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Mechanisms for eosinophil degranulation; release of the eosinophil cationic protein

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Summary. Mechanisms for degranulation in human eosinophils were evaluated. Release of eosinophil cationic protein (ECP), a unique eosinophil granule constituent, was measured upon exposure of purified eosinophils to a large surface consisting of Sephadex beads coated with serum, which leads to complement activation. Extracellular release of approximately 15% of the cellular ECP occurred both with eosinophils from patients with eosinophilia and normal people. Almost all eosinophils isolated from patients with eosinophilia and normal people adhered to serum-treated Sephadex. The data suggest that interaction through C3 receptors is a prerequisite for ECP release from eosinophils when exposed to serum-treated Sephadex. Both cytochalasin B, cytochalasin D and hydrocortisone reduced the release of ECP. Neither the cytochalasins nor hydrocortisone inhibited the adherence of eosinophils to the Sephadex beads. Thus the inhibitory effect of these agents on ECP release is a direct effect on the degranulation process. ECF-A, histamine and colchicine did not affect the release mechanism. No direct relationship was found between degranulation and oxidative burst inasmuch as some soluble mediators induced a high respiratory burst without a concomitant ECP release. Our data suggest that mechanisms for degranulation are not fully identical in eosinophils and neutrophils.

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INTRODUCTION

The eosinophil leucocyte is identified by its crystalloid-containing cytoplasmic granules. Unique components of the granules are proteins such as the major basic protein (MBP; Gleich, Loegering & Maldonado, 1973; Gleich *et al.*, 1974, 1976) and the eosinophil cationic protein (ECP; Olsson *et al.*, 1977), which both show toxicity for schistosomules of *Schistosoma mansoni* (Butterworth *et al.*, 1979; McLaren *et al.*, 1981) and for a variety of mammalian cells (Frigas, Loegering & Gleich, 1980; Fredens, Dahl & Venge, 1982). The reasons for the occurrence of eosinophilia during anaphylactic reactions (Beeson & Bass, 1977) and parasite diseases (Kay, 1979) are not clear. It is possible that studies of unique granule constituents such as MBP and ECP and studies of mechanisms for degranulation may lead to an understanding of the biological function of the eosinophil. We already know that ECP is actually released *in vivo* as judged by studies of serum-ECP in some allergic diseases (Dahl, Venge & Olsson, 1978; Winqvist *et al.*, 1981) and hypereosinophilic syndromes (Spry, 1980).

The present work was done to study mechanisms for degranulation in eosinophils using the release of ECP as a specific marker for that process. Release was induced by exposure of purified eosinophils to a large surface consisting of Sephadex beads coated with serum. Eosinophils were isolated from both healthy individuals and patients with eosinophilia. The results show that the mechanism for release depends on

interaction of the eosinophil with a large surface coated with activated complement. A respiratory burst elicited with certain soluble factors occurred without concomitant degranulation. Pharmacologic modulation indicated somewhat different mechanisms for degranulation in eosinophils and neutrophils.

MATERIALS AND METHODS

Chemicals

Hanks's balanced salt solution (HBSS) was from Flow Laboratories, England. Cytochalasin B and cytochalasin D from Calbiochem-Behring Corp., La Jolla, CA, were dissolved in dimethyl sulphoxide (DMSO) and diluted for use in HBSS to a final maximal DMSO concentration of 0·5%. Calcium ionophore A23187 was from Calbiochem-Behring Corp., La Jolla CA, issued by Eli Lilly Corp. The following chemicals were obtained from Sigma Chemical Corp., St Louis, MO: hydrocortisone-21-sodium succinate, colchicine, histamine, phorbol-12-myristate-13-acetate (PMA), zymosan A, concanavalin A. The synthetic eosinophil chemotactic factor of anaphylaxis (ECF-A,L-Val-Gly-Ser-Glu) was a gift from Dr Edward Goetzl, Robert B. Brigham Hospital, Boston, MA. Sephadex G-15, Dextran 250, Percoll (polyvinylpyrrolidone-coated silica gel) and concanavalin A Sepharose B (Con A Sepharose) were from Pharmacia Fine Chemicals, Uppsala, Sweden. Trypsin was from Kabi, Stockholm, Sweden. Cetyltrimethyl ammoniumbromide (CTAB) was from BDH, Poole, U.K. Polypropylene tubes were from Falcon Plastics, Los Angeles, CA. Human serum was stored at −80° until used unless otherwise stated.

