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THE ENHANCING EFFECT OF CYTOCHALASIN A ON CONCANAVALIN A-INDUCED SUPEROXIDE ANION RELEASE FROM POLYMORPHONUCLEAR LEUKOCYTES*)

By

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ABSTRACT

Superoxide anion release from polymorphonuclear leukocytes stimulated by concanavalin A was markedly enhanced by the simultaneous addition of cytochalasin A at concentrations of 2×10^{-8} to 4×10^{-7}M. These concentrations of cytochalasin A did not affect the phagocytosis of diisodecyl phthalate emulsion. These concentrations were far lower than those of cytochalasins B, C, D and E that also enhanced concanavalin A-induced superoxide anion release. Superoxide anion release stimulated by concanavalin A and cytochalasin A required the binding of concanavalin A on the cell membrane, and was dependent on the existence of extracellular Ca^{2+}. The enhancement by cytochalasin A was reversible. It is suggested that cytochalasin A acts not only as a sulfhydryl reagent as reported previously, but also has actions resembling the other cytochalasins.

INTRODUCTION

Some cytochalasins, which are a group of fungal metabolites with the same fundamental structure, stimulate polymorphonuclear leukocytes (PMNL) to release superoxide anion (O_2^-)[1,2]. Although some cytochalasins are believed to affect the arrangement of the microfilament system[3-5], other actions on cellular systems are variable[6-8]. It has been reported that cytochalasins B, C, D and E largely enhance concanavalin A (con. A)-induced O_2^- release[9-11], but cytochalasin A has an inhibitory effect at a concentration of 10^{-5}M[11]. In the present study, it was found that lower concentrations (2×10^{-8}M–4×10^{-7}M) of cytochalasin A than those examined previously enhanced con. A-induced O_2^- release. The conditions under which cytochalasin A enhancement took place are discussed.

METERIALS AND METHODS

Cytochalasins, con. A, phorbol myristate acetate (PMA) and cytochrome C type III were purchased from Sigma Chemical Co. Formyl-methionyl-leucyl-phenylalanine (FMLP) was obtained from the Protein Research Foundation, and A23187 from Calbiochem. Behring Co. Cytochalasins, PMA and A23187 were dissolved in dimethylsulfoxide (DMSO) and stored at

*) 松浦良二：コンカナバリンA刺激時の多核白血球超酸化物イオン放出に及ぼすサイトカラシンAの増強効果

−20°C. The final concentration of DMSO in the reaction mixture was less than 0.5%.

Human PMNL were isolated from normal volunteers using Conray-Ficoll gradient, 3% dextran sedimentation and hypotonic hemolysis. PMNL were washed twice with Dulbecco's phosphate-buffered saline (PBS), and suspended in ice-cold Dulbecco's PBS at a concentration of 5×10^6/ml.

O_2^- release from PMNL was measured by the reduction of ferricytochrome C at 550–540 nm on a dual wave length spectrophotometer (Hitachi 557) with a constant temperature cuvette holder kept at 37°C. The molar absorption coefficient of cytochrome C was taken as 19.1×10^3 [11]. The reaction mixture contained 5×10^5 PMNL and 100μM cytochrome C in 1 ml of Dulbecco's PBS. After PMNL and cytochrome C were preincubated at 37°C for 5 minutes, various agents were added to the cuvette, and the reduction of cytochrome C was recorded.

The phagocytic activity of PMNL was assayed by the diisodecyl phthalate (DP) emulsion method, a modification of the spectrophotometric one described by Stossel[12], based on quantification of the ingestion of DP particles coated with lipopolysaccharide. The final assay contained the following: 0.01 ml DP emulsion, 0.1 ml pooled human fresh serum, 0.89 ml Krebs-Ringer buffer and 4×10^6 PMNL. After PMNL and cytochalasins were preincubated at 37°C for 10 minutes, the assay of phagocytosis was initialized with the addition of the DP emulsion which was opsonized with pooled human fresh serum. After 15 minutes incuba-

