0.001$). Gelatin (0.005%) was employed in all subsequent experiments.

TABLE I

Cytotoxic activity of the EPO-H_2O_2-halide system[a]

Supplements	^{51}Cr Release		
	pH 7.0		pH 6.0
	0.005% gel	No gel	0.005% gel
	%		
None (background)	19.6 ± 0.9 (25)[b]	27.2 ± 1.9 (11)	18.9 ± 1.2 (13)
EPO + I (10^{-5} M) + H_2O_2	72.2 ± 1.7 (25)	79.2 ± 2.6 (8)	78.1 ± 1.3 (13)
Iodide omitted	17.3 ± 1.4 (12)	31.9 ± 2.5 (8)	14.6 ± 1.4 (5)
H_2O_2 omitted	18.8 ± 1.9 (10)	28.8 ± 1.2 (5)	17.1 ± 0.6 (4)
EPO omitted	18.7 ± 1.3 (13)	27.2 ± 1.1 (7)	15.0 ± 1.6 (4)
EPO + I (10^{-6} M) + H_2O_2	21.1 ± 2.6 (9)	34.3 ± 6.2 (6)	76.5 ± 2.8 (10)
EPO + Cl (10^{-1} M) + H_2O_2	20.1 ± 2.2 (4)	39.1 ± 4.0 (4)	76.6 ± 3.7 (6)

[a] The reaction mixture contained 0.03 M sodium phosphate buffer at the pH indicated, 1.5×10^{-3} M KH_2PO_4, 1.5×10^{-3} M $MgSO_4$, 10^6 LSTRA cells (mean 18,000 cpm ^{51}Cr), and the supplements as follows: EPO, 57.4 munits; H_2O_2, 10^{-4} M; NaCl, 10^{-1} M; NaI, 10^{-5}; or 10^{-6} M; gelatin, 0.005%. When NaCl was not employed, 0.067 M Na_2SO_4 was added to maintain isosmolarity.

[b] Mean ± S.E. (number of experiments).

The cytotoxic activity of the EPO-H_2O_2-halide system at pH 7.0 and with 10^{-5} M iodide was inhibited by azide, cyanide, aminotriazole, and catalase (all $p < 0.001$) with the inhibition by catalase largely abolished by heat treatment (Table II). Under the conditions employed, ^{51}Cr release remained above background only when cyanide was the inhibitor used ($p < 0.02$). Reagent H_2O_2 could be replaced by the H_2O_2-generating system, glucose + glucose oxidase.

The effect of the halide concentration at both pH 7.0 and 6.0 is shown in Figure 1. At pH 7.0, chloride, bromide, and thiocyanate were without effect at all the concentrations employed. Iodide, however, was effective at concentrations ranging from 10^{-5} to 10^{-3} M. Although chloride at 0.1 M was ineffective alone at pH 7.0, it significantly increased cytotoxic activity when combined with iodide at low concentrations, i.e., 10^{-6} M ($p < 0.005$) and 10^{-7} M ($p < 0.01$). At pH 6.0, cytotoxic activity was observed with iodide, bromide, chloride, or thiocyanate ions, with significant ^{51}Cr release observed at concentrations down

to 10^{-7} M iodide or bromide, 10^{-5} M thiocyanate and 10^{-2} M chloride.

Cytotoxic effect of intact eosinophils. Table III demonstrates the cytotoxic effect of intact eosinophils on the LSTRA tumor cell line as measured by ^{51}Cr release. The eosinophils were activated with either preopsonized zymosan or PMA, and the effector:target cell ratio was 5:1. A chloride-containing standard salt solution was employed for suspension of cells.

When zymosan was employed as the stimulus, iodide supplementation was required for an optimum effect; with 10^{-3} and 10^{-4} M iodide, ^{51}Cr release was 46.4 ($p < 0.001$) and 38.1% ($p < 0.001$), respectively, as compared to a background release of 19.8%. Omission of iodide, zymosan, or eosinophils from the complete 10^{-4} M iodide system resulted in ^{51}Cr release, which was not significantly greater than background. Activity was inhibited by 10^{-3} M azide ($p < 0.05$) and 10^{-2} M aminotriazole ($p < 0.01$) but not by 10^{-3} M cyanide. Catalase abolished the cytotoxic activity of the eosinophil-zymosan-iodide system, an effect that was lost on heat inactivation.

