MECHANISM OF THE POTENTIATING EFFECT OF CYTOCHALASIN B ON THE RESPIRATORY BURST INDUCED BY CONCANAVALIN A IN LEUCOCYTES

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1. Introduction

Leucocytes undergo a dramatic increase in respiration during phagocytosis or upon perturbation of the plasma membrane with a variety of soluble factors. This 'respiratory burst' results in the production of large quantities of superoxide anion (O_2^-), of H_2O_2 and indirectly of $OH^\cdot$ and singlet oxygen [1,2]. This impressive phenomenon, which is relevant for the killing activity against invading organisms [1,3,4] and against tumor cells [5], is triggered by the interaction of the stimulatory agents with appropriate receptors or binding sites in the plasma membrane [2]. The ensuing molecular modification of the plasma membrane leads to activation of a piridine nucleotide oxidase that utilizes NADPH as a physiological substrate [2].

We have investigated the mechanism that triggers the respiratory burst by using concanavalin A (con A) shown [6] to stimulate the metabolic activity of leucocytes through a modification of the plasma membrane which follows the interaction of the lectin with the cell surface receptors [6,7]. In the course of that investigation we showed that cytochalasin B (CB) potentiates the respiratory response of guinea pig and rabbit polymorphonuclear leucocytes (PMN) and of rabbit alveolar macrophages to con A [8,9]. A similar effect of CB on rabbit PMN stimulated by the chemotactic peptide formyl-methionyl–leucyl–phenylalanine (FMLP) and by the calcium ionophore A23187 has been shown in [10,11].

This paper deals with the mechanisms underlying the effect of CB and reports data showing that the drug acts by increasing the activation of the NADPH oxidase, the plasma membrane-bound enzyme responsible for the respiratory burst of leucocytes.

2. Materials and methods

Con A, cytochrome *c* (type VI), superoxide dismutase (SOD) (type I), α-methyl-D-mannoside (α-MM) (grade III) and NADPH (grade III) were purchased from Sigma Chemical Co. (St Louis, MO); [^{3}H]con A was purchased from New England Nuclear (Dreieich); cytochalasin B purchased from Serva (Heidelberg) was dissolved in dimethylsulfoxide (DMSO) at 5 mg/ml final conc. Other chemicals were of reagent grade.

2.1. *Cells*

Guinea pig PMN from peritoneal exudate were obtained as in [12]. Human PMN were isolated from blood, taken from normal donors, by dextran sedimentation of red cells and centrifugation of the leucocyte-rich fraction on Ficoll-Paque (Pharmacia, Uppsala). Cells were freed from contaminating erythrocytes by a brief hypotonic shock and suspended in Krebs Ringer phosphate buffer (pH 7.4) containing 5 mM glucose and 0.5 mM $CaCl_2$ (KRP). Both guinea pig and human cell suspensions were composed of 90–95% neutrophils.

2.2. *Oxygen consumption*

Cell respiration was monitored by a Clark-type oxygen electrode attached to a thermostatically controlled plastic vessel (37°C), using $1.5–2 \times 10^7$ cells in 2 ml KRP. To avoid inactivation of the NADPH oxidase the incubation medium contained 2 mM NaN_3 [13].

2.3. *Superoxide release*

O_2^- release was measured by the superoxide-inhibitable reduction of cytochrome *c* [12,14] either by

continuous monitoring or in the same experimental conditions used to measure the oxygen uptake. In the former case the assay medium contained 1 ml KRP, 2.5×10^6 PMN, 100 μM cytochrome *c*, 2 mM NaN_3; con A (100 μg/ml) and SOD (25 μg/ml) were added where required and the reduction of cytochrome *c* was measured at 550 nm in a 576 Perkin Elmer spectrophotometer at 37°C. In the other case O_2^- was measured, as in [12], on samples withdrawn from the electrode vessel, where oxygen uptake was continuously recorded, 4 min after the addition of the stimulant. The assay medium was composed of 2 ml KRP, 2×10^7 cells, 2 mM NaN_3, 200 μM cytochrome *c* and, when necessary, 50 μg SOD.

