EOSINOPHIL PEROXIDASE-INDUCED MAST CELL SECRETION*

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Mast cell secretion of the chemical mediators of immediate hypersensitivity such as histamine is generally initiated in vivo by antigen interaction with IgE antibodies on the mast cell surface (1–4). Mast cell degranulation also may be initiated by the anaphylatoxins C3a and C5a generated by complement activation (5, 6) and by cationic polypeptides from polymorphonuclear leukocyte granules (7, 8). Nonphysiologic stimuli of mast cell mediator release without generalized cytotoxic effects include compound 48/80 (9, 10), polymyxin B (11, 12), the calcium ionophore A-23187 (10, 13), and the hypoxanthine-xanthine oxidase system (14).

Eosinophils are often closely associated with mast cells in tissues, particularly in areas of immediate hypersensitivity. Activation of eosinophils can induce the release of their granule components into the phagocytic vacuole (15–17) or extracellular fluid (18–21). Among these is the eosinophil peroxidase (EPO)[1] which is present in the eosinophil granule matrix in high concentration (22). The substrate for this enzyme, H_2O_2, is also generated by activated eosinophils (23–25). EPO, like the neutrophil peroxidase (myeloperoxidase [MPO]), forms a microbicidal (26–28) and cytotoxic (29) system when combined with H_2O_2 and a halide. EPO, which is a strongly basic protein, also binds firmly to the matrix of isolated mast cell granules (MCG) with retention of peroxidatic activity; indeed, the MCG/EPO complex is more effective than free EPO as the catalyst of the peroxidase-mediated antimicrobial system (30).

These findings prompted a study of the effect of the EPO-H_2O_2-halide system on mast cells. The following dual effect was found: at relatively low EPO concentrations specific secretion occurs, whereas when the EPO concentration is raised, lysis of the mast cells results in the release of all cytoplasmic components. EPO binds to the released MCG to form a complex with retention, indeed potentiation, of secretory activity on mast cells.

Materials and Methods

Special Reagents Guaiacol (anhydrous), β-nicotinaminde adenine dinucleotide, reduced form (β-NADH), pyruvic acid (sodium salt, type II), lactate dehydrogenase (LDH), *o*-phthal-

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[1] *Abbreviations used in this paper* β-NADH, β-nicotinamide adenine dinucleotide, DAB, 3,3-diaminobenzidine tetrahydrochloride, EPO, eosinophil peroxidase, LDH, lactate dehydrogenase, MCG, mast cell granule, MPO, myeloperoxidase, OPT, *o*-phthaldialdehyde, OsO_4, osmium tetroxide

dialdehyde (OPT), human albumin (essentially fatty acid-free), and histamine diphosphate were obtained from Sigma Chemical Co. (St Louis, Mo.), ligroine from Eastman Kodak Co (Rochester, N Y.), glutaraldehyde and uranyl acetate from Polysciences, Inc (Warrington, Pa), osmium tetroxide (OsO_4), Epon 812, and 3,3-diaminobenzidine tetrahydrochloride (DAB) from Scientific Chemical Co. (Huntington Beach, Calif.), and sodium heparin (beef lung) from Upjohn Co., Agricultural Prods MKT (Kalamazoo, Mich.) All other reagents were of the highest commercial grade available

Peroxidase Preparations EPO was partially purified from guinea pig peritoneal eosinophils by gel filtration on Bio-Gel P-150 (Bio-Rad Laboratories, Richmond, Calif) as previously described (28) MPO was prepared in highly purified form from canine pyometral pus by the method of Agner to the end of step 6 (31). EPO and MPO were heated at 100°C for 15 min where indicated. The peroxidase activities of EPO and MPO were determined by guaiacol oxidation (32) 1 U of enzyme is the amount which oxidizes 1 μmol of electron donor/min at 25°C A molar absorbancy of $2.66 \times 10^5\ M^{-1}\ cm^{-1}$ at 470 nm was employed (33). All spectrophotometric analyses were performed using a Cary M-15 spectrophotometer (Cary Instruments, Monrovia, Calif.).

Isolation of Mast Cells. Peritoneal cells were obtained from 200-g male Sprague-Dawley rats by lavage with Tyrode's buffer containing 10 U/ml heparin. The cell suspension which contained 4–5% mast cells was layered on 38% bovine serum albumin, and the mast cell-rich pellet collected by centrifugation. The cell suspension, washed and suspended in Tyrode's buffer contained >90% mast cells with <10% mononuclear cells and <1% eosinophils as determined by toluidine blue staining (34). The mast cells were always greater than 95% viable as assessed by trypan blue dye exclusion (35).

Preparation of MCG/EPO Complexes. MCG free of perigranular membranes were obtained by osmotic lysis of purified mast cells followed by differential centrifugation (36) The MCG were washed twice in 0.1 M phosphate buffer, pH 7 0, to remove histamine and other loosely bound mediators (36) For preparation of MCG/EPO complexes, EPO was incubated with histamine-free MCG in 0.1 M phosphate buffer, pH 7 0 for 30 min at 37°C (30) The complexes were washed three times and suspended in water

Measurement of Histamine Release. Mast cells were preincubated for 5 min at 37°C in a water bath oscillating 80 times/min The components of the reaction mixture (see legends to Figures and Tables) were added to a total volume of 0 5 ml with the MPO or EPO added last to start the reaction Unless otherwise indicated, incubations were for 5 min at 37°C after which the suspensions were placed on ice until centrifugation at 400 *g* for 6 min at 4°C The histamine content of the supernatant fraction and pellet was determined fluorometrically using OPT (37) Fluorometric readings were made between 360 and 480 nm using a model 110 fluorometer (Turner Associates, Div of American Sterilizer Co., Palo Alto, Calif.) The percent histamine release was calculated as follows: (supernatant histamine/pellet plus supernatant histamine) × 100.

