EFFECT OF CYTOCHALASIN B ON RESPONSE OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES TO ZYMOSAN

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INTRODUCTION

Phagocytosis of zymosan particles by human peripheral blood polymorphonuclear leukocytes (PMN) results in the release of lysosomal enzymes into the extracellular medium (1) and enhanced glucose oxidation and lactate production (2). The antibiotic, cytochalasin B (CB), inhibits phagocytosis by PMN (3, 4), presumably by interfering with the membrane-associated network of microfilaments (5). CB is therefore useful in studies of the interrelationship between phagocytosis and metabolic changes in stimulated PMN.

This study confirms earlier reports that CB inhibits phagocytosis by PMN (3, 4, 6) and carbohydrate metabolism in phagocytosing PMN (4, 6, 7). The release of the lysosomal enzyme, β-glucuronidase, however, was moderately stimulated by CB under conditions which resulted in almost total inhibition of phagocytosis.

MATERIALS AND METHODS

Zymosan (Sigma Chemical Co., St. Louis, Mo.) was incubated for 1 h at 37°C with plasma from the donor of cells used in the experiment, collected by centrifugation, and suspended in Krebs-Ringer phosphate buffer (KRP) (8). CB (Imperial Chemical Industries, Macclesfield, Cheshire, England) was dissolved in dimethylsulfoxide (DMSO) before addition to the incubation medium. [1-^{14}C]glucose and [*carboxyl*-^{14}C]inulin were purchased from Amersham/Searle Corp. (Arlington Heights, Ill.).

Heparinized blood (10 U heparin/ml blood) was obtained from normal women. The donors abstained from all dietary sources of methyl xanthines on the morning blood was drawn. After erythrocytes were allowed to sediment at 37°C for 2–3 h, the plasma (containing leukocytes) was removed and centrifuged at 250 *g* for 10 min. The cell pellet was suspended in plasma, and 3 vol of 0.87% NH_4Cl were added to lyse remaining erythrocytes. Leukocytes were then washed twice in KRP and suspended in buffer for incubation. Suspensions (ca. 10^8 cells/ml) contained 95–98% PMN.

Separate aliquots of cell suspensions were used for measurements of inulin uptake and β-glucuronidase release, and for measurement of $^{14}CO_2$ and lactate production. 0.2 ml of cell suspension was added to polyethylene test tubes containing 5 μl of DMSO with the desired concentration of CB and preincubated for 30 min. 0.2 ml of KRP containing glucose (5 mM, final concentration) with or without zymosan (1 mg/ml, final concentration) was then added, and incubation was continued for an additional 30 min for metabolic measurements. Trace concentrations of [1-^{14}C]glucose (0.5 μCi/ml) or [*carboxyl*-^{14}C]inulin (1 μCi/ml) were present in the medium. In experiments testing the effect of glucose on [^{14}C]inulin uptake and β-glucuronidase release, cells were incubated for 30 min in the absence or presence of glucose (5 mM).

Cell suspensions were incubated in polyethylene test tubes at 37°C with continuous shaking. When [^{14}C]inulin was present, the incubation was stopped by chilling the tubes in an ice bath and the addition of chilled KRP containing inulin, 1 mg/ml. The cell suspension was centrifuged at 250 *g* for 5 min, and the supernatant was saved for determination of

β-glucuronidase activity, using *p*-nitrophenyl glucuronide as substrate (9). In some experiments, total enzyme activity was determined in selected tubes by the addition of Triton-X100 (1). For measurement of [^{14}C]inulin uptake, the cell pellet was washed as described by Berger and Karnovsky (10), dissolved in 0.1 ml of 2 N NaOH, and diluted with 1.4 ml H_2O. Aliquots were taken for counting in a 3a40 liquid scintillator (Research Products International Corp., Elk Grove Village, Ill.) and for protein determinations (11). The incubation of cell suspensions containing [1-^{14}C]glucose was stopped by the injection of 0.5 ml of 1 N $HClO_4$ into the medium. $^{14}CO_2$ was collected in hyamine and counted (12). Lactate was measured in the deproteinized fluid (13).

