Evaluation of Opsonic and Leukocyte Function With a Spectrophotometric Test in Patients With Infection and With Phagocytic Disorders

By Thomas P. Stossel

Paraffin oil droplets containing Oil Red O and coated with \textit{Escherichia coli} lipopolysaccharide were ingested rapidly by human peripheral blood phagocytes only if they were pretreated with normal serum. This property formed the basis of a screening test in which lipopolysaccharide-coated particles were opsonized with patients' sera and then were added to autologous leukocytes suspended in two portions, one of which contained nitroblue tetrazolium. After these incubations the cells were washed and extracted with dioxane. Oil Red O and nitroblue tetrazolium formazan in the dioxane extracts were spectrophotometrically assayed, thereby providing simultaneous determinations of the initial rates of ingestion and nitroblue tetrazolium reduction. The test differentiated opsonically-deficient sera and chronic granulomatous disease phagocytes from normals. Serums from individuals with bacterial infections had supernormal opsonic activity, and leukocytes from these patients had increased rates of nitroblue tetrazolium reduction in response to ingestion.

PHAGOCYTOSIS, the major defense mechanism against pyogenic infection, is a complex act that when properly executed includes ingestion and killing of microorganisms. Many microbes resist engulfment unless the opsonins of normal serum alter their surfaces rendering them readily ingestible by polymorphonuclear and mononuclear phagocytes. Increased susceptibility to pyogenic infection is the hallmark of congenital and acquired gamma globulin deficiencies\cite{1} and of certain congenital and acquired disorders of the complement system;\cite{2} and defective opsonization has been documented in these disorders.\cite{3,4} Thus, opsonic impairment is the commonest form of phagocytic dysfunction. The best characterized syndrome in which intraphagocytic killing is deranged is chronic granulomatous disease in which phagocytes do not generate hydrogen peroxide, a major microbicidal agent, during ingestion.\cite{5} Despite normal ingestion,\cite{6,7} normal content of granule enzymes,\cite{8} and normal discharge of these granules into the phagocytic vacuole,\cite{7} granulocytes of these patients fail to kill catalase-positive microorganisms.\cite{9} A biochemical marker that correlates with hydrogen peroxide generation by polymorphonuclear leukocytes is the reduction of nitroblue tetrazolium to nitroblue tetrazolium-formazan,\cite{10} a reaction that occurs in the phagocytic vacuole.\cite{11} The rate of reduction of nitroblue tetrazolium during ingestion of polystyrene spheres by peripheral blood leuko-

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...cytes of patients with chronic granulomatous disease is markedly diminished. Therefore, measurement of this reaction, the quantitative nitroblue tetrazolium test, is an assay for detecting patients with this disorder, but it does not aid in the diagnosis of opsonic deficiencies.

A chemically quantifiable microbial substitute, paraffin oil droplets containing Oil Red 0 and stabilized with bacterial lipopolysaccharide, is rapidly ingested by phagocytes only if pretreated with serum. This paper describes the use of such particles in combination with nitroblue tetrazolium as a screening test that, with 8 ml or more of blood, precisely and rapidly quantitates serum heat-labile opsonic activity, the ingestion rate of phagocytes, and leukocyte oxidative metabolism. It efficiently detects opsonic disorders and chronic granulomatous disease. It also demonstrates differences in phagocytic function between normal and infected individuals and thereby emphasizes the importance of choice of controls in studies of phagocytosis.

MATERIALS AND METHODS

Preparation of Leukocytes

The following adaptation of published procedures for preparation of human leukocytes was utilized. Venous blood was drawn into a syringe containing 1 ml of ACD (NIH formula A) for every 4 ml blood. In general, 8 ml of blood were taken from children and 16 ml were taken from adults in the standard screening assay. Then 6% dextran (500,000 molecular weight) in 0.15 M sodium chloride, 0.5 ml/ml of blood, was taken into the syringe; the syringe was placed on its plunger, and the erythrocytes sedimented for 45-60 min at room temperature. A sample of the supernatant plasma was taken for Wright's-stained smears, from which the percentage of phagocytes (band forms, neutrophils, and monocytes) was enumerated. The remainder was added to plastic, conical 50-ml centrifuge tubes. Subsequent procedures were performed at ice bath temperature. Erythrocytes were removed by addition of 0.8% ammonium chloride, 2 ml/ml of plasma, by mixing for 1 min, and by centrifugation of the cell suspension at 80 g for 10 min. The leukocyte pellet was suspended and washed twice (centrifugation at 80 g for 10 min) with 50 ml of 0.15 M sodium chloride and then was suspended in Krebs-Ringer phosphate medium, pH 7.4, approximately 1 ml/8 ml of blood originally obtained. A sample of this leukocyte suspension was removed for determination of cell number with the electronic Coulter Counter, and the remainder was used for the phagocytic assay. Depending on the white cell count of the original blood sample, this final suspension contained 1-8 × 10⁷ leukocytes/ml. Since completion of these studies, it has been determined that omission of the ammonium chloride lysis step yields leukocytes with less tendency to aggregate and that the degree of erythrocyte contamination arising from this omission does not affect the test results.