Measurement of LDH Release. Incubation of mast cells, termination of the reaction, and centrifugation were performed as described under measurement of histamine release. The cell pellets were suspended in 0.05 M phosphate buffer, pH 7.5, freeze-thawed three times, and centrifuged at 2,000 *g* The LDH activity of this supernate was determined by the fall in absorbance at 340 nm on incubation of the cell preparations with sodium pyruvate and β-NADH in phosphate buffer, pH 7 5 (38) The LDH activity of control cells incubated in buffer alone was determined following comparable treatment, and the percent LDH release was calculated as follows: (control − experimental LDH/control LDH) × 100

Histamine Degradation by the Peroxidase Systems. Histamine was incubated with the components of the peroxidase system as described in the legend to Table III, and the histamine level was determined fluorometrically as described above

Electron Microscopic Studies Pellets of 2×10^6 mast cells incubated with the various components of the reaction mixture (see legends to Figures) were collected by centrifugation at 400 *g*, fixed in 2% glutaraldehyde, postfixed with 1% OsO_4, stained with 0 5% uranyl acetate, dehydrated with ethanol, and embedded in EPON 812 Some pellets were treated with a saturated solution of 3,3-diaminobenzidine and 0 015% H_2O_2 for detection of peroxidase (39) after a brief period of glutaraldehyde fixation and then processed as described above The samples were examined

with a JEOL 100B electron microscope (Japan Electron Optics Laboratory, Tokyo, Japan) at 60 kV

Statistical Analysis. The data are reported as the mean ± SE of the combined experiments. Differences were analyzed for significance using Student's two-tailed t test for independent means except in Table IV where paired analysis was used (not significant, $P < 0.05$)

Results

Degranulation. Greater than 95% of the mast cells incubated in buffer alone for 5 min at 37°C exhibited the usual morphology of unstimulated cells (Fig. 1 A). Unstimulated mast cells are distinguished from other cells by the presence of a number of electron-dense, membrane-bound, cytoplasmic granules. Other cytoplasmic organelles (e.g., mitochondria, endoplasmic reticulum, Golgi vesicles) are not unusual, and the cell is surrounded by a plasma membrane with numerous membrane projections. Less than 5% of control cells showed evidence of degranulation ultrastructurally.

In contrast, 40–60% of the mast cells incubated with EPO (25–30 mU), H_2O_2 (2×10^{-5} M), and iodide (10^{-4} M) had ultrastructural changes characteristic of degranulation (Fig. 1 C). Many granules were swollen and less electron dense; vacuole-like structures containing two or more granules formed, and the altered granules were extruded into the extracellular fluid. There was no evidence of cytotoxic damage at this low level of EPO. When any of the components of the EPO-H_2O_2-iodide system were omitted, mast cell ultrastructure was indistinguishable from that of control cells. A mast cell following incubation with H_2O_2 and iodide (i.e., EPO deletion) is shown in Fig. 1 B.

Noncytotoxic degranulation of mast cells by EPO, H_2O_2 and iodide was confirmed by measurement of histamine and LDH release (Table I). Mast cells incubated in Tyrode's buffer alone for 5 min spontaneously released 7.2% of the granule marker histamine and 4.5% of the cytoplasmic marker LDH into the extracellular fluid (Table I). The further addition of compound 48/80 (1 μg/ml) increased histamine release to 59.3% without affecting LDH release, an effect expected for this agent which initiates mast cell degranulation. A comparable effect was observed when compound 48/80 was replaced by EPO (25–30 mU), H_2O_2 (2×10^{-5} M), and iodide (10^{-4} M); histamine release was increased to 53.2%, whereas LDH release remained at 4.9%. Histamine release was abolished by omission of any of the components of the EPO-H_2O_2-iodide system, by heat inactivation of EPO, and by the addition of the hemeprotein inhibitors azide, cyanide, and aminotriazole. The hemeprotein inhibitors, at the same concentrations, did not affect histamine release induced by 1 μg/ml 48/80 (data not shown). MPO when combined with the same concentrations of H_2O_2 and iodide also induced noncytotoxic mast cell degranulation. As with the EPO system, omission of components, heat inactivation of MPO, and addition of the inhibitors azide (10^{-3} M), cyanide (10^{-3} M), and aminotriazole (10^{-2} M) prevented histamine release (data not shown).

In Table I, chloride at 0.14 M was present in the reaction mixture as a component of the Tyrode's buffer. That chloride alone could not meet the halide requirement under these conditions was indicated by the loss of histamine release on deletion of iodide from the complete peroxidase system. However, when the pH of the Tyrode's buffer was lowered (by omission of bicarbonate and addition of HCl) to 6.5 or 6.0, histamine release in the absence of iodide was observed (Fig. 2), presumably caused

