CYTOTOXICITY FOR TUMOR CELLS OF CATIONIC PROTEINS FROM HUMAN NEUTROPHIL GRANULES

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Among the components of the azurophil or primary granules of the human neutrophilic polymorphonuclear leukocyte (PMN)[1] are myeloperoxidase (MPO), neutral proteases, acid hydrolases, and a group of strongly cationic proteins. Some of these granule constituents participate in the microbicidal activity of the PMN (for review see reference 21). Myeloperoxidase has potent antimicrobial activity when combined with H_2O_2 and a halide cofactor (18–21, 23, 25). Cationic proteins which are present in large amounts in the PMN granules of some species also have microbicidal activity (24, 26, 27, 35, 36). A number of electrophoretically distinct granular cationic proteins have been isolated in high purity from human PMNs by Olsson and Venge (29) and shown to have bactericidal activity againt a variety of microorganisms (26, 27). These proteins also have chymotrypsin-like esterase activity (27, 32), although this appears to be unrelated to bactericidal capacity since heating destroys enzymatic, but not microbicidal activity (26, 27).

PMN granule components gain direct access to ingested micro-organisms by fusion of the granule membrane with the phagocytic vacuole, rupture of the connecting membrane, and discharge of the microbicidal agents into the vacuole (1, 2, 20). Extracellular release of granule constituents including MPO (1, 4, 13, 14, 17) and cationic proteins (31) also occurs. This secretory activity may be initiated by particle ingestion (1, 4, 10, 12–14, 16, 17, 31, 34), contact of the PMN with immune complexes on a noningestible membrane surface (10, 12–14), contact with biologically active complement fragments such as C5a (3), or exposure of cytochalasin B-treated PMNs to particles or C5a (9, 11, 15, 37).

Extracellular release of granule constituents raises the possibility of PMN-mediated tissue damage. Recent attention has focused on the potential role of peroxidases in the destruction of both normal (6, 22, 33) and neoplastic (4–6, 30) mammalian cells. A cytotoxic effect of MPO, H_2O_2, and a halide on mammalian tumor cells has been demonstrated (5, 6), and we have recently documented a role for the myeloperoxidase system in the killing of tumor cells by intact human PMNs in vitro (4). In the latter studies, phagocytosing PMNs released MPO and H_2O_2 which combined with extracellular halides to damage the target cells. Killing of antibody-coated neoplastic cells by human PMNs has also been demonstrated (7, 8), although a potential role for granule constituents in mediating this effect has not been evaluated. In the current report, highly purified human granular cationic proteins are shown to have a cytotoxic effect on mammalian tumor cells.

MATERIALS AND METHODS

Cationic proteins were purified from human PMNs obtained from a patient with chronic myeloid leukemia as previously described (29). A lyophilized fraction designated cationic protein A consisting of a mixture of proteins 1 and 2 (26, 27, 29) and another lyophilized frac-

[1] *Abbreviations used in this paper:* cpm, counts per minute; MPO, myeloperoxidase; PMN, polymorphonuclear leukocyte.

tion designated cationic protein B consisting of a mixture of proteins 3 and 4 (26, 27, 29) were suspended in distilled water at 500 μg/ml and stored at −70°C until just before use. Purified myeloperoxidase from the same cell preparation (28) was used in some experiments; lyophilized MPO was suspended in distilled water at 250 μg/ml and stored at −20°C.

The target cell employed was a well-characterized Moloney virus-induced lymphoma of BALB/c origin designated LSTRA and maintained by serial intraperitoneal passage in BALB/c mice (5). LSTRA cells were harvested from ascites fluid, labeled with ^{51}Cr (sodium[^{51}Cr]chromate, 200–500 μCi/μg Cr, New England Nuclear, Boston, Mass.), washed, and suspended in 0.1 M sodium phosphate buffer, pH 7.0, as previously described (5). Labeled target cells were incubated with the components of the cytotoxicity system (see legends to figures), and the cytotoxic effect was determined as the mean release of ^{51}Cr in duplicate samples. Data are expressed as a percent of maximum releasable activity (5) as follows:

$$\text{Cytotoxicity} = \frac{\text{sample cpm} - \text{control cpm}}{\text{max cpm} - \text{control cpm}} \times 100,$$

where cpm of ^{51}Cr are determined in supernates from experimental samples (sample cpm), control tubes (control cpm, see figure legends), and tubes containing 1% Triton X-100 (max cpm, see reference 5).

Histone (type II, calf thymus) and protamine sulfate were obtained from Sigma Chemical Co., St. Louis, Mo. Preservative-free heparin sodium (146 U/mg) was obtained from Connaught Medical Research Laboratories, Toronto, Canada. Standard error is used throughout as an estimate of variance, and means are compared using the *t*-test.

