FUNCTIONAL AND METABOLIC PROPERTIES OF POLYMORPHONUCLEAR LEUCOCYTES

II. The Influence of a Lipopolysaccharide Endotoxin*

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In recent years many studies have appeared concerning the effects of lipopolysaccharide endotoxins on the resistance of laboratory animals to bacterial infection (1, 2). For the most part these investigations have demonstrated that the administration of small quantities of a variety of lipopolysaccharides results in increased resistance to infection with both homologous and heterologous bacteria (3). The mechanisms underlying these phenomena are imperfectly understood. This is in part related to the multiple effects of these compounds *in vivo* as well as the intrinsic complexity of the infectious process.

Among the more striking physiological effects of endotoxins which have been linked to resistance mechanisms are: stimulation of the phagocytic activity of the reticuloendothelial system (4, 5) and granulocytes (6), enhanced properdin titers (7), alterations in adrenal activity (8), pyrogenicity (9), adjuvant-like effects on antibody production (10), and changes in vascular physiology (11). Because of this broad spectrum of pharmacological activities, it has not been possible to delineate the mode of action of these compounds on mammalian cells *in vivo* nor to distinguish between primary and secondary responses. The present study was designed to investigate in more detail the *in vitro* action of endotoxin upon homogeneous populations of a cell which plays an important role in host defense mechanisms; *i.e.*, the polymorphonuclear leucocyte. This report will deal with alterations in the functional properties of leucocytes as well as the concomitant metabolic changes which are brought about by a highly purified lipopolysaccharide endotoxin.

Materials and Methods

General.—

The preparation of leucocyte suspensions, media, bacterial strains, estimation of phagocytic and bactericidal properties of leucocytes, and the analysis of metabolic functions have been described in previous publications (12, 13).

Lipopolysaccharide:—Two preparations of lipopolysaccharide (AE1688S4 and AE1298S4)

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were obtained through the kindness of Dr. Otto Westphal (Dr. A. Wander Forschunginstitut, Freiburg/Baden, Germany). These were prepared by the phenol-water extraction procedure from *Salmonella abortus equi* and were of equivalent activity in terms of the subsequent findings. The preparation and characterization of this substance have been described by Westphal and Lüderitz (14). This material is available commercially under the trade name of pyrexal.

Immediately prior to each experiment, dried samples of the lipopolysaccharide were weighed and dissolved in sterile balanced salt solution (pH 7.4) to give a concentration of 0.5 to 1.0 mg./ml. Such stock solutions were then further diluted in the same medium to give appropriate experimental concentrations. Solutions containing 1.0 mg./ml. were slightly opalescent whereas lower concentrations were completely clear. No attempt was made to sterilize lipopolysaccharide solutions prior to their addition to leucocyte suspensions. Further details on the use of the lipopolysaccharide will be given under Results.

RESULTS

A. The Influence of Lipopolysaccharide on the Phagocytic and Bactericidal Properties of Polymorphonuclear Leucocytes

Preliminary experiments employing various concentrations of pyrexal revealed that the addition of this substance to leucocyte-bacteria suspensions resulted in an increased rate of killing of *S. albus* (Mendita). Various techniques were used to demonstrate this effect and it was found that pre-incubation of the leucocytes with endotoxin prior to the addition of the bacterial inoculum gave the most consistent results. This method was then employed in all subsequent experiments.

Polymorphonuclear leucocytes were washed and suspended in solution HBG (12). The cells were dispensed to roller tubes and fresh normal rabbit serum added. Various concentrations of endotoxin were next added to the suspensions, mixed thoroughly, and incubated in a constant temperature bath at 37°C. for 20 minutes with constant agitation. The control tubes received an equal volume of solution HBG. At the end of the pre-incubation period a washed suspension of *S. albus* was added to all tubes, and subsequently sampled for viable bacteria. The final concentration of leucocytes was 30 × 10/ml. with a 1 to 3:1 multiplicity of bacteria/leucocyte. The final concentration of endotoxin ranged from 0.1 to 50 μg./ml.

Fig. 1 illustrates the influence of various concentrations of pyrexal on the killing of *S. albus* by polymorphonuclear leucocytes in the presence of 10 per cent fresh normal rabbit serum. All concentrations of endotoxin employed resulted in the increased killing of this organism. The enhanced rate of killing was observed early in the experimental period and at the end of 180 minutes, endotoxin-treated cells had killed a log unit more bacteria than the untreated controls. A consistent finding was the fact that the lower concentrations of endotoxin (0.1 to 1.0 μg./ml.) had a more marked influence on the killing rate than did the higher concentrations. Nevertheless, even a concentration of 50 μg./ml. resulted in more rapid killing when compared to the control. Concentrations as low as 0.01 μg./ml. gave results similar to that obtained with 0.1 and 1.0 μg./ml.