2.4. *NADPH-dependent O_2^- producing activity (NADPH oxidase) in cell free particles*

After the measurement of oxygen consumption guinea pig PMN were withdrawn from the electrode chamber, 5-fold diluted with ice cold KRP, washed, resuspended in 0.34 M sucrose (pH 7.0) containing 1 mM $NaHCO_3$, disrupted by sonication (MSE sonifier) and assayed for NADPH-dependent O_2^- producing activity by monitoring the SOD-inhibitable reduction of cytochrome *c* at 550 nm (Perkin Elmer 576) as in [15].

2.5. *Lectin binding*

A solution of [^{3}H]con A at 4 mg/ml lectin (spec. act. 2300 cpm/μg lectin) was used. The assay was carried out simultaneously with the measurement of oxygen uptake. CB (5 μg/ml) or DMSO 0.1% were added to $1.5–2 \times 10^7$ PMN in 2 ml KRP containing 2 mM NaN_3; the mixture was incubated 3 min at 37°C. After this time the resting respiration was recorded and 2 min thereafter 100 μg/ml labeled con A were added. After 3 min (when the stimulated respiration reached its maximum value, either in the presence or in the absence of CB) 1 ml and 0.5 ml of the cell suspension were withdrawn and rapidly diluted in 9 or 9.5 ml chilled KRP, respectively. The cells were then spun down for 5 min at $400 \times g$ at 0°C, washed with 10 ml KRP and pelletted ($400 \times g$, 5 min). The cell pellets were dissolved in 10 ml scintillation medium composed of 300 ml Triton X-100 (Merck, Darmstadt, scintillation grade), 700 ml toluol and 5.5 g Permablend III (Packard Instrument Co., Downers Grove, IL) and counted in a Beckman LS-100 liquid scintillation counter.

3. Results and discussion

3.1. *Effect of CB on the oxygen uptake of PMN stimulated by con A*

Fig.1 shows the effect of CB on the extra respiration induced by con A on guinea pig PMN. It can be seen that:

(i) The drug causes a marked potentiation of the stimulatory response to con A;
(ii) Both the extra respiration induced by con A and by con A + CB are reversed by α-MM;
(iii) CB induces a slight increase of O_2 consumption also when added alone.

The effect of CB is dose-dependent being already marked at 1 μg/ml and reaching a plateau at 10 μg/ml (not shown). The drug is active in a wide range of concentrations of con A (20–300 μg/ml).

3.2. *Effect of CB on the O_2^- release from PMN stimulated by con A*

The O_2^- formed during the respiratory burst is measured by the reduction of cytochrome *c*. Since this compound does not enter into the cell the O_2^- measured corresponds only to the amount of the radical that is released from the cell into the external medium. This amount is only a part of the O_2^- formed by the stimulated cells [12,16,17]. Fig.2 shows the effect of CB on the respiratory burst of PMN induced by con A, assayed as continuous monitoring of O_2^- release. It can be seen that:

(i) The addition of CB on resting PMN increases the O_2^- production, the increment taking place immediately after the addition of the drug;

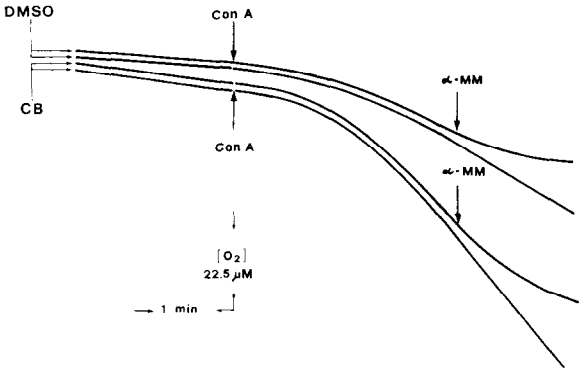

Fig.1. Effect of cytochalasin B on the oxygen uptake by guinea pig PMN stimulated with concanavalin A (con A): CB (10 μg in 2 μl DMSO) or 2 μl DMSO were added 5 min before con A (200 μg in 50 μl KRP); α-MM was 70 mM.