Preparation of Oil Red O in Paraffin Oil

Oil Red O was distributed in approximately 500-mg amounts to 20-ml screw-capped glass tubes containing 0.5 ml of chloroform. The solution of Oil Red A (Allied Chemical Co., Morristown, N.J.) and chloroform was diluted to 20 ml with heavy paraffin oil (Fisher Scientific Co., Pittsburgh, Pa.) (density 0.89) and was shaken overnight. The tubes were centrifuged at 2000 g for 1 hr. The supernatant fluids were pooled in an Erlenmeyer flask with a side arm, and the chloroform was removed by evaporation overnight at 50°C under vacuum. The Oil Red O solution in paraffin oil was centrifuged again, as described above. Analysis of the optical density of the final solution over a period of weeks revealed no change indicative of dye precipitating from the solution. The optical density of this solu-
tion at 525 nm (the absorption peak) was used to compute a constant (K) for converting optical density to milligrams of paraffin oil ingested, where:

$$K = \frac{1}{OD \times 0.89} \text{ ml}$$

**Preparation of Emulsion**

Thirty milligrams of *Escherichia coli* lipopolysaccharide 026:B6, prepared by the Boivin procedure (Difco Laboratories, Detroit, Mich.)\(^{15}\) were suspended in 3 ml of Krebs-Ringer phosphate medium in a thick-walled plastic test tube and were dispersed by sonication for 5 sec. One milliliter of paraffin oil containing Oil Red O was layered over the lipopolysaccharide suspension. The oil was emulsified into droplets by sonication for 45 sec. The tip of the sonifier probe was kept just below the oil-aqueous interface. For convenience, a large amount of emulsion could be prepared in this manner and frozen at $-20^\circ\text{C}$ without alteration of its properties.

**Preparation and Standardization of Nitroblue Tetrazolium Solution**

Nitroblue tetrazolium is relatively insoluble in salt solutions, and the concentrations described in the literature, 1–2 mg/ml, are overestimates of the actual amount of material in solution. This conclusion was verified with several lots of nitroblue tetrazolium from different suppliers. Therefore, the following procedure was utilized to standardize stock nitroblue tetrazolium solutions. Nitroblue tetrazolium was suspended in Krebs-Ringer phosphate at a concentration of 2 mg/ml and then was filtered through a 0.3 $\mu$m Millipore filter. Of the filtrate, 0.1 ml was added to 0.4 ml of 1 mM ascorbic acid in 0.2 N sodium hydroxide. The nitroblue tetrazolium was quantitatively reduced to formazan by this procedure, and the insoluble residue was collected on a 0.3 $\mu$m Millipore filter. The entire filter was dissolved in 2 ml of $p$-dioxane with heating to $85^\circ\text{C}$, and optical density was immediately determined at 580 nm. The actual concentration of the filtrate was approximately 318 $\mu$g/ml. Formazan, prepared as described above, was weighed on tared Millipore filters, and its optical density was determined, thereby providing a conversion factor from optical density to micrograms of formazan (14.14 $\mu$g formazan/optical density unit, for a 1 cm light path in dioxane at $25^\circ\text{C}$).

**Preparation of Serum**

Whole blood was allowed to clot at room temperature; the serum was separated by centrifugation and was analyzed immediately or frozen at $-70^\circ\text{C}$.

**Opsonization of the Emulsion**

Test serum, 0.4 ml, or Krebs-Ringer phosphate medium (control) was incubated with 0.4 ml of the paraffin oil particles at $37^\circ\text{C}$ for 15 min.