Isolation of eosinophils by density gradient centrifugation

Heparinized blood was collected from healthy volunteers with < 300 eosinophils per μl blood and from volunteers with eosinophilia (filariasis, anchylostomiasis, primary hepatocellular carcinoma, eosinophil gastroenteritis, lymphoma).

Five parts of blood were mixed with one part of 6% Dextran 250 in 0·15 M NaCl and left at room temperature 45 min for red cell sedimentation. The dextran-plasma was collected and centrifuged at 450 ***g*** for 5 min and the cells obtained were washed twice in HBSS.

A density gradient was made using a modification of Gärtner (Gärtner, 1980; Winqvist *et al.*, 1982). Percoll and HBSS were mixed to obtain solutions with different densities, pH 7·4. It was important to adjust the osmolarity carefully to 275 mosm/l, otherwise no separation was achieved. Gradients were formed using a peristaltic pump (Minipuls II, Gilson, France) and consisted of Percoll solutions with the following densities: 1·110 (1 ml), 1·095 (3 ml), 1·090 (3 ml), and 1·085 (3 ml), layered on top of each other in 17 × 100 mm polypropylene tubes. Two millilitres of cells (25×10^6/ml) suspended in Percoll, 1·075 g/ml, were layered on each gradient. The tubes were centrifuged in an angle rotor (angle 45°) at 1600 ***g*** for 20 min at room temperature. One-millilitre fractions were collected from the bottom of the tubes using the peristaltic pump. The density of each fraction was measured, the cells were washed in HBSS and counted. Cytocentrifuge smears were prepared for differential counts. The cell suspensions obtained contained 64–98% eosinophils.

Induction of eosinophil degranulation

Serum-treated Sephadex beads. One-tenth of a millilitre of Sephadex beads ($0{\cdot}3 \times 10^6$/ml) suspended in HBSS was preincubated at 37° for 10 min with 0·1 ml human serum and an aliquot of 0·1 ml eosinophils ($0{\cdot}3 \times 10^6$) was added. After incubation at 37° the reaction was terminated by centrifugation for 1·5 min in a Beckman Microphuge (Beckman Instruments, Palo Alto, CA) at 11,600 r.p.m. (8370 ***g***) at room temperature. The supernatant was removed and mixed with CTAB to a final concentration of 0·3%. The cell pellet was extracted in 0·3% CTAB, centrifuged at 8370 ***g*** and the resulting supernatant was used for ECP assay. Samples were stored frozen until assayed. Also for experiments described below the reaction was terminated in this way.

In some experiments the eosinophils were incubated at 37° with cytochalasin B or D (10 μg/ml) for 15 min, with hydrocortisone (10^{-5} M) for 30 min, with colchicine (10^{-5} M) for 30 min, with histamine (10^{-5} M) for 30 min or ECF-A (10 μg/ml) for 40 min before addition of Sephadex beads.

Zymosan-treated serum (ZTS) was prepared by suspending zymosan (5 mg/ml) in 50% serum followed by incubation at 37° for 15 min. After centrifugation, 0·2 ml of the supernatant (ZTS) was immediately mixed with 0·1 ml eosinophil suspension ($0{\cdot}3 \times 10^6$) at 37°.

Concanavalin A Sepharose was washed four times and suspended in HBSS. The suspension, 0·1 ml, was mixed with 0·1 ml serum and 0·1 ml cells ($0{\cdot}3 \times 10^6$ eosinophils) at 37°. The final Con A Sepharose concentration was 100 μg/ml Con A. In some experiments, soluble Con A diluted in HBSS was used.

