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Extracellular release of peroxidase from eosinophils by interaction with immune complexes

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SUMMARY

Much peroxidase is released from eosinophils that ingest complexes formed of human immunoglobulins with specific rabbit antibody. The complex formed of IgE with rabbit antibody, was particularly effective. The amount and rate of release of peroxidase was closely related to the amounts of complex ingested by the eosinophils, and degree of lysis of the cell granules. It is proposed that eosinophils attracted to an allergic lesion ingest complexes of IgE, show lysis of granules with release of peroxidase, and that the peroxidase reduces the allergic reaction.

INTRODUCTION

Despite numerous investigations the specific function of the eosinophils is not yet well understood. Recent reports indicate that the enzymes in their specific granules may play an important role in anti-allergy such as inhibition of histamine release from mast cell and basophil by prostaglandin E1 and E2 (Hubscher, 1975) or inactivation of SRS-A by arylsulphatase (Wasserman, Goetzl & Austen, 1975). Peroxidase, a major enzyme of the specific granule, is also supposed to have a close relation to the function of the eosinophil (Okuda, Takenaka & Ogami, 1973) and is known to be released *in vitro* from these cells following granulolysis (Archer, 1962). The present study describes peroxidase release from eosinophils following the ingestion of immune complexes, especially human IgE-rabbit anti-IgE complex, by chemical determination and electron microscopy.

MATERIALS AND METHODS

Collection of eosinophils. Eosinophils were harvested by the method of Day (1970) from the peripheral blood of patients with eosinophilic granuloma of soft tissue, an atopic disease (Takenaka *et al.*, 1976). The leucocytes were 11400/mm^3 in number and composed of eosinophils 45%, neutrophils 20%, basophils 1%, lymphocytes 31% and monocytes 3%. From 50 ml of heparinized peripheral blood 10^8 leucocytes, were obtained.

Preparation of immune complexes. Gammaglobulin fraction was initially separated from a mixed serum of normal individuals by using ammonium sulfate precipitation and was used to prepare IgG- or IgA-immune complexes. This mixed serum contained 1420 mg IgG/100 ml; 260 mg IgA/100 ml; 95 mg IgM/100 ml and 4·1 g albumin/100 ml. IgG fraction was purified from the gammaglobulin by diethylaminoethyl (DEAE) cellulose column chromatography and was then mixed with an appropriate amount of commercial anti-IgG rabbit serum (Behringwerke) with antibody titre of 1·23 mg/ml. The gammaglobulin fraction, without further purification but after heat inactivation, was also mixed with an appropriate amount of a commercial anti-IgA rabbit serum (Behringwerke) with antibody titre of 0·84 mg/ml.

IgM-rich gammaglobulin fraction was obtained from a patient with macroglobulinemia Waldenström using the ammonium sulphate precipitation method and was then mixed with an appropriate amount of anti-IgM rabbit serum (Behringwerke) after heat inactivation. The antibody titre of anti-IgM used was 3·5 mg/ml.

In order to prepare the IgE immune complex, a pooled serum from the patient with eosinophilic granuloma of soft tissue, containing 70,000 u/ml of IgE, was mixed with the equal volume of commercial anti-IgE rabbit serum (Behringwerke) with antibody titre of approximately 400,000 u/ml. One definite precipitation band was formed between the serum of the above

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patient and each of the following three preparations of anti-IgE sera in the double diffusion test: the commercial anti-IgE (Behringwerke), anti-serum purified by affinity chromatography and provided by Drs Fujita & Ishikawa, and ^{125}I-labelled anti-serum attached Phadebas RAST kit (Pharmacia, Sweden).

Additionally, C3- and albumin-immune complexes were prepared. A fresh human serum was mixed with the equal volume of anti-C3 rabbit serum (Behringwerke) with antibody titre of 1·2 mg/ml. The serum, removed gammaglobulin fraction as described above, was mixed with an appropriate volume of commercial anti-albumin rabbit serum (Behringwerke) with antibody titre of 1·25 mg/ml.

The above mixtures of antigen-antiserum including IgG, IgA, IgE, IgM, C3 and albumin were kept for 24 hr at 4°C and centrifuged at 4°C, 6500 *g* for 30 min. Each resulting precipitate was washed in cold phosphate-buffered saline (PBS) of 0·015 mol, pH 7·4 and was disrupted to form a fine suspension by repeated vigorous pipetting. Two milligrams of total protein of each product, that is of each immune complex, was resuspended in 2·0 ml PBS, 0·015 mol, pH 7·4 for use in the eosinophil incubation tests.