RESULTS

The results of preliminary experiments indicated that preincubation of cells with CB, 50 μg/ml, did not alter baseline β-glucuronidase release from nonphagocytosing cells, although the antibiotic has been shown to inhibit the metabolism of glucose by resting cells (4, 6, 7). Zymosan added to cells preincubated without CB, stimulated the uptake of [^{14}C]inulin and the release of β-glucuronidase from cells, as well as the oxidation of [1-^{14}C]glucose to $^{14}CO_2$ and the production of lactate. Previous incubation with CB in concentrations ranging from 2 to 60 μg/ml resulted in inhibition of zymosan-stimulated [^{14}C]inulin uptake, and $^{14}CO_2$ and lactate production. The release of β-glucuronidase was slightly stimulated at all concentrations of the antibiotic used (Fig. 1).

CB inhibits the transport of 2-deoxy-D-glucose into horse leukocytes (14), and of glucose into hepatoma cells (15). Our findings could have re-

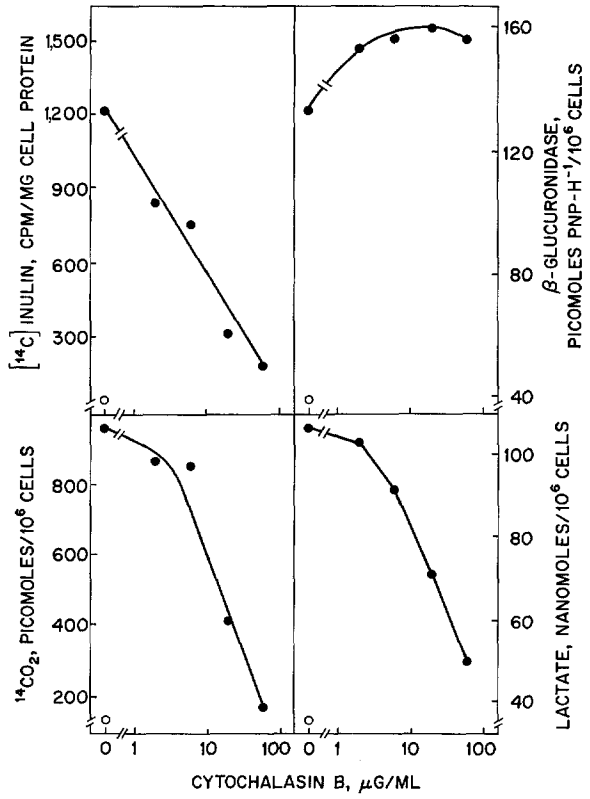

FIGURE 1 Effect of preincubation with CB on [^{14}C]inulin uptake, β-glucuronidase release, glucose oxidation, and lactate production. Human peripheral blood leukocytes (98% PMN) were incubated for 30 min with CB at the concentrations indicated. An equal volume of zymosan suspension containing either [^{14}C]inulin or [1-^{14}C]glucose was then added. Metabolic measurements were then made after an additional 30 min incubation period. Means of triplicate determinations from cells incubated with (closed circles) or without (open circles) zymosan are plotted. β-glucuronidase activity was measured by estimating the rate of production of *p*-nitrophenol (PNP) from *p*-nitrophenyl glucuronide.

TABLE I

Effect of Glucose on [^{14}C]Inulin Uptake and β-Glucuronidase Release

	No zymosan	Zymosan, 1 mg/ml
[^{14}C]Inulin uptake, cpm/mg cell protein (mean ± SE)		
No glucose	16.0 ± 0.62	160 ± 9.0
Glucose, 5 mM	13.5 ± 2.8	191 ± 11
β-Glucuronidase release, % total activity (mean ± SE)		
No glucose	2.37 ± 0.10	11.3 ± 0.15
Glucose, 5 mM	1.87 ± 0.10	11.7 ± 0.13

Human peripheral blood leukocytes (95% PMN) were incubated for 30 min with [^{14}C]inulin. Glucose and zymosan were present where indicated. The means ± standard error of triplicate determinations are given.

sulted from inhibition of glucose utilization by cells incubated with CB. Therefore, we tested the effect of glucose on zymosan-stimulated [^{14}C]-inulin uptake and β-glucuronidase release. Glucose had no significant effect on either inulin uptake or enzyme release by resting or phagocytosing cells (Table I).

