A RAPID QUANTITATIVE ASSAY FOR ACTIVATED NEUTROPHILS

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Abstract—We present here a rapid, sensitive, and convenient assay for activated human PMNs based on detecting the decreased optical density (OD) of aggregated cell suspensions. This quantitative assay uses an ELIZA machine to measure OD changes, with time, of activated cells (5 × 10⁵ cells/well) in microtiter plates. The assay is sensitive, detecting aggregation induced by as little as 0.0001 μg/ml of LPS, or lymphokines in Con-A-activated supernatant diluted 1/500. The assay permits analysis of 400 separate PMN suspensions on the same day starting with less than 80 ml of blood.

INTRODUCTION

Interest in the study of neutrophil (PMN) responses to various stimuli, coupled with the limitations of in vivo methods, have led to the development of a large number in vitro assays of PMN function (1). Assays of PMN activity (e.g., chemotaxis, ingestion, killing) as well as determinations of biochemical correlates of activation (e.g., NBT reduction, superoxide generation, chemoluminescence) characteristically are technically complex procedures that require a significant investment in both time and cell numbers. These realities tend to inhibit more widespread use of the assays and also impose rather severe limitations on the number of variables that can be tested at one time employing cells from modest-size blood samples.

To avoid these limitations and to expand our investigative capabilities, we attempted to develop an enzyme assay for PMN activation employing an ELIZA apparatus which measures the optical density (OD) of the content of microtiter plate wells. Although the enzymatic assay proved unsatisfactory, we noted that the OD decreased with time in those wells that

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contained activated PMNs. Traditionally platelet activation has been assayed as decreased OD due to aggregation (2). Craddock et al. (3, 5) and Hammerschmidt et al. (4) previously reported that stimulated PMNs aggregate and that such aggregation can be detected in modified cuvettes as a decrease in OD. In addition, more recently monocyte activation has similarly been assayed as decreased OD (6). Our finding promised to provide an “order of magnitude” increase in the number of samples that could be evaluated by using microtiter trays and the ELIZA apparatus; therefore we pursued our original observation.

We present here a quantitative assay for PMN activation based on an aggregation-induced drop in OD. Employing this assay, it is possible to repeatedly monitor OD changes in 400 separate 0.25-ml PMN suspensions and thereby analyze kinetics of activation 0–4 h after stimulation. Such an assay can be carried out using cells isolated from 60–80 ml of blood.

**MATERIALS AND METHODS**

*Reagents.* Concanavalin A was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Lipopolysaccharide B (LPS) *salmonella typhosa* 0901 was obtained from Difco Laboratories, Detroit, Michigan. N-Formyl-methionyl-leucyl-phenylalanine (FMLP) was obtained from CalBiochem Behring Corporation, San Diego, California.

All reagents were prepared in Krebs buffer and stored.

*Preparation of Mononuclear Leukocytes (MNCs) and Polymorphonuclear Neutrophils (PMNs).* Blood was obtained from volunteers by means of venipuncture and defibrinated by gentle swirling in a flask containing a siliconized steel wool pad. All glassware used in this study was siliconized. PMN-rich isolates and mononuclear cell (MNC) -rich isolates were prepared by the Ficoll-Hypaque discontinuous gradient method (7). The two isolated fractions typically contained 95% PMNs and MNCs, respectively. Each fraction was washed three times in Krebs buffered saline with 10% autologous serum and resuspended in the same medium. Trypan blue exclusion usually revealed cell viability to be greater than 99%.

*PMN Storage.* PMNs were maintained in 16 × 25-mm plastic tubes (Falcon #2025). Each tube received 8–12 ml of 1 × 10^7 cr in Sorenson’s PBS with 10% autologous serum. Tubes were stored on their sides at room temperature with the caps sealed. PMNs were freshly washed, counted, and resuspended in serum and Krebs buffer just prior to use. Although the total number of cells decreased with time, storage did not decrease the percent viability due to rapid autolysis of dead cells (unpublished observation).

*Mononuclear Cell Culture.* Culture medium was prepared using 100 ml minimal essential medium-suspension (MEM-s); nonessential amino acids (10 mM), 1 ml; Na pyruvate (100 mM), 1 ml; l-glutamine (200 mM), 2 ml; penicillin 5000 units/streptomycin 5000 µg/ml, 1 ml. This basic medium was supplemented with (1) autologous serum 0.5%; (2) mercaptoethanol 10^-2 M, 0.1 ml; (3) calcium chloride 1% solution, 1 ml. Cells were cultured at 2 × 10^5/ml in 16 × 125-mm Corning tissue culture tubes, 5–10 ml/tube, on their sides at 37°C with a 5% CO₂-air mixture renewed daily.

*Supernatant (Lymphokine) Preparation.* Concanavalin A, 5.0 µg/ml, was added to the above cultures to stimulate production of lymphokines. After 40 h of culture, the cells were removed from stimulated and control cultures by centrifugation. The supernatant was then filtered through 0.45 µm filters, and frozen at −20°C until used.