NEUTROPHIL-MEDIATED TUMOR CELL CYTOTOXICITY: ROLE OF THE PEROXIDASE SYSTEM*

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Polymorphonuclear leukocytes (PMN) employ a system comprised of myeloperoxidase (MPO), H_2O_2, and an oxidizable halide cofactor to kill a variety of microorganisms (for reviews see 1, 2). After particle ingestion, MPO is released into the phagocytic vacuole and H_2O_2 is produced in association with the postphagocytic metabolic burst. Both MPO (3, 4) and H_2O_2 (5) are also released into the extracellular fluid during phagocytosis. While the intravacuolar role of the MPO system is well established (1, 2), no extracellular biologic activity of this system other than iodination of nonspecific proteins (1, 6) has been demonstrated. The killing of mammalian tumor cells by the peroxidase system employing isolated components has recently been described (7-9). The current report documents a cytotoxic effect of intact human PMN on neoplastic cells and provides evidence for the involvement of the peroxidase system in mediating this activity.

Materials and Methods

Target Cells. An ascitic Moloney virus-induced lymphoma (LSTRA) (9) maintained by intraperitoneal passage in BALB/c mice was used as the target cell source. Cells were harvested from ascites fluid as previously described (9). All incubations were performed in a 37°C water bath shaker oscillating 60 times/min.

^{51}Cr *Release.* LSTRA cells were labeled with [^{51}Cr]chromate, washed, and suspended in 0.1 M sodium phosphate buffer, pH 7.0 (PB) and ^{51}Cr-release assays performed as previously described (9). Briefly, labeled LSTRA cells were incubated for 2 h with the components of the cytotoxicity system (see table legend), and the mean release of ^{51}Cr in duplicate samples was expressed as a percent of maximum releasable activity.

Oncogenicity. LSTRA cells were incubated at 37°C for 2 h with the components of the cytotoxicity system before injection into the peritoneal cavity of BALB/c mice for the determination of oncogenicity as previously described (9).

Leukocyte Preparation. The Ficoll-Hypaque technique (10) was employed to isolate PMN from the blood of healthy adult volunteers, one male with hereditary MPO deficiency (11), two brothers with leukocyte glucose-6-phosphate dehydrogenase (G6PD) deficiency (12), and four males with chronic granulomatous disease of childhood (CGD). After hypotonic lysis of contaminating erythrocytes, the granulocyte pellet was washed twice in 0.1 M PB and suspended in 0.1 M PB at 5×10^6 PMN/ml. These suspensions averaged 95.2% PMN in normal subjects and 94.4% PMN in patients.

Special Materials. Sodium [^{51}Cr]chromate (200-500 μCi/μg Cr) was obtained from New England Nuclear, Boston, Mass., glucose oxidase (type-V, 200 U/mg) from Sigma Chemical Co., St. Louis, Mo., and catalase (beef liver, crystalline, 54,500 U/mg) from Worthington Biochemical Corp., Free-

hold, N. J. MPO prepared from canine PMN by the method of Agner through the end of step six (13) was assayed by the o-dianisidine method (14); 1 U utilizes 1 μmol of substrate/min at 25°C (15). Zymosan (Nutritional Biochemical Corp., Cleveland, Ohio) was homogenized, incubated for 20 min at 37°C in either fresh frozen (−70°C) human AB serum (preopsonized zymosan) or modified Hanks' solution (9) (nonopsonized zymosan), washed, and suspended in water at 10 mg/ml.

Results

PMN-Zymosan-Halide Cytotoxicity System.   Table I demonstrates the cytotoxic effect of a system comprised of PMN, preopsonized zymosan, and a halide. Significant ^{51}Cr release above control ($P < 0.001$) was observed using either 0.1 M chloride or 10^{-4} M iodide as the halide or using a combination of both halides

