ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1975, p. 736-741

Vol. 7, No. 6
Printed in U.S.A.

Effects of Cytochalasin B on the Intracellular Bactericidal Activity of Human Neutrophils

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Received for publication 18 February 1975

Cytochalasin B (CB) is known to have some inhibitory effects on cytokinesis, single-cell movement, bacterial uptake by phagocytes, and many other processes. The effects of CB on intraleukocytic bactericidal activities in human leukocytes were studied, and the results were summarized as follows. (i) CB inhibited the early stage of the intracellular bactericidal activity of human leukocytes against *Streptococcus pyogenes* D58 (group A). The effect was rapidly eliminated by rinsing the CB solution. (ii) In the late stage of the intracellular bactericidal process, however, CB possessed no effect against *S. pyogenes* D58 (group A) and *Staphylococcus aureus* 209P. (iii) CB also inhibited the translocation of myeloperoxidase granules to the phagosomes of human neutrophils.

Phagocytosis of microbes by neutrophils proceeds by four steps: chemotaxis, attachment, endocytosis, and intracellular digestion. Chemotaxis and endocytosis are the adenosine triphosphate-dependent steps resulting from anaerobic glycolysis (10). The next step, the intracellular bactericidal or digesting process, induces the enzyme burst in aerobic glycolysis (8). In this step, intraphagosomal microbes may be attacked by many lysosomal or peroxisomal enzymes. The precise mechanism of the intracellular bactericidal steps, however, is still uncertain (10).

Cytochalasin B (CB) has been reported to have inhibitory effects on cytokinesis (4), single-cell movement (2, 18, 22), and many other processes (20, 21) by inhibiting the movement of contractile microfilaments (15, 17, 23) and by inhibiting the uptake of glucose (5, 24, 25). But CB has no inhibitory effect on certain cell metabolic activities such as respiration (19) and incorporation of labeled precursors into protein, nucleic acid, or lipids (20, 21). Regarding phagocytosis, many investigators (1, 3) have already reported that CB decreases phagocytosis by inhibiting bacterial uptake. However, the effect of CB on various steps of intracellular bactericidal processes is still uncertain. In this investigation, the effect of CB on various steps of intracellular bactericidal processes was studied first.

It has strongly suggested that bactericidal system composed of myeloperoxidase (MPO), halides, and H_2O_2 has an important role in intraleukocytic bactericidal process (10). In addition, as the intracellular organella of contractile microfilaments has been suggested to regulate intracellular granular translocation (1), the effect of CB on MPO-granular translocation after phagocytosis was also studied in this investigation.

MATERIALS AND METHODS

Organisms. Overnight cultures of *Staphylococcus aureus* 209P in trypto-soy broth (Eiken Chemicals, Japan) and *Streptococcus pyogenes* (group A) in modified Todd-Hewitt media, as well as 72-h cultures of *Candida albicans* ATCC 401 in Sabouraud's agar, were centrifuged at 800 × *g* for 10 min. Then each of the pellets was washed three times with Hanks balanced salt solution (HBSS) and the cell number was adjusted to 10^8/ml in TC 199 containing 20% freshly frozen human sera.

Collection of leukocytes. Peripheral blood from a healthy person was collected in a heparinized syringe and placed in a siliconized tube containing a solution of 6% dextran in physiological saline. After 1 h at room temperature, the leukocyte-rich plasma was withdrawn and the leukocytic fraction was collected. Then the cells were washed twice and the leukocytic number was adjusted to 2×10^6 cells/ml in TC 199 containing 20% freshly frozen human sera. The fraction obtained in this way contained approximately 60% neutrophils.

When this suspension was used as the leukocytic homogenate, residual leukocytes were further removed by osmotic shock in sterilized distilled water for 30 s followed by the addition of an NaCl solution of sufficient strength to make the final concentration 0.9% NaCl.

