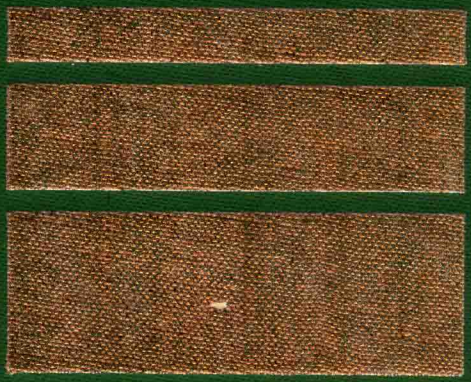
PRACTICAL METHODS IN CLINICAL IMMUNOLOGY



INVESTIGATION OF PHAGOCYTES IN DISEASE

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CHURCHILL LIVINGSTONE

Investigation of Phagocytes in Disease

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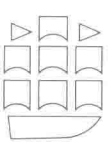

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Foreword

This third volume of the series Practical Methods in Clinical Immunology breaks new ground in that it deals with an immunological function rather than with a single system disease or group of related diseases, as was the case with the first two volumes.

The quantitation of the immunological function of phagocytes in disease is a highly topical excursion into the laboratory investigation of important clinical disorders. The way whereby this substantial experienced team of workers under the leadership of Dr Douglas and Dr Quie has tackled the problem of presenting this new technology is commendable.

In their various contributions, we have chapters ranging from succinct practical laboratory tests for routine diagnostic use to the erudite reviews by Dr Mills and Dr Quie on granulocyte metabolism which adds a glimpse of a not-too-distant future.

Melbourne, 1981 R.C.N.

Preface

Assay of function as well as determination of numbers of phagocytic cells is necessary to evaluate a possible role for abnormal phagocyte response in disease. This book contains descriptions of assays for evaluation of selected functions of polymorphonuclear neutrophil phagocytes, and a description of methods for separation of monocytes from neutrophils and lymphocytes so that similar assays may be carried out with the mononuclear phagocytic cells.

Chapter 9 includes an extensive review of current knowledge concerning the metabolic response of phagocytic cells during stimulated phagocytosis. Research in this area has contributed remarkable new knowledge during the past decade and this information relates to most aspects of phagocyte function.

The team effort by members of the Medicine, Pediatrics, Surgery, Dermatology and Laboratory Medicine-Pathology departments at the University of Minnesota is greatly appreciated. All contributors were members of the University of Minnesota Medical School when this book was prepared; K. L. Cates, S. D. Douglas, and S. H. Zuckerman have recently assumed position in Connecticut, Pennsylvania, and Stockholm. There are many unnamed post-doctoral fellows, technologists, and secretaries whose contributions were essential for the development of the assays described. We express special thanks to Ms Jane Carlson for her competent help with the manuscripts.

Support for work from the contributing laboratories came from many sources, including the National Institutes of Allergy and Infectious Diseases, National Heart, Lung and Blood Institute, National Cancer Institute, The Kroc Foundation, March of Dimes-Birth Defects Foundation, The Minnesota Medical Foundation, the American Lung Association, and the American Legion of Minnesota.

1981 S.D.D., P.G.Q.

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Introduction

S. D. Douglas and P. G. Quie

During the past decade, advances in the understanding of host-parasite interaction have led to the development of clinical immunologic assays for the assessment of phagocyte function. It is now well recognized that the 'professional' phagocytes, the polymorphonuclear neutrophil leukocyte (PMNL) and monocyte, undergo a sequence of physiologic, immunologic and metabolic events in response to inflammatory stimuli. These events can be subdivided and analyzed in the laboratory (Table 1.1).

Table 1.1 Phagocytic cell responses measurable in the laboratory

Aggregation
Adherence
Chemotaxis
Opsonization and phagocytosis
Metabolic response
Bacterial killing

Moreover, several forms of congenital (Table 1.2) and acquired (Table 1.3) disorders of phagocyte function have been identified.