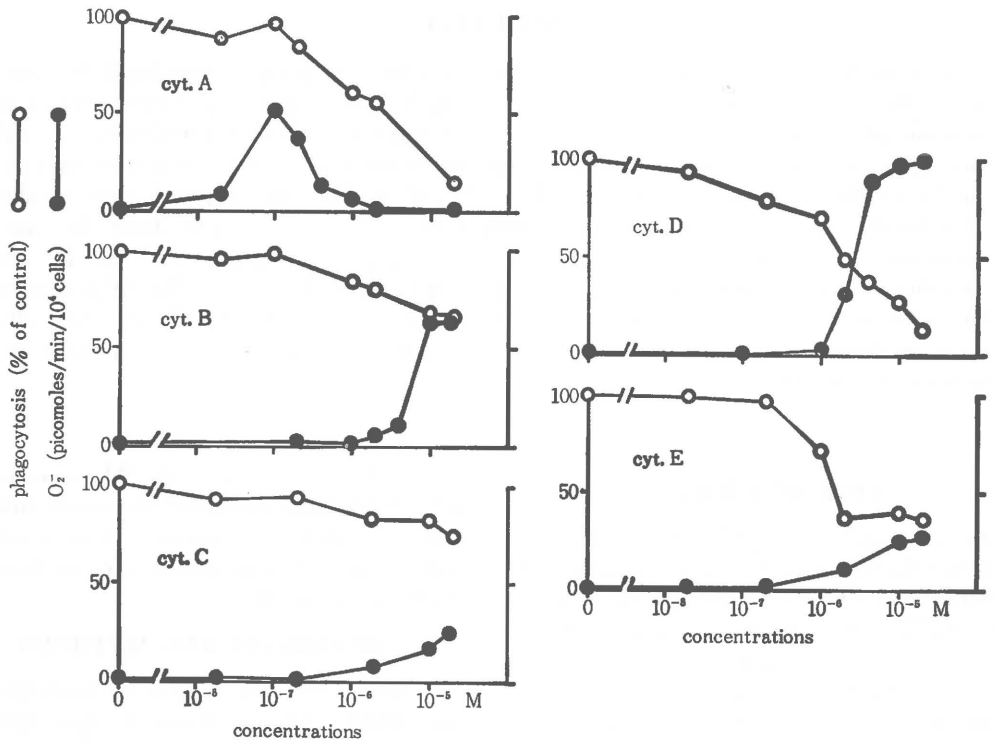

Fig. 1. Effect of various concentrations of cytochalasins on con.A-induced O_2^- release and phagocytosis of DP emulsion. Closed circles (●) indicate O_2^- release. The reaction mixture contains PMNL at the final concentration of 5×10^5/ml. Cytochalasins and con.A (100 μg) are added to the reaction mixture at the same time. Open circles (○) indicate the effect of cytochalasins on the phagocytosis of DP emulsion. The reaction mixture contains PMNL (4×10^6/ml), fresh serum (0.1 ml), DP emulsion (0.01 ml) and cytochalasins. Phagocytosis is expressed as percentages of the control values.

tion, the reaction was stopped by the addition of cold saline. The cells were separated from uningested oil droplets by centrifugation at 250 g for 10 minutes, washed twice and extracted in 1.0 ml of dioxane. Following centrifugation at 1,000 g for 10 minutes, the supernatant was assayed photometrically at a wave length as 525 nm. The effect of cytochalasins on phagocytosis was represented as a percentage of the control value without cytochalasin.

RESULTS

All cytochalasins markedly enchanced con. A-induced O_2^- release (Fig. 1). The concentrations of cytochalasins that had an enhancing effect were as follows: cytochalasin A, from 2×10^{-8} to 4×10^{-7}M, cytochalasin B, C, D and E, greater than 2×10^{-6}M. The concentrations of cytochalasin A that enhanced O_2^- release were far lower than those of the other cytochalasins. At concentrations greater than 2×10^{-6}M cytochalasin A inhibited con. A-induced O_2^- release.