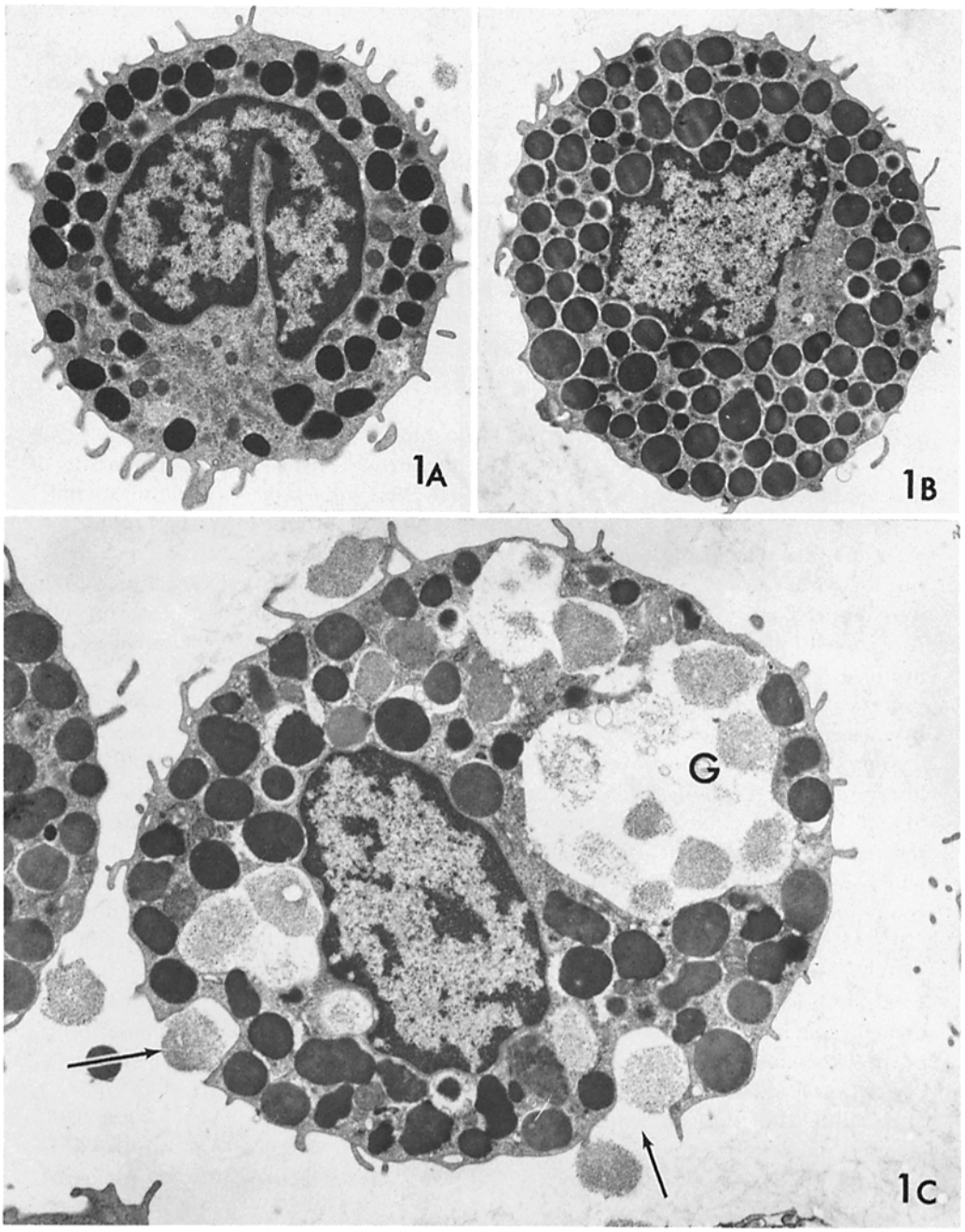

FIG 1 Rat mast cells incubated in Tyrode's buffer, pH 7 4, alone (A, × 6,000) or supplemented as described in Table I with H_2O_2 and iodide (B, × 5,600) or with EPO, H_2O_2 and iodide (C, × 9,500) The cell seen in (B) (EPO deletion) is indistinguishable from the control cell (A) In both, the cytoplasm contains fully developed, electron-dense, homogeneous granules with no evidence of granule secretion. The nucleus, Golgi vesicles, and other organelles appear normal and typical microridges extend from the cell surface The cell in (C) exhibits ultrastructural features characteristic of mast cell secretion. Many swollen and less dense granules (G) are seen in vacuoles or channels, some of which open to the outside of the cell (arrows) Other granules and the intergranular cytoplasm, nucleus, mitochondria, Golgi vesicles, and plasma membrane appear unaffected

TABLE I

*EPO/H_2O_2/I^--induced Histamine Release**

Supplements	Histamine release		LDH release
	%		%
Mast cells (spontaneous release)	7.2 ± 1.0 (8)‡		4 5 ± 1 2 (8)
Mast cells plus 48/80	59 3 ± 5 1 (5)	<0.001	3.7 ± 1.4 (5)
Mast cells plus EPO plus H_2O_2 plus iodide	53 2 ± 4 8 (5)	<0.001	4.9 ± 1.5 (5)
H_2O_2 omitted	9 9 ± 0 7 (5)		2 8 ± 0 9 (5)
Iodide omitted	11 9 ± 2 0 (7)		3.1 ± 1 1 (7)
EPO omitted	10.5 ± 0.8 (8)		3.5 ± 0 8 (8)
EPO heated	7 9 ± 1.6 (4)		4 8 ± 1.5 (4)
Azide (10^{-3} M) added	10.1 ± 2.1 (3)		3.0 ± 1.9 (3)
Azide (10^{-4} M) added	11 6 ± 0.9 (3)		2 3 ± 1 5 (3)
Azide (10^{-5} M) added	54 6 ± 6.3 (3)	<0.001	4.7 ± 2 1 (3)
Cyanide (10^{-3} M) added	10 5 ± 4 1 (3)		5 3 ± 3 1 (3)
Cyanide (10^{-4} M) added	58.3 ± 6.8 (3)	<0 001	4.5 ± 2.1 (3)
Aminotriazole (10^{-2} M) added	10 6 ± 3.1 (3)		3.5 ± 1.7 (3)
Aminotriazole (10^{-3} M) added	17.1 ± 6.1 (3)		2.1 ± 0 9 (3)
Aminotriazole (10^{-4} M) added	59 5 ± 7 1 (3)	<0 001	3 3 ± 1 4 (3)
Mast cells plus MPO plus H_2O_2 plus iodide	38 1 ± 3 5 (5)	<0.001	4 7 ± 1 9 (5)