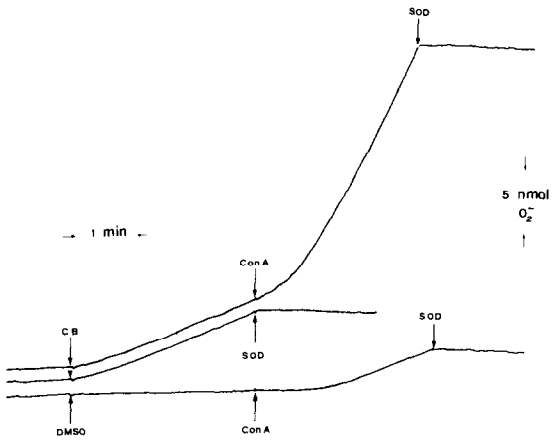

Fig.2. Continuous monitoring of O_2^- release from cytochalasin B-treated guinea pig PMN: CB, 5 μg/ml; con A, 100 μg/ml. For experimental details see section 2.

(ii) Con A causes an increase of O_2^- production after a lag time of 70–80 s;
(iii) Con A added to cells pretreated with CB induces a stimulation of O_2^- production much greater than that induced by con A alone.

To investigate whether the increased recovery of O_2^- in the extracellular medium caused by CB is due to the enhanced formation of the radicals and also to an increased diffusion through the plasma membrane we have studied the effect of the drug by comparing the increment of O_2^- released outside the cells with that of O_2 consumption. The results reported in table 1 show that:

(i) CB added alone induces a slight but significant increase in O_2 consumption and O_2^- recovery. If we consider the ratio O_2^-/O_2 we can see that, for every nmol oxygen consumed, CB-treated cells release 1.05 nmol O_2^- and control cells release 0.41 nmol O_2^-. Taking into account the extra respiration induced by CB for every nmol O_2 consumed, 3.0 nmol O_2^- are released. These data seem to indicate that CB induces in resting cells both an increase in O_2 consumption and an increase in the proportion of O_2^- that is recovered outside the cells;
(ii) CB potentiates both the increase of O_2 uptake and of O_2^- release by con A-treated cells. Taking into account the extra O_2 uptake and the extra O_2^- release it can be seen that the ratio O_2^-/O_2 approximates to unity both in the presence and in the absence of CB, indicating that in con A-treated cells CB does not modify the proportion of O_2^- which is released with respect to the amount which is formed. These findings demonstrate that the greater recovery of O_2^- outside the cells when they are stimulated by con A in the presence of CB, is not linked to an increased diffusion of the radical through the plasma membrane.

3.3. *Effect of CB on the NADPH oxidase activity*

To get a better insight into the effect of CB we have investigated the action of the drug on the activation of the membrane-bound NADPH oxidase, that is the main event responsible for the respiratory burst.

The data of table 2 shows that:

(i) Cell-free particles of cells treated with CB present a higher activity of NADPH oxidase. This indicates that the binding of this drug to the cell membrane represents 'per se' a signal for the activation of the NADPH oxidase, as has been

Table 1
O_2 uptake and O_2^- release by CB-treated guinea pig PMN

	Resting cells			Con A-treated cells		Average increment induced by con A		
	O_2	O_2^-	O_2^-/O_2	O_2	O_2^-	O_2	O_2^-	O_2^-/O_2
Control	28.0 ± 3.0 (10)	11.4 ± 4.0 (6)	0.41	94.4 ± 9.4 (10)	74.6 ± 11.2 (6)	66.4	63.2	0.95
CB	37.3 ± 5.5 (10)	39.4 ± 8.0 (6)	1.05	136.2 ± 15.2 (10)	138.6 ± 19.8 (6)	98.9	98.6	1.02
	$P < 0.005$	$P < 0.025$		$P < 0.001$	$P < 0.025$			
Δ[a]	9.3	28.0	3.0					

[a] CB minus control that is the extra respiration induced by CB

Values are expressed as nmol . 2×10^7 cells^{-1} . 4 min^{-1} ± SEM. Experimental details are in section 2: con A, 100 μg/ml; CB, 5 μg/ml; DMSO, 0.1%. *P* was calculated by Student's *t*-test on paired data; expt in brackets

