RAPID MICROMEASUREMENT OF NEUTROPHIL EXOCYTOSIS

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Abstract—A simple method is described for the visual detection of neutrophil granule exocytosis. The microassay measures release of myeloperoxidase from azurophilic granules following neutrophil stimulation and can be accomplished in less than 30 min. The assay is sensitive, reproducible from day to day, and does not require separation of leukocytes from the reaction before enzyme assay. The procedure is also applicable to the rapid measurement of the suppression of degranulation by antiinflammatory agents.

INTRODUCTION

It has previously been shown by several authors (reviewed by Becker and Henson in reference 1) that neutrophils release inflammatory mediators into the surrounding milieu on contact with a variety of stimuli. The release process involves migration of intracytoplasmic granules to the plasma membrane, fusion of the granule membrane with the plasma membrane and discharge of the contents to the cell exterior (a process of exocytosis). The intracytoplasmic granules contain a wide assortment of lysosomal enzymes capable of hydrolyzing a variety of natural and synthetic substrates. The secretory process does not involve cell lysis, as no evidence of the release of the cytoplasmic enzyme LDH has been observed. To date, all chemotactic agents for neutrophils which have been tested have been shown to cause secretion of enzymes from granules in the presence of surface stimuli or the fungal metabolite cytochalasin B (2–4).

The present communication details briefly a rapid assay of the exocytosis of myeloperoxidase contained in azurophilic granules based on that described earlier by Henson et al. (5). This assay has been routinely adapted in our

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laboratory for screening column chromatography fractions during C5a purification, the quantitation of purified C5a preparations, and assays of other chemotactic factors. The methodology described in this paper also lends itself to the rapid determination of the activity of antiinflammatory agents on neutrophil function.

MATERIALS AND METHODS

Reagents Used. Reagents used routinely in the assay included Hanks' balanced salt solution containing 0.25% bovine serum albumin (Hanks' BSA), cytochalasin B (Aldrich Chemical Co., Milwaukee, Wisconsin) and 3,3'-dimethoxybenzidine (DMB, o-dianisidine HCl, Sigma Chemical Co., St. Louis, Missouri). Several neutrophil activators were tested including N-acetyl-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), (Vega-Fox Biochemicals, Tucson, Arizona), a-casein (Sigma), and bacterial chemotactic factor (a gift from Mr. Henry J. Showell, University of Connecticut). Aggregated human y-globulin was prepared as described by Henson and Oades (6). Purified C5a was obtained from fresh human serum after activation by yeast by a modified procedure (13) of that published by Fernandez and Hugli (7). The C5a was characterized biochemically, resulted in a single band on acid polyacrylamide gel electrophoresis, and showed anaphylatoxin activity when injected into guinea pig skin. Indomethacin (Sigma), hydrocortisone sodium succinate (Upjohn Co., Kalamazoo, Michigan) and prostaglandin E1, which was a gift of Dr. John Pike, Upjohn Co., were used to block enzyme release.

Preparation of Human Leukocytes. Preparations of human leukocytes containing more than 98% neutrophils were obtained from citrated blood by dextran sedimentation followed by Ficoll-Hypaque density centrifugation, osmotic lysis of erythrocytes, and washing in modified Hanks' solution (5). The neutrophils were routinely more than 98% viable by trypan blue exclusion. In some experiments described in the results, a crude leukocyte fraction following dextran sedimentation of the erythrocytes was taken and washed as above.

Description of the Assay. All reactions were performed in U-shaped microtiter plates (Cooke Laboratory Products, Alexandria, Virginia). For quantitative assays of neutrophil activators 90 µl of Hanks' BSA were added to the first well while 50 µl of Hanks' BSA were added to the remaining wells in each row. After addition of 10 µl of neutrophil activator to the first well in each row, serial twofold dilutions were made using 50-µl microdiluters. Next, 25 µl of neutrophils (8 x 10^6/ml) pretreated for 5 min at room temperature with cytochalasin B, 5 µg/ml, were added to each well. The plate was then gently mixed. After incubation of the plate for 10 min at room temperature, 50 µl of 0.2 M sodium phosphate buffer, pH 6.2, were added to each well, followed by 25 µl of an equal mixture of 3.9 mM DMB (1.25 mg/ml in distilled water) and 15 mM H₂O₂ (0.17 ml stock 30% H₂O₂ freshly diluted in 100 ml distilled water). The plate was again gently mixed and incubated at room temperature for 10 min, at which time the plates were scored for enzyme activity. If it was inconvenient to read the plates or if the plates were to be photographed, further color development was halted by addition of 10 µl of 1% NaNO₃. The endpoint for the microtiter assay was the reciprocal of the highest dilution of neutrophil activator in which definite color change was evident. When assaying column fractions for neutrophil activator activity, 10 µl of each test fraction were added to 50 µl of Hanks' BSA and the assay carried out as described above.

In order to determine the activity of anti-inflammatory drugs on neutrophil function,