The intent of this handbook is to provide clinicians, laboratory immu-

Table 1.2 Congenital disorders of phagocyte function

Chronic granulomatous disease
Chediak–Higashi syndrome
Glucose-6-phosphate dehydrogenase deficiency
Familial lipochrome histiocytosis
Myeloperoxidase deficiency
Glutathione system abnormalities
Mannosidosis
Down's syndrome
Pelger-Hüet anomaly
May–Hegglin anomaly
Actin dysfunction
Hyperimmunoglobulin E

Table 1.3 Acquired disorders of phagocyte function

Malnutrition
Irradiation
Thermal injury
Overwhelming infections
Periodontal disease
Leukemia
Hypophosphatemia
Hyperglycemia

nologists, microbiologists and workers in related disciplines with a laboratory manual for the investigation of phagocyte function and the interpretation of results of such investigations.

We recognize that many of the techniques described are still carried out primarily in research laboratories and are under development. It is anticipated that the methodologies utilized for our investigations of phagocytes will soon be widely applied in routine clinical studies. The application of these assays should provide approaches to more precise characterization of host defense mechanisms and hopefully lead to the development of therapeutic modalities which specifically modify defects in these mechanisms.

We are aware of the dangers of isolating particular pathophysiological functions from their broader biomedical implications. Any study of phagocytes in disease must be incomplete without understanding of immune cell and humoral interactions, e.g. the important role of complement in phagocytic activity. However, fastest progress demands maximum specialization and this is why we have restricted this monograph to the phagocytes themselves.

Comprehensive reviews of the immunology of neutrophils and mononuclear phagocytes have appeared recently and it is not our intent to review these fields extensively. For discussion of disorders of phagocyte function including summaries of case reports of individual patients see Klebanoff & Clark (1978).

REFERENCE

Klebanoff S J, Clark R A 1978 The neutrophil function and clinical disorders. North Holland, Amsterdam

2
Granulocyte Aggregometry

P. R. Craddock

INTRODUCTION

One of the earliest recognized manifestations of the acute inflammatory response at the microscopic level is the phenomenon of granulocyte (GR) adhesion to the vascular endothelium adjacent to the inflammatory focus (Virchow, 1897). The accumulation of GR which results from this selective adhesiveness is an important process along with chemotaxis, opsonization and phagocytosis for eradication of inflammatory stimuli. The underlying molecular events leading to increased GR adhesiveness are poorly understood. The pathogenesis of the acute granulocytopenia which occurs during hemodialysis has recently been investigated in the author's laboratory (Craddock et al, 1977a, b) and these studies have produced new insight into the mechanisms and consequences of augmented GR adhesiveness. It was shown that the chemotactic fragment of complement component C5 (C5a) induces significant increments in GR adhesiveness which can be measured *in vitro* by autoaggregation in a platelet aggregometer.

Other assays of GR adhesiveness include adherence to nylon wool (MacGregor et al, 1974) and adhesion to Petri dishes with monolayers of cultured endothelium (Sacks et al, 1978). *In vivo* results of increased granulocyte adhesiveness include pulmonary vascular leukostasis, interstitial edema and lung dysfunction. We were subsequently able to demonstrate that, in addition to GR aggregation *in vitro*, C5a also causes clumping of circulating GR *in vivo* and that the pulmonary leukostasis results from embolization of these aggregates (Hammerschmidt et al, 1979). There is considerable evidence that the accumulation of GR in the microvasculature adjacent to foci of acute inflammation (such as the Arthus re-action) is also a complement-mediated phenomenon, but the fragment(s) responsible remain unknown (Cochrane & Janoff, 1974).

It is evident that GR adhesion is of primary importance in the generation of the acute inflammatory response, and there are a number of techniques presently available for evaluating this aspect of GR function as an isolated event *in vitro*. The method of MacGregor et al (1974) quantitating GR adhesion to nylon fibers, will be discussed. O'Flaherty et al (1977) used particle-sizing electronic equipment to monitor GR auto-aggregation *in vitro* and confirmed the observation of Craddock et al (1977c), that C5a augmented GR adherence.

The aggregometer has been used as a standard assay in our laboratory for evaluating the mechanisms of GR adhesiveness *in vitro* and as a bioassay for C5a activity.

METHODS

Granulocyte preparation

GR must be carefully separated from other blood elements for aggregometry assays. Erythrocytes, which neither participate in nor retard GR aggregation *in vivo* or *in vitro*, mask the increments in light transmission and oscillation amplitude perceived by the aggregometer. Human platelets are unresponsive to activated complement in the absence of specific antibodies, but they might contribute to GR aggregation indirectly by releasing endogenous serotonin or prostaglandin intermediates. Human venous blood with heparin USP (5 u/ml) is mixed with 0.5 vol 6% (w/v) dextran 75 (Gentran 75, Travenol Laboratories, Deerfield, Ill), and allowed to sediment at 20°C for 90 min. The supernatant, leukocyte-rich plasma, is centrifuged (400*g*, 4°C, 5 min) and the cell button suspended in 0.5 ml buffered salt solution (to be described later). After 25 s hypotonic lysis (by adding 12 ml distilled, deionized water), isotonicity is restored by adding 4 ml 3.6% (w/v) physiological saline.

