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Cytophilic and Cytotoxic Properties of Human Eosinophil Peroxidase Plus Major Basic Protein

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The cytophilic and cytotoxic properties of an acetate-buffered solution of human eosinophil peroxidase (EPO) plus major basic protein (MBP) were studied to determine the cytotoxic potential of localized eosinophil degranulation in human tissues. When incubated with EPO + MBP for 5 minutes, viable cells of six unrelated types (Sp 2/0; HeLa; human gastric adenocarcinoma; acute lymphocytic leukemia; IM-9; benign lymphoid hyperplasia) developed varying degrees of cytochemically detectable deposits of EPO on the cell membranes. A single-step propidium iodide exclusion assay was then used to show that EPO + MBP in the absence of hydrogen peroxide is substantially cytotoxic only to the acute lymphocytic leukemia and IM-9 cells. In the presence of 0.003% hydrogen peroxide, EPO + MBP was cytotoxic to five types of cells. It is concluded that human EPO in the presence of MBP has an affinity for the membrane of diverse cell types. The toxicity of EPO + MBP is markedly enhanced by the presence of hydrogen peroxide. (Am J Pathol 1988, 132:455–460)

CERTAIN PATHOLOGIC conditions such as Hodgkin's disease, endomyocardial fibrosis, atopic dermatitis, and chronic myelogenous leukemia are associated with extensive degranulation of eosinophils and deposition of eosinophil peroxidase (EPO) and major basic protein (MBP) into tissues.[1–4] In a variety of experimental systems,[5,6] purified EPO and MBP from animals have been shown to be potent cytotoxins.

Because degranulation of human eosinophils simultaneously releases EPO and MBP,[7] the cytophilic and cytotoxic properties of an acetate-buffered solution containing human EPO plus MBP were investigated, thereby approximating the *in vivo* degranulation of human eosinophils. The objective of the study was to evaluate the potential effects of human eosinophil degranulation in pathologic situations. Viable cells of six types were incubated with EPO + MBP, and cytochemical studies were then performed to detect EPO bound to the cells. In addition, the cytotoxic activity of EPO + MBP in the presence and absence of a low concentration of hydrogen peroxide was measured.

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Materials and Methods

Reagents

Aminoethyl carbazole, propidium iodide, and Percoll were obtained from Sigma Chemical Co. (St. Louis, MO); colorimetric peroxidase substrate (azinobisthiazoline sulfonic acid in pH 4.2 citrate buffer with hydrogen peroxide) and SDS-PAGE supplies were obtained from Bio-Rad (Richmond, CA).

Preparation of Eosinophil Peroxidase Plus Major Basic Protein

To obtain human eosinophils, peripheral blood specimens from donors with hypereosinophilia were

Table 1—Results of Cytotoxicity Assay

Cell type	Description	EPO + MBP cytotoxicity	H_2O_2 cytotoxicity	EPO + MBP + H_2O_2 cytotoxicity
Sp 2/0	Murine myeloma	2 ± 1%	5 ± 4%	35 ± 2%
HeLa	Human cervical carcinoma	1 ± 1%	3 ± 2%	24 ± 3%
Gastric adenocarcinoma	Human pleural fluid	3 ± 3%	−2 ± 1%	19 ± 6%
Acute lymphocytic leukemia	Peripheral blood blasts	27 ± 2%	18 ± 2%	53%*
IM-9	Human B cell line	9 ± 2%	9 ± 2%	22 ± 2%
Lymph node	Benign hyperplasia	ND	ND	ND

* N = 1.
ND, not determined.

incubated with the chemoattractant f-met-leu-phe and then sedimented through a discontinuous Percoll gradient.[8] Residual blood cells contaminating the eosinophil layer were lysed with distilled water, and the eosinophils were washed twice before being cryopreserved. Approximately 10^7 purified, cryopreserved eosinophils obtained by this method from various donors were pooled, washed twice in cold phosphate-buffered saline, and then suspended in 1.5 ml of 0.02 M acetate buffer, pH 4.2. After incubating on ice for 15 minutes, the cells were homogenized by 12 to 15 strokes of a tissue homogenizer at medium speed.