Cytochalasins are thought to suppress pseudopod formation through their effect on the microfilament system and to inhibit the phagocytosis. The phagocytosis of DP emulsion was investigated (Fig. 1). Cytochalasin A had little inhibitory effect on the phagocytosis of DP emulsion at the same concentrations that enhanced release of O_2^-. The concentrations of cytochalasins that inhibited phagocytosis were found to be: for A and D, greater than 1×10^{-6}M, for E, greater than 2×10^{-6}M and for B, greater than 1×10^{-5}M. The degree of inhibition increased with increasing concentration of cytochalasin. The enhancement of O_2^- release by cytochalasins except for cytochalasin A correlated well with the inhibition of phagocytosis. Low concentrations of cytochalasin A enhanced O_2^- release and resulted in a modest degree of inhibition of phagocytosis, while high concentrations inhibited both O_2^- release and phagocytosis.

In the absence of con. A, cytochalasin A (1×10^{-7}M), B and C (2×10^{-5}M) failed to stimulate PMNL to release O_2^-, while cytochalasin D and E (2×10^{-5}M) had a stimulatory effect (Fig. 2). As cytochalasin A did not stimulate PMNL by itself, it was postulated that con. A was necessary for cytochalasin A to show its effect. In this respect, at low

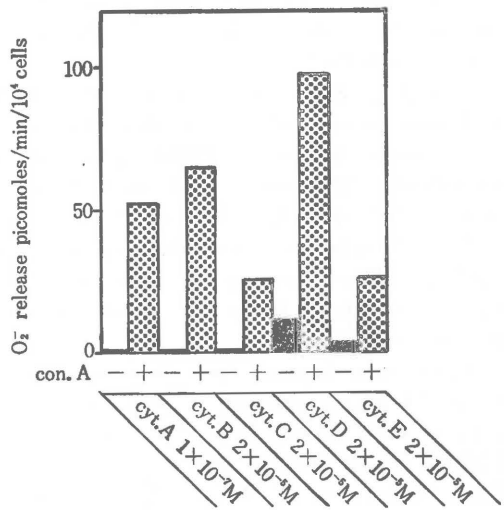

Fig. 2. Stimulatory effect of cytochalasins on O_2^- release when con. A (100 μg) is added simultaneously (dotted columns). The dark columns indicate O_2^- release induced by cytochalasins in the absence of con. A.

concentrations, cytochalasin A resembled cytochalasin B of high concentrations. Therefore, it was compared directly with cytochalasin B.

The stimulatory effect of con. A is related to its physicochemical structure, a tetramer with two binding sites[9]. It was compared with succinyl con. A, which is a dimer with one

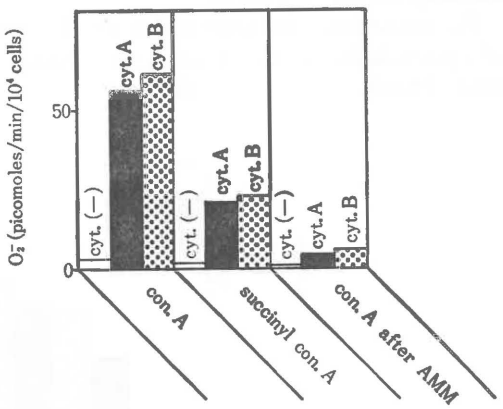

Fig. 3. The first two frames compare con. A with succinyl con. A (100 μg) with respect to O_2^- release. Cytochalasin A (1×10^{-7}M, dark columns) or B (2×10^{-5}M, dotted columns) is added simultaneously, or not added (open columns). The third frame shows the effect of AMM (1×10^{-3}M) on con. A-induced O_2^- release with or without cytochalasin A or B. AMM and PMNL are preincubated for 5 minutes and con. A and/or cytochalasin A or B are added simultaneousy.

binding site. Succinyl con. A showed a weaker ability to stimulate PMNL than native con. A, whether cytochalasin A was added or not (Fig. 3). α-methyl mannoside (AMM) is known to prevent con. A from binding to the mannose receptor on PMNL by competitive inhibition[9]. This agent inhibited O_2^- release from PMNL stimulated by Con. A and cytochalasin A, whether added before or after stimulation. These phenomena were also seen when cytochalasin B was used instead of cytochalasin A (Fig. 3).