Immune complexes. Antibodies against human serum albumin (HSA; Kabi, Stockholm, Sweden) were raised in rabbits. Unfractionated antiserum was mixed with HSA (1 mg/ml) in ratios tested to result in optimal immune complex formation and left at +4° overnight. The precipitate was washed three times in saline and the protein concentration was determined according to Lowry *et al.* (1959). The immune complexes were resuspended to a concentration of approximately 3 mg/ml protein. One-tenth of a millilitre of this suspension, diluted to 0·75 mg/ml, and 0·1 ml serum or 0·1 ml HBSS was after pre-incubation at 37° for 10 min mixed with the cells.

Induction of neutrophil degranulation

Neutrophils (purity >90%) were obtained from the same density gradient as the eosinophils. They were suspended in HBSS, 3×10^6/ml, and 0·1 ml of the suspension was mixed with serum-treated Sephadex as described above for eosinophil degranulation.

Assay for eosinophil cationic protein (ECP)

ECP was measured by a radioimmunosorbent assay (Venge, Roxin & Olsson, 1977). The range of the standard curve was 1·95–500 ng/ml. The supernates were diluted 10–25 times.

Assay for myeloperoxidase (MPO) and lactoferrin

MPO and lactoferrin were determined with immunoradiometric assay (Olsson *et al.*, 1979). The range of the standard curves was 5–60 ng/ml. The supernates were diluted 15 times.

Oxygen consumption

Sephadex beads (0·8 ml; $0{\cdot}3 \times 10^6$/ml HBSS) were incubated with 0·7 ml serum at 37° for 10 min and mixed with 1 ml of a cell suspension to a final concentration of 4×10^6 eosinophils/ml. Thereafter oxygen consumption was measured with a Clark electrode mounted in a 2·24-ml glass-stoppered incubation chamber equipped with magnetic stirring and heating to 37° (Eschweiler & Co, Kiel, Germany; Winqvist *et al.*, 1982).

In some experiments eosinophils were incubated at 37° with cytochalasin B (10 μg/ml) for 15 min or ECF-A (10 μg/ml) for 40 min before addition of serum treated Sephadex.

Oxygen consumption of eosinophils was measured also after addition of ZTS or PMA (100 ng/ml).

Adherence assay

One millilitre of Sephadex G-15 was incubated with normal serum for 10 min at 37° and packed in a 2-ml syringe. Aliquots of 0·4 ml eosinophils were added to the columns, which were incubated for 15 min at 37°. The effluents were then collected and eosinophils counted. To study the effect on adherence eosinophils were preincubated with hydrocortisone (10^{-5} M for 30 min) and cytochalasin B (10 μg/ml for 15 min).

Trypsinization of cells

The eosinophils, 3×10^6/ml, were incubated with 0·1% trypsin for 30 min at 37° and washed with HBSS.

RESULTS

Exposure of eosinophils to Sephadex beads coated with serum resulted in extracellular release of approximately 15% of the cellular content of ECP (Fig. 1). No difference was found in degranulation between eosinophils isolated from patients with eosinophilia as

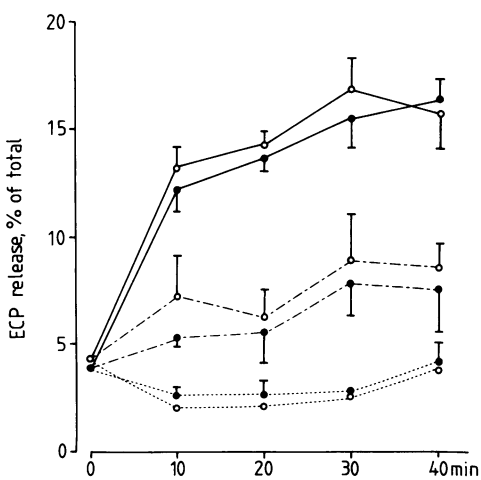

Figure 1. Release of ECP % (±SEM) of total ECP content, with serum-treated Sephadex from eosinophils of healthy individuals (●———●) and eosinophils of patients with eosinophila (○———○). ECP release is also shown after pretreatment of the cells with cytochalasin B, 10 μg/ml (— - — - —), or cytochalasin D, 10 μg/ml (- - - - - - - - - - -)