When PMA was employed as a metabolic stimulus rather than zymosan, iodide was stimulatory but was not essential for cytotoxicity. ^{51}Cr release in the presence of 10^{-3} M and 10^{-4} M iodide was 52.0% ($p < 0.001$) and 39.6% ($p < 0.001$), respectively, whereas in the absence of iodide, 26.5% ($p < 0.01$) of the ^{51}Cr was released as compared to a background of 18.6%. As for the zymosan-dependent systems, cytotoxicity required the presence of eosinophils and PMA and was inhibited by azide ($p < 0.02$), aminotriazole ($p < 0.001$), and catalase ($p < 0.002$), but not by heated catalase or cyanide. When 1% DMSO was substituted for PMA dissolved in DMSO, ^{51}Cr release was not significantly different from background (data not shown).

Cytotoxic effect of intact neutrophils. As shown in Table IV, neutrophils also are toxic to LSTRA cells when stimulated by PMA. The conditions were the same as those employed with eosinophils in Table III. As with eosinophils, iodide supplementation was not essential presumably due to the chloride content

TABLE II

Effect of inhibitors and substitution of glucose oxidase for H_2O_2[a]

Supplements	^{51}Cr Release
	%
Background (no additions)	18.5 ± 1.6 (9)[b]
EPO + I + H_2O_2	70.3 ± 3.0 (9)
Azide added	19.2 ± 1.9 (9)
Cyanide added	29.7 ± 4.3 (6)
Aminotriazole added	17.0 ± 2.2 (6)
Catalase added	15.1 ± 1.9 (4)
Heated catalase added	61.1 ± 7.5 (4)
H_2O_2 omitted; glucose + glucose oxidase added	60.9 ± 2.5 (3)

[a] The reaction mixture was as described in Table I (iodide, 10^{-5} M; 0.005% gelatin) except that sodium azide (10^{-3} M), sodium cyanide (10^{-3} M), aminotriazole (10^{-2} M), or catalase (1,830 units) were added or H_2O_2 was replaced by glucose (10^{-2} M) and glucose oxidase (1.23 units) as indicated.

[b] Mean ± S.E. (number of experiments).

TABLE III

Cytotoxic activity of eosinophils[a]

Supplements	^{51}Cr Release	
	Zymosan	PMA
	%	%
Background	19.8 ± 1.0 (8)[b]	18.6 ± 1.4 (7)
Eos + zym or PMA + I (10^{-3} M)	46.4 ± 2.8 (8)	52.0 ± 4.6 (7)
Eos + zym or PMA + I (10^{-4} M)	38.1 ± 3.0 (6)	39.6 ± 3.4 (5)
Iodide omitted	21.9 ± 2.2 (8)	26.5 ± 1.8 (5)
Zym or PMA omitted	23.2 ± 1.5 (7)	18.9 ± 2.3 (7)
Eos omitted	16.7 ± 1.3 (8)	16.7 ± 2.9 (6)
Azide added	21.6 ± 0.8 (2)	22.8 ± 4.7 (5)
Cyanide added	41.7 ± 3.2 (3)	39.8 ± 3.5 (3)
Aminotriazole added	6.3 ± 4.9 (2)	14.9 ± 2.0 (4)
Catalase added	17.7 ± 3.5 (3)	14.1 ± 2.0 (3)
Heated catalase added	34.8 ± 1.3 (3)	38.0 ± 5.4 (3)

[a] The reaction mixture contained 5×10^{-3} M sodium phosphate buffer, pH 7.4, 1.2×10^{-2} M KCl, 0.11 M NaCl, 2×10^{-3} M $MgCl_2$, 10^{-3} M $CaCl_2$, 10^5 LSTRA cells (mean 18,000 cpm ^{51}Cr), 0.005% gelatin, and the supplements indicated below as follows: eosinophils, 5×10^5; preopsonized zymosan, 1 mg/ml; PMA, 10 ng/ml; NaI, 10^{-3} M or 10^{-4} M; sodium azide, 10^{-3} M; sodium cyanide, 10^{-3} M; aminotriazole, 10^{-2} M; catalase, 12,000 units. The DMSO concentration in reaction mixtures containing PMA was 1%.

[b] Mean ± S.E. (number of experiments).

