INFECTION AND IMMUNITY, Nov. 1980, p. 421-426
0019-9567/80/11-0421/06$02.00/0

Vol. 30, No. 2

Effect of Extracellular Serum in the Stimulation of Intracellular Killing of Streptococci by Human Monocytes

P. C. J. LEIJH, TH. L. VAN ZWET, AND R. VAN FURTH*

Department of Infectious Diseases, University Hospital, 2333 AA Leiden, The Netherlands

This study shows that the intracellular killing of *Streptococcus pyogenes*, *Streptococcus faecalis*, and *Streptococcus pneumoniae* by human monocytes is stimulated by the extracellular presence of both heat-stable and heat-labile serum factors. A similar kind of stimulation of monocytes has been described in respect of catalase-positive microorganisms. However, killing of these bacteria is negligible in the absence of extracellular serum factors, whereas a large proportion of the ingested catalase-negative bacteria are killed in the absence of such extracellular stimuli. Monocytes from patients with chronic granulomatous disease, which are unable to kill *Staphylococcus aureus* even in the presence of extracellular serum, killed *S. pyogenes* equally effectively whether serum was present or absent. This index proved to be the same as that for killing by monocytes of healthy subjects in the absence of serum. Taken together, these results indicate that catalase-negative microorganisms possess some kind of suicide mechanism that leads to the death of these bacteria after their ingestion by monocytes in the absence of an extracellular stimulus. Furthermore, the mechanism by which extracellular serum stimulates intracellular killing probably involves enzymes of the O_2-dependent bactericidal mechanisms of the monocytes.

Recently, we showed that extracellular serum factors are required to stimulate the intracellular killing of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Escherichia coli* by human monocytes (5).

In patients with chronic granulomatous disease (CGD), severe infections with catalase-positive microorganisms are seen more frequently than infections with catalase-negative bacteria (8). The abnormal course of infections with catalase-positive microorganisms is due to a bactericidal defect in the granulocytes and monocytes of these patients; ingestion of the bacteria is normal (1, 2, 4). This bactericidal defect is correlated with a defect in the oxidative metabolism and with the production of bactericidal oxygen products (e.g., H_2O_2) that are obligatory for maximal killing of ingested bacteria (3, 10). It has been postulated that the catalase-negative microorganisms produce H_2O_2 and that because of the lack of catalase, the ingested bacteria are killed by an interaction of this H_2O_2 with the myeloperoxidase of the phagocytes (3, 6, 7, 9, 11).

The stimulation of intracellular killing by extracellular serum factors has been demonstrated so far only for three catalase-positive microorganisms (5). The question arose whether a distinction similar to that between catalase-positive and catalase-negative microorganisms in the bactericidal activity of CGD granulocytes and monocytes exists for the extracellular stimulation of the intracellular killing of these microorganisms by normal monocytes. In the present study the effect of extracellular serum on the intracellular killing of *Streptococcus pyogenes*, *Streptococcus faecalis*, and *Streptococcus pneumoniae* by human monocytes was investigated.

MATERIALS AND METHODS

Monocytes of healthy subjects and patients with CGD. Monocytes were collected by differential centrifugation of blood of healthy donors on Ficoll-Hypaque as described earlier (14). A suspension of 10^7 monocytes per ml in Hanks balanced salt solution (HBSS) plus 0.1% (wt/vol) gelatin was prepared (gelatin-HBSS). The same procedures also were applied to cells from four patients diagnosed as having CGD because their granulocytes and monocytes were unable to kill *S. aureus* in the presence of serum and showed diminished O_2 consumption and H_2O_2 production during phagocytosis.

Microorganisms. *S. faecalis*, *S. pyogenes*, *S. pneumoniae*, and *S. aureus* (type 42D) were isolated from clinical material. All streptococci were catalase negative and were not killed in the presence of fresh serum during 2 h of incubation at 37°C in 100% serum. The microorganisms were held on blood agar plates and transferred every other day. Before use, the bacteria were cultured overnight in Nutrient Broth no. 2 (Oxoid Ltd., London, England) at 37°C, harvested by centrifugation at 1,500 × *g* for 10 min, and washed twice with phosphate-buffered saline. Finally, the microorganisms were suspended in gelatin-HBSS to a concen-

tration of 10^7/ml. In the phagocytosis and killing assays, the number of colony-forming units was determined on blood agar plates.