**Incubations**

To two siliconized glass or polycarbonate plastic 15-ml centrifuge tubes was added 0.015 ml of 0.1 M freshly prepared potassium cyanide in Krebs-Ringer phosphate medium and 0.4 ml of the leukocyte suspension. Krebs-Ringer phosphate medium, 0.4 ml, was added to the first tube, and nitroblue tetrazolium solution, 0.4 ml, was added to the second tube. The tubes were shaken in a water bath at $37^\circ\text{C}$ for 5 min, and then 0.2 ml of particles, opsonized by the patient's serum, was added. After 5 min more of shaking at $37^\circ\text{C}$, 6 ml of ice-cold 1 mM N-ethylmaleimide in 0.15 M sodium chloride was added, and the uningested emulsion was removed by centrifuging the cells at 500 g, discarding the supernatant, disrupting the leukocyte pellet by tapping the bottom of the centrifuge tube, and repeating
the centrifugation with 6 ml of fresh 0.15 M sodium chloride. The tubes were then drained by inversion, and the inner surfaces were wiped with tissue. Oil Red O and formazan were extracted from the pellets by adding 1 ml of dioxane and heating the extracts at 85°C for 15 min. The optical densities of the solutions clarified by centrifugation at 1000 g for 15 min were determined at 525 nm and 580 nm.

**Calculation of Results**

For 5-min incubations, milligrams paraffin oil ingested/min/10⁷ phagocytes was quantified from the dioxane extract of the incubation that did not contain nitroblue tetrazolium:

\[
\frac{\text{OD}_{525} \times 50}{\text{leukocytes/ml} \times \text{(bands, PMN, and monocytes)}} \times K
\]

Where K is the conversion constant described above and 50 is a factor accounting for the time of incubation and bringing the final result to a base of 10⁷ phagocytes.

Nitroblue tetrazolium reduction, micrograms formazan generated/min/10⁷ phagocytes was derived from:

\[
\frac{(\text{OD}_{580} \text{ of extract with formazan}) - (\text{OD}_{580} \text{ of extract without formazan}) \times 50 \times 14.14}{\text{cells/ml} \times \text{(bands, PMN, and monocytes)}}
\]

The formazan-paraffin oil ratio was the quotient of the second calculation divided by the first.

**Patients**

Apparently healthy subjects were designated as normals. Cord blood was obtained from six full-term newborns. These sera had normal IgG concentrations, nearly normal C3 concentrations, but low levels of IgM and IgA. Blood was also obtained from febrile patients with a variety of pyogenic infections. In this study, note was not made of duration of illness or of treatment. Five patients with chronic granulomatous disease, three males and two females, were studied. The diagnosis was established by the failure of their leukocytes to augment hexose monophosphate shunt activity and quantitative nitroblue tetrazolium reduction during phagocytosis of latex beads. Two of four female carriers of chronic granulomatous disease had previously been identified by the quantitative nitroblue tetrazolium test. Serum and leukocytes were obtained from patients with normal serum immunoglobulin concentrations but with C3 levels of less than 1000 mg/liter: from four with systemic lupus erythematosis, one patient with acute glomerulonephritis, and one patient with Type I essential hypercatabolism of C3. Serum and leukocytes were collected from one patient with untreated congenital agammaglobulinemia and from two patients with acquired hypogammaglobulinemia. These patients had normal C3 levels.

**Other Studies**

The quantitative nitroblue tetrazolium test was performed by the method of Baehner and Nathan, and histochemical estimation of nitroblue tetrazolium reduction by individual phagocytes ingesting zymosan was made by the technique of Nathan et al. Immunglobulin and complement determinations were performed in the laboratory of Dr. C. A. Alper by means of quantitative immunoprecipitation.

**RESULTS**

Human leukocytes ingested lipopolysaccharide-coated oil droplets at an extremely slow rate unless the particles were first preincubated with fresh normal serum (opsonized) (Fig. 1, left). If the particles were opsonized, a
rapid rate of ingestion, constant with time for 5 min, was instantly initiated. If the cells, rather than the particles, were preincubated with serum, the maximal rate of uptake was attained after a delay. This phenomenon is illustrated in Fig. 2 (left), where the kinetics of nitroblue tetrazolium reduction during phagocytosis of lipopolysaccharide emulsion are shown to parallel those of the uptake of Oil Red O shown in Fig. 1.

The opsonic process was completed within 15 min at 37°C and was inhibited by incubation of the system at 0°C, or in the presence of 10⁻³ M EDTA, or if the serum was pretreated with zymosan or heated at 56°C for 30 min. When the concentration of opsonized particles added to the system was doubled, the initial rate of ingestion was not altered, indicating that the standard concentration of particles was saturating and that true initial rates of ingestion, independent of particle concentration, were measured. Figure 2 (right) shows that the nitroblue tetrazolium concentration was not limiting under the assay conditions, where the final nitroblue tetrazolium concentration was approximately 150 µg/ml. Figure 1 (right) reveals that the opsonic activity of human serum, as determined in the standard assay system, was limiting and was proportional to serum concentration. Under standard assay conditions, the optical density at 580 nm of extracts containing Oil Red O alone was approximately 20% of extracts of paired incubations that contained nitroblue tetrazolium as well. The former was subtracted from the latter to quantify nitroblue tetrazolium reduction. The optical density of formazan at 525 nm was almost 80% of that of Oil Red O. Therefore, it was not possible to perform the entire assay with a single incubation containing both Oil Red O particles and nitroblue tetrazolium.