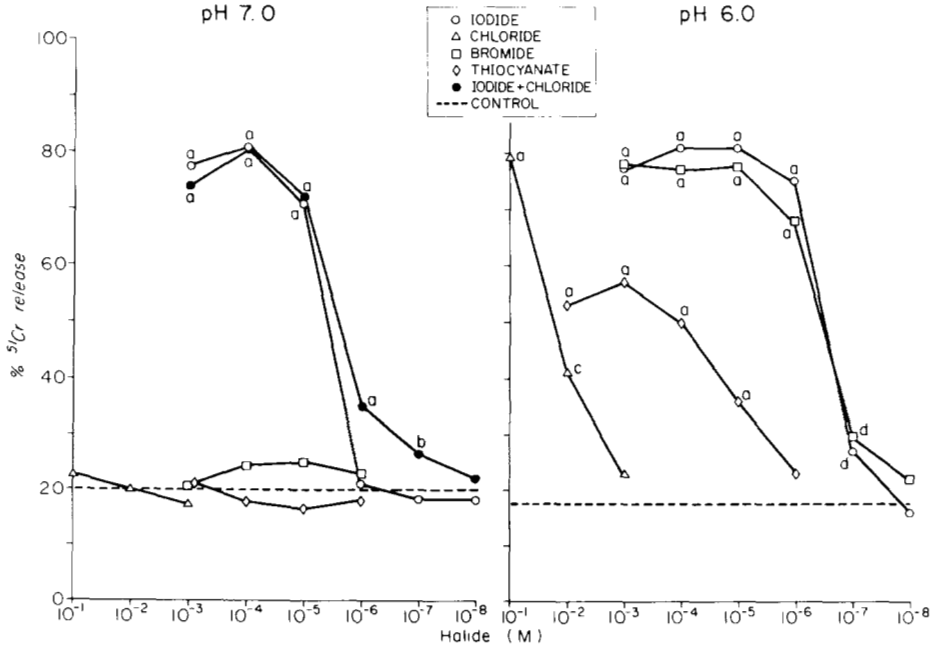

Figure 1. Effect of halide concentration of pH 7.0 and 6.0. The reaction mixture was as described in Table I except that the pH and the iodide, bromide, chloride, and thiocyanate concentrations (sodium salts) were varied as indicated. In experiments where both iodide and chloride were employed, the chloride concentration was constant at 0.1 M and the iodide concentration was varied as indicated. The results are the mean of three or more experiments. ^{51}Cr release in control samples (background) are indicated by the *horizontal broken line*. Significance of difference from control: [a] $p < 0.001$; [b] $p < 0.005$; [c] $p < 0.01$; [d] $p < 0.025$. All others not significant.

TABLE IV

Cytotoxic effect of PMA-stimulated neutrophils[a]

Supplements	^{51}Cr Release
	%
Background	16.4 ± 1.6 (4)[b]
Neutrophils + PMA + I (10^{-3} M)	47.6 ± 2.5 (4)
Neutrophils + PMA + I (10^{-4} M)	45.1 ± 3.2 (4)
Iodide omitted	37.0 ± 1.8 (4)
PMA omitted	18.1 ± 1.8 (4)
Neutrophils omitted	17.9 ± 1.2 (4)
Azide added	14.5 ± 2.3 (4)
Cyanide added	20.3 ± 4.1 (4)
Aminotriazole added	19.5 ± 3.3 (4)

[a] The reaction mixture was as described for Table III except that 5 × 10^5 neutrophils were used instead of eosinophils.

[b] Mean ± S.E. (number of experiments).

of the suspension medium. ^{51}Cr release in the presence of 10^{-3} M and 10^{-4} M iodide was 47.6% and 45.1%, respectively, whereas in the absence of iodide, 37.0% of the ^{51}Cr was released. All three values were significantly greater than the background ^{51}Cr release of 16.4% ($p < 0.001$). Cytotoxicity was dependent on the presence of neutrophils and PMA, and was inhibited by azide (10^{-3} M), cyanide (10^{-3} M), and aminotriazole (10^{-2} M).

