PEROXIDASE-MEDIATED MAMMALIAN CELL CYTOTOXICITY*

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Nonphagocytic destruction of animal cells can be accomplished by a variety of cellular and humoral mechanisms (for review, see ref. 1). Serum antibodies may be cytotoxic either in the presence of complement, or thymus-independent lymphocytes. Lymphocytes may also act directly against target cells, either through a soluble lymphotoxin or through direct cell to cell contact of viable, metabolically active thymus-dependent lymphocytes. More recently, a lymphocyte-macrophage interaction has been implicated in the growth inhibition of murine tumor cells, and this may represent yet another cytotoxic mechanism.

The T cell-mediated cytotoxic process is independent of RNA and protein synthesis, but requires energy and divalent cations. Low temperature, prostaglandin E_2, cytochalasin B, and proteolytic enzymes have been reported to inhibit the reaction. The mechanisms underlying cell damage in all these reactions remains obscure. In addition, many studies employ heterogeneous cell populations and require high effector-target cell ratios.

In this article we wish to call attention to a possible cytotoxic mechanism for the extracellular destruction of target cells, the major reactants of which are derived from cells commonly found at inflammatory sites. It is based upon the prior observations of Klebanoff and his colleagues that peroxidase, hydrogen peroxidase, and halide ions form a potent system for the intracytoplasmic killing of microbial species (2).

Methods

Mouse lymphoma cells L1210 (obtained from Dr. Doris Hutchinson through the courtesy of Dr. Alan Goldberg) were maintained in a modified Eagle's minimum essential medium containing calf and fetal calf serum (3). Human lymphoid cells (no. 8866) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum, and were provided by Dr. Gary Hoffman. 20–40 million viable cells were suspended in 1 ml Hanks' balanced salt solution containing 20% fetal calf serum and 200 μCi $Na_2{}^{51}CrO_4$ (Amersham Searle Corp., Arlington Heights, Ill., specific activity 50–150 mCi/mg Cr) and incubated for 30 min at 37°C with continuous agitation as described by Canty and Wunderlich (4). 400 thousand labeled target cells were washed, resuspended in phosphate-buffered saline (pH 7.2) containing 0.3% glucose, and incubated for 90 min at 37°C with various test solutions. The tubes were then centrifuged at 1,000 × g for 10 min, and an aliquot of the

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supernatant was removed for counting in a gamma-ray spectrometer. Total releasable chromium was estimated by counting the supernatants of cells lysed in 5% Nonidet-P40 (Shell Oil Co., New York). Over 90% of the total cell associated label was releasable in this way. Approximately 50,000 cpm were incorporated per 10^6 cells. Human red blood cells were collected in heparin, washed, and similarly labeled with 200 μCi $Na_2{}^{51}CrO_4$ at a concentration of 25×10^7 cells/ml. Human peripheral blood mononuclear cells were concentrated on a mixture of Ficoll (Pharmacia, Sweden) and sodium diatriazoate (Hypaque, Winthrop Laboratories, New York) (5), washed, and labeled with 100 μCi $Na_2{}^{51}CrO_4$ at a concentration of 10^7 cells/ml.

Lactoperoxidase, grade B, was purchased from Calbiochem, San Diego, Calif. Glucose oxidase, aspergillus niger, type V was obtained from Sigma Chemical Co., Saint Louis, Mo. Purified human myeloperoxidase was generously provided by Dr. Julius Schultz. Peroxidase activity was measured by the *O*-dianisidine method as described by Steinman and Cohn (6). 1 U of enzyme activity is defined as that amount capable of decomposing 1 μmol H_2O_2 per minute at 25°C (7).

Percent ^{51}chromium released was calculated from the expression:

$$\% \text{ release} = \frac{\text{cpm supernatant (experimental)}}{\text{cpm supernatant (NP-40-treated cells)}} \times 100.$$

Results represent the averages of triplicate determinations. No correction was made for background counts, which were less than 5% of the counts in any tube. Cell viability was assessed with Trypan blue, in phosphate-buffered saline containing 2% fetal calf serum, as described by Boyse et al. (8).