The cell button produced by centrifuging (400*g*, 4°C, 5 min) this preparation is suspended in 5ml buffered salt solution and applied to the upper surface of 5 ml cold (4°C) Ficoll-Hypaque solution (Boyum, 1968). After this gradient preparation has been centrifuged (400*g*, 4°C, 30 min) three cell layers are apparent. The two uppermost layers contain erythrocyte ghosts which, if they had been intact would have co-sedimented with the GR, and mononuclear cells and platelets. GR precursors (myeloblasts, promyelocytes and myelocytes) if present in the initial blood sample are found in the mononuclear layer below the erythrocyte ghosts. The cell button contains 95–99% pure GR with neutrophil/eosinophil/basophil differential counts the same as the blood donor. The button is washed once in the buffered salt solution and finally suspended in buffer at a concentration of approximately 1×10^7 cells/ml. The overall neutrophil yield is $43 \pm 15\%$. Increasing the centrifugation speed of the density gradient step to 1000*g* reduces the yield to $39 \pm 13\%$, and any shortening of its duration to less than 30 min also reduces overall efficiency. Erythrocyte contamination is negligible ($< 0.1\%$) and the platelet/GR ratio is invariably less than 1/10. Platelet contamination can be further minimized by using blood from patients with uncomplicated idiopathic thrombocytopenic purpura on minimal therapy.

Aggregometry

The nephelometric equipment used to monitor GR aggregation must be capable of detecting GR clumps containing only 5–20 cells. Platelet aggregates are considerably larger, so that techniques routinely applied in the study of platelet aggregation must be modified and the signal amplified wherever possible. We presently use a Payton aggregometer–recorder system (Models 300 BD and Pf 10HOD, Payton Associates, Buffalo, NY) and siliconized glass cuvettes which hold 0.5 ml cell suspension together with the stimulus under study. Under these conditions, approximately 20 individual aggregation waves can be generated in cells prepared from 30 ml blood.

In order to standardize this technique, minimal and maximal light transmis-

sion limits are always calibrated with GR suspensions containing 5×10^6 cells/ml and 10^7 cells/ml respectively. The full-scale deflection of 10 mV thus represents the difference between the light transmission of these two standard preparations and all assays are run at a final cell count of 10^7/ml. A 0.45 ml vol of GR suspension (at cell count 1.11×10^7/ml) is placed in the cuvette and after 60 s delay to allow warming to 37.4°C and adequate mixing at 150*g*, 0.05 ml of the appropriate stimulus is added (Fig. 2.1). The optimum stimulus volume is in the range 0.02–0.05 ml because larger volumes produce an excessively greater dilution artefact and it is difficult to achieve consistent values when smaller volumes are used. The cell count of the GR suspension is modified according to the stimulus volume to achieve a final count in the cuvette of 10^7cells/ml. The increments in light transmission and oscillation amplitude develop immediately after the dilution artefact (Fig. 2.1).