* The reaction mixture contained 2 × 10^5 mast cells in Tyrode's buffer, pH 7 4 and where indicated, 1 μg/ml compound 48/80, 25–30 mU EPO or MPO, 2 × 10^{-5} M H_2O_2, 10^{-4} M NaI and azide, cyanide, and aminotriazole at the concentrations indicated Probability values for the difference from the mast cells alone (spontaneous release) are shown where significant ($P < 0$ 05)

‡ Mean ± SE of (n) experiments

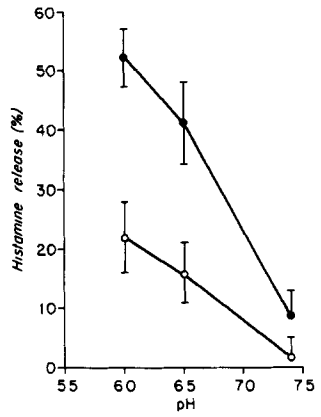

FIG 2. Effect of pH on histamine release by the EPO (MPO)-H_2O_2-chloride system. The reaction mixture contained 2 5 × 10^5 mast cells, 25–30 mU EPO (●) or MPO (○), and 2 × 10^{-5} M H_2O_2 in Tyrode's buffer at the pH indicated The pH of the buffer was varied by deletion of $NaHCO_3$ and adjustment with 6 N HCl. Histamine release above background (mean ± SE of four experiments) is shown. The background was 7.6 ± 1.8% at pH 7.4, 9.6 ± 2.8% at pH 6.5; and 13.7 ± 3.4% at pH 6 0, and LDH release was <5 0% at each data point

by the presence of chloride in the reaction mixture. MPO also was effective under these conditions, although the histamine release induced by the EPO-mediated system was greater than that induced by the MPO system at equivalent guaiacol units of peroxidase activity. In Table II, Tyrode's buffer was replaced by phosphate buffer, pH 7.4, containing $CaSO_4$. Background histamine release was higher under these conditions; however, the further addition of EPO, H_2O_2, and iodide at 10^{-4} or 10^{-5} M significantly increased release Iodide at 10^{-6} M and chloride at 0.1 M were ineffective individually; however, when combined, they could meet the halide requirement of the peroxidase system The choride-free phosphate buffer could not be employed at pH 6.5 or 6.0 because spontaneous histamine release increased to unacceptable levels (>20%) under these conditions.

It has been reported that histamine is deaminated by MPO when supplemented with H_2O_2 (4×10^{-3} M) and chloride (0.124 M) at pH 5.3, but not at pH 7.0 (40). In this study, iodide could not substitute for chloride in the MPO-dependent system, and EPO at both pH levels had no activity with either halide (40). These findings raised the possibility that our results may be influenced by the degradation of histamine by the peroxidase systems. As seen in Table III, however, there was no degradation of histamine under the conditions employed here (pH 7.4; H_2O_2, 2×10^{-5} M; EPO or MPO, 25–30 mU; iodide, 10^{-4} M; chloride, 0.14 M) as measured fluorometrically. The results were comparable at pH 5.3. We confirmed the degradation of histamine at the higher H_2O_2 concentration (4×10^{-3} M) employed by Fabian and Aronson (40). However, under our conditions, H_2O_2 alone significantly decreased histamine levels at pH 5.3, and the further addition of MPO and chloride increased the degradation seen with H_2O_2 at both pH 5.3 and 7.4 (data not shown).

EPO binds to isolated MCG to form a complex which retains the capacity to induce mast cell degranulation when supplemented with H_2O_2 and iodide (Table IV). Indeed, the MCG/EPO complex-H_2O_2-iodide system produced greater histamine release than the comparable granule-free EPO system when standardized to the same guaiacol units of peroxidase activity. When the MCG/EPO complexes were incubated

TABLE II

*Effect of Halide Concentration**

Supplements	Histamine release	
	%	
None	10 2 ± 3 4‡	
EPO plus H_2O_2 plus iodide (10^{-4} M)	44 5 ± 4 9	<0 002
EPO plus H_2O_2 plus iodide (10^{-5} M)	36 2 ± 3 6	<0 002
EPO plus H_2O_2 plus iodide (10^{-6} M)	9 6 ± 1 9	
EPO plus H_2O_2 plus chloride (10^{-1} M)	8 9 ± 3 8	
EPO plus H_2O_2 plus iodide (10^{-6} M) plus chloride (10^{-1} M)	27 3 ± 4 0	<0 02

* The reaction mixture was as described for the mast cells plus EPO plus H_2O_2 plus iodide system in Table I except that the Tyrode's buffer was replaced by 0 1 M sodium phosphate buffer, pH 7 4, containing 0 7 mM $CaSO_4$, and the halide was varied as indicated Probability values for the difference from the mast cells alone (spontaneous release) are shown where significant ($P <$ 0 05)

‡ Mean ± SE of four experiments

TABLE III

*Peroxidation of Histamine**

Supplements	Histamine	
	pH 5 3	pH 7 4
	μg	
None	4.3 ± 0.2‡	4 2 ± 0 2
H_2O_2	4 6 ± 0 3	4.4 ± 0 2
H_2O_2 plus EPO plus iodide	4.4 ± 0.2	4 2 ± 0 1
H_2O_2 plus EPO plus chloride	4 5 ± 0 2	4 2 ± 0 3
H_2O_2 plus MPO plus iodide	4 5 ± 0.2	4 4 ± 0 2
H_2O_2 plus MPO plus chloride	4.3 ± 0.4	4 3 ± 0.5

* The reaction mixture contained 4 μg histamine in 0.1 M phosphate buffer at pH 5.3 or 7 4, and where indicated, 2×10^{-5} M H_2O_2, 10^{-4} M NaI, 0.14 M NaCl, and 25–30 mU EPO or MPO