Table 2
Effect of CB on the NADPH-oxidase activity of subcellular particles from resting and con A-treated guinea pig PMN

Additions	(nmol . min^{-1} . mg protein^{-1}) ± SEM	
	DMSO	CB
None	2.6 ± 0.2 (7)	3.4 ± 0.4 (7) $P < 0.05$
Con A	9.0 ± 1.2 (10)	22.8 ± 3.8 (5)
Con A + α-MM[a]	3.8 ± 0.5 (6)	4.2 (2)

[a] α-MM was added 2 min after the addition of the lectin, when the cell reached the maximal activation. Experimental details are in section 2: con A, 100 μg/ml; CB, 5 μg/ml; DMSO, 0.1%; α-MM, 70 mM. P was calculated by Student's t-test on paired data; no. expt in brackets

shown for other members of the cytochalasin family [18];

(ii) CB potentiates the activation of the oxidase induced by con A;

(iii) The activation of the enzyme is reversible. In fact when α-MM is added to cells stimulated by con A or con A plus CB, during the linear phase of the respiratory excitation, the activity of the NADPH oxidase resumes its resting value.

These results demonstrate that the action of CB consists in an actual increase of the responsiveness of leucocytes to the stimulatory agent and that the target of the drug is the mechanism of the activation of the NADPH oxidase in intact PMN. When added to cell free particles from either resting or stimulated PMN, CB does not modify the activity of the oxidase (not shown).

3.4. *Effect of CB on the interaction between the lectin and the PMN plasma membrane*

On the basis of the different effect of native and dimeric lectin, on the enhancing effect of anti-con A antibodies [7] and on the reversibility of the respiratory burst following the displacement, by α-MM, of the lectin molecules from their receptors on the plasma membrane, we have suggested that the triggering of the metabolic stimulation is due to the ligand–receptor interaction followed by redistribution of the ligand–receptor complexes on the cell surface and that the metabolic stimulation would be proportional to the extent of receptor crosslinkage induced by the lectin [6,7]. Thus the greater responsiveness of the NADPH oxidase caused by CB might be explained in terms of an increase of the binding of con A to leucocyte plasma membrane. We have tested this possibility and the first two experiments show that CB did not modify the binding of con A. During this phase of our work it was shown [19] that, in human PMN, CB increased the binding of the lectin to cell receptors. These results prompted us to continue the experiments on the effects of CB on the binding of con A by using both the cell types. Table 3 shows that guinea pig neutrophils bind virtually the same amount of lectin either in the presence or in the absence of CB. On the contrary CB increases the binding of con A to the cell surface of human polymorphs. These findings indicate that the effect of CB on the respiratory burst and on the extent of con A-binding can be unrelated phenomena. At least in guinea pig PMN the effect of CB on the respiratory burst is not due to a greater binding of the lectin to the cell receptors. This conclusion is consistent with the data in [11] on the enhancing effect of CB on lysosomal enzyme release and on superoxide production by rabbit PMN stimulated with FMLP.

On the basis of these findings we suggest that CB exerts its action by modifying the molecular events that occur after the binding of the lectin to cell receptors on the plasma membrane. The nature of this modification can be only a matter of speculation. In general terms it can be said that the drug facilitates and/or accelerates the molecular modifications of the plasma membrane that trigger or that are the signals for, the activation of the NADPH oxidase, such as ion fluxes across the PMN plasma membrane [10], the

Table 3
Effect of cytochalasin B on the binding of con A to guinea pig and human PMN

	Guinea pig PMN (6)	Human PMN (4)
Con A + DMSO	0.315 ± 0.030	0.244 ± 0.030 $P < 0.05$
+ CB	0.317 ± 0.028	0.343 ± 0.060
+ DMSO + α-MM[a]	0.065 ± 0.020	0.053 ± 0.010
+ CB + α-MM[a]	0.077 ± 0.021	0.060 ± 0.013

[a] The amounts of lectin bound in the presence of α-MM are reported to show the specificity of the con A binding; in the presence of α-MM con A was unable to stimulate the PMN metabolism

Values are expressed as μg con A/10^6 cells. The means ± SEM are reported. Experimental details are in section 2: con A, 100 μg/ml; CB, 5 μg/ml; DMSO, 0.1%; α-MM, 70 mM. P was calculated by Student's t-test on paired data; no. expt in brackets

aggregation of glycoproteins in the plane of the membrane [20] and the assembly of the postulated subunits of the oxidase system [21].