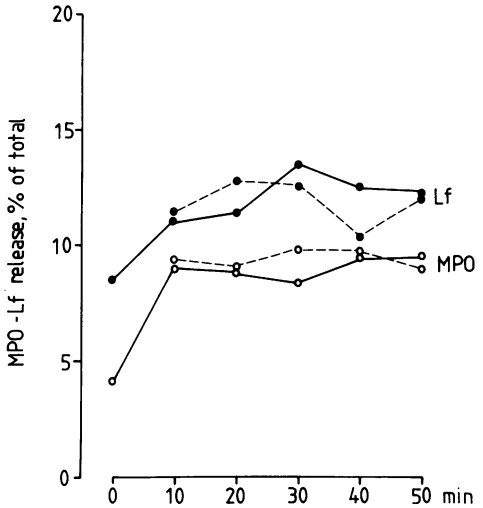

Figure 2. Release (% of total content) of myeloperoxidase (MPO) and lactoferrin (Lf) from neutrophils with serum-treated Sephadex (———). Release from cells preincubated with cytochalasin B, 10 μg/ml, is also shown (— — — — —).

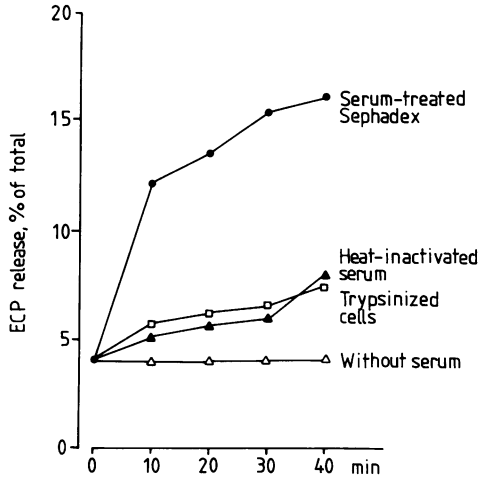

Figure 3. Release of ECP, % of total, with serum-treated Sephadex (●———●), Sephadex treated with heat-inactivated (56°) serum (▲———▲), untreated Sephadex (△———△) and with serum-treated Sephadex after trypsinization (0·1%, 30 min) of the cells (□———□).

compared to eosinophils from normal individuals. Cytochalasin B (10 μg/ml), and cytochalasin D (10 μg/ml) both inhibited the ECP-release, cytochalasin D being the most potent inhibitor.

For comparison, neutrophil release of a primary granule constituent (myeloperoxidase) and a secondary granule constituent (lactoferrin) were measured upon exposure of neutrophils to serum-coated Sephadex beads (Fig. 2). The relative release of myeloperoxidase and lactoferrin was lower than the relative ECP release from eosinophils and, contrary to ECP release, it was not inhibited by cytochalasin B.

ECP release was reduced when eosinophils were exposed to Sephadex beads pretreated with heat-inactivated serum instead of fresh serum (Fig. 3). Untreated Sephadex did not induce degranulation at all. Furthermore, trypsinized eosinophils showed a decrease in ECP-release during incubation with serum treated Sephadex. These results suggest that interaction of eosinophils with serum-treated Sephadex through C3-receptors is a prerequisite for degranulation since C3-receptors on the cell surface are destroyed by trypsin (Tai & Spry, 1980).

Hydrocortisone reduced the Sephadex-stimulated ECP release (Fig. 4). Other pharmacological agents tested, such as colchicine, ECF-A and histamine, did not seem to affect the ECP release (data not shown). Immune complexes consisting of albumin–anti-albumin also induced release of ECP from eosinophils, provided that fresh serum was present (Fig. 5). Soluble factors such as zymosan-treated serum (ZTS), Con A and PMA did not by themselves induce ECP release

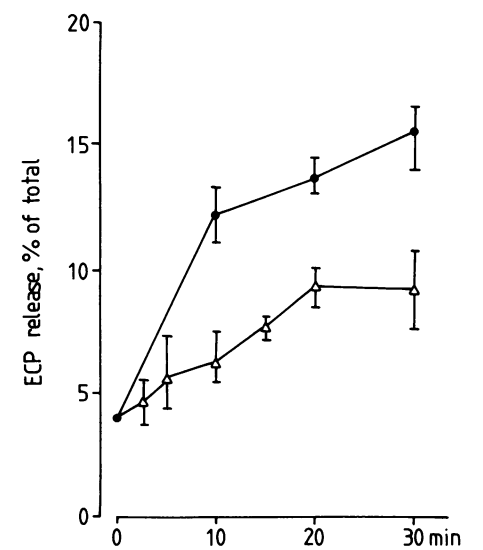

Figure 4. Effect of preincubation of eosinophils with hydrocortisone (10^{-5} M, 30 min) on release of ECP, % (±SEM) of total, with serum-treated Sephadex (△———△). Untreated cells (●———●).