DISCUSSION

CB has been shown to inhibit phagocytosis by human PMN as measured by the uptake and killing of bacteria (3, 4, 6) or by the direct examination of cells incubated with particles (4, 6). The difficulties inherent in these methods have been discussed (4, 10). We have elected to use the uptake of [^{14}C]inulin into cells as an index of phagocytosis, as described previously (10). This large molecular weight compound presumably enters cells in the water taken into the phagocytic vesicle. Water also enters cells during the process of pinocytosis. The uptake of [^{14}C]inulin, therefore, serves as an index of endocytosis, and is not specific for either phagocytosis or pinocytosis (10). Since endocytosis of either type is associated with metabolic changes (16), it is reasonable to relate PMN metabolism and function to [^{14}C]-inulin uptake.

Three possible mechanisms have been proposed for the release of lysosomal enzymes from phagocytosing cells (1). The first, release of enzymes after cell death, cannot account for release of lysosomal enzymes from PMN phagocytosing zymosan, since lactic dehydrogenase, a nonlysosomal cytoplasmic enzyme, is not released from cells in response to zymosan (1).

A second mechanism which has been proposed is that of "regurgitation during feeding." Electron microscope examination of rabbit neutrophils phagocytosing zymosan particles (17) has suggested that lysosomes fuse with the phagocytic vesicle as it forms during particle uptake. The lysosomes appear to discharge their content of enzymes into the vesicle. Enzymes are then extruded through the unclosed "mouth" of the phagocytic vesicle. Henson (17) has suggested that neutrophils which have phagocytosed many particles "run out of energy" and become inefficient in closing off developing phagocytic vesicles. Lysosomal enzymes which have been extruded into the vesicle are allowed to leak out into the medium. Since CB inhibits glucose metabolism (14, 15), it is possible that the early phases of phagocytosis occur in the presence of CB, but that the antibiotic deprives the cells of sufficient energy to close off the mouth of the vesicle. However, the observation that complete deprivation of the cells of exogenous glucose does not mimick the effects of CB argues against this possibility.

We favor a third mechanism, "directed exocytosis," or "reverse endocytosis," proposed for the stimulation of release of enzyme by zymosan. Phagocytosis is apparently not required for the particles to stimulate enzyme release. It thus appears possible that zymosan, by coming into contact with cell membrane receptors, triggers lysosomal enzyme release without the requirement for the development of a phagocytic vesicle.

Orci et al. observed that CB enhances glucose-induced secretion of insulin by isolated rat pancreatic islets (18). They suggested that the network of microfilaments of the beta cell might act as a barrier which denies the insulin-secretory granules access to the cell membrane. CB, by disrupting the function of this network, could cause enhancement of insulin secretion. A similar mechanism could explain the moderate enhancement of zymosan-stimulated β-glucuronidase release from PMN.

SUMMARY

Phagocytosis of zymosan by human peripheral blood PMN, measured by the uptake of [^{14}C]inulin from the incubation medium, was inhibited by CB. CB also decreased zymosan-stimulated oxida-

tion of [1-^{14}C]glucose to $^{14}CO_2$ and production of lactate, but moderately stimulated the release of β-glucuronidase. The results suggest that release of β-glucuronidase from cells exposed to zymosan does not require phagocytosis of the particles.