RESULTS

Results of an illustrative experiment are shown in Table I, demonstrating the ability of the complete lactoperoxidase-glucose oxidase-glucose-iodide system to lyse L1210 cells. Significant chromium release could be detected at enzyme activities of 7.5 mU of lactoperoxidase, and 7.5 mU of glucose oxidase in the presence of 0.05 μmol of iodide but was not seen at enzymes levels of 5 mU or below of lactoperoxidase and glucose oxidase. Additional cytolysis

TABLE I

Lactoperoxidase-Mediated Cytotoxicity for L1210 Target Cells

Reagent added	^{51}Chromium released
	%
LPO (30 mU), GO (30 mU), KI (0.05 μmol)	36.0
LPO (15 mU), GO (15 mU), KI (0.05 μmol)	30.3
LPO (7.5 mU), GO (7.5 mU), KI (0.05 μmol)	26.3
LPO (30 mU), GO (30 mU)	18.8
LPO (30 mU), KI (0.05 μmol)	11.5
GO (30 mU), KI (0.05 μmol)	15.2
None	13.0

4×10^5 L1210 cells were incubated for 90 min at 37°C in phosphate-buffered saline (pH 7.2) containing 15 μmol glucose, and additional reagents as indicated in a total volume of 2 ml. Abbreviations are as follows: LPO, lactoperoxidase; GO, glucose oxidase; KI, potassium iodide.

was seen at higher enzyme concentrations. In various experiments using 30 mU of each enzyme, between 35 and 86% release of label was observed after a 90 min incubation. When either enzyme was omitted, only background levels of chromium release were measured. Omission of the iodide also considerably reduced the cytotoxicity. Similar results were obtained with no. 8866 human lymphoid cells, human erythrocytes, and human peripheral blood mononuclear cells (Table II). All three lymphoid cell types showed comparable sensitivity, while considerably more label was released from the red blood cells.

Sequential assays over several hours (Fig. 1) showed that significant chromium release is detectable by 30 min, and proceeds at a nearly linear rate for the first 2 h of incubation. Little additional release was seen after up to 5 h of incuba-

TABLE II

Comparison of the Susceptibility of Various Cell Types to the Complete Lactoperoxidase (or Myeloperoxidase)-Hydrogen Peroxide-Iodide System, or to its Components

Cell type	^{51}Chromium released			
	Complete system	Glucose oxidase omitted	Peroxidase omitted	Spontaneous release
	%			
L1210	36.0	11.5	15.2	13.0
L1210 (MPO)	95.6	—	—	15.4
8866	37.3	15.4	13.5	16.2
Human red blood cells	89.4	2.7	2.2	2.3
Human peripheral blood Mononuclear cells	56.6	18.5	19.5	17.3

4–5 $\times$ 10^5 cells (5 $\times$ 10^6 cells in the case of erythrocytes) were incubated for 90 min at 37°C in phosphate-buffered saline (pH 7.2) containing 15 μmol glucose, 30 mU peroxidase, 30 mU glucose oxidase, and 0.02 μmol potassium iodide, as appropriate, in a total volume of 2 ml. ^{51}Chromium release computed as in text.

tion (not shown). Less than 15% of the cell label was spontaneously lost during the first 2 h of incubation, after which time spontaneous release rose considerably. All three lymphoid cell types showed similar degrees of spontaneous release. Erythrocytes showed considerably less spontaneous release.

Although maximum chromium release was observed only after 90 min of incubation, it was not necessary for the halogenating system to be active for the entire time. Exposing the cells to the complete system for 15 min was sufficient to achieve maximum chromium release after an additional 75 min incubation. A 5 min exposure resulted in release of about 75% of the maximum after a total incubation of 90 min.

Parallel experiments using Trypan blue showed an identical time-course for cell death. However, at each time, considerably more cells were judged to be nonviable by this technique than as indicated by chromium release, suggesting a greater sensitivity for the dye method.

Myeloperoxidase readily substituted for lactoperoxidase in releasing chromium from L 1210 cells (Table III). Inclusion of 15 mU of myeloperoxidase and 15 mU of glucose oxidase in the presence of 0.05 μmol iodide led to 90% release of chromium after 90 min. Cytotoxicity was nearly the same when the iodide