Serum. Serum of healthy donors with blood group AB was used throughout the experiments. The blood was allowed to clot for 1 h at room temperature, centrifuged for 20 min at 1,100 × *g*, and stored in 2-ml aliquots at −20°C. Heat-inactivated serum was obtained by a 30-min incubation of serum at 56°C. Heat-inactivated serum adsorbed with streptococci to remove specific antibodies to these microorganisms was prepared as described elsewhere (5).

Preopsonization of microorganisms. Preopsonized bacteria were obtained by incubation of 5×10^6 bacteria per ml in gelatin-HBSS with 10% serum for 25 min at 37°C under rotation (4 rpm). The bacteria were then washed twice with HBSS and suspended to a concentration of 10^7/ml in gelatin-HBSS.

Phagocytosis assay. The phagocytosis of bacteria was measured as a decrease in the number of viable extracellular bacteria, as previously described (14). Phagocytosis at a given time point (t) was expressed as the percent decrease in the initial number of viable extracellular bacteria according to the formula $F(t) = ([N_0 - N_t]/N_0) \times 100$, in which N_0 is the number of viable extracellular bacteria at zero time and N_t is the number of viable extracellular bacteria at time t.

Killing assay. The intracellular killing of the three species of microorganisms was determined as described elsewhere (5, 14). In brief, after 3 min of phagocytosis of preopsonized bacteria at 37°C, the noningested bacteria were removed by differential centrifugation (4 min at 110 × *g*) and two washes at 4°C. Next, monocytes containing ingested bacteria were incubated at 37°C, and after lysis of the monocytes the number of viable intracellular bacteria was determined at various time points. The level of intracellular killing at a given time point (t) was expressed as the percent decrease in the number of viable intracellular bacteria according to the formula: $K(t) = ([N_0 - N_t]/N_0) \times 100\%$, in which N_0 is the number of viable intracellular bacteria at zero time and N_t is the number of viable intracellular bacteria at time t.

Statistical analysis. All values in the text and tables represent the mean and standard deviation of at least four experiments. The standard deviations of the killing indices ranged from 3 to 15% of the observed values. Statistical analysis was performed with Student's t test.

RESULTS

Phagocytosis of streptococci by monocytes. First, the effect of serum on the ingestion of streptococci by monocytes was investigated. Incubation of 5×10^6 monocytes per ml and 5×10^6 bacteria per ml in HBSS at 37°C under rotation (4 rpm) gave no decrease in the number of viable extracellular bacteria (Fig. 1), which indicates that no appreciable phagocytosis of streptococci occurs in the absence of serum. Incubation of monocytes and streptococci under the same conditions but in the presence of 10% serum resulted in a rapid decrease in the number of viable extracellular bacteria for all three bacterial species studied (Fig. 1). Since incubation of bacteria plus 10% serum led to an increase in the number of viable bacteria (Fig. 1), it could be concluded that this decrease was not due to a bactericidal effect of serum on the bacteria.

From these findings it may be concluded that the ingestion of all three species of *Streptococcus* requires the opsonization by serum factors.

Number of viable intracellular bacteria after phagocytosis. The number of viable intracellular bacteria was determined during incubation of 5×10^6 monocytes per ml with 5×10^6 preopsonized bacteria at 37°C under rotation to determine the duration of the period of ingestion giving the maximum number of viable intracellular bacteria, as measured after lysis of the monocytes. The results show that for all three bacterial species a maximum yield of viable cell-associated bacteria is obtained after incubation periods of about 5 to 10 min (Fig. 2). Phagocytosis of preopsonized streptococci at a bacteria-to-monocyte ratio of 1:1 in the presence of phenylbutazone (2 mg/ml), a drug which has almost no effect on the rate of phagocytosis but inhibits intracellular killing, (3, 12, 13, 15), resulted in an initial increase in the number of viable intracellular bacteria during the first 5 min (Fig. 2). The difference between the numbers of viable intracellular bacteria determined after incubation with and without phenylbutazone represents the number of bacteria killed during the ingestion period. Since this difference was virtually nil during the first few minutes of phagocytosis, we decided to perform our killing assay after an ingestion period of 3 min.