Figure 3 summarizes the results of the phagocytic screening test for normal individuals and for patients with different diseases. Serums of patients, which
Fig. 3. Initial rates of ingestion of lipopolysaccharide-coated paraffin oil droplets by leukocytes engulfing particles pretreated with autologous serum are shown in left of each column. Ratios of initial rate of nitroblue tetrazolium reduction to initial rate of ingestion are shown in right-hand portion of each column. Leukocytes and serum were obtained from normal and infected controls (black circles), patients with C3 deficiency (open circles), immunoglobulin deficiency (open squares), chronic granulomatous diseases (black triangles), chronic granulomatous disease "carriers" (black squares), and newborn infants (open triangles). The range of normal is evident from this figure.

were deficient in C3 or which lacked immunoglobulins, promoted subnormal phagocytic rates, as did serums of six newborn full-term infants. The lesion was localized to the serum by appropriate mixing experiments. While the range of normal ingestion rates was quite large, patients with bacterial infections tended to have ingestion rates greater than the normals, although overlap with the apparently normal population was considerable. Mixing experiments (Table 1) implicated primarily but not totally the serum as the source of this enhancement. In these mixing experiments, the serum used to opsonize the particles was often ABO incompatible, but it was shown in other studies that the activity of a given individual's serum was essentially equally effective with incompatible or compatible cells.

Nitroblue tetrazolium reduction is a function of ingestion (Fig. 2). Therefore, the rates of nitroblue tetrazolium reduction shown in Fig. 3 are corrected for ingestion rates. Patients with abnormal serums, despite having diminished absolute nitroblue tetrazolium reduction rates, had normal reduction rates when corrected for the impaired ingestion. Leukocytes of patients with chronic granulomatous disease had subnormal rates of nitroblue tetrazolium reduction even after correction for ingestion. Mothers of two of these patients had nitroblue tetrazolium reduction rates intermediate between normals and the

Table 1. Comparison of Serum and Cells of Normal Controls and Infected Patients

<table>
<thead>
<tr>
<th>Serum</th>
<th>Rate of Ingestion (mg paraffin oil/10^7 phagocytes/min)</th>
<th>Rate of Nitroblue Tetrazolium Reduction (mg/10^7 phagocytes/min)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>0.138 (0.121-0.157)*</td>
<td>0.148 (0.133-0.175)</td>
<td>&lt;0.05†</td>
</tr>
<tr>
<td>Infected</td>
<td>0.353 (0.202-0.406)</td>
<td>0.425 (0.266-0.501)</td>
<td>&lt;0.05†</td>
</tr>
</tbody>
</table>

*Mean and range for six separate samples.
†Significance of difference between normal and infected serum based on Student's t test.
‡Significance of difference between normal and infected cells.
patients. Two others consistently had reduction rates in the low normal range, and the same results were obtained with the quantitative nitroblue tetrazolium test. Histochemical studies showed that these mothers had two populations of leukocytes, one that reduced nitroblue tetrazolium in phagocytic vacuoles containing zymosan particles and one that did not. The leukocytes of patients with bacterial infections tended to have nitroblue tetrazolium reduction rates greater than the normals, even after correction for the enhanced ingestion rates (Fig. 3).

In all of these studies, the data have been expressed in terms of phagocytes defined as band forms, segmented neutrophils, and monocytes. Eosinophils were excluded. When Wright's-stained smears of leukocytes ingesting opsonized lipopolysaccharide-paraffin oil droplets were examined, engulfed particles were clearly apparent as lucent vacuoles within the cytoplasm. Eosinophils rarely ingested the particles. Band forms, segmented neutrophils, and monocytes appeared to participate equally in ingesting the droplets, although precise quantitation of relative rates of uptake could not be assessed by this morphologic technique. At least 90% of the phagocytes of the tests summarized in Fig. 3 were mature segmented neutrophils.

DISCUSSION

Analysis of phagocytic function in the evaluation of patients with susceptibility to pyogenic infection requires assessment of opsonization, ingestion, and killing of microorganisms. The nature and method of activation of all serum opsonins are not yet established. Nevertheless, the importance of gamma globulin and of complement, particularly of activated C3, in promoting phagocytosis of microorganisms is well established. The opsonic activity of serum for the lipopolysaccharide-paraffin oil particles is heat labile. In studies described elsewhere, it has been determined that opsonization of these particles involves C3 fixation by means of the alternate complement pathway, i.e., the properdin system. This fixation is enhanced by antibody to lipopolysaccharide. Not unexpectedly, therefore, the screening test introduced in this paper detected opsonic deficiencies associated with hypogammaglobulinemia, and with diminished serum levels of C3 secondary to various disorders.