The homogenate was centrifuged at 10,000*g* for 10 minutes to pellet the insoluble debris, and the clear, pale yellow supernatant was aliquoted into polypropylene Eppendorf tubes and stored in the vapor phase of liquid nitrogen. The OD_{415nm} of the EPO preparation derived by this method was 0.2, as determined on a Beckman DU7 spectrophotometer. To assay the peroxidase activity, 10 μl of the thawed EPO extract were added to 110 μl of peroxidase substrate. The solution was incubated at 20 C for 1 minute, and 120 μl of 2% oxalic acid was added to stop the formation of the blue color, which had an absorbance of 1.1 at 413 nm, as measured on a Titertek Multiscan ELISA reader (Flow Labs, McLean, VA).

The acetate-buffered extract of the eosinophil homogenate then was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions, using a Bio-Rad Mini-PROTEAN II cell and a 12.5% SDS-polyacrylamide gel. The gel was stained in coomassie brilliant blue R-250 and washed in methanol-acetic acid before photography.

Cell Lines and Clinical Specimens

The cell lines and clinical specimens used in this study are listed in Table 1. The Sp 2/0 and IM-9 cell lines were derived from cultures obtained from the American Type Culture Collection (Bethesda, MD) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin and streptomycin, and nonessential amino acids (Irvine Scientific, Santa Ana, CA). The HeLa cell line was provided by Dr. Leslie Redpath, Radiation Biology Division, University of California at Irvine. Cryopreserved clinical specimens were obtained from the University of California, Irvine, Hematopathology Tissue Bank.

Treating Cells With EPO + MBP and Cytochemical Studies

The treatment and subsequent cytotoxicity studies are modifications of procedures originally described by Nathan and Klebanoff.[5] Approximately 10^7 cryopreserved cells of each type were thawed, washed twice in phosphate-buffered saline (PBS), filtered through a Nytex mesh in a Pasteur pipette, and then suspended in 1 ml of PBS. Half of the cells (500 μl) from each specimen were mixed with 50 μl of the EPO + MBP solution (treated cells), and the other half of the cells were added to 50 μl of acetate-buffer without EPO + MBP (untreated cells). After 5 minutes of incubation at room temperature, the cells were washed twice with serum-free medium and then resuspended in 500 μl of DMEM with 10% fetal calf serum and 10^{-4} M sodium bromide (assay medium). For comparative purposes, Sp 2/0 and HeLa cells were also obtained directly from cell culture and were coated in an identical fashion.

Aliquots of treated and untreated cells then were suspended in 1% crystalline bovine serum albumin and deposited on glass slides with a cytocentrifuge (Cytospin 2, Shandon, UK). The air-dried cells were stained for peroxidase activity with aminoethylcarbazole in acetate buffer[1] and were counterstained with Mayer's hematoxylin before rinsing in Scott's tap water substitute. Photomicrographs were taken with a Nikon Optiphot microscope equipped with planapochromat objectives and a UFX-II camera.

Cytotoxicity Assay

For the cytotoxicity studies, treated and untreated cells of each type were prepared as described above. Duplicate assays were performed on 100-μl aliquots of each specimen in 1.5 ml polypropylene Eppendorf tubes.

Untreated cells were incubated for 15 minutes at 37 C in assay medium alone (background control) and in assay medium containing 0.003% hydrogen peroxide (H_2O_2 toxicity). Treated cells were incubated similarly in assay medium alone (EPO + MBP toxicity) and in assay medium containing 0.003% hydrogen peroxide (EPO + MBP + H_2O_2 toxicity).

After the incubation, the cells were added to 50 μl of a 2 μM solution of propidium iodide in PBS for 3 minutes. Dead cells develop a bright red nuclear fluorescence due to irreversible intercalation of the dye into DNA.[9] The cells then were washed once, suspended in 200 μl of PBS, and sonicated at setting #20 for 1 second with a Vibracell ultrasonic sonicator to disperse cell clumps.

From each specimen, 8000 cells were analyzed for nuclear fluorescence at 580 nm on an EPICS C flow cytometer (Coulter, Hialeah, FL). The percent cytotoxicity for each tube was calculated by determining the percentage of dead cells in that tube and subtracting the mean percentage of dead cells in duplicate measurements of the background control. Results are expressed in Table 1 as the mean percent cytotoxicity of duplicate assays ± 1 standard deviation.