When endotoxin was added at the same time as the bacterial inoculum there

was a lag period of approximately 20 to 30 minutes during which time killing rates were similar to those in the control tube. Thereafter, the rate at which endotoxin-treated cells killed the staphylococci was increased. The concentrations of endotoxin employed in these experiments had no effect on the viability or growth rate of *S. albus* in leucocyte-free medium.

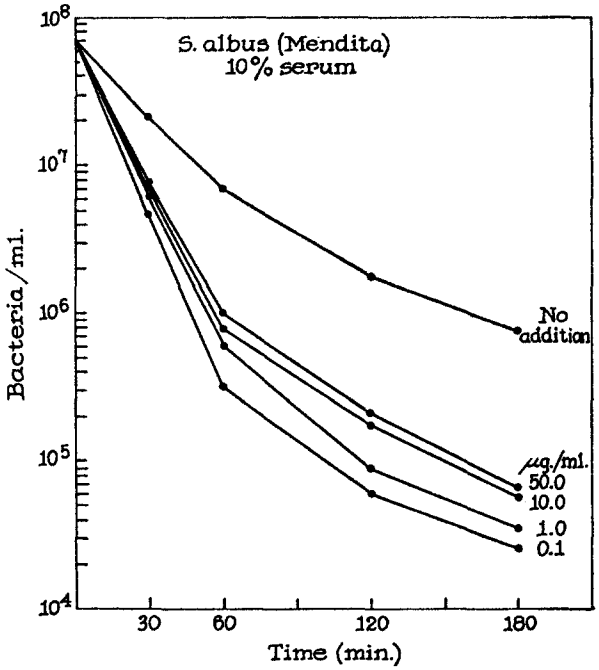

FIG. 1. The influence of pyrexal on the killing of *S. albus* by polymorphonuclear leucocytes in the presence of 10 per cent serum.

When similar experiments were conducted at lower serum concentrations, markedly different results were obtained. Fig. 2 illustrates one such experiment which was conducted at a serum concentration of approximately 1 per cent. The rate of killing exhibited by the control was less than with 10 per cent serum; a reflection of the suboptimal concentration of thermolabile opsonin. The addition of 0.1 to 1.0 μg./ml. of endotoxin resulted in an enhanced killing rate similar to that seen in the presence of 10 per cent serum. At a concentration of 10.0 μg./ml. the number of viable staphylococci remained essentially constant during the 3 hours of incubation. At 50.0 μg./ml. the bacteria were able to multiply in the presence of the leucocytes following a short lag period. This degree of multiplication was similar to that observed with this strain in the absence of leucocytes.

The difference in the behavior of endotoxin at various serum concentrations suggested that there was a factor in normal rabbit serum which neutralized its action and secondly that high concentrations of this material had an adverse effect on the leucocytes.

From these results it was possible to conclude only that endotoxin was capable of increasing the bactericidal properties of leucocyte-bacteria mixtures. Two possible explanations were that there was an increase in the bactericidal

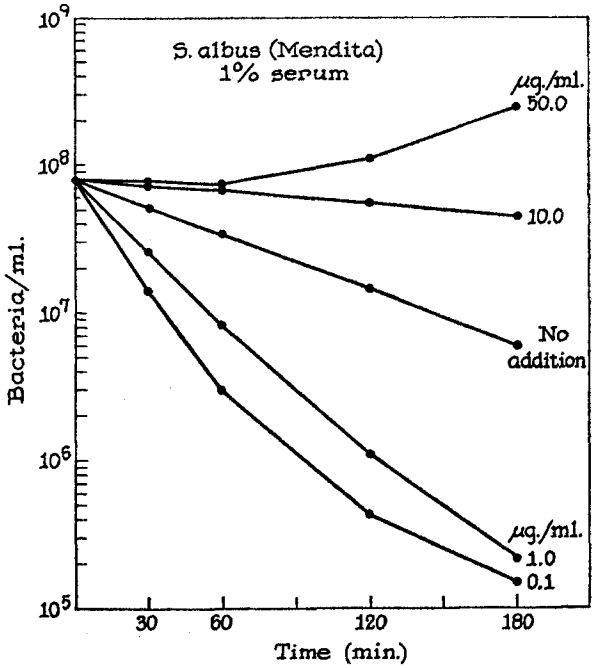

FIG. 2. The influence of pyrexal on the killing of *S. albus* by polymorphonuclear leucocytes in the presence of 1 per cent serum.

potency of the medium with extracellular destruction of bacteria or that enhanced phagocytosis occurred with subsequent intraleucocytic inactivation.