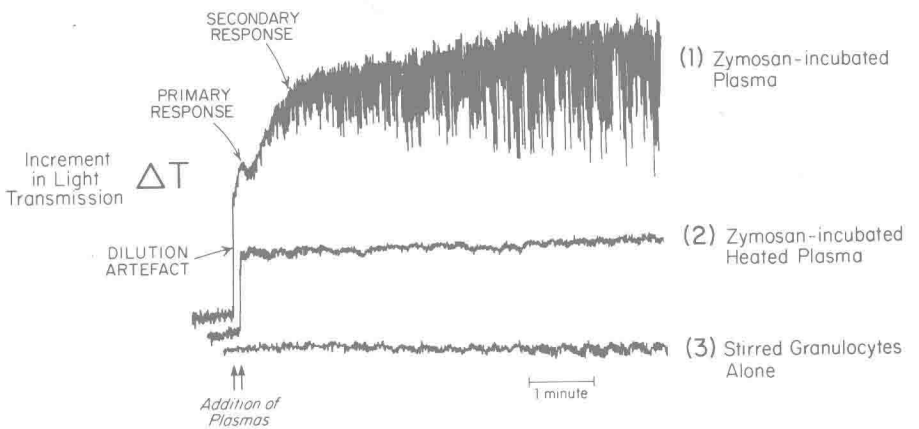

Fig. 2.1 (1) The aggregation response produced by addition of 0.05 ml zymosan-activated plasma C (0.2 mg zymosan/ml plasma incubated at 37°C for 30 min) to 0.45 ml of GR suspension containing 1.11×10^7 cells/ml and stirring in an aggregometer at 900 rpm and 37.4°C. The addition of 0.05 ml zymosan incubated but heat (56°C, 30 min) decomplemented plasma (2) produces a dilution artefact only, and (3) cells stirred alone under similar conditions remain stable in all respects

When chemotactic stimuli such as C5a or n-formyl-methionine-leucine-phenylalanine are used there is invariably slurring of the aggregation tracing which is possibly a manifestation of the sudden pseudopod-related shape change which precedes maximal cell–cell association (Craddock et al, 1977c); non-chemotactic stimuli such as the ionophore A23187, polylysine and sodium fluoride do not produce this feature. The increments in light transmission and oscillation amplitude peak 3–4 min after the addition of the aggregating stimulus can be quantitated in one of four ways, the simplest of which is direct visual inspection.

More objective methods include direct measurement (in mV) of the overall increase in light transmission after a fixed interval of 2–3 min, or of the minimal rate of light transmission increments (in mV/sec). These methods are routinely used in platelet aggregometry. However, our recently designed inexpensive digital integrator which quantitates the area under the aggregation tracing and,

in units of mV.sec/min, provides a direct and objective measurement of both the rate and the amplitude of aggregation responses (Craddock et al, 1980). Each assay takes approximately 3–4 min, but if a 2-channel aggregometer is available, the second channel can be used to prepare the cell sample for the next assay while another is in progress. The granulocyte suspension is kept at 4°C and mixed intermittently.

Technical variables

There are several important considerations in the selection of the most appropriate buffer for washing and final suspension of the cells. Initially we used Hanks' Balanced Salt Solution (HBSS) containing 0.5 g/100 ml (w/v) human serum albumin (HBSS/A), which provides optimal aggregation responses (Fig. 2.2). It can be purchased commercially as a sterile solution, and has been used widely in the study of other parameters of GR function. Citrated plasma, the medium most often used in platelet aggregometry, attenuates aggregation responses (Fig. 2.2), presumably by causing moderate depletion of divalent cation availability.

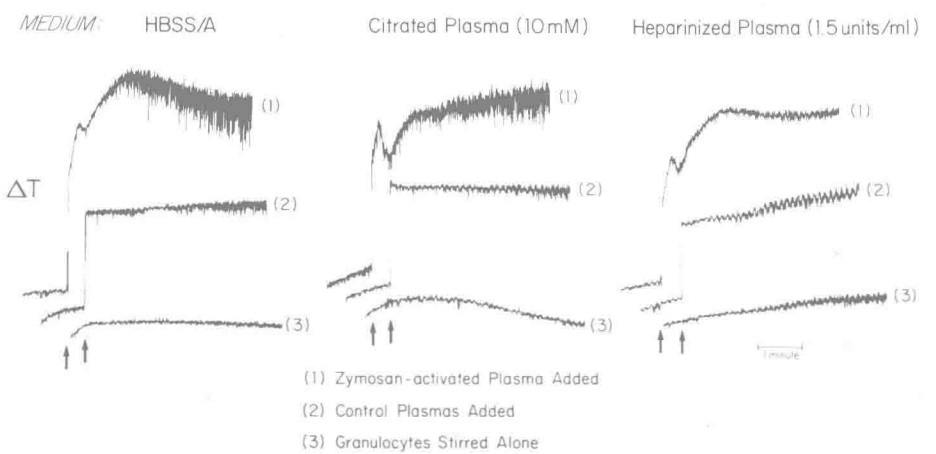

Fig. 2.2 Effects of various suspending media, including Hanks Balanced Salt Solution containing 0.5% human serum albumin (HBSS/A) (left), citrated plasma (center), and heparinized plasma (right) on C5a-induced GR aggregation