Above and beyond the effect of con. A, 1×10^{-7}M of cytochalasin A enhanced the release of O_2^- induced by other agents such as FMLP (1×10^{-6}M), A23187 (5×10^{-6}M) and PMA (10 ng) (Fig. 4). Cytochalasin B enhanced the release of O_2^- induced by FMLP, A23187, but inhibited PMA-induced O_2^- release (Fig. 4). The necessity of extracellular divalent cations for O_2^- release from PMNL was examined when stimulated by con. A and cytochalasin A (Fig. 5). The complete removal of extracellular Ca^{2+} almost abolished O_2^- release, but that of extracellular Mg^{2+} did not influence O_2^- release. The release of O_2^- stimulated by con. A and cytochalasin A was dependent on extracellular Ca^{2+}, but independent of extracellular Mg^{2+}. Cytochalasin B was affected similarly by Ca^{2+}. (Fig. 5)

To investigate the reversibility of the effect of cytochalasin A, O_2^- release was examined after PMNL were preincubated with cytochalasin A or B and washed (Fig. 6). PMNL were incubated with various concentrations of cytochalasin A or 2×10^{-5}M of cytochalasin B at 37°C for 10 minutes, and centrifuged at 250 g for 10 minutes. The sedimented PMNL were washed three times with Dulbecco's PBS, and resuspended to the initial cell concentration.

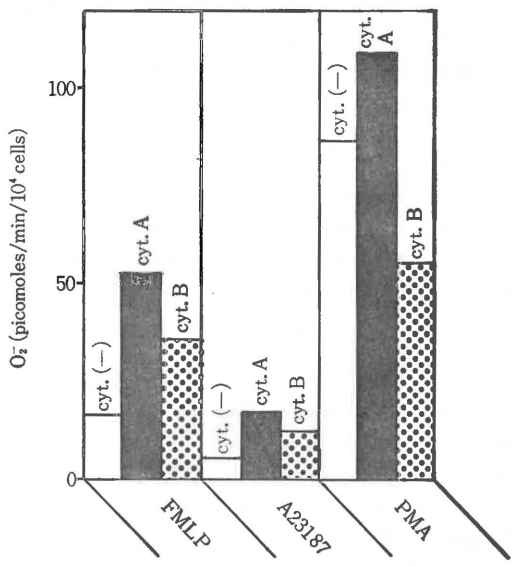

Fig. 4. Effect of cytochalasin A (1×10^{-7}M, dark columns) and B (2×10^{-5} M, dotted columns) on O_2^- release induced by FMLP (1×10^{-6}M), A23187 (5×10^{-6}M) and PMA (10 ng). Cytochalasins and each of the aforementioned agents are added simultaneously.

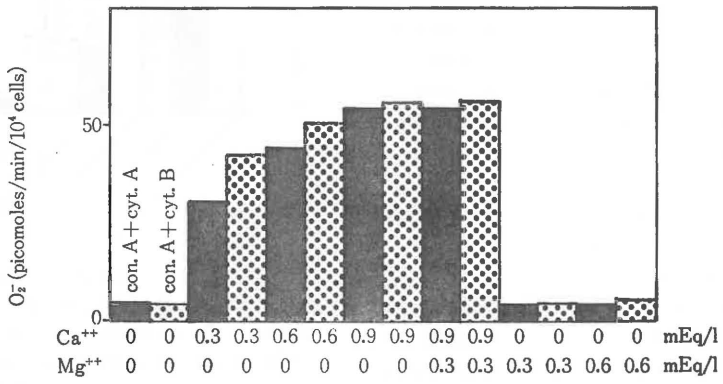

Fig. 5. Effect of Ca^{2+} and Mg^{2+} on O_2^- release induced by con. A (100 μg) plus cytochalasin A (1×10^{-7}M, dark columns) or B (2×10^{-5}M, dotted columns). PMNL are suspended in Ca^{2+}—and Mg^{2+}—free PBS. Before measurement of O_2^-, Ca^{2+} and Mg^{2+} are added as indicated to the reaction mixture.

