ULTRASTRUCTURAL CHARACTERIZATION AND MORPHOMETRIC ANALYSIS OF HUMAN EOSINOPHIL DEGRANULATION

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SUMMARY

Human eosinophil degranulation induced by the calcium ionophore A23187 was examined by transmission and scanning electron microscopy, and morphometric analysis. After incubation with A23187, eosinophil degranulation was characterized by granule movement to the cell periphery and pentalaminar membrane fusion between perigranular and plasma membranes. As adjacent granules fused they became swollen and vesiculated; their contents were released into large cytoplasmic vacuoles, which communicated extracellularly through surface pores. Extracellular release of eosinophil peroxidase without release of lactate dehydrogenase occurred after treatment with the ionophore. Morphometric analysis of the transmission electron micrographs indicated a significant reduction of cytoplasmic granules in the degranulated cells. There was a loss primarily of larger granules and alternatively an increase in the smaller-sized granules (less than 0.1 μm²), suggesting the possibility that exocytosis of mature granules is accompanied by new cytoplasmic granule formation.

INTRODUCTION

Eosinophils are normally present in small numbers in the circulation but typically accumulate in areas of immediate hypersensitivity reactions, helminthic infestations and certain neoplasms. Eosinophils, like neutrophils, when stimulated by a variety of soluble agents or appropriately opsonized particles respond with an increase in oxygen consumption, which is associated with the release of superoxide and hydrogen peroxide (H₂O₂) (Baehner & Johnston, 1971). Concomitant with the respiratory burst is the release of granule contents either into the phagosome (Cotran & Litt, 1969) or the extracellular fluid (Archer & Hirsch, 1963; McLaren, Mackenzie & Ramalho-Pinto, 1977). Eosinophils contain high concentrations of peroxidase (eosinophil peroxidase, EPO) in the matrix of their large cytoplasmic granules (Bainton & Farquhar, 1970). The EPO–H₂O₂–halide system can induce mast cell secretion (Henderson, Chi & Klebanoff, 1980), inactivate chemical mediators of immediate hypersensitivity (Henderson, Jörg & Klebanoff, 1982) and is cytotoxic to a variety of target cells (Migler, DeChatelet & Bass, 1978; Henderson, Chi, Jong & Klebanoff, 1981). In addition, basic proteins with toxic properties (Gleich,
Loegering & Maldonado, 1973) are released in high concentrations during the degranulation process. These and other enzyme systems released by stimulated eosinophils may contribute to the activity of these cells in tissues.

In comparison to other phagocytes, much less is known about the ultrastructure of eosinophil degranulation and the factors that regulate this secretory process. In this report we examine the effects of the calcium ionophore A23187 on the morphology of eosinophils obtained from the peripheral blood of normal human individuals. A23187-induced degranulation was characterized by the fusion and vesiculation of adjacent granules with the formation of large intracellular channels, which communicated with the extracellular space through surface pores. Non-cytotoxic extracellular release of EPO was found. Morphometry was used to quantitate the changes in numbers and size distribution of cytoplasmic granules occurring as a consequence of degranulation; morphometric analysis supported the ultrastructural observation that ionophore treatment may also stimulate new granule formation in eosinophils.

MATERIALS AND METHODS

Special reagents

Guaiacol (anhydrous) was obtained from Sigma Chemical Co., St Louis, MO, metrizamide from Nygaard and Co., Oslo, Norway, and the calcium ionophore A23187 from Calbiochem-Behring Corp., San Diego, CA.

Preparation of eosinophils.

Eosinophils were purified from blood obtained from normal individuals with 2–3% peripheral eosinophilia as previously described (Vadas et al. 1979). In brief, cell suspensions prepared by dextran sedimentation were layered over 23% (w/v) metrizamide and centrifuged at 1500 g for 35 min at 20°C. The eosinophil-containing cell pellets were combined, the red blood cells hypotonically lysed and the eosinophils suspended in a standard salt solution (pH 7.2) consisting of 154 mM-NaCl, 2.7 mM-KCl, 4 mM-NaH2PO4, 2.7 mM-KH2PO4, 0.9 mM-CaCl2, 0.8 mM-MgSO4 and 6 mM-glucose. The cell preparations typically contained greater than 97% eosinophils (less than 3% neutrophils) and were greater than 95% viable as measured by Trypan Blue exclusion.