‡ Mean ± SE of four experiments

TABLE IV

*Comparison of Histamine Release by EPO and MCG/EPO Systems**

Peroxidase activity	Histamine release		
	EPO	MCG/EPO	
mU	%		
2 5	15 1 ± 1 9 (5)‡	19 7 ± 1 6 (5)	<0.02
5 0	20 9 ± 1 4 (8)	27 6 ± 1 8 (8)	<0.002
12 5	34 6 ± 3 0 (5)	39 5 ± 4 4 (8)	

* The reaction mixture was as described for the mast cells plus EPO plus H_2O_2 plus iodide system in Table I except that the peroxidase activity of the EPO and MCG/EPO complexes were varied as indicated Probability values for the difference between the EPO and MCG/EPO systems as determined by paired analysis are shown where significant ($P < 0.05$)

‡ Mean ± SE of (n) experiments

with intact mast cells, the complexes bound to the cell surface (Fig. 3). The isolated granules appeared to break up into smaller units each with a dense core and less dense periphery. Adherence of the peroxidase-positive granule segments to the cell surface and to the tips of the cytoplasmic protrusions were seen.

Cytolysis. The EPO-H_2O_2-halide system was cytotoxic to mast cells when the EPO concentration was raised to relatively high levels. As shown in Fig. 4, when the EPO level was increased to 100 mU, combination with H_2O_2 (2×10^{-5} M) and iodide (10^{-4} M) resulted in the release of both histamine (68.9%) and the cytoplasmic marker LDH (59.0%) from the mast cells. Omission of EPO, H_2O_2, or iodide abolished this cytotoxic effect.

The cytotoxic activity of the EPO-H_2O_2-halide system at high EPO levels was confirmed by ultastructural studies (Fig. 5). Greater than 90% of the cells showed nonselective loss of cytoplasmic constituents, mitochondrial swelling, and disruption of nuclear and cytoplasmic membranes. Some granules were released with intact perigranular membranes and retained their electron density Other extracellular granules were membrane-free and swollen, similar to normally secreted granules. A prominent ring of electron-dense peroxidase-positive material was seen on the surface

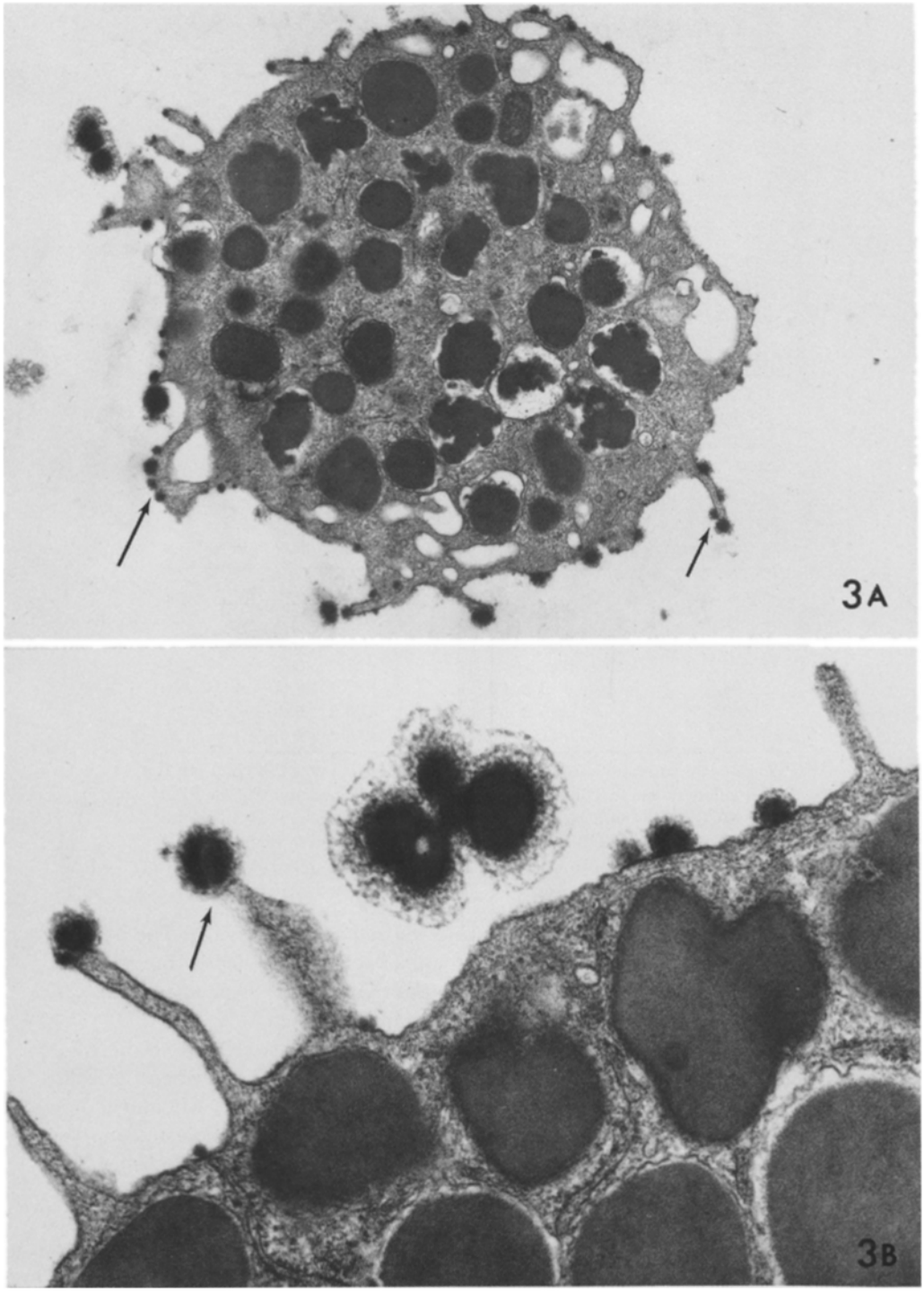

FIG 3 Mast cell incubated with MCG/EPO complexes in Tyrode's buffer at 37°C for 1 min (A, × 15,000) or 5 min (B, × 47,200) The cells were examined cytochemically for peroxidase The heterogeneity in size of the DAB-positive complexes and their binding to the cell surface and the tips of the microridges (arrows) are shown There is no evidence of degranulation as would be expected from the absence of H_2O_2 and iodide from the reaction mixture