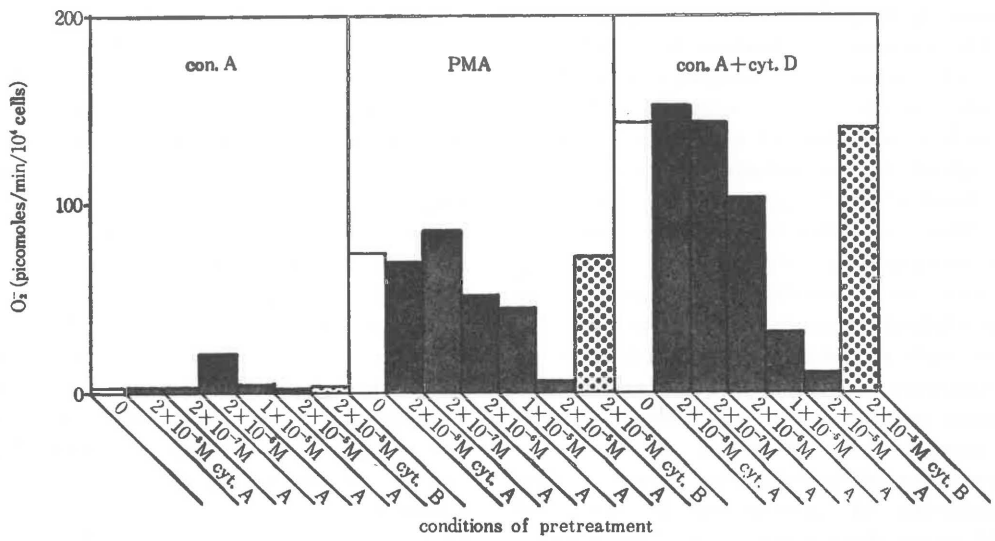

Fig. 6. O_2^- release of PMNL pretreated with cytochalasin A or B and washed. PMNL are incubated with various concentrations of cytochalasin A or 2×10^{-5}M of cytochalasin B at 37°C for 10 minutes, and washed three times with Dulbecco's PBS. O_2^- release from these PMNL is measured by stimulation with con. A (100 μg), PMA (20 ng) and con.A plus cyochalasin D (2×10^{-5}M).

O_2^- release from these PMNL was measured during stimulation by con. A, PMA or con. A plus cytochalasin D.

An enhancing effect of pretreatment with cytochalasin A on con. A-induced O_2^- release was found only when 2×10^{-6}M of cytochalasin A was employed. Pretreatment with concentrations other than 2×10^{-6}M cytochalasin A did not enhance the induction of O_2^- release by con.A. PMNL pretreated with greater than 2×10^{-6}M of cytochalasin A had less O_2^- releasing activity when stimulated by PMA, or con. A plus cytochalasin D. Pretreatment with 2×10^{-8}M to 2×10^{-7}M of cytochalasin A had no influence. These data indicate that washing procedures cannot abolish completely the effect of preincubation with more than 2×10^{-6}M cytochalasin A. O_2^- release from non-treated PMNL was not inhibited by the addition of PMNL treated with 2×10^{-5}M of cytochalasin A (data not shown). This fact indicates that cytochalasin A remains combined with PMNL and was not included in the medium. In contrast to cytochalasin A, the effect of cytochalasin B was reversible.

DISCUSSION

It was confirmed in this study that cytochalasin B, C, D and E markedly enhance con. A-induced O_2^- release. With these cytochalasins, the enhancement of O_2^- release correlated well with the inhibition of the phagocytosis of DP emulsion. These agents are known to affect the microfilament system[3-5], and the relationship between O_2^- release and the microfilament system has been debated[11]. However, the precise mechanism of interaction remains unknown.