The assessment of ingestion requires readily quantifiable substrates for phagocytosis. This substrate must be easily separable from the phagocytes so that true internalization may be differentiated from nonspecific adhesion and that saturating concentrations of particles can be employed. When these criteria are met, it is possible with a single time point to determine initial rates of ingestion. A short incubation period is preferable when dealing with phagocytes in suspension, since clumping of these cells occurs rapidly at 37°C. The ability of paraffin oil droplets in general to satisfy these criteria has been discussed, and data pertinent to the kinetics of this system for use with human phagocytes have been presented in this paper. The findings that no ingestion of lipopolysaccharide-paraffin oil particles occurred at zero time, at 0°C, or in the presence of 1 mM N-ethylmaleimide and that the initial rate of
engulfment was saturable with respect to particle concentration strongly indicate that nonspecific adherence of particles to cells was not included in the measurement of particle uptake.

The most direct method for assessment of killing of microbes by leukocytes involves measurement of loss in viability of a microbial population suspended with phagocytes. This technique has provided much useful information about phagocytosis and originally detected a bactericidal defect in chronic granulomatous disease. This method, however, is time-consuming, is relatively insensitive and imprecise, and does not provide optimal quantitative resolution. The importance of hydrogen peroxide for intraphagocytic microbial killing has made it reasonable to utilize reactions that correlate with hydrogen peroxide generation, such as nitroblue tetrazolium reduction, as an indirect quantitative approach for detection of clinically relevant killing defects. Other processes that are associated with hydrogen peroxide generation and can be quantitated reliably include hexose monophosphate shunt activity and fixation of iodide to protein. The latter reaction is particularly pertinent because it requires the presence of peroxidase-containing granules and normal degranulation. Hence, it can detect deficiency of myeloperoxidase, an adjunct to hydrogen peroxide-mediated microbial killing, as well as absence of hydrogen peroxide. These biochemical tests, however, do not differentiate impaired ingestion from deranged leukocyte metabolism because the rates of the metabolic reactions are functions of the ingestion rate. The screening test presented in this paper does differentiate immediately between diminished ingestion and impaired oxidative metabolism.

In addition to detecting opsonic deficiencies and chronic granulomatous disease, this test also revealed increased opsonic activity of serum from patients with bacterial infections. Enhanced serum opsonic activity has previously been observed with serum from patients with subacute bacterial endocarditis and from heroin addicts. Leukocytes of infected patients demonstrated super-normal ingestion and nitroblue tetrazolium reduction rates (Fig. 3). Resting leukocytes in whole blood of patients with certain infections may spontaneously reduce nitroblue tetrazolium, producing formazan precipitates in or on the cells. The basis of this type of nitroblue tetrazolium reduction and its relationship to the reduction occurring during particle ingestion remain to be defined. However, the influence of the clinical state of the subject on test results is noteworthy, because it emphasizes the importance of defining the control group. The difficulties encountered in this study and by others in detecting carriers of chronic granulomatous disease by quantitative methods may represent an example of this problem. In the report that introduced the quantitative nitroblue tetrazolium test, the reduction of nitroblue tetrazolium by female carriers of chronic granulomatous disease was considerably closer to the patient population than to the controls. The control cells were derived from patients with infections in that study, and the lower limit of nitroblue tetrazolium reduction for normal controls may not have been appreciated. This lower limit leaves little room for carriers. Histochemical methods first suggested by Windhorst et al. are useful for verifying the presence of the carrier.
state. These techniques, unlike quantitative tests, have the advantage of not being influenced by compensation on the part of the normal cell population. They are not totally satisfactory because they rely on subjective factors, and the morphology is not always optimal.

The normal initial rates of ingestion found for leukocytes of patients with chronic granulomatous disease engulfing lipopolysaccharide-coated particles opsonized with autologous serum confirm and extend the findings obtained previously using albumin-coated paraffin oil droplets, and by others using bacteria as test particles. Although the patients were not actively infected when the studies were performed, it is notable that the ingestion rates were at the lower range of normal. It is unlikely that a subtle impairment in ingestion accounts for the abnormal metabolic responses of these patients’ leukocytes, but it will be of interest to determine whether failure to enhance ingestion in the presence of bacterial infection is another feature of this syndrome.

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