Effect of serum on the intracellular killing of streptococci by monocytes. To find out whether extracellular serum is obligatory for the intracellular killing of catalase-negative microorganisms, monocytes containing streptococci (after phagocytosis for 3 min) were incubated in the presence and absence of extracellular serum. After phagocytosis of *S. pyogenes* at a bacteria-to-monocyte ratio of 1:1, incubation of monocytes in the presence of 10% serum led to a rapid decrease in the number of viable intracellular bacteria (Fig. 3). Incubation of monocytes containing ingested *S. pyogenes* in the presence of gelatin-HBSS gave killing indices of 61.0 and 61.9% at 60 and 120 min, respectively, which are significantly lower ($P < 0.05$) than the indices obtained in the presence of serum (Fig. 3). Incubation of monocytes containing ingested bacteria in the presence of heat-inactivated serum resulted in indices between those obtained in the presence and absence of serum (Fig. 3). The number of viable intracel-

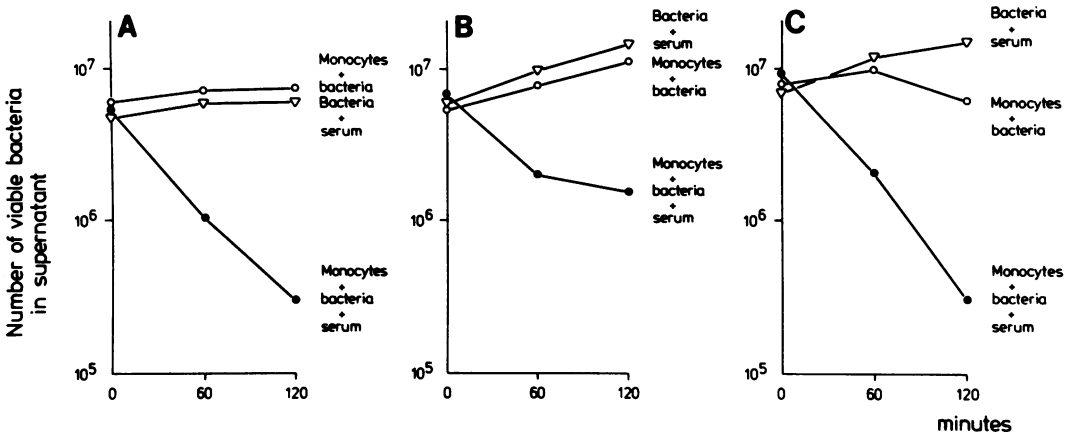

FIG. 1. *Kinetics of the phagocytosis of (A) S. pyogenes, (B) S. faecalis, and (C) S. pneumoniae by human monocytes. Monocytes (5 × 10^6/ml) were incubated with 5 × 10^6 streptococci per ml and 10% serum at 37°C under rotation (●). No decrease in the number of viable extracellular bacteria occurred when serum was absent (○) or where the bacteria were incubated with 10% (▽) serum without monocytes.*