Eosinophil peroxidase (EPO) and lactate dehydrogenase (LDH) release from eosinophils

Duplicate eosinophil preparations were preincubated for 5 min at 37°C in an oscillating water bath in the standard salt solution. A23187 was then added to make a final volume of 1.0 ml and the incubation continued for an additional 20 min unless otherwise indicated. The samples were then centrifuged at 400 g for 5 min at 4°C and the supernatants and pellets were stored at −70°C until EPO (Henderson et al. 1982) or LDH (Henderson et al. 1980) activity was measured as previously described.

Electron microscopic studies

Pellets of 5×10⁶ eosinophils incubated with the various components of the reaction mixture (see legends to figures) were collected by centrifugation at 400 g and prepared for transmission (Henderson et al. 1980) and scanning (Henderson et al. 1981) electron microscopy as previously described.
Human eosinophil degranulation

Morphometry

The size and distribution of membrane-bound cytoplasmic granules of eosinophils incubated in buffer in the absence or presence of the calcium ionophore A23187 were examined by morphometric analysis. Seventy five transmission electron micrograph cross-sections containing a portion of the nucleus of control and A23187-stimulated cells were studied. Morphometric granule size analysis was performed as previously described (Weibel, 1979; Hammel, Lagunoff, Bauza & Chi, 1983), with the mean axial ratio calculated as the length of the long axis divided by the length of the short axis; the short axis length was measured perpendicular to the long axis. Granule sizes were calculated using an Apple III computer.

Statistical analysis

The data are reported as the mean ±S.E. of the combined experiments. Differences were analysed for significance using Student's two-tailed t-test for independent means (not significant, P > 0.05).

RESULTS

Degranulation

Greater than 95 % of the eosinophils from normal individuals incubated for 5 min at 37°C in the standard salt solution exhibited the usual morphology by transmission and scanning electron microscopy of unstimulated cells (Fig. 1). The eosinophils were characterized by large membrane-bound cytoplasmic granules with a crystallloid core and surrounding matrix (Fig. 1A). The plasma membrane surrounding the cell contained numerous villous projections and ridges (Fig. 1A,B) and other cytoplasmic organelles such as Golgi vesicles, mitochondria and endoplasmic reticulum were not unusual.

Alterations in granule morphology were seen 1 min after incubation of eosinophils with A23187 (10 µg/ml). The central crystalloid core material of the granules disappeared and intragranular vesicular structures formed after ionophore stimulation (Fig. 2). Vesiculation of the granules was observed in greater than 80 % of the

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Fig. 1. Human eosinophils. Eosinophils were incubated in 1·0 ml of the standard salt solution (pH 7·2) for 15 min. Transmission electron microscopy (A, ×10000) revealed cells containing numerous membrane-bound, cytoplasmic granules (CG) with an electron-dense, crystalline core typical of the specific granules of human eosinophils. The heterochromatin of the nucleus had the characteristic peripheral distribution of eosinophils (A). Golgi vesicles, mitochondria and other organelles appeared normal. Transmission (A) and scanning electron microscopy (B, ×9000) revealed microvilli (MV) uniformly distributed on the surface of the cell.

Fig. 2. Ionophore-treated human eosinophils (1 min). Eosinophils were incubated with the calcium ionophore A23187 (10 µg/ml) in the standard salt solution (pH 7·2) for 1 min at 37°C (A, ×36000; B, ×67500; C, ×56000; D, ×64000). In (A), close apposition of granules is seen. Vesicular structures (V) are present in the matrix of one granule, which also shows a loss of electron density (arrow) in the crystalline core. In (B), the matrix of one granule is completely vesiculated; and in (C), the central crystalline core of a vesiculated granule is no longer evident; fusion (arrows) of the adjacent granule membranes occurs. In (D), adjacent granule matrices interconnect (arrows) and large vacuoles (V) are noted within the combined structure.
Fig. 1. For legend see p. 35.
Fig. 2. For legend see p. 35.
eosinophils 1 min after stimulation with A23187. Cytoplasmic granules that fused with adjacent granules typically showed evidence of vesiculation, whereas those that fused directly with the surface membrane (Fig. 3) did not. Intragranular vesiculation was not usually observed more than 5 min after incubation with the ionophore. Fusion occurred between adjacent perigranular membranes and secretory sacs formed, which contained the altered material from the neighbouring granules. Vacuoles were also formed within these secretory sacs. At the same time, cytoplasmic granules were noted to be in close proximity to the plasma membrane (Fig. 3).