TABLE I
PMN-Zymosan-Halide Cytotoxicity System

Supplements	^{51}Cr release		
	Chloride (10^{-1} M)	Iodide (10^{-4} M)	Chloride (4×10^{-2} M) + iodide (10^{-5} M)
	%	%	%
Control (no supplements)	24.6 ± 0.7 (28)*	24.6 ± 0.7 (28)*	24.6 ± 0.7 (28)*
PMN + ZYM + halide(s)	38.8 ± 1.1 (38)‡	40.4 ± 1.1 (30)‡	44.5 ± 0.9 (31)‡
PMN omitted	19.4 ± 1.2 (9)	21.4 ± 0.8 (6)	22.0 ± 2.4 (4)
ZYM omitted	26.5 ± 1.5 (10)	23.9 ± 1.1 (7)	26.0 ± 1.3 (5)
Chloride omitted	18.8 ± 1.1 (11)	—	27.2 ± 1.2 (7)
Iodide omitted	—	18.8 ± 1.1 (11)	27.0 ± 1.1 (7)
PMN heated	20.0 ± 0.5 (2)	19.6 (1)	21.5 (1)
PMN homogenized	22.1 ± 0.8 (2)	20.8 (1)	21.3 (1)
PMN sonicated	18.2 (1)	18.9 (1)	26.5 (1)
ZYM nonopsonized	24.9 ± 1.0 (2)	22.5 ± 0.4 (2)	24.2 ± 1.2 (2)
Azide added	24.2 ± 1.4 (6)	23.2 ± 0.9 (4)	22.0 ± 2.3 (3)
Cyanide added	21.0 ± 0.6 (4)	19.3 ± 0.9 (3)	18.1 ± 1.2 (3)
Catalase added	22.3 ± 1.6 (5)	22.3 ± 1.0 (5)	19.5 ± 0.2 (2)
Heated catalase added	32.0 ± 1.6 (4)‡	41.6 ± 1.6 (5)‡	36.8 ± 2.3 (2)‡

The complete reaction mixture contained 0.03 M sodium phosphate buffer pH 7.0, 1.5 × 10^{-3} M KH$_2$PO$_4$, 1.5 × 10^{-3} M MgSO$_4$, 10^5 LSTRA cells, and the supplements indicated below as follows: PMN, 2.5 × 10^5; zymosan (ZYM), 0.1 mg (preopsonized except as indicated); chloride and iodide as the sodium salts in the concentrations indicated; azide and cyanide as the sodium salts at 10^{-4} M; and catalase, 1,400 U. In tubes containing either no NaCl or 4 × 10^{-2} M NaCl, Na$_2$SO$_4$, 0.067 M or 0.04 M, respectively, was added to maintain isosmolarity. Total vol was 0.5 ml. PMN or catalase were preheated at 100°C for 15 min where indicated.
* Mean ± SE (number of experiments).
‡ Significantly increased above control, P < 0.001; all others NS (*t* test).

at concentrations at which either one alone showed only minimal activity. Under these latter conditions the combined effect of chloride and iodide was greater than additive. Cytotoxicity was abolished by deletion of each component of the system, by pretreating the PMN with heat, homogenization or sonication, or by the use of nonopsonized zymosan. These experiments all employed a ratio of effector to target cells of 2.5:1. A cytotoxic effect was present at a ratio of 1.25:1 but was not detectable at 0.5:1.

A cyctotoxic effect of PMN was also demonstrated by loss of oncogenicity of LSTRA target cells for mice. Nearly all animals (22 of 24) receiving 10^5 control LSTRA cells exposed to the buffer system alone developed tumors and died. In contrast, only 6 of 24 mice receiving cells exposed to the complete cytotoxicity system (PMN, zymosan, 0.04 M chloride, and 10^{-5} M iodide as in Table I) died with tumors ($P < 0.05$, chi-square test). Omission of PMN, zymosan, or both halides resulted in tumors and death in six of six, six of six, and five of six animals, respectively.

Involvement of the Peroxidase System. The mechanism of the cytotoxic effect of phagocytosing PMN was initially investigated with the use of specific inhibitors (Table I). Cytotoxicity was abolished by the heme enzyme inhibitors, azide and cyanide. Catalase caused similar inhibition, an effect which was largely reversed by preheating the enzyme. Since these studies suggested involvement of MPO and H_2O_2, experiments were performed employing PMN from patients with leukocytic defects including a patient with hereditary MPO deficiency and six patients with defective H_2O_2 production (leukocyte G6PD deficiency and CGD). No significant release of ^{51}Cr above controls was observed when PMN from any of these patients were substituted for normal cells (Fig. 1). Attempts were then made to restore cytotoxic activity by adding purified MPO, reagent H_2O_2 or a peroxide generating enzyme system (glucose and glucose oxidase) (Fig. 1, inset). MPO-deficient PMN had a potent cytotoxic effect in the presence of added MPO (p < 0.001, t test) but remained without activity when H_2O_2 or glucose oxidase was added. In contrast, PMN from CGD patients remained inactive when MPO was added, but significant cytotoxic activity was restored by

