Activation of the respiratory burst of guinea pig neutrophils by dicyclohexylcarbodiimide

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DCCD activates the respiratory burst in guinea pig peritoneal neutrophils. The onset of the superoxide producing activity is preceeded by a lag, inversely proportional to the dose of the stimulant and to the temperature. Initial rates of superoxide formation exhibit different dependencies on the concentrations of DCCD and on temperature. Activation of NAD(P)H oxidase is inhibited by preincubation of neutrophils with 2-deoxyglucose and does not require the presence of extra cellular Ca^{2+}.

Dicyclohexylcarbodiimide *Superoxide* *Respiratory burst* *Neutrophil*

1. INTRODUCTION

Phagocytizing neutrophils exhibit a sharp increase in the rate of their oxygen consumption [1,2]. This respiratory burst is a manifestation of the activation of a membrane-bound reduced pyridine nucleotide oxidase that catalyzes the reduction of molecular oxygen to produce superoxide ions [3]. The latter give rise to other reactive oxygen forms (e.g., hydrogen peroxide and hydroxyl radicals) that participate in the oxygen-dependent killing of ingested microorganisms [3,4].

The respiratory burst may also be elicited by non-physiological, particulate [5,6] or soluble stimuli [7,8]. They interact initially with the cell membrane, but the molecular basis of activation of the oxidase is unknown.

We have found that dicyclohexylcarbodiimide (DCCD) activates the respiratory burst in guinea pig peritoneal neutrophils.

2. MATERIALS AND METHODS

Guinea pig neutrophils were isolated from (casein-induced) peritoneal exudate 18 h after the injection of sodium caseinate (Difco) by a standard procedure [9] and resuspended in Ca^{2+}-free Krebs-Ringer phosphate solution containing 2 mM $MgCl_2$ and 2 mM glucose (KRP). Cytochrome *c* (type II), superoxide dismutase, 2-deoxyglucose and phorbol myristate acetate (PMA) were the products of Sigma. DCCD was obtained from Fluka. PMA and DCCD were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the assay mixtures was $\leqslant 0.8\%$. Superoxide production was measured as superoxide dismutase inhibitable reduction of cytochrome *c* [10]. For DCCD activation, suspensions of cells in KRP were prewarmed for 3 min at 37°C in the presence of 0.05 mM cytochrome *c*. DCCD was then added and cells were further incubated for 5 min, spun down in an Eppendorf microfuge and absorbances of the supernatants at 550 nm measured. Controls contained either dimethyl sulfoxide or DCCD in the presence of 30 μg/ml superoxide dismutase. The changes at 550 nm were also measured continuously against a reference, containing cells, cytochrome *c*, superoxide dismutase and the appropriate activating agent. The extent of cytochrome *c* reduction was calculated using 18.5 as the millimolar difference extinction coefficient between oxidized and reduced forms of cytochrome *c* at 550 nm [11].

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Results are given as mean values of representative experiments performed in duplicates or triplicates. Experiments were repeated with different preparations of cells. To account for variations in absolute rates of superoxide production, rates obtained at given conditions were calculated relative to the respective values at optimal conditions for stimulation (40 μg DCCD.2.5 $\times$ 10^6 cells^{-1}.ml^{-1}, 37°C). The inter-experimental variability was $\leqslant$20% of mean relative values. Spectrophotometric measurements were carried out in a Cary 118 recording spectrophotometer.

3. RESULTS

Addition of DCCD to resting guinea pig neutrophils resulted in a rapid production of superoxide anions as shown by the superoxide dismutase inhibitable reduction of cytochrome *c* (fig.1). The onset of the enzymic activity of the cells was preceded by a short lag time, measured by extrapolation of the linear portion of the curve to the initial baseline [12] (fig.1). The figure also in-

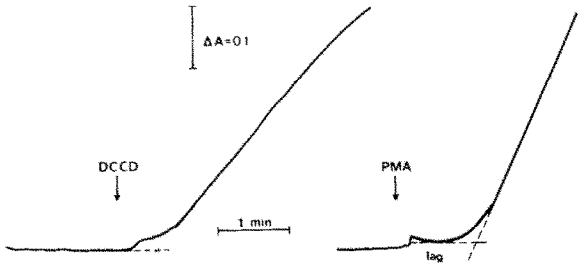

Fig.1. Activation of the respiratory burst by DCCD (40 μg/ml) and PMA (0.2 μg/ml) measured as superoxide dismutase inhibitable reduction of cytochrome *c* at 550 nm. Guinea pig neutrophils were suspended in KRP at 2.5 $\times$ 10^6 cells/ml; 37°C.