DISCUSSION

The eosinophil peroxidase is shown here to be cytotoxic to LSTRA tumor cells when combined with H_2O_2 and a halide. The cytotoxic system was dependent on each component (EPO, H_2O_2, halide) and was inhibited by catalase and by the peroxidase inhibitors azide, cyanide, and aminotriazole. The halide requirement varied with the pH and the presence of gelatin. Only iodide of the halides employed was effective with gelatin (0.005%) at pH 7.0; however, chloride, although ineffective alone, significantly increased cytotoxic activity at otherwise ineffective iodide concentrations. Thus, with chloride at physiologic concentration (0.1 M), iodide was active at concentrations down to 10^{-7} M. The synergistic effect of iodide and chloride on the cytotoxic activity of the MPO-H_2O_2-halide system on LSTRA cells was previously reported (25). When gelatin was omitted, chloride was effective alone at pH 7.0. This is consistent with our earlier finding that the bactericidal activity of the EPO-H_2O_2-halide system can be inhibited by gelatin (6). When the pH was lowered to 6.0, cytotoxicity was observed when EPO and H_2O_2 were supplemented with either iodide, bromide, chloride or thiocyanate, even in the presence of gelatin (Fig. 1). Gelatin (0.005%) was employed in most of the studies as it significantly decreased background ^{51}Cr release.

Intact guinea pig eosinophils activated either by phagocytosis of opsonized zymosan or by PMA also are capable of mediating tumor cell cytotoxicity. In each instance, cytotoxicity was abolished by deletion of either the eosinophils or the activating agent (zymosan, PMA). Our findings are compatible with the involvement of the EPO-H_2O_2-halide system in the cytotoxicity of eosinophils under these conditions. Chloride was present in the reaction mixture as part of the standard salt solution and iodide was added. In the phagocytosis model, deletion of the iodide abolished cytotoxicity, whereas when PMA was used, iodide deletion decreased but did not abolish activity. Thus, a halide was required for optimum activity. Catalase was inhibitory, whereas heated catalase was not, indicating a requirement for H_2O_2. The hemeprotein inhibitors azide and aminotriazole abolished cytotoxicity with both activating agents, a result compatible with a requirement for peroxidase. Cyanide, however, was ineffective under our experimental conditions. Cyanide was the least effective of the hemeprotein inhibitors when tested on the bactericidal (6) or cytotoxic (Table II) activity of the cell-free EPO-H_2O_2-halide system. The relative insensitivity of EPO to cyanide as compared to azide has been described under other conditions (3, 26).

Earlier studies indicated that human neutrophils are cytotoxic to the LSTRA cells when combined with opsonized zymosan (25) or concanavalin A (27) and a halide, and evidence has been presented which implicates the peroxidase-H_2O_2-halide system in this toxicity (25, 27). The involvement of the peroxidase system in the cytotoxic activity of PMA-activated neutrophils was recently reported in preliminary form (28). We also report here on the toxicity of PMA-activated neutrophils on the LSTRA target cells. As with eosinophils, activity was dependent on both neutrophils and PMA, was decreased but not lost on the deletion of iodide (chloride was present in the standard salt solution), and was inhibited by azide and aminotriazole. Cyanide also was inhibitory, in contrast to our findings with eosinophils.

Our findings confirm that under certain conditions of stimulation, eosinophils can release their granule peroxidase into the extracellular fluid. Phagocytosis of zymosan and PMA were employed as the stimuli here; in other studies, interaction with antibody-coated schistosomula (19, 20) or immune complexes (29) resulted in peroxidase release. Peroxidase release by PMA requires special comment. Earlier reports have shown that specific granule markers (e.g., lysozyme, alkaline phosphatase) are preferentially released on exposure of neutrophils to PMA (30–32); comparable release of the azurophil granule markers, myeloperoxidase, and β-glucuronidase were not detected. The studies reported here and those of Clark (28), however, indicate that PMA can induce the extracellular release of peroxidase from neutrophils or eosinophils as evidenced by its involvement in the cytotoxic reaction.

Eosinophils do not appear to be critical to the host defense against bacterial invasion; they are present in small numbers, are not a striking feature of the acute inflammatory response, and are less bactericidal *in vitro* than are neutrophils (7–11). Eosinophils, however, accumulate in areas of helminthic infestations and in association with some neoplasms. Our findings raise the possibility that EPO released from eosinophils [and possibly stabilized by adsorption to mast cell granules [33]] may combine with H_2O_2 from the same or other phagocytes (neutrophils, mononuclear phagocytes) and a halide to form a system toxic to the adjacent cells.

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