Intracellular bactericidal test. Intracellular bactericidal test was performed by a modified Holmes method (7, 13). Reaction mixtures composed of 0.5 ml of leukocytic suspension and 0.1 ml of staphylococcal or streptococcal suspension were put into a siliconized tube, and phagocytosis was initiated by incubating

the reaction mixtures in a 5% CO_2 atmosphere at 37 C. After 5 min, 10 ml of HBSS was added to the reaction mixtures followed by slight pipetting. Then centrifugation at 50 × *g* for 5 min was carried out. This step was performed twice to remove extracellular unphagocytized bacteria. After washing, bacteria-containing leukocytes were resuspended in TC 199 containing 20% inactivated calf serum and antibiotics (penicillin, 100 U/ml, and streptomycin, 100 μg/ml).

CB was dissolved in ME_2SO and added to the leukocytic medium at a final concentration of 5 μg of CB per ml and 0.1% (vol/vol) ME_2SO. CB-treated or CB-free reaction mixtures were cultured in the above CO_2 incubator at given intervals (0, 15, 30, 60, 90, 120, and 150 min), and then the viability of bacteria in CB-treated and CB-free groups were assayed. After appropriate times, 0.2 ml of each reaction mixture was washed once with HBSS and the pellet was transfered to 1.0 ml of distilled water followed by vigorous pipetting. The viable bacterial number of the solution obtained in this way was measured by colony formation of the pour plate method.

Washing experiment. The reaction mixtures were composed of 0.5 ml of leukocytic suspension and 0.1 ml of streptococcal suspension. After a 5-min incubation period, 10 ml of HBSS was added to the reaction mixture and pipetted slightly. The mixture was then centrifuged at 50 × *g* for 5 min and the viable bacterial numbers of the supernatant were assayed by colony formation, as described above. Viable bacterial numbers of the supernatant from the second to the sixth washing were plotted (see Fig. 4).

MPO staining. The effect of CB on translocation of MPO granules was also studied. A reaction mixture composed of leukocytic suspension and 0.05 ml of *Candida* suspension was incubated to start phagocytosis. After 5 min, one group of leukocytes containing *Candida* was transferred to the medium containing CB (5 μg/ml) and another group to CB-free medium. After 30 min, leukocytes from these two groups were smeared and stained by the method of Sato and Sekiya (14). Briefly, the slide specimen was reacted with 0.5% cupric sulfate for 1 min. Then it was reacted upon 0.1% benzidin containing 5 μmol of hydrogen peroxide for 4 min. One percent safranin staining was performed for contrast.

Bactericidal test using leukocytic homogenate. The erythrocyte-free leukocytic fraction described above, containing 1.3×10^9 cells, was suspended in 2 ml of HBSS and was then homogenized (Marsan Homo Blender, Sakuma. Ltd.) for 60 min. Thereafter, the viable bacterial number was counted by colony formation.

RESULTS

Influence of CB on intracellular bactericidal effects. At first, the effect of CB on intraleukocytic bactericidal activity against *S. pyogenes* was examined at appropriate times (Fig. 1). In this experiment, CB treatment was stopped after 60 min by washing the leukocytes twice with CB-free medium. After rapid washing, the same medium was added and intraleukocytic bactericidal activity was again assayed. In the second group CB treatment was still continued. The third group represents the time course experiment of the intraleukocytic survival number of bacteria in which leukocytes received no CB treatment. As shown in this figure, intraleukocytic bactericidal activity is significantly inhibited by CB treatment. But this inhibitory effect seems to disappear rapidly by rinsing the CB solution.

The next experiment was performed to determine where (whether in translocation of the lysosomal and peroxisomal enzymes to the phagosome or in enzymatic release into phagosome) CB exerts an inhibitory effect on intraleukocytic bactericidal activity. Experimental conditions were the same as in Fig. 1. In this experiment, CB treatment was initiated 15 or 30 min later, compared with the broken line of the Fig. 1. Fifteen minutes after extracellular bacteria had been washed away CB inhibited bactericidal activity to a slighter degree than in the early CB treatment (Fig. 1 and 2). When CB treatment was performed 30 min after washing, some enhancement of intracellular bactericidal activity was observed. It seems likely from these results that CB decreases intraleukocytic bactericidal activity in the early stage but has no effect in the late stage.