The first possibility was rendered unlikely by the results of the following experiments.

Pyrexal at concentrations of 0.1, 1.0, and 10.0 μg./ml. was added to leucocytes suspended in solution HBG-10 per cent serum. The suspensions were incubated at 37°C. with constant agitation. At time 0, 60, and 180 minutes aliquots were removed and the leucocytes sedimented at 500 R.P.M. (International Centrifuge No. 2) for 5 minutes. The cell-free supernatant fluids were then dispensed to screw-capped tubes and a washed inoculum of *S. albus* was added. The tubes were then incubated at 37°C. and sampled for viable bacterial counts at 0, 60, and 180 minutes.

The results of such experiments indicated that the supernatant fluids obtained from endotoxin-treated leucocytes did not exhibit any intrinsic bactericidal activity on the staphylococci. In addition, the organisms after 180 minutes' incubation exhibited growth which was identical with that seen in solution HBG-10 per cent serum. From these results it was concluded that endotoxin did not cause the release of demonstrable bactericidal substances into the medium.

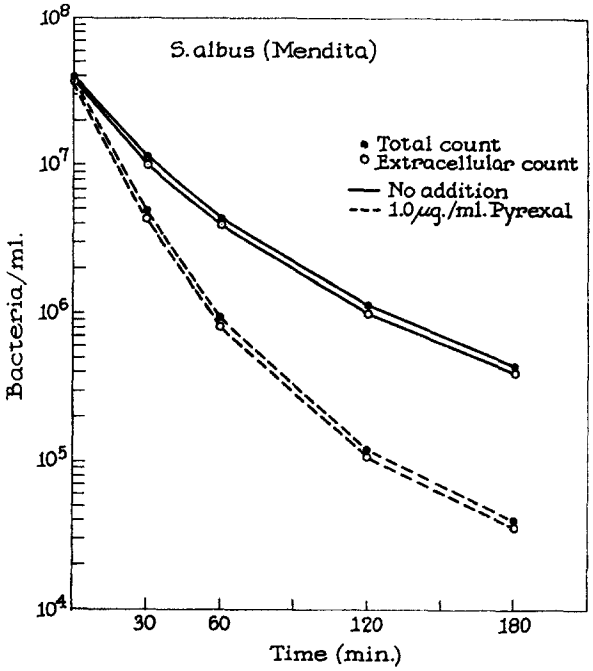

FIG. 3. The localization and fate of *S. albus* in the presence and absence of pyrexal.

The possibility of enhanced phagocytosis was next examined. The procedure employed to evaluate phagocytosis has been described in previous publications (12, 13). Fig. 3 demonstrates the influence of pyrexal, at a concentration of 1.0 μg./ml. on the total and extracellular viable counts of *S. albus* (Mendita) in the presence of leucocytes. In both the control and treated suspensions the fall in extracellular viable bacteria was identical with the reduction in the total viable count. This finding, in the absence of extracellular killing, indicated increased phagocytosis.

Further studies were conducted on the localization and fate of bacteria in leucocyte suspensions containing the two concentrations of serum employed previously (Table I). The studies with 10 per cent serum indicated that the killing of bacteria at various endotoxin concentrations was accompanied by a

similar reduction in extracellular organisms. No significant changes in the leucocyte-associated fraction were noted. In 1 per cent serum the inhibitory effects of large amounts of endotoxin were related to decreased phagocytic activity of the leucocytes and not intracellular survival of the staphylococci.

The next series of experiments examined the relationship between serum factors and the effects of pyrexal on the phagocytic activity of leucocytes.

TABLE I

The Effect of Various Concentrations of Lipopolysaccharide on the Phagocytosis and Killing of S. albus (Mendita)

Medium*		Bacteria/ml. after incubation at 37°C.‡					
		60 min.			180 min.		
Lipopoly-saccha-ride	Serum	Total	Extracellular	Leucocyte-associated	Total	Extracellular	Leucocyte-associated
μg./ml.	*per cent*						
0	10	7.0×10^6	6.7×10^6	3.2×10^4	8.5×10^5	8.3×10^5	3.2×10^4
1.0	10	6.0×10^5	6.0×10^5	1.8×10^4	3.2×10^4	3.0×10^4	1.2×10^4
10.0	10	8.3×10^5	8.5×10^5	4.2×10^4	6.4×10^4	6.2×10^4	2.0×10^4
50.0	10	1.2×10^6	1.0×10^6	5.1×10^4	9.5×10^4	9.6×10^4	1.8×10^4
0	1.0	4.1×10^7	3.6×10^7	4.8×10^4	7.0×10^6	6.4×10^6	4.5×10^4
1.0	1.0	8.2×10^6	7.0×10^6	6.2×10^4	1.8×10^5	9.5×10^4	2.8×10^4
10.0	1.0	7.0×10^7	5.2×10^7	3.8×10^4	6.5×10^7	7.0×10^7	4.8×10^4
50.0	1.0	7.5×10^7	8.2×10^7	7.2×10^4	2.5×10^8	3.1×10^8	7.2×10^4

* Balanced salts +0.0056 M glucose + 0.01 per cent crystalline bovine serum albumin.