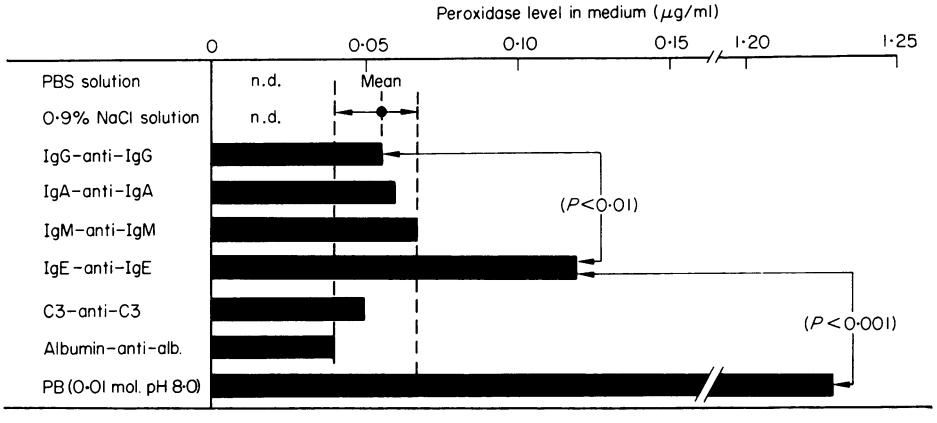

FIG. 1. Different peroxidase concentrations released in different incubation media. The concentration is highest in incubation with IgE immune complex of six different kinds of immune complexes. The differences of peroxidase between IgE-anti-IgE and the other classes of immune complex were statistically significant ($P<0·01$) according to the χ^2-test. n.d. = Not detectable.

Eosinophil incubation. 7×10^6 leucocytes preparations were incubated in 2·0 ml PBS of 0·015 mol, pH 7·4, saline, phvsphate buffer (PB) of 0·01 mol, pH 8·0 and six kinds of immune complexes for 1 hr at 37°C, shaking gently, and centrifuged at 300 *g* for 10 min. The supernatant obtained was used for determining peroxidase amount and the precipitate, the cell pellet, was prepared for electron microscopy.

Peroxidase determination. The determination of peroxidase was carried out by the guaiacol method of Jermyn & Thomas (1954). Briefly, 2·0 ml samples were mixed with 2·0 ml of a mixture of equal volume of saturated guaiacol solution and 0·1 mol peroxide solution. After incubation for 10 min at 37°C, 2·0 ml of 1 N NaOH was added. The optical density of the above reaction product was measured at 400 μm wave length by means of a spectrophotometer. Horseradish peroxidase, type 1 (Sigma), was used as standard.

Electron microscopical study. Cell pellets were prefixed at 4°C with 1% glutaraldehyde for 1 hr and washed with PBS twice. Then they were immersed in 3-3′ diaminobenzidine (DAB) Tris-HCl-buffered solution for 1 hr at 4°C. After washing with PBS the pellets were added to Graham–Karnovsky solution (Graham & Karnovsky, 1966) at room temperature for 30 min. They were sufficiently washed again in PBS and fixed with 1% OsO_4 for 2 hr. After the subsequent procedure of dehydration and embedding in Epon 812, ultrathin sections were made. These sections, contrasted with uranyl acetate and lead citrate or without any further treatment, were observed under the electron microscope (JEM-T7S, Japan).

RESULTS

Much peroxidase, e.g. 1·23 μg/ml, was released from eosinophils completely destroyed by 0·01 mol phosphate buffer pH 8·0. Destruction of the eosinophils was confirmed by loss of specific granules, formation of Charcot–Leyden crystals and complete loss of intracellular peroxidase activity. No peroxidase was released when eosinophils were incubated in phosphate-buffered saline or saline.

When eosinophils were incubated with the different classes of human immunoglobulin and rabbit antibody, various amounts of peroxidase, from 0·041–0·123 μg/ml, were released into the medium. The greatest amount was released when complexes containing IgE were tested; about twice the amount (0·123 μg/ml) than in the presence of the other classes of immunoglobulin (0·041–0·068 μg/ml) (Fig. 1).