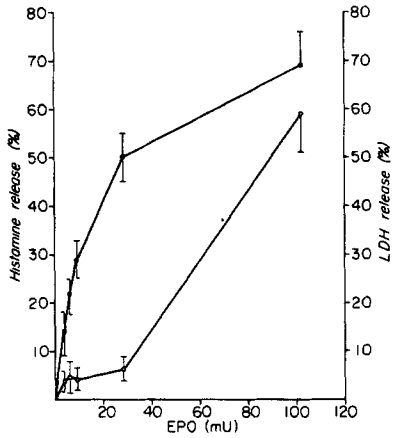

FIG 4 Histamine and LDH release as a function of EPO concentration The reaction mixture was as described for the mast cells plus EPO plus H_2O_2 plus iodide system in Table I except that the EPO concentration was varied as indicated The data represent histamine (●) and LDH (○) release above background (histamine release 8.4 ± 2.5%; LDH release 4 3 ± 1 9%) and are the mean ± SE of four to six experiments

of membrane-free extracellular granules as well as on the cell surface by diaminobenzidine cytochemistry when the high level of EPO (100 mU) was used (Fig. 5). Omission of H_2O_2 from the staining procedure abolished the reaction. Peroxidase-positive material could not be detected on the granule surface when less EPO (25–30 mU) was employed under our experimental conditions.

The effect of serum albumin on histamine release by the EPO-H_2O_2-iodide system at the low (25 mU) and high (100 mU) EPO concentrations is shown in Table V. Albumin at 1,000 or 2,500 μg/ml significantly inhibited histamine release by the noncytotoxic 25 mU EPO system (each $P < 0.001$ as compared with complete EPO system without albumin), whereas albumin at 10 or 100 μg/ml did not. Histamine release, however, remained significantly greater than background (spontaneous release) at all the albumin concentrations employed (Table V). The results were comparable at the higher EPO concentrations (100 mU); histamine release was inhibited by albumin at 100, 1,000, and 2,500 μg/ml ($P < 0.05$, <0.002, and <0.001, respectively, as compared with the complete EPO system without albumin), but in each instance release remained significantly above background (Table V). LDH release, which is high at this level of EPO indicating cytotoxic activity, remained high when albumin at 10 or 100 μg/ml was added. However, at the higher albumin concentrations (1,000 and 2,500 μg/ml) LDH release was at the background level. Thus, the addition of protein under these conditions converted this cytotoxic histamine release system to a noncytotoxic secretory one. Normal rat serum had a comparable inhibitory effect on histamine release at the same protein concentrations, whereas a protein-free ultrafiltrate of serum (10%) was without effect (data not shown).

Discussion

Specific secretion of MCG components is induced by the peroxidase-H_2O_2-halide system. EPO (or MPO) at relatively low levels (4–30 mU) when combined with H_2O_2 and a halide increased the release of the granule marker, histamine, without affecting