‡ Zero counts

Total ..... 7.2×10^7

Extracellular ..... 6.8×10^7

Leucocyte-associated ..... 7.6×10^4

Exudate leucocytes were washed and dispensed to roller tubes. The cells were then pre-incubated for 30 minutes at 37°C. in the presence or absence of both serum and pyrexal. The basic medium was solution HBG and each tube contained 60×10^6 leucocytes in a total volume of 2.0 ml. At the end of the pre-incubation period, the leucocytes were sedimented at 450 R.P.M. for 4 minutes, the supernatant fluid carefully removed, and the cells washed twice with 10 ml. of solution HBG. The cells were then resuspended in 1.8 ml. of solution HBG (tubes A, B) or in 1.8 ml of solution HBG-10 per cent serum. An 0.2 ml. aliquot of washed *S. albus* (Mendita) was then added, the tubes stoppered and incubated at 37°C. Aliquots were removed at 0, 60, and 180 minutes for total viable counts.

Fig. 4 illustrates the results of one such experiment. The leucocytes in tubes A and B were maintained in the absence of serum during both the pre-incubation and assay period. Neither the control, nor treated cells killed an appreciable number of staphylococci. Tube C represents the serum control in which there

was a 2 log unit reduction in viable bacteria. Tube D represents leucocytes which were pre-incubated with pyrexal in the absence of serum. The cells were then thoroughly washed to remove the endotoxin and resuspended in a serum-containing medium. Enhanced killing occurred and was quantitatively similar to that observed in tube E in which serum had been present in the

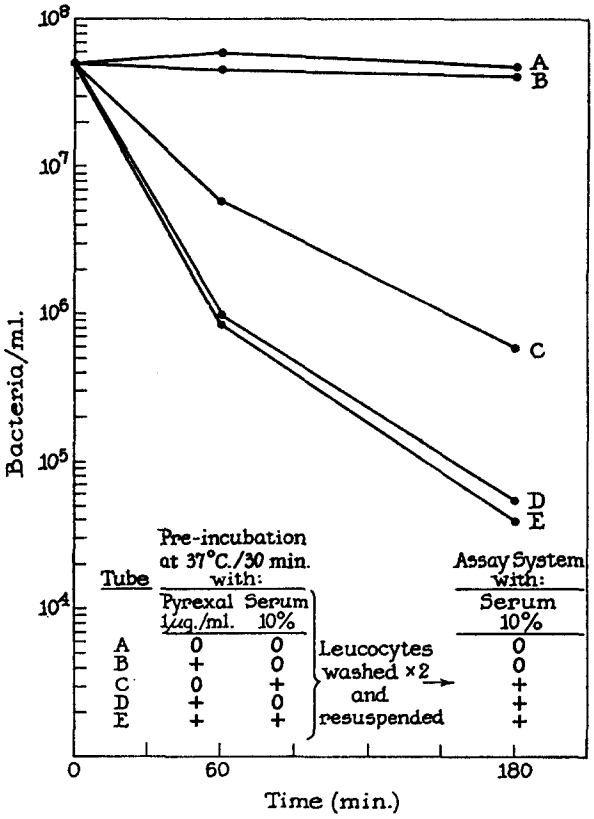

FIG. 4. The relationships between serum and pyrexal on the phagocytic activity of polymorphonuclear leucocytes.

pre-incubation period. From these results it appeared that endotoxin could interact with leucocytes in the absence of serum and increase the phagocytic activity of the cells. However, the enhanced phagocytic activity of endotoxin-treated cells could only be demonstrated in the presence of the opsonizing components of serum.

The demonstration that endotoxin enhanced the phagocytic activity of leucocytes could be based upon (*a*) a primary effect upon the phagocyte, or (*b*) the release of opsonizing materials from the leucocyte into the medium. In

attempting to differentiate between these two possibilities, the following experiment was performed.