In other studies we have shown that complete deletion of calcium or magnesium from the suspending buffer results in almost total (>90%) and only partially reversible inhibition of C5a- and nFMLP-induced aggregation responses (Craddock et al, 1980). The importance of the divalent cations is further substantiated by the studies with the cationophore A23187. If it becomes necessary, however, to use plasma as a support medium, heparin USP is a satisfactory anticoagulant (Fig. 2.2). Within the concentration range 1–12 u/ml plasma, heparin neither interferes with the generation of C5a in zymosan-activated plasma nor with the aggregation responses produced by C5a or nFMLP. Serum is unsatisfactory because of the generation of C5a which invariably accompanies activation of the coagulation cascade. This activation is insufficient to produce significant depletion of C components measured by the usual assays involving

sheep erythrocyte hemolysis, but does give rise to significant quantities of C5a-aggregating activity in this much more sensitive nephelometric assay.

For most purposes, HBSS/A is the most satisfactory medium. Better buffering can be achieved with Hepes-buffered salt solution (HSS) described by Boucek & Snyderman (1976). The use of HSS buffer is not essential because chemotaxin-stimulated aggregation responses are consistent in the pH range 7.2–9.2 and are only significantly (>30%) impaired when the pH falls

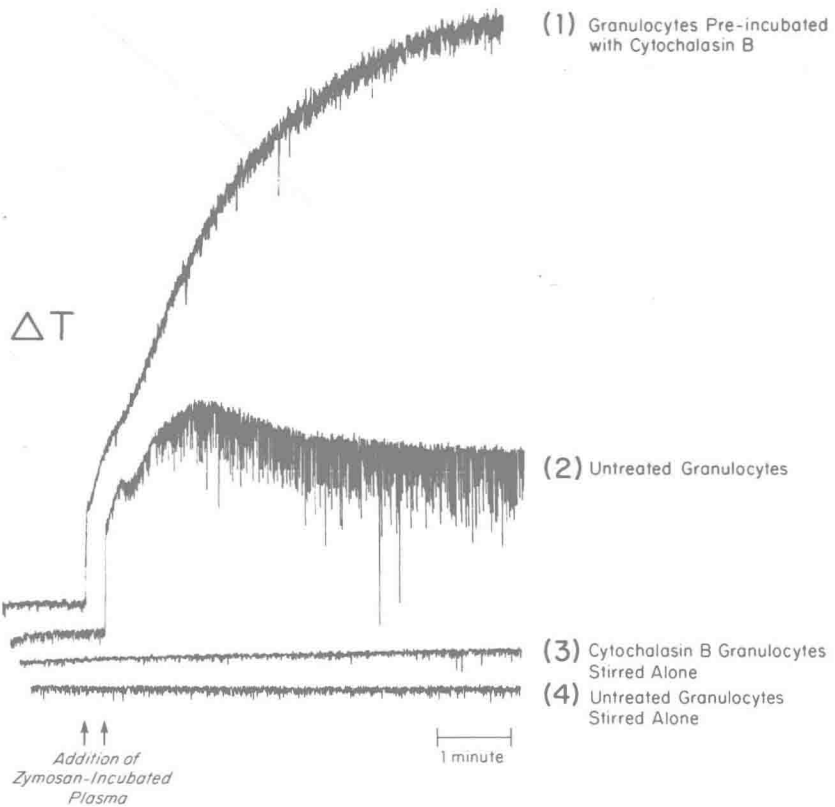

Fig. 2.3 Exposure of GR to cytochalasin B (5 μg/ml, 60 s, 37.4°C) before adding zymosan-incubated plasma gives aggregation approximately 5-fold greater than when untreated cells are similarly stimulated

below 6.2. The optimal calcium and magnesium concentrations are 1.5 mM and 1.3mM respectively. Although aggregation (in contrast to random locomotion and chemotaxis) is not inhibited by glucose depletion or by inhibition of glycolysis with 2-deoxyglucose, we retain glucose in the buffer so that an aliquot of the cell preparation can be used for parallel studies of these and other functional parameters. Regardless of the buffer solution used, the presence of 0.5 g/100 ml human serum albumin promotes stability of the aggregation responses. Although it has no immediate effect on GR responses, deletion of albumin impairs aggregation by as much as 50% after the cells have been standing on the bench for 3 h after their initial preparation. In contrast, cells suspended