The effect of CB on the intraleukocytic bactericidal activity against *S. aureus* was also studied. CB decreased the intraleukocytic bactericidal activity against *Staphylococcus* in the early stage but enhanced it in the late stage (Fig. 3). Though this pattern was similar to that

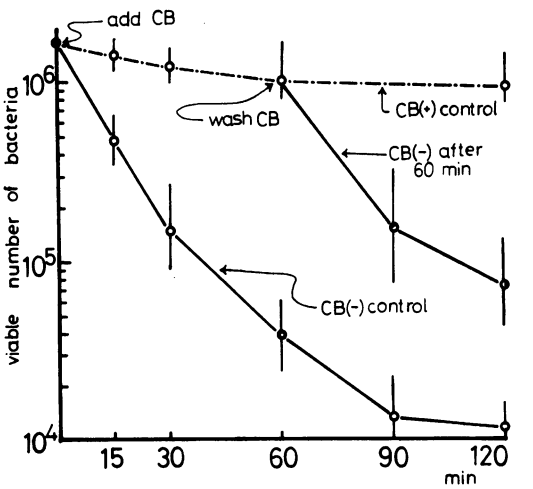

FIG. 1. *Effect of the intraleukocytic bactericidal activity against S. pyogenes. These lines represent the mean values of three experiments.*

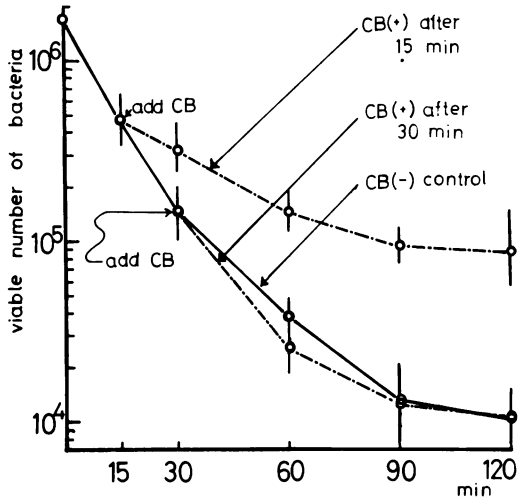

FIG. 2. *Comparison of intraleukocytic bactericidal activities among various stages of CB treatment against S. pyogenes. These data are obtained from three experiments.*

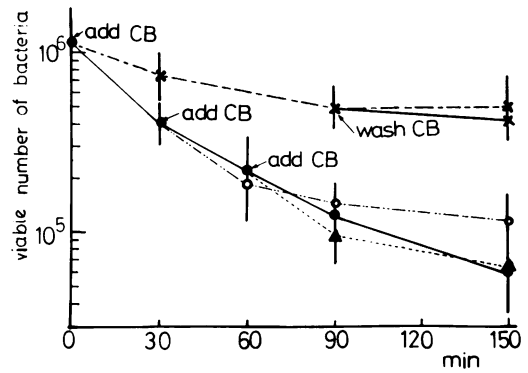

FIG. 3. *Effect of CB on the intraleukocytic bactericidal activity against S. aureus. Symbols: × (dashed line), add CB at 0 time; × (solid line) wash CB after 90 min; O, add CB after 30 min; ▲, add CB after 60 min; ●, CB-free control group. These lines represent the mean values of three experiments.*

of *Streptococcus*, for the most part *Staphylococcus* showed more resistant to the intraleukocytic bactericidal systems than *Streptococcus*. In Fig. 3, no significant difference was detectable by this technique between the CB-treated and CB-washed groups as was shown in Fig. 3.

Validity of intraleukocytic bactericidal test. Whether the method employed in this study only reveals CB as inhibiting the uptake rate of bacteria, as other investigators have reported (1, 3), remain to be investigated. If washing procedures are insufficient to rinse the extracellular unphagocytized or cell-binding bacteria, this method would not detect the intracellular bactericidal activity exactly. To determine the validity of the procedure, the viable number of bacteria in the washed supernatant was assayed. As shown in Fig. 4, viable numbers of bacteria reached a plateau by the third washing. In addition, I could barely find extracellular or cell-binding bacteria in the smeared and stained specimens of washed samples. To prevent further engulfing effect on unphagocytized bacteria by neutrophils, attention was also paid to the following points. After phagocytosis started, freshly frozen human serum (opsonin) was excluded from the medium and antibiotics were employed to kill extracellular unphagocytized bacteria. With regard to these deliberations, it is likely that CB (Fig. 1, 2, and 3) decreases intraleukocytic bactericidal activities rather than the bacterial uptake.