Leucocytes were exposed to endotoxin at concentrations of 1.0, 10.0, and 50.0 μg./ml. in the presence of 10 per cent serum. After a preliminary incubation period of 60 minutes the leucocytes were thoroughly washed with large volumes of solution HBG to remove endotoxin, and resuspended in the same medium. The cells were then incubated at 37°C. and sampled at 60 and 180 minutes. The leucocytes from these aliquots were then sedimented at 450 R.P.M. for

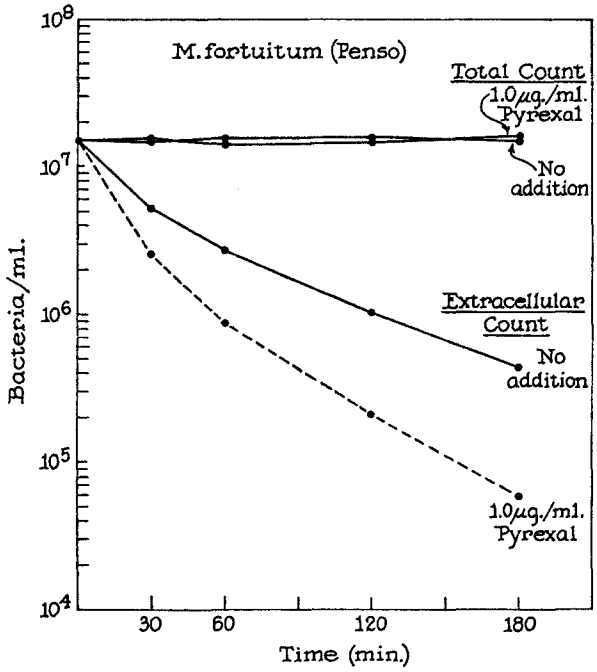

FIG. 5. The effects of pyrexal on the phagocytosis and intracellular fate of *Myco. fortuitum* (Penso).

5 minutes. The cell-free supernatant fluids were collected and used as medium for a fresh lot of washed leucocytes. *S. albus* (Mendita) was added and the tubes sampled for viable counts.

The results of such an experiment indicated that the cell-free supernatant fluids from endotoxin-treated leucocytes had no greater opsonizing activity than those from untreated leucocytes.

It was next of interest to examine the effect of endotoxin on an organism which had previously been shown to resist the intracellular milieu of the leucocyte and survived within leucocytes for periods of 180 minutes. Figure 5 represents an experiment performed with *Mycobacterium fortuitum* (Penso). The upper two lines represent the total number of viable acid-fast bacilli in the

leucocyte suspension. Since more than 90 per cent of these organisms are engulfed by the leucocytes and are intracellular after the first 30 minutes, it is apparent that endotoxin treatment did not alter their intracellular fate. The lower two lines represent the number of viable extracellular bacteria. Here, endotoxin-treated leucocytes showed a greater rate of phagocytosis as indicated by the more rapid reduction in extracellular bacteria.

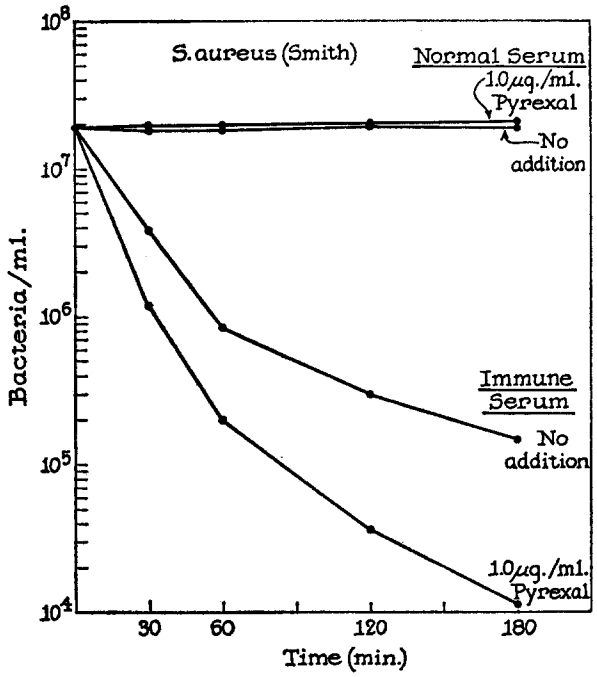

FIG. 6. The effect of pyrexal on the fate of *S. aureus* (Smith) in the presence and absence of homologous immune serum.