Results

Preparation of Eosinophil Peroxidase Plus Major Basic Protein

The reduced SDS-PAGE of the stock solution of the extract of human eosinophil homogenate is illustrated in Figure 1, along with the molecular weight standards. The protein bands at approximately 52 kD and 16 kD correspond to the heavy and light chain subunits of eosinophil peroxidase,[10] and the dense band at approximately 15 kD is presumed to represent eosinophil major basic protein. Three very faint bands also are present between 22 kD and 42 kD.

Cytochemical Studies

Representative results of the cytochemical studies are illustrated in Figures 2 to 5. Except for very rare neutrophils and eosinophils in the clinical specimens, there was no detectable endogenous peroxidase activity among the untreated cells (Figure 2).

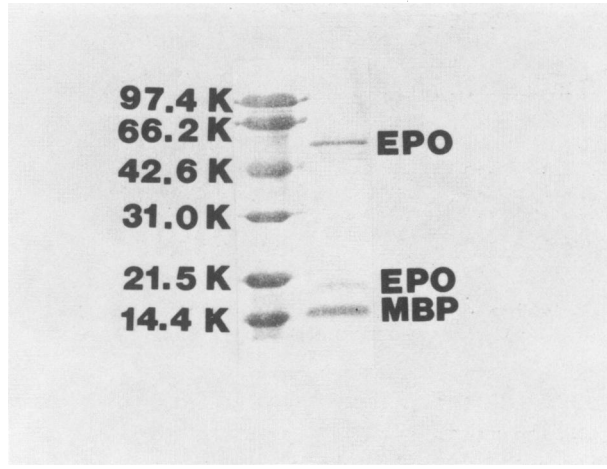

Figure 1—SDS-PAGE under reducing conditions of stock solution of human eosinophil extract (right lane). Molecular weight markers are in the left lane. The predominant proteins are the heavy and light chain subunits of eosinophil peroxidase and major basic protein.

In contrast, all of the treated specimens had cells with detectable peroxidase activity, which varied considerably from specimen to specimen. The peroxidase activity on the Sp 2/0 cells (Figure 3) and HeLa cells was present uniformly in discrete granules and as a cytoplasmic blush on almost every cell. The distribution of peroxidase on the gastric adenocarcinoma cells (Figure 4) and on the cells from benign lymphoid hyperplasia was more heterogeneous. Some cells were densely coated with peroxidase, while many smaller cells had only scattered granules on their membrane. The IM-9 cells (Figure 5) and acute lymphocytic leukemia cells had distinct linear and granular deposits of peroxidase activity on the membranes of almost every cell.

Treated and untreated cells obtained directly from cell culture were also examined for their peroxidase activity. Untreated cells had no detectable peroxidase activity, but morphologic degeneration of the cells was present and was presumed secondary to the brief incubation of the fresh cells in acetate buffer. Treated cells from cell cultures had substantial peroxidase activity and similar degenerative changes.

Cytotoxicity Assays

The results of the cytotoxicity assays are presented in Table 1. The background control cytotoxicity was less than 20% in all specimens except in the cells from the case of benign lymphoid hyperplasia, for which cytotoxicities were not determined. EPO + MBP cytotoxicity was greater than 5% for the IM-9 cells and the acute lymphocytic leukemia cells. These two cell

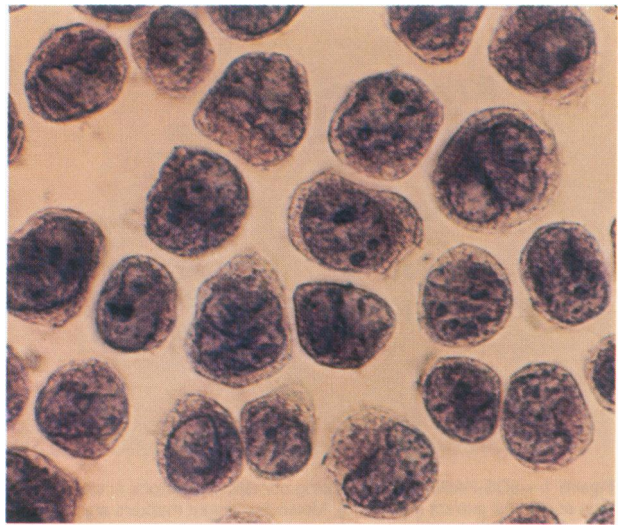

Figure 2—Cytopreparation of untreated Sp 2/0 cells. There is no evidence of endogenous peroxidase activity. Original magnification ×600, Mayer's hematoxylin counterstain with rinsing in Scott's tap water substitute