Effect of CB on MPO granular translocation. Klebanoff (9) proposed an intracellular bactericidal system composed of MPO, halides, and H_2O_2. I have also observed the importance of the translocation of MPO granules to the phagosome (13). In the next experiment, the effect of CB on this enzyme translocation was studied by means of histochemical MPO reaction. Figure 5a shows the accumulation of MPO granules to the phago-

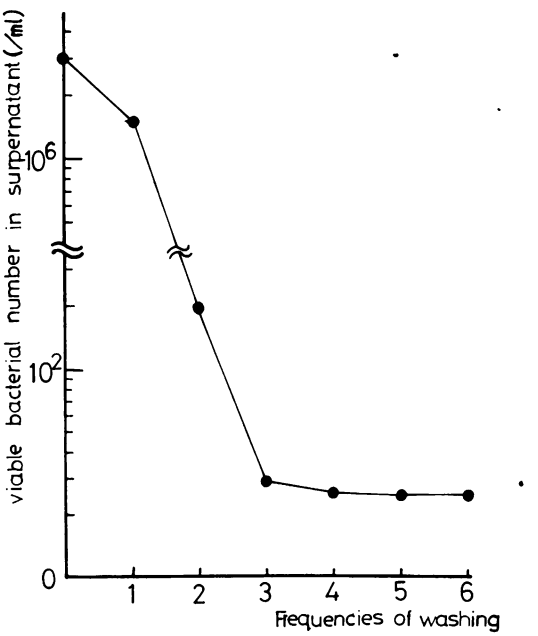

FIG. 4. *Viable bacterial numbers in washed solutions. The number of extracellular unphagocytized bacteria of each washed solution was measured and plotted.*