The next model system investigated was the interaction of a mouse-virulent, coagulase-positive strain of *S. aureus* with rabbit polymorphonuclear leucocytes. Previous studies had shown that this organism was not appreciably phagocyted by leucocytes in the presence of normal rabbit serum (12). This was demonstrated by the fact that there was no reduction in total viable counts during a 3 hour period and that essentially all the organisms could be accounted for as extracellular bacteria. When homologous immune serum was introduced, phagocytosis took place and there was rapid intracellular destruction of the virulent staphylococci. Fig. 6 illustrates an experiment in which endotoxin was added to suspensions of leucocytes and *S. aureus* (Smith) in the presence of normal and immune rabbit serum. In the presence of 10 per cent normal rabbit serum there was no change in the total number of viable organisms either in

the presence or absence of endotoxin. When homologous immune serum was employed at a final concentration of 1:50 there was a marked reduction in the number of staphylococci. This was more pronounced in the endotoxin-treated suspension with approximately a tenfold greater reduction in viable staphylococci during the 180 minute period. This result again suggested that endotoxin enhanced the phagocytic activity of the leucocytes. In this instance, however, the effect could be demonstrated only in the presence of a specific opsonin contained in immune serum.

B. Metabolic Effects of Endotoxin on Polymorphonuclear Leucocytes

In view of the influence of minute quantities of endotoxin on the phagocytic activities of polymorphonuclear leucocytes it was of interest to examine the effects of this compound on their carbohydrate metabolism. Since the ingestion of various Gram-positive and Gram-negative organisms was associated with a number of metabolic alterations, it seemed possible that at least a portion of these changes could be related to components of the bacteria; *e.g.*, lipopolysaccharides. A review of the literature revealed only scanty information on this subject. The work of Martin *et al.* (15) showed an effect of pyromen in increasing the production of lactic acid by human blood leucocytes as well as decreasing the consumption of oxygen.

The effects of pyrexal on the metabolic activity of leucocytes was conducted in a manner similar to that employed for the study of particle ingestion (12, 13). Glucose utilization, lactic acid formation, and glycogen metabolism were simultaneously studied on suspensions of leucocytes (20 to 30 $\times$ 10^6/ml.) agitated in siliconed test tubes. Oxygen consumption was measured in a conventional Warburg apparatus. Concentrations of pyrexal ranging from 0.1 to 50.0 μg./ml. were used. The medium employed in all studies consisted of solution HBG containing 0.0056 M glucose. In the majority of the experiments fresh normal rabbit serum was present at a final concentration of 10 per cent. Endotoxin was added at zero time, the tubes immediately sampled, and subsequently incubated at 37°C. Samples for analysis were also obtained at 60 and 180 minutes in all cases.

The addition of endotoxin at concentrations of 0.1 to 50 μg./ml. had no influence on the oxygen consumption of leucocytes. Fig. 7 illustrates the lack of effect at 1.0 and 10.0 μg./ml. respectively. In contrast, the addition of a phagocytable particle produced a marked increase, similar to that described in the previous paper. This difference in the response of leucocytes to endotoxin and particles was the most marked difference in their respective metabolic effects.

Whereas oxygen consumption was not affected by endotoxin under these conditions, there was a striking increase in glycolysis. Figs. 8 and 9 demonstrate the influence of graded doses of pyrexal on glucose utilization and lactic acid production. At the lowest concentrations employed (0.1 μg./ml.) both glucose utilization and lactic acid production were enhanced. The per cent

difference between treated and control tubes was approximately 50 per cent during a 3 hour incubation. As the endotoxin concentration was increased 100-fold to 10.0 μg./ml., no further stimulation was noted. At 50 μg./ml. there was a less marked response than at lower concentrations. In certain respects this type of dose response relationship was similar to that observed in the bactericidal system.