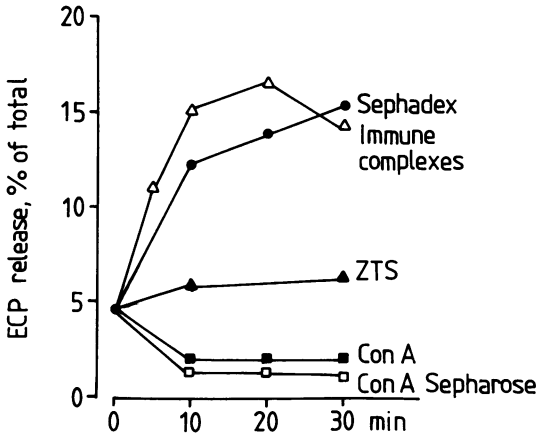

Figure 5. Release of ECP, % of total from eosinophils incubated with: (●———●) serum-treated Sephadex; (△———△) immune complexes (albumin–anti-albumin); (▲———▲) zymosan-treated serum (ZTS); (■———■) Con A, 100 μg/ml; (□———□) Con A Sepharose, 100 μg/ml.

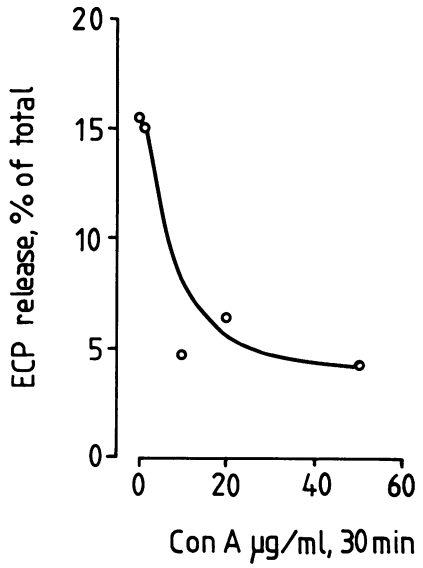

Figure 6. Effect of preincubation of eosinophils with Con A (0·1–50 μg/ml, 30 min) on release of ECP, % of total, upon incubation with serum-treated Sephadex.

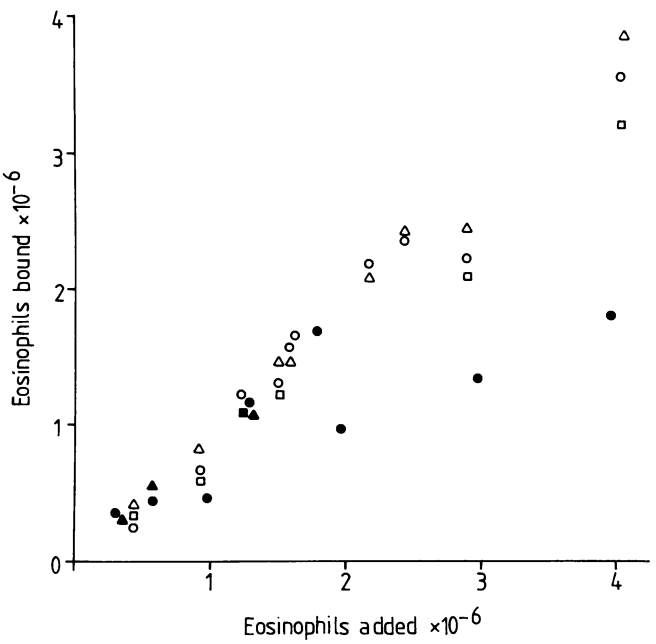

Figure 7. Adherence of eosinophils to serum-treated Sephadex. The number of eosinophils added to the Sephadex was varied and the number of eosinophils bound was calculated. Eosinophils obtained from four patients with eosinophilia (○) and three healthy individuals (●) were studied. Results are also presented for eosinophils pretreated with cytochalasin B (△,▲) and hydrocortisone (□,■); *open symbols* represent eosinophils from patients with eosinophilia and *closed symbols* represent eosinophils from normal people.