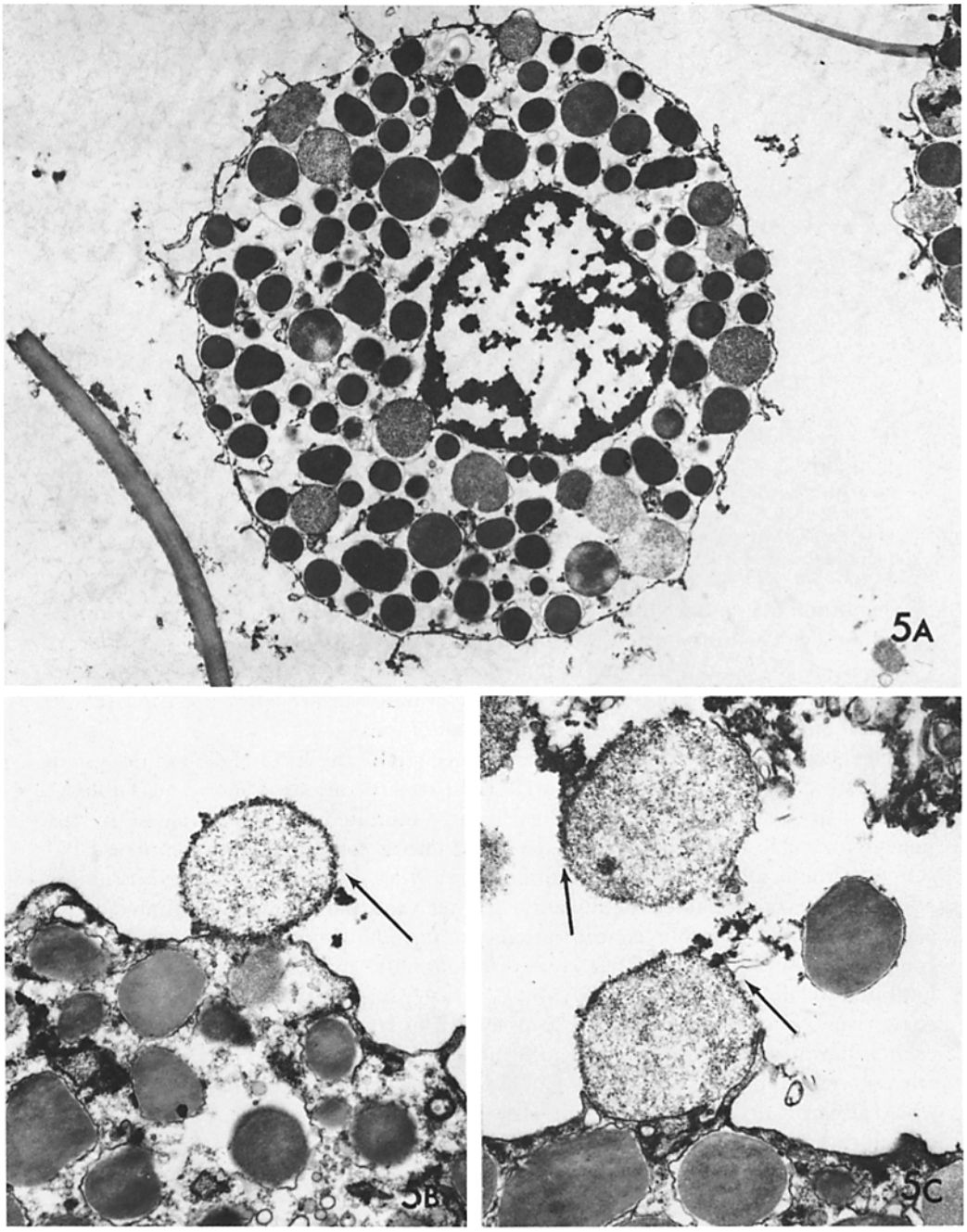

FIG 5 Mast cells were incubated for 5 min at 37°C in 0.5 ml Tyrode's buffer, pH 7 4, containing 100 mU EPO, 10^{-4} M iodide, and 2×10^{-5} M H_2O_2 The preparations were treated with DAB In (A) (× 8,000), the cytotoxic changes are readily apparent aggregation of the nuclear chromatin, nuclear and cytoplasmic membrane breakage, loss of cytoplasm and cytoplasmic constitutents, and cell swelling The cell surface contains electron-dense deposits not seen on cells similarly treated except that H_2O_2 was omitted from the DAB reaction mixture In (B) (× 15,700) and (C) (× 16,600), swollen and membrane-free extracellular granules with peroxidase-positive material on their surface (arrows) are seen Other extracellular granules were membrane bound and uniformly electron dense comparable with normal intracellular granules (C) They are presumably released from disrupted cells

TABLE V

*Effect of Albumin on Histamine Release by the EPO Systems**

Supplements	Histamine release		LDH release	
	%		%	
Mast cells (spontaneous release)	8 4 ± 2.3‡		3 8 ± 1 0	
Mast cells plus EPO (25 mU) plus H_2O_2 plus iodide	50 4 ± 3.7	<0 001		
Albumin (10 μg/ml) added	51.2 ± 6.2	<0 001		
Albumin (100 μg/ml) added	36 7 ± 5 3	<0.002		
Albumin (1,000 μg/ml) added	21 0 ± 3 0	<0.02		
Albumin (2,500 μg/ml) added	17.2 ± 1 8	< 0 02		
Mast cells plus EPO (100 mU) plus H_2O_2 plus iodide	66 9 ± 4 5	<0.001	71 6 ± 6 1	<0 001
Albumin (10 μg/ml) added	61 9 ± 3.3	<0.001	77 6 ± 8.3	<0 001
Albumin (100 μg/ml) added	47 3 ± 5 8	<0 001	58 3 ± 4 2	<0 001
Albumin (1,000 μg/ml) added	35 9 ± 4 9	<0.002	3.6 ± 2 1	
Albumin (2,500 μg/ml) added	27 3 ± 5 1	<0.02	2 8 ± 1 4	

* The reaction mixture was as described for the mast cells plus EPO plus H_2O_2 plus iodide system in Table I except that the peroxidase activity of the EPO was varied, and human serum albumin was added at the indicated concentrations Probability values for the difference from the mast cells alone (spontaneous release) are shown where significant ($P < 0.05$).

‡ Mean ± SE of five experiments

the release of the cytoplasmic marker, LDH. At pH 7.4, iodide was active at concentrations down to 10^{-5} M, whereas chloride at 0.1 M was ineffective. A combination of 0.1 M chloride and 10^{-6} M iodide, however, could meet the halide requirement. This synergism between iodide and chloride has been previously reported with other target cells (29, 41). Histamine release with chloride as the sole available halide was observed when the pH was lowered to 6.5 or 6.0.

Degranulation of mast cells without cytolytic activity was confirmed by ultrastructural studies. Degranulation, with secretion of granule-associated mediators such as histamine, is characterized by a change in granule morphology with swelling and loss of electron density. Vacuoles which form around individual granules fuse to form larger vacuoles and channels which open to the outside of the cell where morphologically altered granules are detected. These changes have been described for mast cells stimulated to secrete by agents such as antigen specific for cell membrane-bound IgE (2), compound 48/80 (9), and polymyxin B sulfate (11, 12). They also are seen when mast cells are exposed to the EPO-H_2O_2-iodide system at relatively low EPO concentrations (Fig. 1). The cell membrane remains intact, other intracellular organelles appear unaffected, and the cell surface continues to exhibit the numerous membrane projections characteristic of normal nonsecreting cells.

Xanthine oxidase when incubated with hypoxanthine has been reported to induce noncytotoxic histamine release from mast cells (14). Histamine release by this oxygen radical and H_2O_2-generating system was inhibited by catalase but was unaffected by either superoxide dismutase or mannitol suggesting a role for H_2O_2. In their study, concentrations of reagent H_2O_2 equal to or greater than 5×10^{-5} M were found to induce significant histamine release above background (14). We employed H_2O_2 at a concentration (2×10^{-5} M) which was ineffective alone (14; Table I) or in combination

with either iodide or EPO; the complete EPO-H_2O_2-halide system was required (Table I).