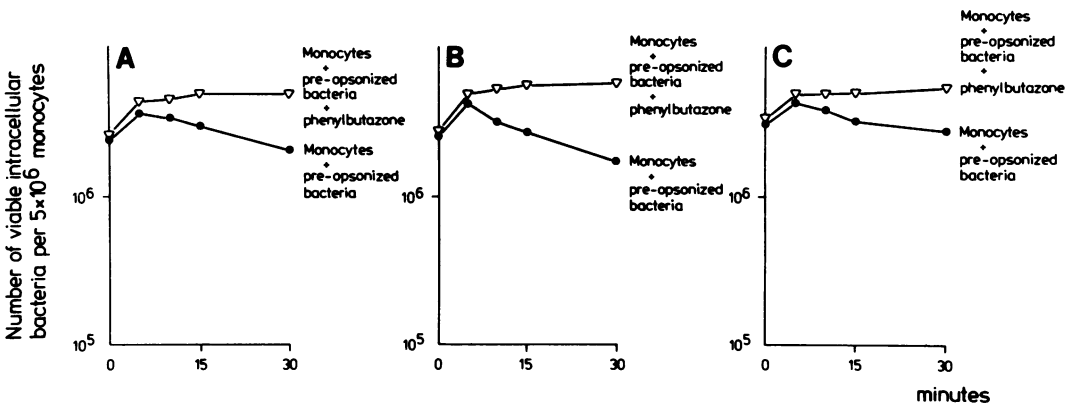

FIG. 2. *Number of viable intracellular (A) S. pyogenes, (B) S. faecalis, and (C) S. pneumoniae after the phagocytosis of preopsonized bacteria alone (●) and in the presence of 2 mg of phenylbutazone per ml (▽). Phagocytosis occurred at a bacteria-to-monocyte ratio of 1:1.*

lular bacteria showed a decrease in the presence of adsorbed and inactivated serum similar ($P > 0.4$) to that found with inactivated serum, which indicates that specific opsonic antibodies are not required for the stimulation of the killing process (Fig. 3). These results show that the intracellular killing of *S. pyogenes* is stimulated by both heat-labile and heat-stable extracellular serum factors.

Incubation of monocytes containing ingested *S. pyogenes* after phagocytosis at a bacteria-to-monocyte ratio of 10:1 gave similar results: a higher intracellular killing index in the presence of serum compared with incubation without serum (Fig. 3).

To see whether the effect of serum found for the intracellular killing of *S. pyogenes* is similar for the killing of other catalase-negative microorganisms by monocytes, the intracellular killing of *S. faecalis* and *S. pneumoniae* was measured. Incubation of monocytes containing ingested *S. faecalis* after phagocytosis at a bacteria-to-monocyte ratio of 1:1 in the presence of 10% fresh serum, 10% inactivated serum, and 10% adsorbed inactivated serum and without serum gave a maximum decrease in the number of viable intracellular bacteria after incubation in the presence of fresh serum (Fig. 4). This decrease is significantly higher than those obtained in the absence of serum ($P < 0.01$) and in the presence

of inactivated or adsorbed-inactivated serum ($P < 0.05$) (Fig. 4). After phagocytosis at a bacteria-to-monocyte ratio of 10:1 instead of 1:1, the rate of killing of *S. faecalis* by monocytes in the presence of serum was higher than that in the absence of serum (Fig. 4).

Incubation of monocytes containing *S. pneumoniae*, after phagocytosis at bacteria-to-monocyte ratios of 1:1 and 10:1, in the presence of serum gave a significantly greater ($P < 0.05$) decrease in the number of viable intracellular bacteria than that in the absence of serum. The intracellular killing in the presence of extracellular inactivated serum or adsorbed inactivated serum was similar to the killing in the absence of serum (Fig. 4).

From these results we may conclude that the catalase-negative microorganisms investigated are killed by monocytes to a certain extent but that maximum killing requires stimulation of the monocytes by extracellular serum.

Effect of serum on the intracellular killing of *S. aureus* and *S. pyogenes* by monocytes of patients with CGD. Monocytes of four patients with CGD were first investigated with respect to their ability to kill *S. aureus* in the presence and absence of extracellular serum. The number of *S. aureus* ingested by CGD monocytes after 3 min of incubation (Table 1) was in the range for normal monocytes (5). The negligible intracellular killing of *S. aureus* by CGD monocytes was virtually not stimulated by extracellular serum (Table 1) compared with the effect on normal monocytes, which resulted in the killing of about 80% of the intracellular *S. aureus* (Table 2).

The mean number of viable intracellular *S. pyogenes* after incubation of CGD monocytes and preopsonized bacteria for 3 min at 37°C (Table 3) was similar to the value after ingestion by normal monocytes under similar conditions. This indicates normal ingestion of *S. pyogenes* by CGD monocytes. Re-incubation of these CGD monocytes containing *S. pyogenes* in the presence of serum at 37°C gave an average killing index of 67.0% at 60 min, which does not differ ($P > 0.2$, paired observations) from the killing index obtained during the re-incubation in the absence of extracellular serum (Table 3).

