# EFFECT OF CYTOCHALASIN B ON THE OXIDATIVE METABOLISM OF HUMAN PERIPHERAL BLOOD GRANULOCYTES.

Dirk Roos*, Joke W.T. Homan-Müller* and Ron S. Weening+

*Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, P.O.B. 9190, Amsterdam; and
+Pediatric Clinic, Binnengasthuis, University of Amsterdam, Amsterdam, The Netherlands.

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SUMMARY: Pretreatment of human granulocytes with cytochalasin B before addition of opsonized zymosan particles resulted in strong inhibition of the oxygen consumption, the hydrogen peroxide production, and the hexose monophosphate shunt activity as compared to normal phagocytosing cells. In contrast, however, no effect of cytochalasin B was found on the generation of superoxide anions. These seemingly controversial results can be explained by the action of cytochalasin B on the cell membrane.

## INTRODUCTION

Cytochalasin B (cyto B)*, a metabolite of the mold Helminthosporium dematioideum, interferes with the function of cytoplasmic microfilaments (1,2) and inhibits membrane transport of sugars and nucleosides in cultured cells (3-5). In granulocytes, this drug suppresses the capacity to ingest particles without inhibiting the attachment of the particles to the cell surface (6,7). Nevertheless, cyto B-treated granulocytes selectively release lysosomal enzymes after stimulation with immune (8) or non-immune (9) stimuli.

Recently it has been shown that under exactly the same conditions the generation of superoxide anions ($O_2^-$) is also stimulated (10). Although we have shown previously that superoxide is a quantitatively important intermediate in the production of hydrogen peroxide (11), it is still unknown whether granulocyte stimuli activate all oxidative processes in cyto B-treated cells. The action of cyto B on several of these reactions is the subject of this report.

*Abbreviations: cyto B = Cytochalasin B, STZ = Serum-treated zymosan.



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## MATERIALS AND METHODS

Cytochalasin B (I.C.I. Research Laboratories, Alderley Park, Cheshire, England) was prepared as a 50 µg/ml stock solution in 1% dimethylsulfoxide (J.T. Baker Chemicals N.V., Deventer, The Netherlands). The granulocytes were preincubated during 10 min. at 37°C in a 1:10 dilution of the drug before addition of other compounds and stimuli.

Zymosan (kindly provided by Dr. K.W. Pondman) was suspended in fresh human serum at a concentration of 4 mg/ml, homogenized in a Potter homogenizer and incubated for 30 min. at 37°C. After centrifugation and washing twice, this preparation of serum-treated zymosan (STZ) was suspended in 154 mM NaCl at a concentration of 5 mg/ml. Granulocytes were incubated with a 1:10 dilution of this preparation.

Granulocytes were isolated from fresh, defibrinated human blood, as described elsewhere (12) and suspended in a buffer consisting of 138 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.5 mM glucose and 0.5% (w/v) human albumin, pH 7.4. The final cell suspensions, containing more than 95% granulocytes, were split into several aliquots for the assay of the metabolic parameters. It must be emphasized, however, that the different parameters were not determined under identical conditions, since addition of cytochrome c (for the $O_2^-$ assay) resulted in strong inhibition of the other parameters (unpublished results). A theoretical explanation is shown in fig.1.

Oxygen consumption was measured polarographically with an oxygen electrode as described before (12). Superoxide generation was determined by measuring the superoxide dismutase-inhibitable reduction of cytochrome c, as previously described (11). Hydrogen peroxide production was measured with a method based on the oxidation of leucodiacetyl-2,7-dichlorofluorescein to a fluorescent compound by $H_2O_2$ in the presence of peroxidase (13). Hexose monophosphate shunt activity was assayed as $^{14}CO_2$ production from [1-$^{14}$C]glucose by the method of Pachman (14,15). First, the cells were preincubated with [1-$^{14}$C]glucose in the presence of 5.5 mM glucose for 15 min. at 37°C. Next, cyto B was added while the cells were kept at 37°C for another 10 min. Finally, the experiment was started with the addition of STZ or NaCl (154 mM). Release of lysozyme was determined as previously described (10). Extracellular lactate dehydrogenase was measured as a control for cell integrity. No significant release of lactate dehydrogenase was found under any condition.

## RESULTS

The inhibitory action of cytochalasin B could be demonstrated by adding this compound to a suspension of STZ-stimulated granulocytes in an oxygraph. Fig. 2 shows that 80% inhibition of the oxygen consumption of these cells was obtained in about 5 min. This inhibition was dose-dependent (not shown) while DMSO (0.1%, v/v) alone had no effect or inhibited only slightly. In subsequent experiments we preincubated the cells with cyto B for 10 min. to be certain of complete action of this drug. The cyto B-treated cells were still capable of completely normal release of lysosomal enzymes when challenged with opsonized zymosan (fig. 3).