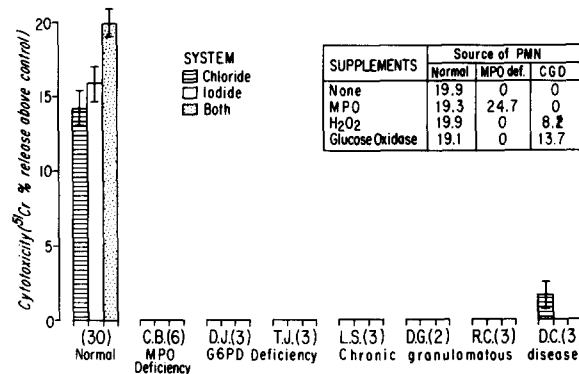| SUPPLEMENTS	Source of PMN		
	Normal	MPO def.	CGD
None	19.9	0	0
MPO	19.3	24.7	0
H₂O₂	19.9	0	8.2
Glucose Oxidase	19.1	0	13.7

FIG. 1. Cytotoxicity system using PMN from patients with leukocytic defects. The reaction mixtures were as described for the complete PMN-zymosan-halide systems in Table I. Halide concentrations were as follows: chloride system, 0.1 M NaCl; iodide system, 10^{-4} M NaI; and chloride plus iodide system, 0.04 M NaCl and 10^{-5} M NaI. For ease of interpretation, control values have been subtracted from experimental values and results expressed as mean percent release of ^{51}Cr above controls ($\pm$ SE). The diagnosis, patients' initials, and number of observations in parentheses are indicated on the abscissa. The data in the inset represent the means of nine studies with normal PMN, three studies with PMN from the patient with hereditary MPO deficiency, and a total of nine studies with PMN from the four CGD patients. For these experiments the complete reaction mixture was as described above for the chloride plus iodide system with the supplements as follows: MPO, 16 mU; H_2O_2, 5×10^{-5} M; and glucose oxidase, 0.14 U. When glucose oxidase was used, 0.01 M D-glucose was also added.

either H_2O_2 (p < 0.01) or the glucose oxidase system (p < 0.001), the latter being more effective. This restoration of activity is consistent with reports of normal or increased H_2O_2 production by MPO-deficient PMN (16) and normal secretion of MPO in CGD leukocytes (3, 17).

Discussion

These studies demonstrate the ability of human PMN to act as effector cells against mammalian tumor cells in vitro using a relatively low ratio of effector to target cells and two cytotoxicity assays: ^{51}Cr release and loss of oncogenicity. Cytotoxicity depends on the presence of intact PMN, phagocytosable particles, and a halide cofactor. Studies employing inhibitors and leukocytes from patients with neutrophil dysfunction syndromes clearly implicate MPO and H_2O_2 in mediating this cytotoxic effect. A mechanism involving the reaction of secreted MPO and H_2O_2 with extracellular halides to form a cytotoxic system is proposed. Since the blood monocyte also contains MPO and forms H_2O_2 during phagocytosis (for review see 18), the MPO-mediated cytotoxicity system may also be operative with this cell type.

While the data presented point out the cytotoxic potential of PMN via the MPO-H_2O_2-halide system, the effects observed are not target cell specific. In particular there is currently no evidence for increased susceptibility of tumor cells to peroxidase-mediated damage and toxic effects of the isolated peroxidase system on normal mammalian cells have been clearly demonstrated (7,15,19). Neutrophil-mediated cytotoxicity could acquire target cell specificity through the participation of humoral or cellular immune responses to tumor antigens. Immunologically directed neutrophil accumulation might be expected to result from the release of chemotactic lymphokines or the interaction of neoplastic cells and tumor-specific antibody with associated formation of complement-derived chemotactic agents. Attachment of antibody molecules to the tumor cell surface would also provide a mechanism for immunologically specific release of peroxidase system components. While particle ingestion served as the stimulus for MPO and H_2O_2 release in the current study, secretion of enzymes (4, 20) including MPO (4) and metabolic stimulation (17, 21) also occur when neutrophils come in contact with antigen-antibody complexes on a nonphagocytosable membrane surface. Evidence has been presented for in vivo attachment of immunoglobulins to the surface of tumor cells (22) and a report of in vitro killing of antibody-coated malignant cells by human neutrophils has recently appeared (23). The demonstration of peroxidase-mediated cytotoxicity for mammalian tumor cells using both cell-free systems (7,9) and intact human PMN raises the possibility that the neutrophil is involved via the peroxidase system in the host defense against neoplastic disease.

Summary

A cytotoxic effect of human neutrophils on mammalian tumor cells is demonstrated. Cytotoxicity depends on the presence of intact neutrophils, phagocytosable particles, and a halide cofactor and is inhibited by azide, cyanide, and catalase. Neutrophils from patients with myeloperoxidase (MPO)

deficiency or defective H_2O_2 production are not cytotoxic, but activity is restored by addition of purified MPO or H_2O_2 respectively. The findings support a mechanism involving the phagocytosis-induced extracellular release of MPO and H_2O_2 and their reaction with a halide cofactor to damage the target cells.

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