Table 1

Activation of the respiratory burst by PMA and DCCD[a]

Stimulus	Lag time (s)	Linear rate (nmol O_2^-/min)
DCCD (20 μg/ml)	40	9.15
PMA (0.2 μg/ml)	67	13.2
DCCD + PMA	46	11.5

[a] 2.5 $\times$ 10^6 cells/ml

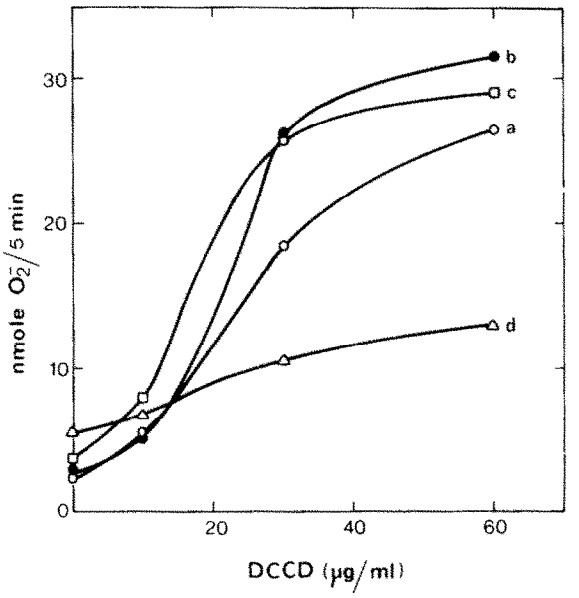

Fig.2. The dependence of superoxide production rates on [DCCD] at different cell densities: (a) 10^6/ml; (b) 4 $\times$ 10^6/ml; (c) 8 $\times$ 10^6/ml; (d) 2 $\times$ 10^7/ml.

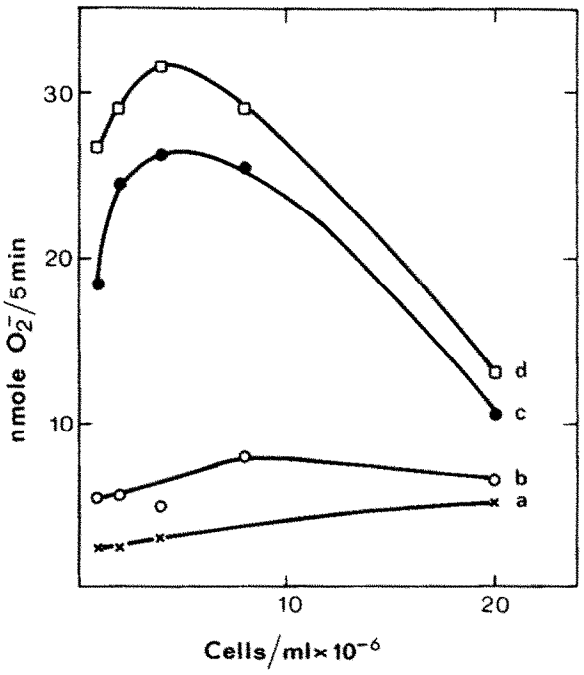

Fig.3. The dependence of superoxide production rates on [cells] at different [DCCD]: (a) 0; (b) 10 μg/ml; (c) 30 μg/ml; (d) 60 μg/ml.

Table 2

The dependence of the respiratory burst on [DCCD][a]

DCCD (μg/ml)	Lag time (s)	Linear rate (nmol O_2^-/min)
10	73	5.3
20	56	5.8
40	37	17.0
60	24	13.0

[a] Continuous assays at 2.5×10^6 cells/ml

cludes the response of neutrophils to phorbol myristate acetate. The superoxide generating activity of the cells could not be further augmented by a simultaneous stimulation with DCCD and PMA (table 1).

The rate of superoxide production at a given concentration of cells increased with the dose of DCCD, approaching saturation (fig.2). When DCCD was kept constant and the concentration of cells varied, the superoxide forming activity passed through a maximum, dropping at high cell densities (fig.3). Table 2 summarizes the dependence of lag times and rates of superoxide formation on the dose of the activating agent. At 40 μg DCCD/ml, the superoxide generation rate was maximal; further increases in [DCCD] resulted in lower rates. The response, however, continued to be faster, as indicated by shorter lag times. Activation was also favoured by higher temperatures, the enzymic activity being impaired above 40°C (table 3).

Table 3

The effect of temperature on the DCCD-elicited respiratory burst[a]

Temp. (°C)	Lag time (s)	Linear rate (nmol O_2^-/min)	Total O_2^- (nmol/5 min)
26	94	8.0	26.3
31	58	12.4	41.3
38	37	17.0	45.5
45	22	6.0	13.5

[a] 2.5×10^6 cells/ml were stimulated with 40 μg/ml DCCD

Lag times and linear rates were measured in a continuous assay; total superoxide formation in single point determinations

Table 4

The effect of 2-deoxy-D-glucose on the DCCD-elicited respiratory burst[a]

Additions	Lag time (s)	Linear rate (nmol O_2^-/min)
None	42	9.0
2-Deoxy-D-glucose		
2 mM	53	4.9
10 mM	65	3.0

[a] 2.5×10^6 cells/ml were preincubated for 3 min with a given [2-deoxy-D-glucose] and stimulated with 40 μg DCCD/ml

Lag times and linear rates were measured in a continuous assay

Preincubation of cells in the presence of the energy metabolism inhibitor deoxy-D-glucose affected both activation and activity, as shown in table 4.