When the EPO level was increased to 100 mU, combination with H_2O_2 and iodide resulted in lysis of mast cells with release of LDH as well as histamine and morphological evidence of cell disintegration In contrast with noncytotoxic secretion, the cell membrane was disrupted, and the cellular organelles including cytoplasmic granules were seen in the extracellular fluid. Some of the extracellular granules were swollen and had a decrease in electron density characteristic of secreted granules; others were electron opaque and were similar in appearance to the cytoplasmic granules of intact, unstimulated mast cells. The latter were presumably released by cell disruption rather than secretion.

We have recently demonstrated that EPO, which is a strongly positive protein, binds to the negatively charged MCG matrix to form a complex (30). The peroxidase is bound largely to the surface of the granule and requires high sodium chloride concentrations (0.75 M or greater) for dissociation. Peroxidases have bactericidal and cytotoxic activity when combined with H_2O_2 and a halide. EPO retained its toxic activity after binding to mast cell granules; indeed, the MCG/EPO complex had significantly greater bactericidal (30) and cytotoxic (W. R. Henderson, E. C. Jong, E. Y. Chi, and S. J. Klebanoff. Unpublished data.) activity than did the granule-free EPO system when compared at equal guaiacol units of peroxidase activity. The MCG/EPO complex when combined with H_2O_2 and iodide also was more effective than an equivalent amount of free EPO in the stimulation of mast cell secretion (Table IV).

When mast cells were exposed to the EPO-H_2O_2-iodide system at EPO levels (100 mU) that mediated cytotoxic release of granules, peroxidase activity was detected on the surface of the extruded granules as well as on the cell surface by reaction with diaminobenzidine. Peroxidase was not detected at these sites when smaller amounts of EPO (30 mU), which induced noncytotoxic histamine release, were used. The concentration of EPO under these conditions presumably was below the threshold required for detection by our electron microscope procedure.

These findings suggest that the following sequence of events may occur at sites of inflammation characterized by an eosinophil and mast cell response. Eosinophils attracted to the site of inflammation may be activated by reaction with an appropriately opsonized particle or other stimulus to secrete EPO. A particle too large to be ingested, such as a helminth, would favor extracellular release (19); however, peroxidase also could be released when cell lysis occurs (42) or when granule rupture precedes the completion of the act of engulfment. H_2O_2 generated by activated eosinophils (or other phagocytes in the region) would be expected (23–25), and the peroxidase and H_2O_2 may combine with a halide to initiate mast cell secretion. At pH 7.4, iodide, when combined with chloride at physiological levels, was active at concentrations down to 10^{-6} M. This is above the physiological level in extracellular fluid; however, under in vivo conditions, a steady-state iodide concentration would be expected in which iodide utilized by the peroxidase system would be immediately replaced by an equivalent amount from the total body pool with the potential for the utilization of larger amounts by the peroxidase system. If the pH falls to 6.5 or 6.0, chloride at physiological concentrations is effective without iodide. A fall in pH of this magnitude may occur in an inflammatory locus. Other cells in the region or

soluble proteins would be expected to compete with mast cells for the products of the peroxidase system. The outcome of this competition would depend on the relative concentrations of the target cells or proteins, their affinity for and proximity to the peroxidase system, and the level of activity of that system. The total protein content of interstitial fluid in which mast cells and eosinophils interact is unknown but is probably much lower than the concentration found in serum (43). Under the conditions employed here, albumin at concentrations up to 2,500 μg/ml inhibited but did not abolish histamine release. LDH release by the EPO system was abolished by albumin under conditions in which histamine release remained high. Thus under these conditions, a cytotoxic system was converted to a noncytotoxic secretory system by extracellular protein. The mast cell granules released by the EPO-H_2O_2-halide system would be expected to form a complex with EPO with retention, indeed, potentiation of the secretory activity of the peroxidase on mast cells. Mast cell secretion may be influenced by neutrophils and MPO in a similar fashion although MPO does not bind as firmly to MCG as does EPO (30). Release of chemical mediators of immediate hypersensitivity and the formation of stable MCG/EPO complexes with augmented bactericidal and cytotoxic activity would be expected to influence the subsequent inflammatory response.

Summary

Eosinophil peroxidase (EPO) at relatively low levels (4–30 mU), when supplemented with H_2O_2 and a halide, induced mast cell degranulation. Histamine release occurred without concomitant release of the cytoplasmic marker lactic dehydrogenase (LDH), and this, together with ultrastructural studies, indicated a noncytotoxic effect comparable with that induced by other mast cell secretagogues. At pH 7.4, iodide was effective at concentrations down to 10^{-5} M, and although chloride alone was ineffective at 0.1 M, a combination of 0.1 M chloride and 10^{-6} iodide could meet the halide requirement. Chloride alone was effective at pH 6.5 and 6.0. EPO could be replaced by myeloperoxidase.

When the EPO level was increased to 100 mU, combination with H_2O_2- and iodide-induced cytotoxic histamine release as indicated by concomitant LDH release and ultrastructural evidence of cell disruption. This cytotoxic response reverted to a secretory one on the addition of albumin. Peroxidase was detected on the surface of extruded granules by diaminobenzidine cytochemistry. The mast cell granule (MCG)/EPO complex when supplemented with H_2O_2 and iodide was more effective than free EPO in the stimulation of mast cell secretion. The stimulation of mast cell mediator release by the EPO-H_2O_2-halide system and the formation of MCG/EPO complexes with augmented cytotoxic activity may influence the adjacent inflammatory response.

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