Pentalaminar membrane fusion between perigranular and plasma membranes was observed. At 5 min after stimulation (Fig. 4) there was prominent bulging of the cell surface due to submembranous secretory sacs or vacuoles. Some of these secretory vacuoles still contained granule material (Fig. 4A). Pores were noted in the surface membrane by scanning electron microscopy (Fig. 4c). At 20 min after incubation with A23187, intracellular vacuoles fused to form large channels, some of which opened to the outside of the cell through surface pores (Fig. 5). Surface microridges were greatly reduced at this stage of secretion (Fig. 5a,b). No intact granule matrices were noted extracellularly, suggesting that eosinophil granule contents are completely solubilized in the extracellular fluid. By transmission electron microscopy, small, electron-dense cytoplasmic granules (less than 0.3 μm in diameter) with
perigranular membranes were frequently observed around the centriole and Golgi apparatus 20 min after ionophore stimulation (Fig. 6). Such small granules were rarely seen in unstimulated cells (Fig. 6). Greater than 75% of eosinophils from normal individuals exhibited these various morphological features after treatment with ionophore A23187. These morphological changes were not observed when eosinophils were incubated with ionophore in the absence of calcium in the reaction mixture (data not shown).

Non-cytotoxic degranulation was confirmed by measurement of EPO and LDH release. As shown in Fig. 7, EPO release from human eosinophils increased from 1-1 to 20-3% as the A23187 concentration was increased from 1 to 10 μg/ml, with LDH release remaining less than 5%. LDH release, however, increased to 13-2% when the A23187 concentration was increased to 20 μg/ml, indicating cytotoxic damage to the cells.
Fig. 5. Ionophore-treated human eosinophils (20 min). Eosinophils were incubated as described for Fig. 2 for 20 min. Large intracellular channels (ch) were noted by transmission electron microscopy in normal (A, ×17 000) eosinophils. A loss of surface projections was seen by both transmission (A) and scanning microscopy (B, ×5400). Surface pores (arrows) can be seen in B.

The transmission electron micrographs of human eosinophils before and after A23187 treatment were studied by morphometric analysis. A total of 712 eosinophil granules from unstimulated cells and 441 granules from ionophore-treated cells were counted. Eosinophil granules are varied in shape ranging from spheres to prolate ellipsoids (Fig. 1A). Since the mean axial ratio for control cells was 1.13 and for ionophore-treated cells was 1.09, it could be assumed by the criteria of Weibel (1979) that the granule profiles approximated spheres for determination of granule size; the percentage error incurred by this approximation of granule shape (Weibel, 1979) was less than 2% for control cells and less than 1% for ionophore-stimulated cells. The number of granules per transmission electron micrograph cross-section

Fig. 6. Cytoplasmic granule formation after ionophore stimulation. Eosinophils were incubated either in buffer alone (A, ×29 000) or in the presence of A23187 for 20 min as described for Fig. 2 (B, ×35 000). A centriole (c) and Golgi apparatus (G) are seen in the control cell (A). In (A), numerous characteristic, large (greater than 0.1 μm² granule profile size area) cytoplasmic granules (cg) are present surrounding the centriole with only an occasional small granule (less than 0.1 μm²) noted (arrow). In (B), after stimulation with A23187, there is loss of the larger size granules as secretory vacuoles (v) are formed. An increased number of granules less than 0.1 μm² (arrows) can be seen in the region of the centriole (c) and Golgi apparatus (G) in B.
Fig. 6
Fig. 7. Effect of A23187 on peroxidase release. The reaction mixture contained $5 \times 10^6$ eosinophils and the calcium ionophore A23187 at the concentrations indicated, in 1.0 ml of the standard salt solution. Incubation was for 20 min at 37°C. EPO (●●●) and LDH (○○○) release above background (1.2 ± 0.4% for EPO; 1.9 ± 0.8% for LDH) is shown. The results are the mean ± S.E. of three experiments.

was reduced 44.2% in the A23187-treated eosinophils (18.7 granules/cross-section) compared to control cells (33.5 granules/cross-section; $P < 0.05$). The size distribution of eosinophil granule profile areas indicated that fewer granules from 0.1–0.5 $\mu$m$^2$ in size were found in secreted cells compared to control eosinophils whereas more granules from 0.01–0.1 $\mu$m$^2$ in size were seen in the ionophore-treated cells (Fig. 8). When the percentage distribution of granule profile areas was determined (Fig. 9), 42.8% of the granules in the ionophore-stimulated cells were in the 0.01–0.1 $\mu$m$^2$ size range compared to only 19.1% in the control cells with a preponderance of granules from the secreted cells found in the smallest size range (0.02–0.03 $\mu$m$^2$ size range, data not shown).