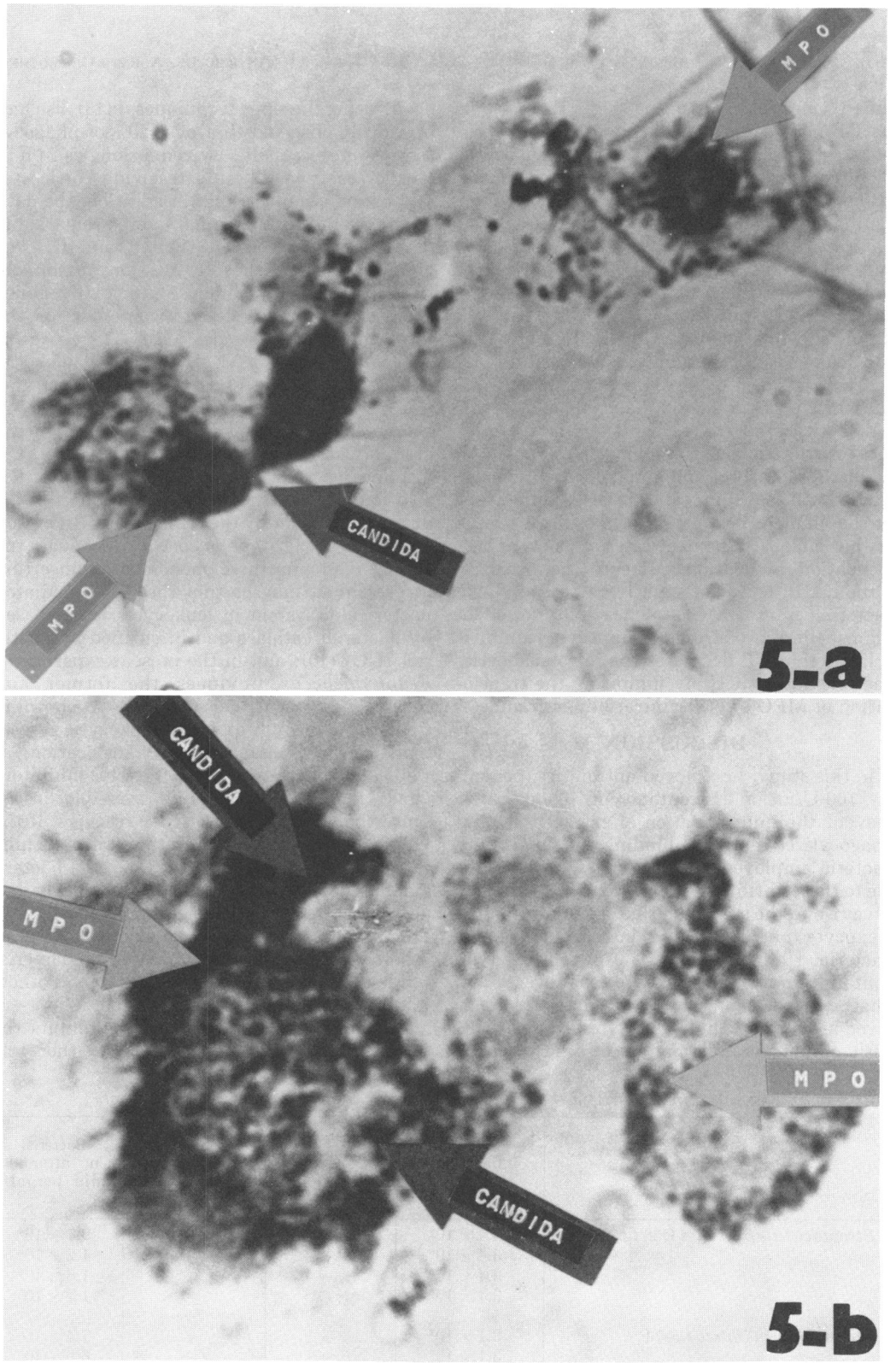

FIG. 5. (a) *MPO staining of neutrophils phagocytizing Candida albicans. No CB treatment was carried out in this group. Two neutrophils are phagocytizing three Candida spores. Dark grains represent MPO granules. Note the accumulation of MPO granules around the phagocytized spores.* ×640. (b) *CB-treated MPO staining of neutrophils phagocytizing C. albicans. A neutrophil is phagocytizing two candida spores. Dark grains represent MPO granules. Note that there is no accmulation of MPO granules around two phagocytized spores.* ×640.

some (*Candida*). This figure shows the data on the CB-nontreated control group which represents the normal step in the intraleukocytic microbiocidal process. The leukocytes in the CB-containing medium, however, showed no significant translocation of MPO granules in the majority of samples (Fig. 5b). From these results, it seems likely that CB significantly inhibits the translocation of MPO granules.

Effect of CB on bactericidal tests using a leukocytic homogenate. If CB decreases the intraleukocytic bactericidal activity by inhibiting the translocation of MPO or other intracellular granules, it should have no effect in a leukocytic homogenate. The effect of the mixture of the leukocytic homogenate and CB against streptococci and staphylococci was also studied. As shown in Table 1, CB had no effect on the viable cell numbers of these two strains in the leukocytic homogenate, after 60 min of incubation. Incubation of streptococci in HBSS without CB, however, slightly decreased viable bacterial number. CB alone, had no effect against these two strains. From these results, it is likely that CB decreases the intraleukocytic-bactericidal activity by inhibiting the translocation of MPO or other intracellular granules.