Cells activated with DCCD in KRP with or without Ca^{2+} exhibited similar rates (not shown) indicating that the activation of the NAD(P)H oxidase of guinea pig neutrophils by DCCD was independent of extracellular Ca^{2+}.

4. DISCUSSION

Activation of the respiratory burst of guinea pig neutrophils by DCCD resembled in many respects activation by other stimuli. At saturating concentrations of DCCD, superoxide formation detected as superoxide dismutase-inhibitable reduction of cytochrome *c*, proceeded at rates comparable to those induced by saturating doses of PMA (fig.1, table 1). The finding that no additional activity could be induced by a simultaneous exposure of cells to both DCCD and PMA (table 1) indicated that each of them induced the same enzymatic activity.

The respiratory burst elicited by DCCD followed the kinetic pattern observed with other stimuli [12–14]. It consisted of two phases: a lag, during which NAD(P)H oxidase underwent activation; and, a superoxide-producing phase (fig.1). As in

the case of digitonin and PMA [12–14], activation and activity exhibited different dependencies on [DCCD] and temperature (tables 2,3). It appears that while the cellular pathway connecting the interaction of DCCD with the cell to the enzymatic response is temperature resistant, at least up to 45°C, the activated enzyme is temperature sensitive.

Both the activation and activity were inhibited by the presence of energy metabolism inhibitor deoxyglucose (table 4). Similar inhibition of the respiratory burst of digitonin and PMA-stimulated cells was correlated with depletion of ATP content of the cells [13,14]. As longer latencies under suboptimal conditions for stimulation with a given agent lead to lower activities [15], the inhibition of the superoxide generation rates by deoxyglucose may reflect a requirement for energy in the activation process only.

The extent of the respiratory burst activated by DCCD is governed not by the initial concentration of the stimulant but by the amount of DCCD/cell. This is borne out by the decrease in the magnitude of the response at higher cell densities (fig.3). When the results of fig.3 are replotted to describe the extent of O_2^-/cell, as a function of DCCD/cell, an initially linear relationship is obtained. This is due to the high hydrophobicity of DCCD, partitioning preferentially into the membranes of the cells.

DCCD is well recognized for its chemical reactivity with various functional groups [16] and its effects on the activity of membrane-bound enzymes [17]. Further studies are planned to characterize its interaction with neutrophils and to establish whether the activation of the dormant oxidase is due to a specific reaction of DCCD with the enzyme or with its activity determining apparatus.

REFERENCES

[1] Baldridge, C.W. and Gerard, R.W. (1933) Am. J. Physiol. 109, 235.
[2] Karnovsky, M.L. (1962) Physiol. Rev. 42, 143.
[3] Babior, B.M. (1978) New Engl. J. Med. 298, 659.
[4] Badwey, J.A. and Karnovsky, M.L. (1980) Annu. Rev. Biochem. 49, 695.
[5] Hohn, D.C. and Lehrer, R.I. (1975) J. Clin. Invest. 55, 707.
[6] Segal, A.W. and Coade, S.B. (1980) Biochem. Biophys. Res. Commun. 84, 611.
[7] Respine, J.E., White, J.G., Clawson, C.C. and Holmes, B.M. (1974) J. Lab. Clin. Med. 83, 911.
[8] Graham, R.C., Karnovsky, M.J., Shafer, A.W., Glass, E.A. and Karnovsky, M.L. (1967) J. Cell Biol. 32, 629.
[9] Stahelin, H., Suter, E. and Karnovsky, M.L. (1956) J. Exp. Med. 104, 121.
[10] Babior, B.M., Kipnes, R.S. and Curnutte, J.T. (1973) J. Clin. Invest. 52, 741.
[11] Margoliash, E. and Frohwirt, N. (1959) Biochem. J. 71, 570.
[12] Cohen, H.J. and Chovaniec, M.E. (1978) J. Clin. Invest. 61, 1081.
[13] Cohen, H.J. and Chovaniec, M.E. (1978) J. Clin. Invest. 61, 1088.
[14] Newburger, P.E., Chovaniec, M.E. and Cohen, H.J. (1980) Blood 55, 85.
[15] Lehrer, R.I. and Cohen, L. (1981) J. Clin. Invest. 68, 1314.
[16] Linnett, P.E. and Beechey, R.B. (1979) Methods Enzymol. 55F, 495.
[17] Beechey, R.B., Linnett, P.E. and Fillingame, R.H. (1979) Methods Enzymol. 55F, 426.