This study showed that high concentrations of cytochalasin A largely inhibited O_2^- release and the phagocytosis of DP emulsion. For cytochalasin A no correlation was found between the inhibition of phagocytosis and the enhancement of O_2^- release. This phenomenon seems to be related to the fact that cytochalasin A is the most reactive with sulfhydryl (SH) groups among the cytochalasins tested[13]. In cytochalasin A only one of the hydroxyl groups of cytochalasin B is replaced by a keto group. It is acknowledged that at high concentrations cytochalasin A has several properties that differ from cytochalasin B in the lymphocyte system[14,15]. One of them is that the action of the former is irreversible and that of the latter is not. This irreversibility of action was also seen on PMNL at high concentrations of cyto-

chalasin A, in good contrast to cytochalasin B. The irreversible inhibition by cytochalasin A of O_2^- release and phagocytosis may be explained by the reaction with SH groups on the surface membrane or cytoplasmic proteins. One reason is that cytochalasin A is much more reactive with SH groups than cytochalasin B[16]. Moreover, other SH reagents mimick the action of cytochalasin A[17].

It was also shown that a low concentration of cytochalasin A enhanced con. A-induced O_2^- release with a modest degree of inhibition of DP emulsion phagocytosis. The other cytochalasins never enhanced O_2^- release at such low concentrations. However, neither a low concentration of cytochalasin A nor a high concentration of cytochalasin B stimulated PMNL in the absence of con. A. The enhancement of O_2^- release by cytochalasin A was lost after washing PMNL. A similar response was found with a high concentration of cytochalasin B. It seems likely that cytochalasin A has at least two modes of action depending on its concentration.

As to the enhancement of O_2^- release, several mechanisms have been postulated, and cytochalasin A has been compared with cytochalasin B.

One explanation for the enhancement by cytochalasin B is an inhibition of the formation of phagocytizing vacuoles by a disturbance of the microfilament system, which may result in the efficient trapping of O_2^- by cytochrome C[19]. This explanation is probably not applicable to cytochalasin A since a low concentration of cytochalasin A had a modest inhibitory effect on phagocytosis. The difference between the concentrations of cytochalasin A which enhance O_2^- release and those which inhibit phagocytosis supports the possibility that these two actions are derived from different mechanisms in the other cytochalasins too.

On the other hand, the binding of con. A to the plasma membrane was verified to be essential for enhancement of O_2^- by cytochalasin A, because AMM prevented con. A from binding and abolished O_2^- release by con. A and cytochalasin A. The weaker ability of succinyl con.A to stimulate PMNL than native con.A indicates that the tetramer structure of con. A is more suitable than the dimer. These phenomena seen with cytochalasin A are similar to those seen with cytochalasin B. In a recent study, cytochalasin B was shown to promote the binding of con. A on PMNL and to enhance O_2^- release[9]. The same mechanism may be effective in the case of cytochalasin A.

It has been reported that Ca^{2+} influx may play an important role in triggering oxidative metabolism, lysosomal enzyme release and chemotaxis of PMNL[20-23]. Our results indicate that extracellular Ca^{2+} may be essential for the enhancement of con. A-induced O_2^- release by cytochalasin A or B. It is acknowledged that cytochalasin B enhances Ca^{2+} influx[23]. Cytochalasin A may have the same action.

Cytochalasin A also enhanced the release of O_2^- induced by FMLP, A23187 or PMA, while cytochalasin B inhibited slightly PMA-induced O_2^- release. The reason for this difference remains unexplained. However, at low concentrations cytochalasin A resembles a high concentration of cytochalasin B in many other respects with regard to the enhancement of O_2^- release. Elferink referred to its enhancing effect on lysosomal enzyme release, which resembled that seen with cytochalasin B[17]. It appears, then, that cytochalasin A has not only an inhibitory action as SH reagent on O_2^- release but also an enhancing action resembling cytochalasin B.

The binding sites for low concentrations of cytochalasin A remain unidentified. Cytochalasin A has been found not only to bind the plasma membrane, but also to penetrate and bind to microsomes. High affinity binding sites for low concentrations of cytochalasin A have been shown to exist on red cell membrane[24], but there have been no reports on PMNL. Further investigation of high affinity binding sites or apparatus for cytochalasin A is needed to clarify the mechanisms of enhancement. A survey of the activation mechanism may provide a clue to the etiology of chronic granulomatous disease, in which PMNL of patients display defective activation of O_2^- producing enzyme[25].

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