(Fig. 5); nor did Con A Sepharose. Con A, however, reduced the Sephadex-stimulated release of ECP (Fig. 6).

Almost all the eosinophils isolated from patients with eosinophilia and normal people adhered to serum-treated Sephadex (Fig. 7). Neither cytochalasin B nor hydrocortisone inhibited the adherence of the cells.

No correlation was found between the respiratory burst measured as oxygen consumption and the ECP release. In particular ZTS and PMA, which both elicited a marked respiratory burst (Fig. 8), did not induce degranulation. Actually, incubation of eosinophils with opsonized Sephadex produced only a slight increase in oxygen consumption. Pretreatment of the cells with ECF-A increased and cytochalasin B decreased the Sephadex stimulated respiratroy burst.

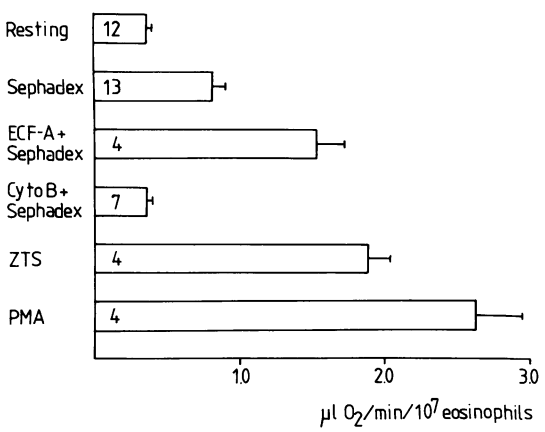

Figure 8. Oxygen consumption, $\mu l\ O_2/min/10^7$ eosinophils, in resting eosinophils as well as after incubation with serum-treated Sephadex, ECF-A plus serum-treated Sephadex, cytochalasin B plus serum-treated Sephadex, zymosan-treated serum (ZTS) or PMA (100 ng/ml). The number of experiments is indicated in the figure. *Bars* indicate SEM.

DISCUSSION

The discovery of ECP (Olsson *et al.*, 1977) has provided a tool for studies of release of a specific eosinophil constituent as a result of degranulation. Furthermore, development of methods for purification of eosinophils has made it possible to study eosinophils from healthy individuals. The present study showed that eosinophils release ECP when exposed to a large surface (Sephadex) coated with activated complement (Metcalfe *et al.*, 1977). Adherence of eosinophils to serum-treated Sepharose also results in release of arylsulphatase (Metcalfe *et al.*, 1977). Phagocytosis of opsonized zymosan by eosinophils results in release of histaminase, arylsuphatase and β-glucuronidase (Zeiger & Colten, 1977). Our results suggest some differences in mechanisms for induction of degranulation in eosinophils and neutrophils. Despite the fact that eosinophils exhibit fewer receptors for C3b than neutrophils (Anwar & Kay, 1978), the former cell shows higher granule protein release on opsonized Sephadex than the latter.

Degranulation of neutrophils can be induced by a number of mechanisms such as lysis, regurgitation during phagocytosis, reversed endocytosis and soluble factor-induced secretion (Goldstein & Weissman, 1979). Of these mechanisms, reversed endocytosis may be the most important for eosinophils; serum-coated Sephadex used in the present study provides an opsonized surface too large for ingestion and induces extracellular release of granule constituents. An identical mechanism may operate on the surface of opsonized parasites leading to eosinophil degranulation and parasite killing.