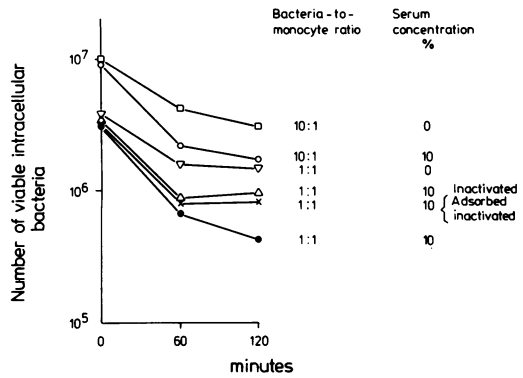

FIG. 3. *Intracellular killing by human monocytes after 3 min of ingestion of preopsonized S. pyogenes at bacteria-to-monocyte ratios of 1:1 and 10:1. The killing assay was performed without serum (▽, n = 10; □, n = 4) and with 10% serum (●, n = 10; ○, n = 4), 10% inactivated serum (△, n = 4), and 10% adsorbed inactivated serum (×, n = 4).*

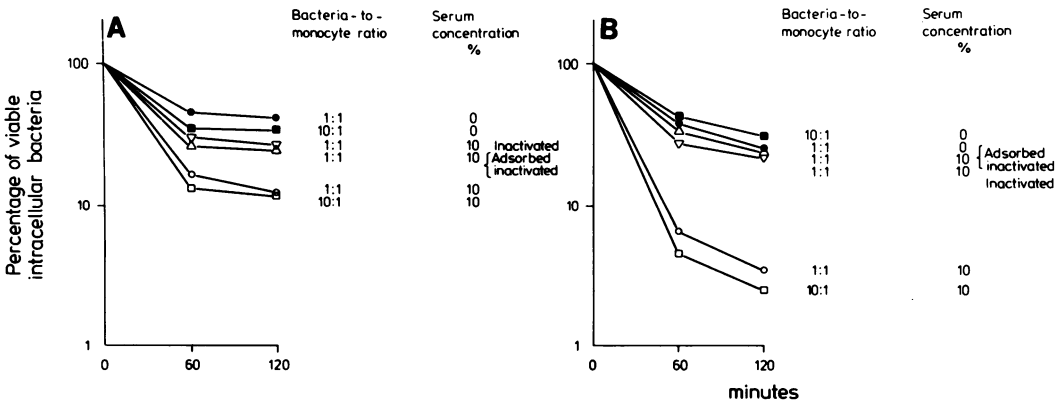

FIG. 4. *Intracellular killing of (A) S. faecalis and (B) S. pneumoniae by human monocytes. The killing assay was performed, after phagocytosis for 3 min at bacteria-to-monocyte ratios of 1:1 and 10:1, in the presence of 0, 10, 10% inactivated and 10% adsorbed serum. (Each point represents four experiments, except killing in the presence of 0 or 10% normal serum after phagocytosis at a bacteria-to-monocyte ratio of 1:1 for S. faecalis [n = 10] and for S. pneumoniae [n = 7].)*

DISCUSSION

The rate of intracellular killing of *S. pyogenes*, *S. faecalis*, and *S. pneumoniae* by human monocytes proved to be dependent on the presence of extracellular serum. In the absence of serum, about 50 to 70% of the ingested bacteria were killed, whereas when fresh serum was present extracellularly during the killing phase, maximum killing of ingested streptococci was obtained. Since heat-inactivated serum gave only a slight stimulation of intracellular killing compared with the effect of fresh serum, a stimulation by heat-labile serum factors, probably complement components, is indicated (5). The fact that adsorbed heat-inactivated serum, which lacks opsonic activity for the various microorganisms under study, stimulated intracellular killing as much as heat-inactivated serum did, makes stimulation by specific opsonic antibodies unlikely. Furthermore, the results obtained with absorbed heat-inactivated serum make it very unlikely that the increase of the killing index in the presence of serum is due to the ingestion of cell-associated bacteria at the onset of the killing assay.

TABLE 1. *Phagocytosis and intracellular killing of S. aureus by monocytes of patients with CGD*

Patient no.	No. of viable intracellular *S. aureus*[a]	Intracellular killing (%) at 60 min[b]	
		In the absence of serum	In the presence of serum
1	2.1×10^6	3.2	13.1
2	9.1×10^5	6.7	13.3
3	9.3×10^5	0.0	0.0
4	1.3×10^6	4.3	6.2
Avg	1.3×10^6	3.5 ± 2.8	8.2 ± 6.4

[a] A total of 5×10^6 monocytes per ml was incubated with 5×10^6 *S. aureus* and 10% serum for 3 min at 37°C under rotation.