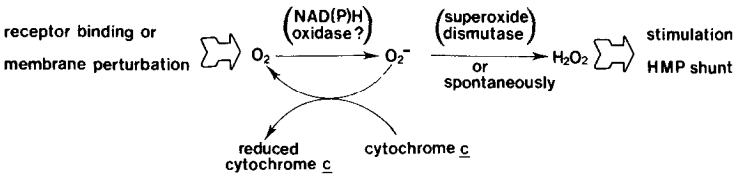


Fig. 1 Schematic representation of the oxidative metabolism of granulocytes, also showing the detection method of superoxide anions ($O_2^-$).

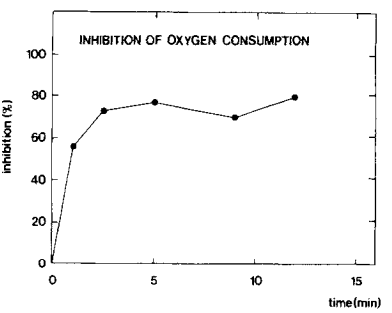


Fig. 2 Inhibition of oxygen consumption by cyto B. The curve shown was obtained by measuring the rate of oxygen consumption at different time intervals after addition of cyto B (5 µg/ml) to a granulocyte suspension stimulated with STZ (0.5 mg/ml). Cyto B was added 7 min. after addition of STZ.

The effect of 10 min. preincubation with cyto B on several parameters of the oxidative metabolism is shown in Table 1. The stimulated generation of $O_2^-$ was not affected by cyto B, whereas the other 3 parameters were inhibited for 70-90%. To investigate the lack of inhibition of cyto B on the superoxide generation more closely, we have varied both the cyto B concentration (fig. 3) and the cytochrome c concentration (fig. 4). Except at very high cytochrome c concentrations, no substantial differences between cyto B-treated and non-treated cells were found.

Since cyto B has also been reported to inhibit the transport of sugars into cells, we have preincubated granulocytes with 5.5 mM glucose at 37°C for 15 min. before addition of cyto B. After another 10 min. at 37°C, the experiment was started by the addition of STZ. This pretreatment did not, however, prevent the cyto B inhibition on the oxygen consumption (without preincubation with glucose: 67% inhibition by cyto B, with preincubation: 66% inhibition).

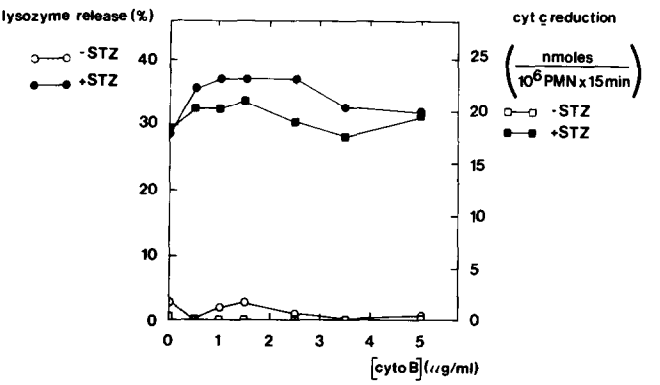


Fig. 3 Effect of cyto B on lysozyme release and cytochrome c reduction by human granulocytes. Reaction time: 15 min. Lysozyme release expressed as percent of total activity (100% = 1.4 ± 0.5 µg lysozyme/$10^6$ cells, n = 7) released by Triton-X-100. Lysozyme release: o-o, -STZ; ●-●, +STZ. Cytochrome c reduction: □-□, -STZ; ■-■, +STZ.

TABLE I - EFFECT OF CYTOCHALASIN B ON SEVERAL PARAMETERS OF OXIDATIVE METABOLISM.

| Parameter | Additions | | Response (nmoles/$10^6$ granulocytes x 15 min.) (Mean ± S.E.M.) | |
|---|---|---|---|---|
| | | (n) | Normal cells | Cyto B-cells |
| Oxygen consumption | - | 10 | 2.4 ± 0.5 | 1.9 ± 0.4 |
| | STZ | 9 | 46.6 ± 4.9 | 12.5 ± 1.0 |
| Superoxide generation | - | 7 | 1.6 ± 0.3 | 1.4 ± 0.2 |
| | STZ | 7 | 14.8 ± 0.7 | 14.4 ± 1.9 |
| $H_2O_2$ production | - | 4 | 0 | 0 |
| | STZ | 4 | 36.9 ± 2.6 | 8.7 ± 0.5 |
| $[1\text{-}^{14}C]$glucose oxidation | - | 3 | 0.3 ± 0.1 | 0.2 ± 0.1 |
| | STZ | 3 | 7.4 ± 1.2 | 0.8 ± 0.1 |