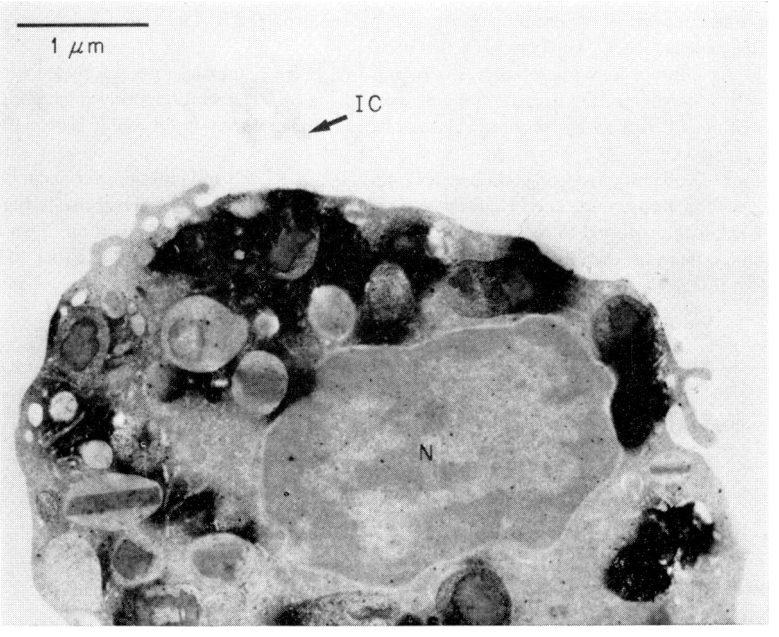

FIG. 2. Eosinophil incubated in IgG-immune complex shows diffusions of peroxidase activity and an electron dense reaction product from the matrix of the specific granules into the surrounding parts of the cytoplasm and near the margin of the cell membrane. IC = flecks of extracellular immune complex.

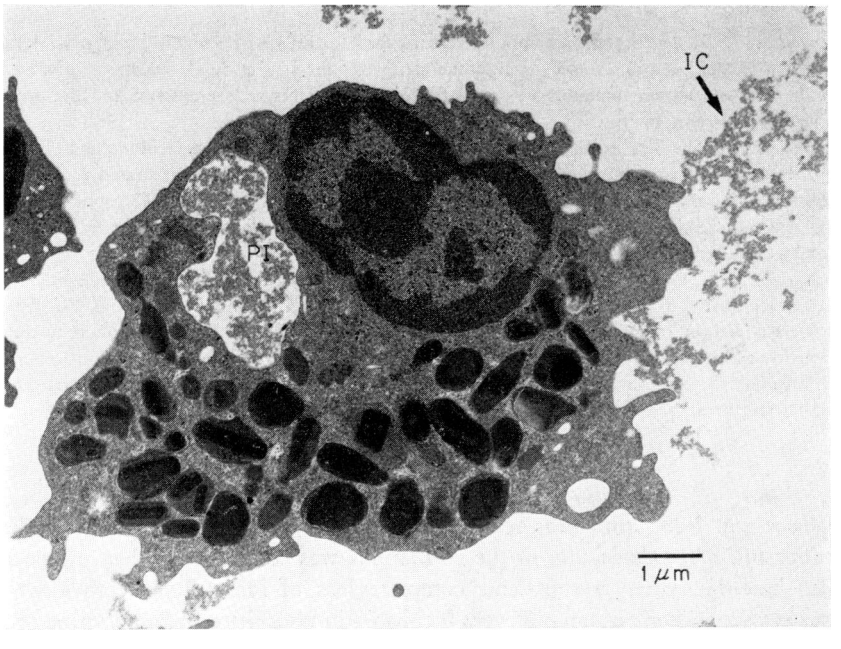

FIG. 3. Eosinophil incubated in IgE-immune complex shows fine cut surfaces of phagosomes in different sizes and shapes which contain moderate electron dense materials similar in appearance and electron density to flecks of the immune complex used for incubation. IC = flecks of extracellular immune complex; PI = phagosome with immune complex.

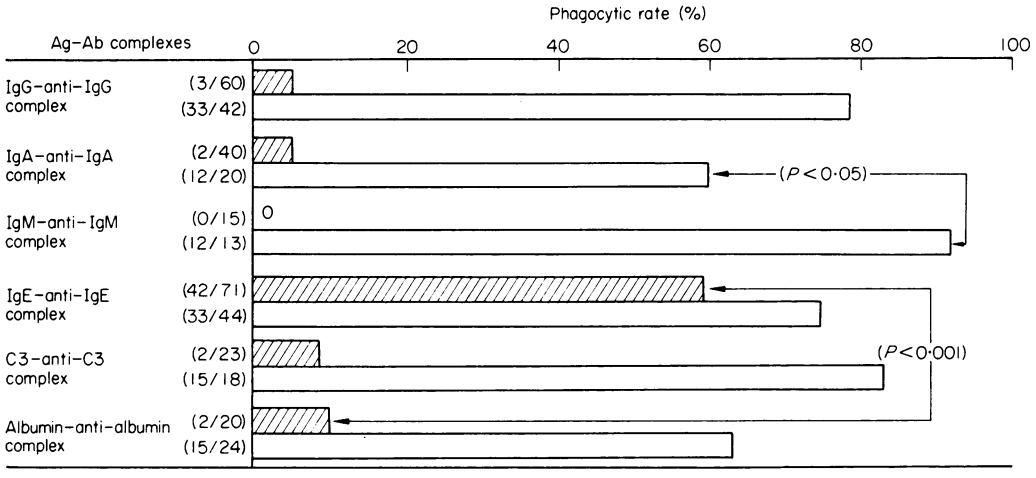

FIG. 4. Appearances of phagosome in the eosinophils and neutrophils incubated with different kinds of immune complexes. The eosinophils with phagosomes are seen more frequently in the incubation with IgE complex than that with other complexes, which the neutrophils with phagosomes show little variation according to the kind of immune complex. The differences of phagocytic rate between IgE,-anti-IgE and the other classes of immune complex were statistically significant ($P < 0{\cdot}001$) according to the χ^2-test. ▨ Eosinophil; □ neutrophil. Figures in parentheses are the analysed cell count.