RESULTS

Cationic Protein B

Fig. 1 illustrates the effect of various concentrations of human granule cationic protein B on LSTRA tumor cells employing a 2-h incubation period. A significant cytotoxic effect was observed at 25 μg/ml (9.6% ± 2.3, $n = 5$, $P < 0.02$, *t*-test), although ^{51}Cr release was more striking at 50 μg/ml (45.9% ± 8.4, $n = 11$, $P < 0.001$). Fig. 2 shows the dependence of the cytotoxic effect on the time of exposure to granule cationic proteins. Also depicted in Fig. 2 is a comparison of the cytotoxic effects of cationic protein B and MPO from the same PMNs. MPO was used in the presence of H_2O_2 and chloride since previous studies had demonstrated a requirement for each of these components for cytotoxic activity (5). No cytotoxicity was observed

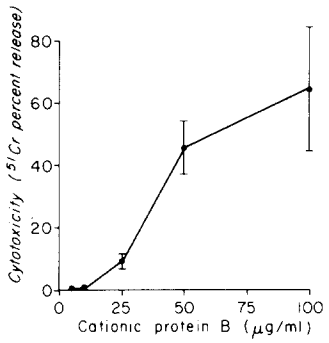

FIGURE 1 Cytotoxic effect of human granule cationic protein B on LSTRA cells. The complete reaction mixture contained 0.07 M sodium phosphate buffer, pH 7.0; 1.5×10^{-3} M KH_2PO_4; 1.5×10^{-3} M $MgSO_4$; 10^5 LSTRA cells (mean 4,700 cpm ^{51}Cr); and cationic protein B, 0 (control tubes)–100 μg/ml. Total volume was 0.5 ml. Incubations were performed in 10 × 75-mm glass test tubes for 2 h in a 37°C water bath shaker agitating 60 times per minute. Results are expressed as mean percent (±SE) ^{51}Cr release.

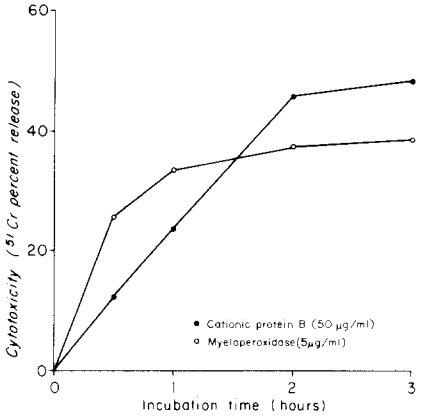

FIGURE 2 Kinetics of the cytotoxic effect of granular cationic protein B and myeloperoxidase. The conditions for testing cationic proteins were the same as described in Fig. 1, except that a constant amount of cationic protein B (50 μg/ml) was employed. The myeloperoxidase system consisted of 0.03 M sodium phosphate buffer, pH 7.0; KH_2PO_4, $MgSO_4$, and LSTRA cells as in Fig. 1; MPO 5 μg/ml (enzyme activity 24 mU/ml [see reference 4]); H_2O_2 5×10^{-5} M and NaCl 0.1 M. The incubation period was varied as indicated. Mean percent ^{51}Cr release in 9 experiments with MPO and 4–11 experiments with cationic proteins is shown.

with H_2O_2 and chloride in the absence of MPO. Even though the amount of MPO employed (5 μg/ml) was 10% the amount of cationic proteins, comparable cytotoxic activity was observed in the

TABLE I

Effects of Heating, Ionic Strength, and Heparin

Conditions*	Cytotoxicity (^{51}Cr percent release)		
	Cationic protein B	Cationic protein A (Lot 1)	Cationic protein A (Lot 2)
Complete system	45.9 ± 8.4 (11)‡	93.1 ± 6.9 (3)	3.8 ± 3.8 (5)
Cationic protein heated§	78.9 ± 9.3 (4)	82.4 ± 17.6 (2)	68.3 ± 1.7 (3)
Ionic strength decreased‖	47.0 ± 2.9 (2)	100.0 ± 0.0 (2)	31.2 ± 7.4 (2)
Ionic strength increased‖	40.4 ± 2.9 (2)	100.0 ± 0.0 (2)	0.0 (1)
Heparin added (30 μg/ml)	0.0 ± 0.0 (2)	1.8 ± 1.8 (2)	–

* Conditions as specified in Fig. 1 (cationic protein 50 μg/ml) with alterations as noted.

‡ Mean ± SE, number of experiments in parentheses.

§ 90°C for 10 min.

‖ Ionic strength altered by employing phosphate buffer at 0.05 M (decreased) or 0.10 M (increased) in place of the usual 0.07 M concentration.

two systems. Although the MPO system had a more rapid initial rate of activity and a lower final activity relative to the cationic proteins, these differences were not significant. Since cationic protein B at 10 μg/ml or less had no significant effect, the data suggest that MPO in the presence of H_2O_2 and chloride constitutes a more potent cytotoxic system than the granule cationic proteins.

The cytotoxic effect of cationic protein B was not diminished by heating at 90°C for 10 min. In fact, with the conditions employed (50 μg/ml of cationic protein B, 2-h incubation period), significant enhancement ($P < 0.05$) was observed (Table I). Changing the ionic strength in the cytotoxicity assay by using 0.05 M and 0.1 M phosphate buffer in place of the usual 0.07 M concentration (see figure legends) had no significant effect on the activity of cationic protein B (Table I).