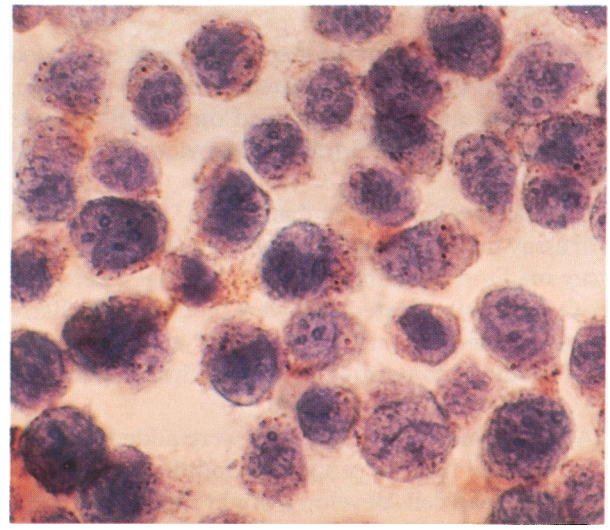

Figure 3—Cytopreparation of treated Sp 2/0 cells. Peroxidase activity is present as discrete granules and a diffuse blush. Original magnification ×600

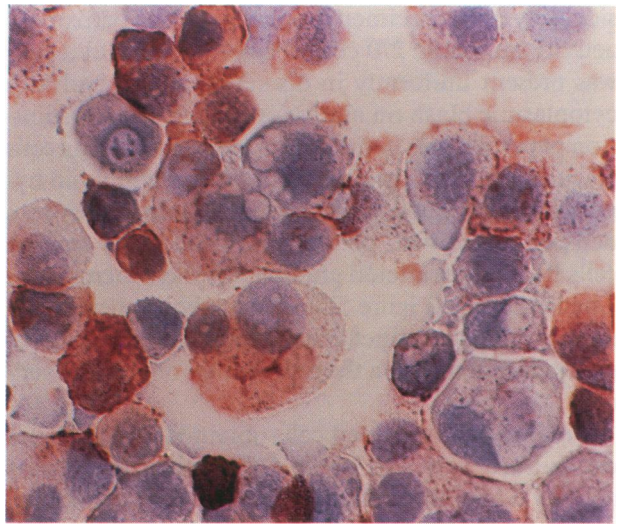

Figure 4—Cytopreparation of treated gastric adenocarcinoma cells incubated in peroxidase substrate. There is substantial cell-to-cell variation in the degree of peroxidase activity. Original magnification ×600

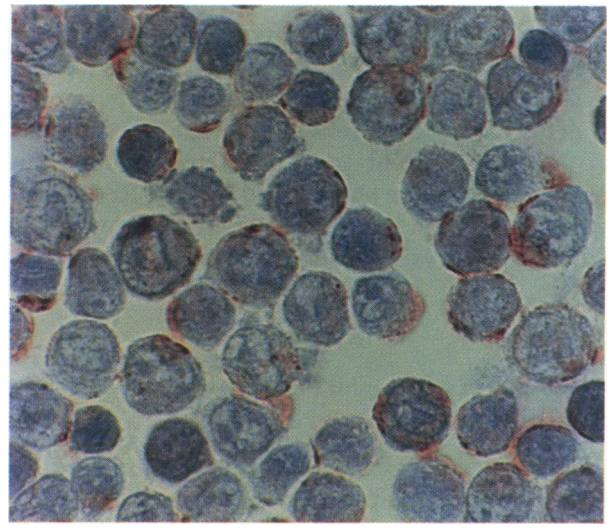

Figure 5—Cytopreparation of treated IM-9 cells. Discrete linear and granular deposits of peroxidase are evident on the cell membrane of most cells. Original magnification ×600

types also had the greatest degree of sensitivity to 0.003% hydrogen peroxide alone, which was essentially nontoxic when used alone on the other three types of cells in the study. EPO + MBP + hydrogen peroxide was cytotoxic to all of the types of cells tested; for the Sp 2/0, HeLa, and gastric adenocarcinoma cells, the cytotoxicity of EPO + MBP + peroxide substantially exceeded the additive toxicities of EPO + MBP alone and hydrogen peroxide alone.