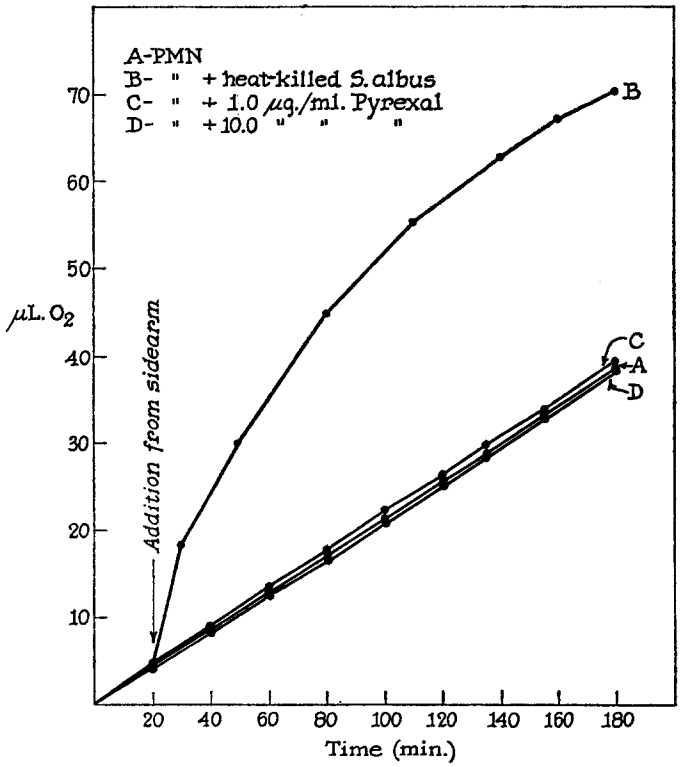

FIG. 7. The effect of pyrexal and heat-killed *S. albus* on the oxygen consumption of polymorphonuclear leucocytes.

Time course studies revealed that endotoxin produced a linear increase in glucose utilization and lactic acid formation. The metabolic rates of the treated cells were always greater than the controls. In contrast, the activation of glycolysis in phagocyting leucocytes was always greatest during the period of maximal particle ingestion.

Fig. 10 shows the influence of similar concentrations of endotoxin on glycogen synthesis. Only minor quantitative effects were noted, but it was consistently observed that some depression of glycogen synthesis occurred, particularly with the higher doses of endotoxin.

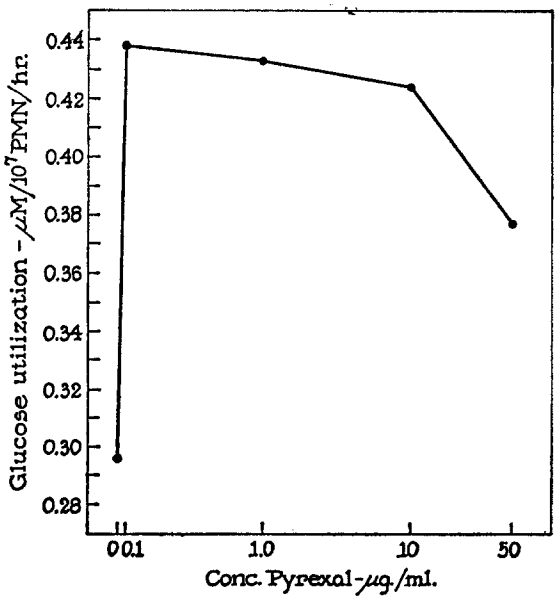

FIG. 8. The effect of pyrexal on the glucose utilization of leucocytes.

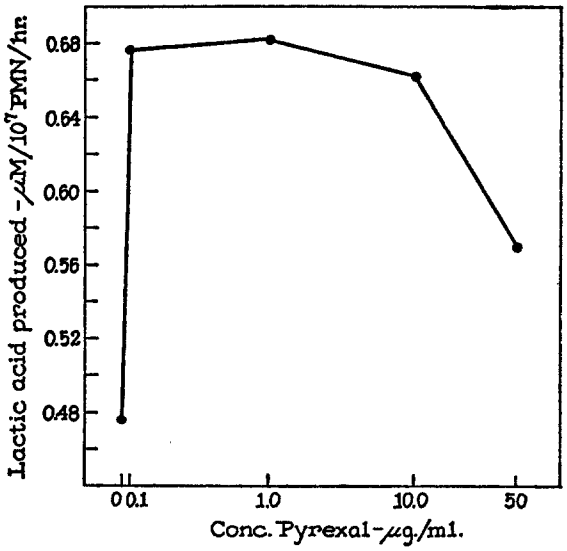

FIG. 9. The effect of pyrexal on the formation of lactic acid by leucocytes.

Only a limited number of experiments were conducted in the absence of serum. In three separate experiments it was found that pyrexal at a concentration of 1.0 μg./ml. had similar qualitative effects as in the presence of serum.

Untreated leucocytes in the absence of serum consumed 0.219 μM glucose/10^7 PMN/hr. whereas treated cells utilized 0.328 μM/10^7 PMN/hr. Complete dose response curves were not carried out in the absence of serum.

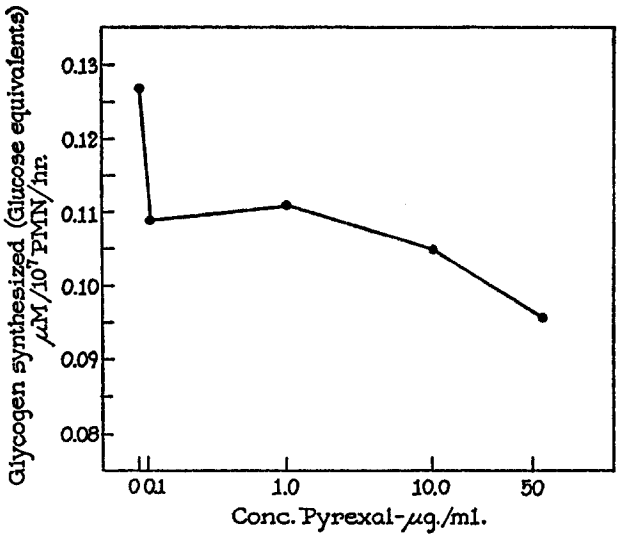

FIG. 10. The effect of pyrexal on the glycogen metabolism of leucocytes.