Cationic Protein A

Two separate lots of cationic protein A from PMNs of two different patients were studied (Table I). Lot 1 was highly potent with a mean ^{51}Cr release of 93.1% ± 6.9 in three experiments ($P < 0.01$) using 50 μg/ml and a 2 h incubation period. At 25, 10, and 5 μg/ml ^{51}Cr release was 39.8, 16.8, and 2.6%, respectively. With the same conditions, lot 2 was markedly inferior in cytotoxic activity (mean 3.8% ^{51}Cr release with 50 μg/ml in five experiments). Similar differences in bactericidal and esterase activities of these two preparations were also observed.[2] Heating at 90°C for 10 min had no significant effect on the more potent preparation (Table I). Heating the weaker preparation resulted in a significant increase in cytotoxic activity to a mean of 68.3% ($P < 0.001$, Table I). It was also possible to detect activity in the weaker cationic protein A preparation by decreasing the ionic strength (mean 31.2% with 0.05 M phosphate buffer, $P < 0.02$, Table I). This latter finding may parallel the inverse relationship noted between ionic strength and bactericidal activity of the granule cationic proteins (26).

Heparin, Histone, and Protamine

Heparin, a strongly anionic agent, inhibits the bactericidal activity of the granule cationic proteins (35). In the current studies 30 μg/ml of heparin completely inhibited the cytotoxic effect of 50 μg/ml of cationic protein (Table I). Incomplete inhibition was observed with 6 μg/ml of heparin. These concentrations of heparin alone had no detectable effect on the target cells. Histone and protamine, as examples of other strongly basic proteins, were also tested for cytotoxic activity. Histone was very potent, with 10 μg/ml giving 66.8% ± 10.0 ^{51}Cr release ($P < 0.01$); protamine was less active, with 25 μg/ml giving 22.9% ± 7.2 ^{51}Cr release ($P < 0.05$). The cytotoxic effect of both histone and protamine was unaffected by heating (90°C for 10 min), was completely inhibited by heparin (30 μg/ml), and was diminished approximately 50% by an increase in osmotic strength from 0.07 M to 0.10 M phosphate.

DISCUSSION

The data presented document the ability of cationic proteins from granules of human PMNs to kill mammalian tumor cells as measured by a ^{51}Cr

[2] Olsson, I. Unpublished observations.

release assay. This cytotoxic effect was time and concentration dependent. The reasons for the differences in cytotoxic, bactericidal, and enzymatic activity between the two cationic protein A preparations are unknown. The quantities or properties of these granule constituents may differ in the PMN preparations from two different chronic myeloid leukemia patients. Similar studies are planned for the basic proteins which are currently being purified from the PMNs of normal subjects.

The mechanism of the cytotoxic activity of granule cationic proteins has been partially characterized. It does not depend on the esterase activity of these proteins since heating at 90°C for 10 min destroys this enzymatic activity (26, 27) but did not decrease cytotoxicity. Heating, in fact, enhanced the cytotoxic activity. A similar enhancing effect of heating has been reported for the bactericidal effect of human granule cationic proteins (27). The presence of a heat-labile inhibitor in the cationic protein preparations cannot be excluded. The anionic agent heparin, an inhibitor of cationic protein bactericidal activity (35), completely blocked the cytotoxic effect as well. Thus, the cytotoxic and bactericidal activities of these proteins may share common mechanisms. It is probable that binding of the highly positively charged molecules to the negatively charged cell surface is an important initial step. The cytotoxic activity of the other basic proteins tested, histone and protamine, is consistent with an ionic interaction of this type.

The current studies also extend our earlier work on the cytotoxic effect of a system comprised of canine MPO, H_2O_2, and a halide (5) to human MPO (also see reference 6). Thus, at least two microbicidal systems of the human PMN are capable of killing mammalian tumor cells. Although the myeloperoxidase system has been implicated in the cytotoxic effect of phagocytosing PMNs (4), further studies will be required to assess the role of granule cationic proteins in the extracellular killing of tumor cells by intact neutrophils.

SUMMARY

Cationic proteins purified from human polymorphonuclear leukocyte granules exert a cytotoxic effect on mammalian tumor cells. This effect is time and concentration dependent, is inhibited by the anionic agent heparin, and is enhanced by preheating the cationic proteins. Other strongly basic proteins (histone, protamine) also exhibited cytotoxic activity. Myeloperoxidase isolated from human leukocytes is cytotoxic when combined with H_2O_2 and chloride. Under these conditions, the potency of the myeloperoxidase-mediated system is greater than that of the cationic proteins.

The excellent technical assistance of Ms. Coralie Baker and Mrs. M. B. Håkansson is gratefully acknowledged.

This work was supported by grants AI07763 and CA18354 from the United States Public Health Service and grant 75:140 from the Swedish Cancer Society. Dr. Clark is the recipient of Research Career Development Award CA00164 from the National Cancer Institute.

Received for publication 22 December 1975, and in revised form 3 May 1976.

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