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REFERENCES

1. Weissmann, G., R. B. Zurier, P. J. Spieler, and I. M. Goldstein. 1971. Mechanisms of lysosomal enzyme release from leukocytes exposed to immune complexes and other particles. *J. Exp. Med.* **134**:149 s.
2. Karnovsky, M. L., R. L. Baehner, S. Githens III, S. Simmons, and E. A. Glass. 1971. Correlations of metabolism and function in various phagocytes. *In* Immunopathology of Inflammation. B. K. Forscher and J. C. Houck, editors. Excerpta Medica Foundation, Publishers, Amsterdam. 121–132.
3. Davis, A. T., R. Estensen, and P. G. Quie. 1971. Cytochalasin B. III. Inhibition of human polymorphonuclear leukocyte phagocytosis. *Proc. Soc. Exp. Biol. Med.* **137**:161.
4. Zigmond, S. H., and J. G. Hirsch. 1972. Effects of cytochalasin B on polymorphonuclear leukocyte locomotion, phagocytosis and glycolysis. *Exp. Cell Res.* **73**:383.
5. Wessels, N. K., B. S. Spooner, J. F. Ash, M. O. Bradley, M. A. Luduena, E. L. Taylor, J. T. Wrenn, and K. M. Yamada. 1971. Microfilaments in cellular and developmental processes. *Science (Wash. D. C.).* **171**:135.
6. Malawista, S. E., J. B. L. Gee, and K. G. Bensch. 1971. Cytochalasin B reversibly inhibits phagocytosis. Functional, metabolic and ultrastructural effects in human blood leukocytes and rabbit alveolar macrophages. *Yale J. Biol. Med.* **44**:286.
7. Skosey, J. L., E. Damgaard, and D. Chow. 1972. Effect of cytochalasin B upon glucose oxidation by human peripheral blood leukocytes. *Fed. Proc.* **31**:802. (Abstr.)
8. Cohen, P.P. 1957. *In* Manometric Techniques. W. W. Umbreit, R. H. Burris, and S. F. Stauffer, editors. Burgess Publishing Company, Minneapolis. 3rd edition. 149–150.
9. Baehner, R. L., M. J. Karnovsky, and M. L. Karnovsky. 1969. Degranulation of leukocytes in chronic granulomatous disease. *J. Clin. Invest.* **48**:187.
10. Berger, R. R., and M. L. Karnovsky. 1966. Biochemical basis of phagocytosis. V. Effect of phagocytosis on cellular uptake of extracellular fluid, and on the intracellular pool of L-α-glycerophosphate. *Fed. Proc.* **25**:840.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the phenol reagent. *J. Biol. Chem.* **193**:265.
12. Rodbell, M. 1964. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239**:375.
13. Hohorst, H.-J. 1963. L-(+)-Lactate. Determination with lactic dehydrogenase and DPN. *In* Methods of Enzymatic Analysis. H.-U. Bergmeyer, editor. Academic Press Inc., New York. 266–270.
14. Zigmond, S. H., and J. G. Hirsch. 1972. Cytochalasin B inhibition of 2-deoxy-D-glucose transport into leukocytes and fibroblasts. *Science (Wash. D. C.).* **176**:1432.
15. Estensen, R. D., and P. G. W. Plagemann. 1972. Cytochalasin B: inhibition of glucose and glucosamine transport. *Proc. Natl. Acad. Sci. U. S. A.* **69**:1430.
16. Graham, R. C. Jr., M. J. Karnovsky, A. W. Shafer, E. A. Glass, and M. L. Karnovsky. 1967. Metabolic and morphological observations on the effect of surface-active agents on leukocytes. *J. Cell Biol.* **32**:629.
17. Henson, P. M. 1971. The immunological release of constituents from neutrophil leukocytes. II. Mechanisms of release during phagocytosis, and adherence to non-phagocytosable surfaces. *J. Immunol.* **107**:1547.
18. Orci, L., K. H. Gabbay, and W. J. Malaisse. 1972. Pancreatic beta-cell web: its possible role in insulin secretion. *Science (Wash. D. C.).* **175**:1128.