These results obtained from chemical determination corresponded with these from electron microscopical observations. The peroxidase activity, an electron dense reaction product, was localized in the specific granules and occasionally observed around their matrix when eosinophils were incubated in control PBS or saline. Some peroxidase activity was diffused out of the granular matrix into the cytoplasm and to the outer surface of the cell membrane. Such diffusion of peroxidase activity was presumed to be functional and not degenerative since the limiting membrane of the specific granules was not destroyed (Fig. 2). The intensity of peroxidase activity in the eosinophil was rather less in incubation with IgE immune complex than with other complexes, suggesting that a greater amount of peroxidase was released from the cell into the incubation medium with IgE complex.

Phagosomes appeared in the cytoplasm of the eosinophils in different sizes and shapes when these cells were incubated with different kinds of immune complexes (Fig. 3). The phagosomes contained moderate electron dense materials, similar in appearance to the immune complexes suspended in the incubation media. The percentage of eosinophils with phagosomes to total cells was calculated. Eosinophils with phagosomes were seen more frequently in the incubation with IgE complex (59·2%) than with other complexes (0–10%) (Fig. 4).

DISCUSSION

In this study the release of peroxidase, a major component of the specific granules of the eosinophils, was confirmed by the interaction of these cells with different classes of immune complexes, especially the IgE-immune complex. A considerable amount of peroxidase was determined in the incubation media and peroxidase activities were observed not only in the matrix of the granules but in the cytoplasm around the granules and the outer surface of the plasma membrane of the cells when these cells were incubated with immune complexes, but not found when incubated with control saline or PBS.

Though several technical details limit the strength of the evidence, we nevertheless believe that our conclusions are valid.

The immune complexes do not offer a strict comparison between different classes of human immunoglobulin, because they are formed of human globulin with the specific rabbit antibody, and cells could react to some extent with rabbit Fc. However the human albumin-rabbit anti-albumin complex affords an

appropriate control. If the amount of peroxidase released by complexes containing IgG and IgM is not considered significant compared to the control, the amount of peroxidase released by the IgE-rabbit anti-IgE (Fig. 1) is undoubtedly significant. However the differences between the activities of the immunoglobulin classes does not take into account any difference in concentration of each immunoglobulin, because the complexes were used at a concentration of 2 mg/ml, which does not take into account the proportion of rabbit antibody according to its variable affinity for antigen. The small amounts of impurities of each of the human immunoglobulin preparations are not considered significant.

The eosinophil preparations were contaminated with a small number of neutrophils and lymphocytes, but the proportion of contaminating cells was constant in the different tests, and the amount of peroxidase in neutrophils is negligible.

If peroxidase release is specific, what is its mechanism? IgE immune complex caused the greatest amount of peroxidase release as well as a simultaneous and frequent appearance of phagosomes in the cytoplasm. This observation suggests an important role of phagocytosis as a trigger mechanism of the peroxidase release, since many investigators (Henson, 1971; Hawkins & Peeters, 1971; Tew, Hess & Donaldson, 1969; Parish, 1969; Janoff & Zeligs, 1968) documented the release of constituents of leucocytes to the outside of the cells during their phagocytosis of immune complexes. Peroxidase release was not the result of cell destruction since the limiting membrane of the specific granules as well as the cell plasma membrane showed no sign of destruction. Peroxidase activities were diffused around the specific granule in the cytoplasm. The cells must be killed if peroxidase leaks from the granules in the cytoplasm, since peroxidase is toxic to cells. Peroxidase, therefore, must pass through membrane-limited channels to the outside of the cell, as reported by Okuda, Kawabori & Takenaka (1976, submitted for publication).

What is the biological significance of the peroxidase release? Rytomaa (1960) emphasized an important role of peroxidase in eosinophil function without any definite evidence. Archer & Jackas (1965) reported a disruption of mast cells by peroxidase rich component of eosinophil granule in an *in vitro* experiment. Eosinophils are attracted to sites of antigen–antibody reaction and granulo-lysis occurs during phagocytosis of immune complexes by eosinophils. Then, liberation of peroxidase from the cell to the surrounding tissues is induced and this enzyme may act against allergic change. Further study is needed to define the anti-allergic role of peroxidase.

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