DISCUSSION

The present investigation has demonstrated that minute quantities of a lipopolysaccharide endotoxin have a direct influence on both the functional and metabolic properties of the rabbit polymorphonuclear leucocyte. These effects were discernible by increased phagocytic activity of the cells and by the stimulation of aerobic glycolysis. The mechanisms underlying these reactions are not clear. Previous investigators have observed that the *in vitro* effects of endotoxin on blood leucocytes are associated with changes in the surface properties of the cells. Bergmann *et al.* (16) have described increased electrophoretic mobility of endotoxin-treated leucocytes, and Meier and Schär (17) have shown more rapid migration of leucocytes from plasma clots. A related *in vivo* phenomenon may be the increased adhesiveness of circulating leucocytes following the intravenous injection of endotoxin. Such effects, as well as those reported in the present study, are brought about by similar concentrations of lipopolysaccharide. It is not known whether these alterations can be produced by a direct interaction with the cell surface or whether the endotoxins must penetrate the cell. In any event, the expression of the lipopolysaccharide effect appears to be on the surface activity of the leucocyte and/or on the properties of its limiting membrane.

The association between enhanced phagocytosis and an increased rate of glycolysis has been commented upon previously in reference to the ingestion of heat-killed bacteria. A similar relationship is also true for the endotoxin-treated leucocyte. Whether this metabolic stimulation is an expression of increased membrane permeability or a direct enzymatic interaction is unknown. Future experiments with cell-free homogenates of leucocytes may resolve this question. Although the increased permeability of the cell to substrate has not been established, it is clear that endotoxin-treated leucocytes liberate intracellular products into the medium. Such effects were first reported by Kerby (18), who showed the release of a lysozyme-like enzyme from blood leucocytes. More recently, Collins and Wood (19) have demonstrated the *in vitro* liberation of endogenous pyrogen from similarly treated rabbit peritoneal exudate polymorphonuclear leucocytes. In a sense, many of the responses of leucocytes to endotoxin may be considered a form of cell "injury," and ultimately quite non-specific in etiology. Nevertheless, as a consequence of both particle ingestion and endotoxin treatment, such "injury" is associated with enhanced functional activity.

The stimulation of particle ingestion in the endotoxin-treated animal is apparently more complex than in these *in vitro* conditions. Many other factors may serve to yield apparent increases in phagocytic activity without being associated with a direct influence upon the phagocytes. One of the more important of these factors is the appearance of opsonins in the serum. The recent studies of Whitby and Rowley (20) suggest that the sera of mice which have been injected with endotoxin exhibit an increased capacity to opsonize certain Gram-negative bacilli. It seems possible, therefore, that the *in vivo* administration of endotoxin can result in both the direct stimulation of the phagocyte, as well as the production of "specific" and/or "non-specific" opsonic materials. Both effects would act in a complementary fashion and thereby enhance the efficiency and rate of phagocytosis.

SUMMARY

The effects of a purified bacterial lipopolysaccharide endotoxin on homogenous populations of rabbit polymorphonuclear leucocytes have been studied *in vitro* under defined conditions. Employing a 500-fold range of concentration (0.1 to 50.0 μg./ml.), it was shown that endotoxin enhanced the rate at which staphylococci were killed by leucocytes. The mechanism underlying the increased killing was found to be a direct stimulation of the phagocytic activity of the leucocyte and not mediated by the release of bactericidins or opsonins from the treated cells. In the presence of 10 per cent serum all concentrations of endotoxin enhanced phagocytosis, whereas at lower serum concentrations, the higher doses of lipopolysaccharide inhibited the phagocytic activity of the cells.

Similar concentrations of endotoxin were capable of increasing the utilization of glucose and the production of lactic acid. Endotoxin treated leucocytes exhibited no change in oxygen consumption, and only a slight depression in glycogen synthesis.

It appeared that endotoxin could interact and alter the functional and metabolic properties of leucocytes in the absence of serum.

The demonstration of enhanced phagocytic activity of endotoxin-treated cells was dependent upon the particular opsonic requirements for the organism under study.

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