## DISCUSSION

Our results confirm earlier reports (6,16,17) about a dose-dependent inhibition of the $[1\text{-}^{14}C]$glucose oxidation by cyto B. Recently, both Rossi et al. (18) and Schell-Frederick et al. (19) reported inhibition of the oxygen consumption in cyto B-treated, zymosan-stimulated granulocytes as compared to non-treated cells. Finally, Curnutte and Babior (20) found 60% stimulation of the superoxide

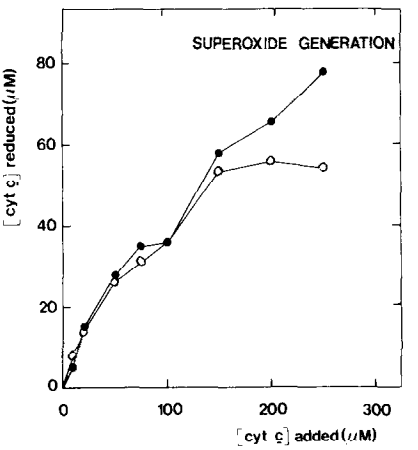


Fig. 4 Influence of cytochrome c concentration on cytochrome c reduction by granulocytes incubated for 15 min. with STZ, with (●-●) or without (o-o) cyto B.

generation in suspensions of cyto B-treated granulocytes incubated with E.Coli. No attention, however, has been paid so far to the discrepancy of inhibition of oxygen consumption without inhibition (even stimulation) (10,20) of the generation of its product, superoxide.

Our results cannot be explained by a non-specific reduction of cytochrome c, since we always found strong inhibition (90-95%) of this reaction by superoxide dismutase. Previously, we have shown (11) that superoxide is an important intermediate in the reaction from oxygen to hydrogen peroxide in granulocytes (fig. 1). Therefore, it is also hardly imaginable that cyto B only inhibits a hypothetical direct conversion of oxygen into $H_2O_2$ without interfering with a superoxide-containing pathway. Finally, competition between cytochrome c and cyto B is ruled out by the experiments shown in figs. 3 and 4.

More likely, the explanation must be sought at the level of the particle attachment to the membrane. It has been shown that cyto B-treated cells show impaired engulfment of particles as compared to normal cells (6,8,21). Thus, it is highly probable that particles like zymosan bind to less receptors on the cell membrane of cyto B-treated granulocytes. Since the metabolic reactions in granulocytes are initiated by binding of stimuli to the cell surface receptors

(10), this inhibition of receptor binding may well be the cause of the metabolic inhibition. From the experiment shown in fig. 2 it may even be derived that cyto B should be able to disconnect particle-cell bonds.

The lack of inhibition of superoxide production may simply be explained by the method used for its determination (reduction of cytochrome c). Previously, we have presented evidence that superoxide is generated by a system located in the cell membrane (9,10). Thus, under conditions of normal phagocytosis, this agent is produced also in the membranes of the phagosomes, since these organelles originate from the outer cell membrane. Once the superoxide diffuses from the phagosomes into the cytosol, however, it will be inactivated rapidly by the enzyme superoxide dismutase. Since cyto B-treated granulocytes are completely unable to ingest zymosan particles (8), these cells will release a much larger part of their superoxide into the medium than normal cells. Thus, a much more efficient trapping of $O_2^-$ is possible in cyto B-treated cells, since there is less competition between cytochrome c and other electron acceptors for $O_2^-$. Such arguments do not exist for the other parameters.

In short, we think that cyto B treatment leads to less cell-particle binding, resulting in impaired stimulation of all oxidative reactions. The finding that $O_2^-$ generation seems to be excluded from this inhibition can be explained by a more efficient reaction of this agent with cytochrome c under these conditions.

This concept of granulocyte stimulation is in accordance with our observations and those of others (6,20) that cyto B has only little effect on the metabolism of non-stimulated granulocytes. Also, the finding of Rossi et al. (18) that cyto B only inhibits the oxygen consumption of cells stimulated with solid stimuli and not when soluble activators are used, completely fits into our explanation. It has to be proven, however, that less particle-cell bonds are formed with cyto B-treated cells and that the rate of oxidative reactions is directly proportional to the number of bonds formed or to the number of cells that have bound a certain threshold amount of ligands.

Finally, it may be derived from Table 1 that STZ stimulates all parameters of the oxidative metabolism in cyto B-treated cells. Our earlier conclusion (10) that attachment of particles to the cell membrane is sufficient for stimulation of the oxidative reactions in these cells, based upon observations of superoxide generation only, have now been confirmed with other parameters: cytochalasin B inhibits the oxidative metabolism, but does not prevent the stimulation by STZ. Thus, granulocyte membrane receptors not only serve in particle recognition, but may also initiate the biochemical events which accompany phagocytosis and killing.

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