[b] Expressed as percent decrease in the number of viable intracellular bacteria.

Comparison of the results of the present study with our earlier findings on the effect of extracellular serum on the intracellular killing of catalase-positive microorganisms (5) showed a striking difference in the killing indices between catalase-positive and catalase-negative microorganisms during incubation in the absence of extracellular serum (Table 2). Whereas there was almost no killing of ingested catalase-positive microorganisms under these conditions, a large proportion of the catalase-negative microorganisms were killed. The difference between the killing indices obtained in the presence and absence of extracellular serum shows that for catalase-positive microorganisms the addition of extracellular serum results in an increase of the killing index by on average 75%, whereas for catalase-negative microorganisms this increase is on the average only 25%.

Monocytes and granulocytes from CDG patients cannot kill catalase-positive microorganisms, but can eliminate catalase-negative species (1, 2, 6). The explanation of this discrepancy is probably that H_2O_2, which is normally produced by the phagocytes during phagocytosis and is necessary for optimal functioning of the myeloperoxidase-peroxide bactericidal system, is lacking in CGD phagocytes but is formed by the catalase-negative microorganisms itself (4, 6, 7, 11). In the present study the similar indices obtained for the killing of *S. pyogenes* by monocytes of CGD patients as well as monocytes of healthy donors in the absence of extracellular serum supports this view and indicates a kind of suicide of the microorganisms, since the H_2O_2 formed by the bacteria interacting with the my-

TABLE 2. *Intracellular killing of microorganisms by monocytes after phagocytosis at a bacteria-to-monocyte ratio of 1:1*[a]

Microorganism	Catalase production by microorganisms	No. of expt	Intracellular killing (%) at 60 min[b]		Differences (B − A)
			In the absence of serum (A)	In the presence of serum (B)	
S. aureus[c]	+	20	−6.0 ± 21.0	75.3 ± 9.5	81.3
S. epidermidis[c]	+	5	26.3 ± 5.6	91.3 ± 8.1	65.0
E. coli[c]	+	9	15.1 ± 12.0	93.2 ± 3.0	78.1
S. pyogenes	−	10	61.0 ± 7.7	79.3 ± 13.0	18.3
S. faecalis	−	10	53.1 ± 15.2	82.8 ± 6.3	29.7
S. pneumoniae	−	7	68.8 ± 13.1	93.6 ± 2.3	24.8

[a] Phagocytosis for 3 min at 37°C.

[b] Expressed as percent decrease in the initial number of viable intracellular bacteria with standard deviations.

[c] Data taken from reference 5.

TABLE 3. *Phagocytosis and intracellular killing of S. pyogenes by monocytes of patients with CGD*

Patient no.	No. of viable intracellular *S. pyogenes*[a]	Intracellular killing (%) at 60 min[b]	
		In the absence of serum	In the presence of serum
1	9.0×10^5	52.8	71.1
2	1.3×10^6	54.6	74.0
3	1.9×10^6	65.4	62.9
4	1.8×10^6	68.4	61.0
Avg	1.5×10^6	60.3 ± 7.8	67.0 ± 6.3

[a] A total of 5×10^6 monocytes per ml was incubated with 5×10^6 *S. pyogenes* and 10% serum for 3 min at 37°C under rotation.

[b] Expressed as percent decrease in the number of viable intracellular bacteria.

eloperoxidase resulted in the killing of these catalase-negative bacteria. Since catalase-positive species destroy produced H_2O_2, such a mechanism is unlikely for this group of microorganisms.

The similarity of the indices for the killing of *S. pyogenes* by CGD monocytes in the presence and absence of extracellular serum, as well as the fact that extracellular serum does not stimulate the intracellular killing of *S. aureus* by CGD monocytes, suggests that the stimulation of intracellular killing by extracellular serum probably involves components of the O_2-dependent bactericidal system of the phagocytes.