Neutrophils are known to respond to soluble C5a with extracellular secretion from azurophil and specific granules provided that the cell has been treated with cytochalasin B (Goldstein & Weissman, 1979). Contrary to this, eosinophil degranulation on exposure to serum-treated Sephadex was reduced both by cytochalasin B and cytochalasin D. Zymosan-induced histaminase release from eosinophils, but not from neutrophils, was also reduced in the presence of cytochalasin B (Zeiger & Colten, 1977). Cytochalasin B interferes with the function of cytoplasmic microfilaments and also inhibits hexose transport (Zigmond & Hirsch, 1973) but cytochalasin D only affects microfilaments (Miranda *et al.*, 1974a, b).

Cytochalasin B reversibly inhibited binding of eosinophils to IgG antibody-coated red cells but not to red cells coated with C3b (Tai & Spry, 1980). Therefore treatment of eosinophils with cytochalasins was not expected to interfere with binding to serum-treated Sephadex. Actually, we found no inhibition of eosinophil adherence upon treatment with cytochalasins. These agents seem to inhibit directly the degranulation process in eosinophils. Also hydrocortisone reduced the ECP release without interfering with adherence of the eosinophils. Oral prednisone administration has been shown to reduce whole blood eosinophil adherence but isolated eosinophils had normal adherence both after oral prednisone administration and after

addition of hydrocortisone *in vivo* (Altman *et al.*, 1981).

Con A and PMA provoke secretion from neutrophil-specific granules (Goldstein & Weissman, 1979) but these agents did not provoke ECP-release from eosinophils. It was, however, reported that Con A gives rise to perinuclear vacuoles containing granule contents in eosinophils but without release to the exterior (Tai & Spry, 1981). Ultrastructural changes were most marked in granule crystalloid material. In the light of our own results on inhibition of ECP release with cytochalasins, it is of interest that cytochalsin B was found to inhibit the effects of Con A-induced vacuolization (Tai & Spry, 1981). On the other hand, we found that pretreatment of the cells with Con A reduce the Sephadex-stimulated ECP release. This effect may depend on either inhibition of adherence or degranulation.

Eosinophil chemotactic factor of anaphylaxis (ECF-A) has been reported to enhance the expression of human eosinophil receptors for C3b (Anwar & Kay, 1978) and to initiate release of arylsulphatase (Wasserman, Goetzl & Austen, 1975). We did not find a similar effect for release of ECP, which may depend on different compartmentalization of arylsulphatase and ECP. We, however, used an ECF-A tetrapeptide and Wasserman utilized a crude Sephadex G-25 preparation.

We have clear evidence that ECP can be released *in vivo* detected as an increased level of serum-ECP. This was found for example during antigenic challenge in certain allergic diseases (Dahl *et al.*, 1978; Winqvist *et al.*, 1981). It is not clear if increased serum-ECP is the result of release from circulating eosinophils or eosinophils activated in the tissues. Circulating immune complexes could induce some degranulation which is supported by the present finding of ECP release upon interaction between eosinophils and immune complexes. It is, however, more likely that ECP is released in the tissues upon exposure to surfaces coated with immunoglobulin and complement with the escape of some ECP to the circulation.

Eosinophils from patients with eosinophilia have been reported to have a decrease of cell surface charge, activation of acid phosphatase and an increase in membrane hexose transport when compared with normal eosinophils (Bass *et al.*, 1980). We found, however, no difference in ECP release between such eosinophils and eosinophils from healthy individuals.

The finding of release of only 15% of total ECP content was not a result of adherence and degranulation of only a fraction of eosinophils since almost all eosinophils added adhered to serum-treated Sephadex.

No direct relationship was found between degranulation and oxidative burst inasmuch as some soluble mediators induced a high respiratory burst in eosinophils without a concomitant ECP release. Studies with non-phagocytic stimuli indicate that these two phenomena can occur independently in neutrophils too (Goldstein & Weissman, 1979). It is most evident with C5a, which in the absence of cytochalasin B stimulates neutrophils to generate superoxide without provoking enzyme release (Goldstein & Weissman, 1979). Contrary to findings with neutrophils (Goldstein & Weissman, 1979), pretreatment of eosinophils with cytochalasin B decreased Sephadex-stimulated enhancement of the oxidative metabolism. It is emphasized that this effect is not caused by inhibition of eosinophil adherence since that process was unaffected by cytochalasin.

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