DISCUSSION

Our studies indicate that normal human eosinophils undergo non-cytotoxic degranulation after incubation with the calcium ionophore A23187 as determined both biochemically by the release of EPO without the release of the cytoplasmic marker, LDH, and morphologically by transmission and scanning electron microscopy. The calcium ionophore A23187 initiates degranulation of a variety of secretory cells by transporting extracellular calcium across the surface membrane or by mobilization of intracellular calcium stores (Foreman, Mongar & Gomperts, 1973). In mast cells,
Fig. 8. Size distribution of eosinophil granule profile areas. The number of granules from control cells (A) and cells stimulated with A23187 as described for Fig. 5 (B) for each 0.05 μm² size range from 0 to 1.0 μm² is shown. The horizontal axis shows the upper limit of the size range for each group. A total of 712 granules from control cells (29 cross-sections analysed) and 441 granules from A23187-treated cells (46 cross-sections analysed) were measured.
for example, the ionophore induces morphological changes of degranulation similar to those induced by antigen; biochemically, ionophore stimulation resembles the later stages of antigen-induced histamine release (Foreman et al. 1973; Cochrane & Douglas, 1974; Lichtenstein, 1974). We have recently described the morphological and chemical changes induced by the calcium ionophore A23187 in horse eosinophils (Henderson, Chi, Jörg & Klebanoff, 1983), and have also shown that ionophore-stimulated horse and human eosinophils are a potent source of leukotrienes (LT) B₄, C₄ and D₄, which are 5-lipoxygenase products of arachidonic acid metabolism (Jörg, Henderson, Murphy & Klebanoff, 1982; Henderson, Harley & Fauci, 1984).

In the present study, characteristic ultrastructural features seen after ionophore stimulation of human eosinophils were the movement of granules to the periphery of the cytoplasm, with fusion of perigranular membranes with one another and with the plasma membrane. As granules fused, their crystalline cores appeared to dissolve and their matrices became vesiculated. Vesiculation occurred within the matrix of centrally located cytoplasmic granules 1–5 min after ionophore stimulation. Intragranular vesiculation may be an important step in the formation of intracellular vacuoles and channels into which granule components are fully solubilized before extracellular release. Although it is possible that vesiculation of granules is an artifact of ionophore treatment, similar granule matrix vesiculotubular structures have been noted previously in eosinophils undergoing degranulation. Peripheral
Human eosinophil degranulation

blood eosinophils from a patient with eosinophilic granuloma demonstrated intra-granular vesicular tubular structures after incubation with immune complexes (Okuda, Takenaka, Kawabori & Ogami, 1981). Okuda et al. (1981) also reported the same tubular intragranular changes in tissue eosinophils present in nasal mucosa obtained from allergic individuals. These intragranular vesicular structures have also been observed in tissue eosinophils of patients with Hodgkin’s disease (Parmley & Spicer, 1974), in guinea-pig eosinophils infiltrating the small intestinal lamina propria in response to infection with the nematode Trichostrongylus columbriformis (Huxtable & Rothwell, 1975), in rat eosinophils after intraperitoneal injection of foetal calf serum (Komiyama & Spicer, 1975), and in guinea-pig eosinophils disrupted by the surface-active agent, Aerosol-OT (El-Hashimi, 1971).

One factor that may affect vesiculation of the granule matrix is the presence of an intact crystalloid core. Horse eosinophils do not contain a central crystalloid body and the formation of intragranule vesiculotubular structures is not a prominent feature of their degranulation induced by A23187 (Henderson et al. 1983). Intragranular vesiculation was also not observed in human bone marrow eosinophils that showed in vivo evidence of degranulation (Skinnider & Ghadially, 1974). These eosinophils obtained from a patient with chronic active hepatitis and several episodes of disseminated intravascular coagulation were similar to horse eosinophils in the virtual absence of crystalline core granular material. Similarly, primary granules that lack a crystalline core in bone marrow eosinophil promyelocytes from patients with chronic myelogenous leukaemia, adenocarcinoma and idiopathic thrombocytopenic purpura do not exhibit vesiculation changes when their perigranular membranes fuse with those of adjacent granules and with the surface membrane during secretion (Hyman et al. 1978).