Before sonication, the treated cells incubated in hydrogen peroxide formed large clumps. Similar clumping was not observed in any of the other tubes.

Discussion

Human eosinophil peroxidase in the presence of major basic protein has been shown to have affinity for the membranes of viable cells obtained from six diverse sources. The binding required only 5 minutes of incubation and had a variable distribution on the surface of the cells.

The results of these cytochemical studies, therefore, suggest that human EPO from degranulating eosinophils can bind rapidly to a variety of unrelated mammalian cells; this observation is consistent with previ-

ous reports that EPO isolated from horses and guinea pigs has affinity for a variety of murine cell lines.[5,11] The strong cytophilia of human EPO may be due to an attraction between highly cationic EPO (isoelectric point greater than pH 11) and the mostly anionic membrane of cells.[5] Alternatively, the localization of EPO on cell surfaces may indicate binding of EPO by mannose receptors on the cell membrane, because EPO is known to have a high content of mannose.[12] Because human EPO had a variable binding affinity for the heterogeneous cells in clinical specimens, it is likely that additional, as yet not understood factors are involved in the binding of human EPO to cells.

The significance of the cytochemical observation about the affinity of EPO for cell membranes is that it complements previous reports of EPO-specific trapping systems.[13-15] Ultrastructural studies have been used to demonstrate convincingly that basophils, mast cells, and granular lymphocytes internalize EPO.[13,14] Zabucchi et al[15] showed that neutrophils deficient in myeloperoxidase rapidly bind human EPO and internalize it in small vesicles. The authors' studies indicate that human EPO in the presence of MBP also has affinity for the membrane of many other types of cells, providing yet another mechanism for the "trapping" and subsequent localization of this enzyme at sites of eosinophil degranulation. Ultrastructural studies will be needed to determine if membrane-bound EPO on these other types of cells is also internalized in a manner similar to that of neutrophils and basophils.

In the cytotoxicity assay, human EPO + MBP had a somewhat limited potential for cell killing in the absence of hydrogen peroxide. By contrast, three types of treated cells (Sp 2/0; HeLa; gastric adenocarcinoma) incubated in hydrogen peroxide had cytotoxicities that substantially exceeded the additive cytotoxicities of EPO + MBP alone plus hydrogen peroxide alone. This finding is compatible with a previous report that the cytotoxic effect of cell-bound EPO obtained from guinea pigs is markedly enhanced by the presence of 0.003% hydrogen peroxide.[5] Because the free radicals generated by EPO are highly reactive and rapidly neutralized by biologic materials,[16,17] direct binding of the EPO to cells is probably necessary for effective cell killing in the presence of hydrogen peroxide.

An important unresolved issue raised by these studies is the possibility of an interaction between the various eosinophil granule proteins in producing cytotoxicity. An analysis of such interaction would require the use of purified eosinophil granule proteins. The authors have thus far encountered difficulties in producing useable quantities of stable preparations of purified human eosinophil proteins. Substantial and unavoidable losses of cationic eosinophil proteins occur during each step of the purification process because of the strong nonspecific adsorption of eosinophil proteins to gels and dialysis membranes.[12] Moreover, purified human eosinophil granule proteins polymerize rapidly and require stabilization with detergents or salts or reduction with dithiothreitol and alkylation with iodoacetamide.[6,10] Nonetheless, interaction between eosinophil granule proteins may occur and should be the subject of future investigations.

In conclusion, this study has documented that human EPO from an eosinophil extract has affinity for a wide variety of cell types. The authors speculate, therefore, that human EPO is likely to remain sequestered at the pathologic sites of eosinophil degranulation by binding almost immediately to cells in the vicinity of the degranulation. Any cells adjacent to degranulating eosinophils in effect may function as an EPO trap. The cytotoxic effects of eosinophil degranulation are enhanced by hydrogen peroxide and are probably limited to the cells that are actually coated with EPO and MBP.

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