ACKNOWLEDGMENTS

This study was partially supported by the J. A. Cohen Institute of Radiopathology and Radiation Protection. The cooperation of C. Weemaes (Pediatric Clinic, University Hospital, Nijmegen, The Netherlands), R. Schuurman (Sophia Kinderziekenhuis, Rotterdam, The Netherlands), and R. Weening (Pediatric Clinic, Binnengasthuis, Amsterdam, The Netherlands), made it possible to study monocytes of CGD patients. The metabolic studies done in these CGD patients were performed by D. Roos (Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands).

LITERATURE CITED

1. **Holmes, B., A. R. Page, and R. A. Good.** 1967. Studies of the metabolic activity of leucocytes from patients with a genetic abnormality of phagocytic function. J. Clin. Invest. **46**:1422-1432.
2. **Kaplan, E. L., T. Laxdall, and P. G. Quie.** 1968. Studies of polymorphonuclear leucocytes from patients with chronic granulomatous disease of childhood; bactericidal capacity for streptococci. Pediatrics **41**:591-599.
3. **Klebanoff, S. J.** 1970. Myeloperoxidase-mediated antimicrobial systems and their role in leucocyte function, p. 89-110. *In* E. Schultz (ed.), Biochemistry of the phagocytic process: localization and the role of myeloperoxidase and the mechanisms of the halogenation reaction. North Holland Publishing Co., Amsterdam.
4. **Klebanoff, S. J., and L. R. White.** 1969. Iodination defect in the leucocytes of a patient with chronic granulomatous disease of childhood. N. Engl. J. Med. **280**: 460-466.
5. **Leijh, P. C. J., M. Th. van den Barselaar, Th. van Zwet, M. R. Daha, and R. van Furth.** 1979. Requirement of extracellular complement and immunoglobulin G for intracellular killing of micro-organisms by human monocytes. J. Clin. Invest. **63**:772-784.
6. **Mandell, G. L., and E. W. Hook.** 1969. Leucocyte bactericidal activity in chronic granulomatous disease: correlation of bacteria hydrogen peroxide production and susceptibility to intracellular killing. J. Bacteriol. **100**: 531-532.
7. **Pitt, J., and H. P. Bernheimer.** 1974. Role of peroxide in phagocytic killing of pneumococci. Infect. Immun. **9**: 48-52.
8. **Quie, P. G.** 1972. Clinical manifestations of chronic granulomatous disease of childhood: a congenital defect in phagocyte function, p. 139-150. *In* R. C. Willimans and H. D. Fudenberg (ed.), Phagocyte mechanisms in health and disease. Intercontinental Medical, New York.
9. **Repine, J. E., J. G. White, C. C. Clawson, and B. M. Holmes.** 1974. Effects of phorbol myristate acetate on the metabolism and ultrastructure of neutrophils in chronic granulomatous disease. J. Clin. Invest. **54**:83-90.
10. **Root, R. K., and T. P. Stossel.** 1974. Myeloperoxidase-mediated iodination by granulocytes: intracellular site of operation and some regulating factors. J. Clin. Invest. **53**:1207-1215.
11. **Shohet, S. B., J. Pitt, R. L. Baehner, and D. G. Poplack.** 1974. Lipid peroxidation in the killing of phagocytized pneumococci. Infect. Immun. **10**:1321-1328.
12. **Solberg, C. O.** 1975. Influence of therapeutic concentrations of phenylbutazone on granulocyte function. Acta Pathol. Microbiol. Scand. Sect. B **83**:100-102.
13. **Strauss, R. R., B. B. Paul, and A. J. Sbarra.** 1968. Effect of phenylbutazone, an anti-inflammatory agent, on phagocytosis and intracellular killing by guinea pig polymorphonuclear leucocytes. J. Bacteriol. **96**:1982-1990.
14. **van Furth, R., T. van Zwet, and P. C. J. Leijh.** 1978. In vitro determination of phagocytosis and intracellular killing by polymorphonuclear and mononuclear phagocytes, p. 32.1-32.19. *In* D. M. Weir (ed.), Handbook of experimental immunology. Blackwell Scientific Publications, Oxford.
15. **van Schaik, M. L. J., R. S. Weening, and D. Roos.** 1979. Phenylglyoxal is not a selective inhibitor of phagocytosis. J. Cell. Sci. **38**:331-343.