The nature of the secretory stimulus may also be an important factor in the formation of vesiculotubular granule structures. Phagocytosis of zymosan particles induces different secretory changes in human eosinophils than does stimulation with the soluble secretagogue A23187. After ingestion of zymosan particles intracellularly, eosinophil granule contents are discharged individually and directly onto the zymosan surface to form a phagolysosome (Henderson & Chi, 1985). Intragranular vesiculation is not observed. Fusion and swelling of other cytoplasmic granules and granule movement to the periphery of the cell with bulging of the cell membrane also do not occur after phagocytosis of zymosan particles. Similar ultrastructural features of fusion of the granule membrane with the phagocytic vacuole membrane and release of granule contents into the intracellular vacuole have been described in rabbit (Zucker-Franklin & Hirsch, 1964) and guinea-pig (Cotran & Litt, 1969) eosinophils ingesting zymosan particles, and human eosinophils phagocytosing Candida organisms (Ishikawa, Yu & Arbesman, 1972); intragranular vesiculation was not described in these studies.

Dvorak et al. (1981) have shown that antigen or concavalin A-induced degranulation of guinea-pig basophils is characterized by the formation of cytoplasmic vesicles, which fuse with the membranes of secretory granules to enable the fusion of neighbouring granules and formation of degranulation sacs. These vesicles are
thought to be generated from plasma membrane as a consequence of basophil activation (Dvorak et al. 1981). It is not clear in human eosinophil degranulation whether vesiculation of the granule matrix arises from the perigranular membrane or from plasma membrane vesicles. We did not observe vesicle formation arising from the plasma membrane in the stimulated eosinophils, though we cannot exclude this possibility. Eosinophil intragranular vesiculotubular structures may possibly arise from lipids of the perigranular membranes, and promote intergranular fusion and vacuolation.

Other prominent changes in A23187-induced human eosinophil degranulation were bulging of the surface membrane by underlying vacuoles that communicated with the extracellular fluid through surface pores, and a marked reduction in surface membrane projections. Surface bulging is most probably the result of swelling of peripheral granules caused by the entrance of extracellular fluid through surface pores into the granule matrix. Alternatively, some surface vacuoles may represent peripheral movement of degranulation sacs formed initially in more central regions of the cells by vesiculation, vacuolation and fusion of adjacent granules.

In summary, A23187-induced human eosinophil degranulation exhibits many ultrastructural features in common with those observed in horse eosinophils stimulated with A23187 (Henderson et al. 1983), and in rat and human mast cells stimulated either immunologically or non-immunologically (Chi, Lagunoff & Koehler, 1976; Lawson et al. 1979; Henderson et al. 1980; Chi, Henderson & Klebanoff, 1982; Caulfield, Lewis, Hein & Austen, 1980). Similar changes seen in both eosinophils and mast cells include fusion of the membranes of adjacent cytoplasmic granules, swelling of granules and formation of intracellular vacuoles and channels. Pores in the surface membrane that communicate with the vacuoles and channels enable extracellular release of granule contents.

Morphometric analysis confirmed the striking changes in the number and size distribution of eosinophil granules that occurred with degranulation. Control cells contained nearly twice as many granules as found in cells treated with ionophore for 20 min. The size distribution of the granule profile areas indicated that there was a loss of larger size granules (greater than 0.1 \( \mu \text{m}^2 \)) in the A23187-treated cells compared to control eosinophils, suggesting that secretion involves release of larger and presumably more mature cytoplasmic granules. Alternatively, the degranulated cells contained more granules in the smaller size range (0.01–0.5 \( \mu \text{m}^2 \)) both in absolute numbers (Fig. 8) and as the percentage of the distribution of total granules (Fig. 9) compared to unstimulated eosinophils. It is possible that the smaller granules are obscured in the plane of electron microscope sections by the larger granules in unstimulated cells and thus the small granules are not increased in numbers in degranulated cells but are only more readily counted. Alternatively, the increase in small-sized granules may reflect formation of newly synthesized, immature granules as a consequence of the secretory process. Although eosinophils have only a small ribosome content, there is evidence for new granule formation after secretion in both basophils and mast cells. New cytoplasmic granule formation has been observed in guinea-pig basophils after anaphylactic degranulation (Dvorak
Human eosinophil degranulation

et al. 1982). Mature mast cells also regranulate following degranulation as shown by their synthesis of new secretory material and formation of mature cytoplasmic granules (Ginsburg et al. 1978; Kruger & Lagunoff, 1981; Burwen, 1982). Further studies will be required to determine whether a similar process of regranulation following degranulation occurs in human eosinophils.

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