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References

[1] Klebanoff, S. J. and Clark, R. A. (1978) The Neutrophil: Function and Clinical Disorders, pp. 283–488, Elsevier/North-Holland, Amsterdam, New York.
[2] Rossi, F., Patriarca, P. and Romeo, D. (1980) in: The Reticuloendothelial System: A Comprehensive Treatise (Friedman, H. et al. eds) vol. 2, pp. 153–188, Plenum, New York.
[3] Sbarra, A. J., Selvaraj, R. J., Paul, B. B., Poskitt, P. K. F., Mitchell, G. W. jr, Levis, F. and Asbell, M. A. (1977) in: The Granulocyte: Function and Clinical Utilization, pp. 29–48, A. R. Liss, New York.
[4] Johnston, R. B. jr, Keele, B. B. jr, Misra, H. P., Lehmeyer, J. E., Webb, L. S., Bachner, R. L. and Rajagopalan, K. V. (1975) J. Clin. Invest. 55, 1357–1372.
[5] Nathan, C. F., Silverstein, S. C., Brukner, L. H. and Cohn, Z. A. (1979) J. Exp. Med. 149, 100–113.
[6] Romeo, D., Zabucchi, G. and Rossi, F. (1973) Nature New Biol. 243, 111–112.
[7] Romeo, D., Zabucchi, G., Berton, G. and Schneider, C. (1978) J. Membr. Biol. 44, 321–330.
[8] Zabucchi, G., Soranzo, M. R., Berton, G., Romeo, D. and Rossi, F. (1978) J. Reticuloendothel. Soc. 24, 451–460.
[9] Rossi, F., Bellavite, P., Dobrina, A., Dri, P. and Zabucchi, G. (1980) in: Mononuclear Phagocytes, Functional Aspects (Van Furth, R. ed) pt II, pp. 1187–1217, Martinus Nijhoff, The Hague.
[10] Becker, E. L., Sigman, M. and Oliver, J. M. (1979) Am. J. Pathol. 95, 81–98.
[11] Bennet, J. P., Cockcroft, S. and Gomperts, B. D. (1980) Biochim. Biophys. Acta 601, 584–591.
[12] Dri, P., Bellavite, P., Berton, G. and Rossi, F. (1979) Mol. Cell. Biochem. 23, 109–122.
[13] Jandl, R. C., Andrè-Schwartz, J., Borges-DuBois, L., Kipnes, R. S., McMurrich, B. J. and Babior, B. M. (1978) J. Clin. Invest. 61, 1176–1185.
[14] Babior, B. M., Kipnes, R. S. and Curnutte, J. T. (1973) J. Clin. Invest. 52, 741–744.
[15] Bellavite, P., Berton, G., Dri, P. and Soranzo, M. R. (1981) J. Reticuloendothel. Soc. 29, 47–60.
[16] Root, R. K. and Metcalf, J. A. (1977) J. Clin. Invest. 60, 1266–1279.
[17] Goldstein, I. M., Roos, D., Kaplan, H. B. and Weissmann, G. (1975) J. Clin. Invest. 56, 1155–1163.
[18] Nabi, Z. F., Takeshige, K., Hatae, T. and Minakami, S. (1979) Exp. Cell Res. 124, 293–300.
[19] Cohen, M. S., Metcalf, J. A. and Root, R. K. (1980) Blood 55, 1003–1010.
[20] Schlessinger, J., Koppel, D. E., Axelrod, D., Jacobson, K., Webb, W. W. and Elson, E. L. (1976) Proc. Natl. Acad. Sci. USA 73, 2409–2413.
[21] Cohen, H. J., Chovaniec, M. E. and Ellis, S. E. (1980) Blood 56, 23–29.