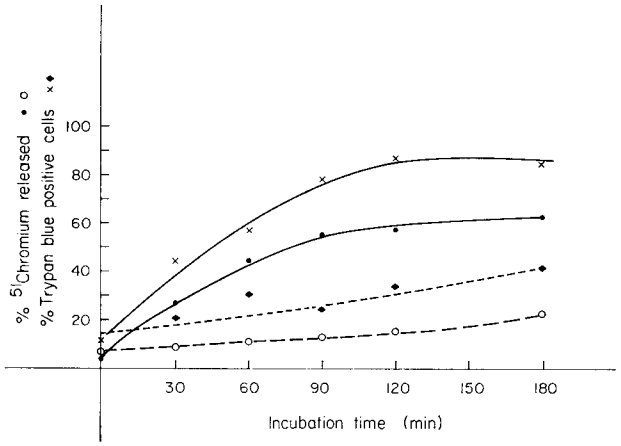

FIG. 1. Time-course of ^{51}chromium release, and Trypan blue nuclear staining, for 4 × 10^5 L1210 cells incubated in phosphate-buffered saline (pH 7.2) containing 15 μmol glucose, 30 mU lactoperoxidase, 30 mU glucose oxidase, and 0.05 μmol potassium iodide (complete system) or glucose-containing phosphate-buffered saline alone (control), in a total volume of 2 ml. Reactions stopped by the addition of two volumes of cold medium 199. Percent chromium released calculated as in text. ●—● complete system, ^{51}chromium released; ×—× complete system, Trypan blue uptake; ○--○ control, ^{51}chromium released; ◆--◆ control, trypan blue uptake.

TABLE III

Effectiveness of Myeloperoxidase in Mediating ^{51}Chromium Release from L1210 Cells in the Presence or Absence of Potassium Iodide

Enzyme activities	MPO, GO, KI	MPO, GO	LPO, GO, KI	LPO, GO
15 mU	90.7	84.1	86.3	28.8
30 mU	95.6	92.9	84.6	35.0
45 mU	92.3	85.4	86.1	39.4

4–5 × 10^5 L1210 cells were incubated for 90 min at 37°C in phosphate-buffered saline (pH 7.2) containing 15 μmol glucose, equal activities of peroxidase and glucose oxidase, as indicated, and 0.05 μmol potassium iodide as appropriate in a total volume of 2 ml. ^{51}Chromium release calculated as in text.

was omitted. 60 mU of myeloperoxidase alone released 14.2% of the ^{51} chromium, compared to a spontaneous release of 13.6%.

DISCUSSION

Our work shows that a peroxidase-hydrogen peroxide-halide system is cytotoxic for a variety of mouse and human cells. The reactants needed are avail-

able from inflammatory cells or are present in the extracellular fluid. Myeloperoxidase may amount to 5% of the dry weight of human neutrophils (9). About 25% of the total cellular myeloperoxidase is released from neutrophils during phagocytosis (10). Thus, about 1.5×10^6 neutrophils could provide the 2 ng of myeloperoxidase used in our experiments. Macrophages (11) or lymphocytes (12) are unlikely to contribute significant amounts of myeloperoxidase, although monocytes do contain myeloperoxidase and eosinophils have a related peroxidase (13).

Peroxide production by stimulated human (14) or guinea pig (15) neutrophils has been estimated at 0.03–0.26 m μmol H_2O_2 generated/10^6 cells per minute. These figures, obtained either by a fluorometric assay of dialysed cell lysates, or by oxygen electrode measurements of the amount of oxygen evolved by catalase treatment of phagocytizing cells are likely to represent minimum values only. Further, close cell contact may considerably increase the local concentration of peroxide at the target cell surface. Peroxide does escape from stimulated neutrophils (16), and these measurements suggest that cytotoxic levels could be reached. We cannot estimate the additional contributions of peroxide from such other cell types as the macrophage or lymphocyte.

Inflammatory exudates are invariably a mixture of neutrophils, eosinophils, lymphocytes, macrophages, and other cell types. Together, these cells may provide the components of a complete cytotoxic system. Close cell-to-cell contact could magnify their contributions and contribute to the specificity of the reaction. Such a system would not necessarily be effective only against foreign cells, but might also account for the host tissue destruction which accompanies such inflammatory responses as the Arthus phenomenon (17). In unpublished work, we have observed that macrophages are also susceptible to the cytotoxic effects of this system. This observation raises the possibility that such a mechanism could be effective also in limiting an inflammatory response by destroying immigratory phagocytes once they had reached a critical level in an inflammatory focus. Our experiments show that biologically achievable levels of myeloperoxidase and hydrogen peroxide, in the presence of the ubiquitous chloride ion form a potent system for extracellular cell killing. We are currently exploring the potential of various inflammatory cell types to participate in such a reaction.

SUMMARY

Lactoperoxidase, in the presence of hydrogen peroxide and iodide is cytotoxic for human and mouse lymphoid cells, and human erythrocytes. Myeloperoxidase, in amounts equivalent to 1.5×10^6 neutrophils, readily replaces lactoperoxidase, and allows the substitution of the iodide ion by chloride. The myeloperoxidase-mediated reaction is rapid, and highly efficient, leading to 85–90% cell death in 90 min, as measured by ^{51}chromium release and dye exclusion. The mixture of granulocytes, monocytes, and lymphocytes present in an

inflammatory exudate, and the intimate cell-to-cell association characteristic of cytotoxic phenomena may provide the in vivo requirements for such a system.

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