DISCUSSION

In this study, I employed antibiotics (penicillin, 100 U/ml, and streptomycin, 100 μg/ml) to prevent the multiplication of extracellular unphagocytized and cell-binding bacteria. Both bacteria employed in this study were susceptible to these antibiotics. However, this technique gave rise to the suspicion that antibiotics in the leukocyte might kill the intracellular viable bacteria. Holmes et al. (7), using the medium containing antibiotics, reported that over 80% of phagocytized bacteria (*S. aureus* 502A) survived as long as 24 h in leukocytes from patients with chronic granulomatous disease of childhood. Using the same techniques, I (13) also had previously observed that over 20% of phagocytized *S. aureus* 209P survived as long as 24 h in leukocytes from patients receiving corticosteroid or radiation therapy. In addition, the data presented in Fig. 1, 2, and 3 showed remarkable differences between CB-treated and CB-free groups. From these results, it seems reasonable to conclude that antibiotics in the extracellular fluid do not play a significant role in the intracellular bactericidal test. There was a slight difference between the intraleukocytic bactericidal effects of CB on the two strains (Fig. 2 and 3). CB had a weaker effect on *Staphylococcus* than on *Streptococcus*. In the case of *Streptococcus*, the influence of CB disappeared completely by the removal of CB solution. On the other hand, the influence of CB did not disappear by the removal of CB solution in the case of *Staphylococcus*. Although the precise reason for these phenomena is uncertain at present, it may be that the MPO-mediated bactericidal system in leukocytes is decreased by bacterial catalase and intensified by bacterial H_2O_2 (10); and in the present experiment, *Staphylococcus* produces the former and *Streptococcus* does the latter (13). As shown in this study, CB inhibits intraleukocytic lysosomal or peroxisomal enzyme translocation, as well as the bactericidal activity. This inhibitory effect, however, seems to be reversible, in the sense that bactericidal activity recovers. If the CB effect is from the pioncytized site, washing alone cannot rapidly recover the initial bactericidal activity. From these results it is likely that CB acts directly on a membrane receptor or membrane-binding organella (microfilament or others). Reversible effects of CB on bacterial uptake (11) as well as on cytokinesis and others (20, 21) have already been reported.

As shown in Fig. 2 and 3, CB inhibits the intracellular bactericidal activity in the early

TABLE 1. *Effect of CB on the bactericidal activity using the leukocyte homogenate*

Strains	Initial bacterial no. (per ml)	Added with:		Bacterial no. after 60 min (per ml)
		Leukocyte homogenate	CB (5 μg/ml)	
Streptococcus pyogenes D58 (group A)	3.4×10^6	+	+	9.7×10^4
	3.4×10^6	+	−	1.2×10^5
	3.4×10^6	−	+	1.6×10^5
	3.4×10^6	−	−	1.7×10^5
Staphylococcus aureus 209 P	3.1×10^6	+	+	7.4×10^5
	3.1×10^6	+	−	6.9×10^5
	3.1×10^6	−	+	1.2×10^6
	3.1×10^6	−	−	1.6×10^6

stage, whereas it somewhat enhances the intracellular bactericidal activity in the late stage. My explanation for this phenomenon is a possibility that there are two steps in intracellular bacterial killing, the first being the translocation of the lysosomal or peroxisomal enzymes to the phagosome and the second being enzyme release to the phagosome. CB may inhibit the first step and further enhance the second step. The first possibility was suggested from the data on the MPO staining and on the leukocytic homogenate, as well as on the observation of Allison et al. (1). Davis et al. (3) reported that CB increased exocytosis of rabbit polymorphonuclear leukocytes. Henson and Oades (6) and Skosey et al. (16) reported that CB increased the release of lysosomal enzymes from polymorphonuclear leukocytes, when these leukocytes became attacked to aggregated gamma globulin or zymosan particles. These observations support my second hypothesis.

Estensen and Plagemann (5), Zigmond and Hirsh (24, 25), and Wagner et al. (19) reported that CB inhibited glycolysis. One interpretation of the data on phagocytosis is that decreased levels of glycolysis might cause a low production of intracellular lactic acid and H_2O_2. But I (12) observed that CB significantly increased the reducing activity of nitroblue tetrazolium dye by human leukocytes. Consequently, the effect of CB through anaerobic glycolysis might be operated by many complicated systems.

However, the precise mechanism of the intraleukocytic bactericidal process remains obscure. Thus, substances that specifically inhibit certain phagocytic function will contribute a great deal to our understanding of the phagocytic process.

ACKNOWLEDGMENT

I am greatly indebted to Dr. I. Tadokoro for his generous advice concerning this study.

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