INHIBITORY EFFECT OF DI-ISOPROPYL FLUOROPHOSPHATE ON ACTIVATION OF AN O_2^--FORMING ENZYME OF POLYMORPHONUCLEAR LEUKOCYTES WITH C3b-ZYMOSAN

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1. Introduction

Polymorphonuclear leukocytes promote superoxide anion (O_2^-) production when stimulated with appropriate stimuli [1–6]. The enhanced O_2^- production requires intact, viable cells and proceeds independently of phagocytosis [6–8]. Furthermore, the reaction was found to be inhibited with various potent inhibitors of serine proteases such as di-isopropyl fluorophosphate (Dip-F) [9–12]. Although there is no direct evidence, the availabe information suggests that a certain chymotrypsin-like serine protease is involved in the activation of an O_2^--forming enzyme, probably a NAD(P)H oxidase which is embedded in the surface membrane of leukocytes [3,13–19].

To clarify the inhibitory mechanism of Dip-F, we studied the effect of the inhibitor on activation of the O_2^--forming enzyme by measuring its activity in a particulate fraction isolated from sonicated guinea pig polymorphonuclear leukocytes [18,19]. The O_2^--forming activity of the leukocytes stimulated with complement-treated zymosan (C3b-zymosan) in the presence of Dip-F was found to be markedly lower than that stimulated in the absence of Dip-F, though the inhibitor did not influence the isolated enzyme at all. These results, indicating that Dip-F inhibits strongly the activation process of a certain O_2^--forming enzyme, probably a NAD(P)H oxidase, with C3b-zymosan, are reported here.

2. Materials and methods

2.1. *C3b-zymosan*

A suspension of zymosan (30 mg in 10 ml saline) was treated by heating for 10 min at 90°C and washed 3 times with 20 mM phosphate-buffered saline (pH 7.4). The zymosan was then reacted with 6.0 ml freshly isolated guinea pig serum for 30 min at 37°C [7]. After washing with Krebs-Ringer phosphate buffer supplemented with 5.6 mM D-glucose (pH 7.4) (buffer 1), the C3b-bound zymosan thus formed was suspended in buffer 1 and used as a stimulus.

2.2. *Measurement of O_2^- production by intact leukocytes*

Guinea pig polymorphonuclear leukocytes were isolated from the peritoneal cavity of animals intraperitoneally injected with casein [12]. The determination of O_2^- production was performed by measuring the superoxide dismutase-inhibitable reduction of equine ferricytochrome *c* by O_2^- [12]. For this purpose, leukocytes (6×10^6 cells) were incubated in the presence or absence of 0.9 mg C3b-zymosan in 1.0 ml buffer 1 supplemented with 0.25% bovine serum albumin and 100 nmol cytochrome *c* for 15 min at 37°C [12]. After incubation, the reaction mixtures were centrifuged at 4°C, and the absorption spectra of supernatants were measured from 540–560 nm. The amounts of reduced cytochrome *c* were calculated, using an absorption coefficient of 21.1 mM^{-1} cm^{-1} at 550 nm (reduced–oxidized) [20].

2.3. *Solubilization of the O_2^--forming enzyme*

The O_2^--forming enzyme of leukocytes was isolated as in [18,19] for isolation of NAD(P)H oxidase from human neutrophils. The C3b-zymosan-treated or non-treated leukocytes (1.5×10^8 cells) were washed 2 times with buffer 1 and resuspended in 8.0 ml cold 0.34 M sucrose in 0.15 M NaCl (pH 7.5). The cells were then disrupted by sonication at 0°C (two 20 s pulses at 160 W interrupted by a 30 s cooling period)

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using a Heat System W220F instrument fitted with a cuphorn. After removal of zymosan together with cell debris and nuclei by centrifugation at 400 × *g* for 10 min, the particulate fraction from the disrupted cells was sedimented by centrifugation at 100 000 × *g* for 30 min. The particles sedimented were washed 2 times with 0.35 M sucrose in 0.15 M NaCl and suspended in the same medium.

Solubilization of the O_2^--forming enzyme from the particles was performed using 20 mM glycine buffer supplemented with 0.4% Triton X-100, 2 mM NaN_3 and 1.6 μm $CaCl_2$ (pH 8.4) (buffer 2). The particle suspension (5.0 ml) was mixed with 5.0 ml buffer 2 and sonicated at 0°C (three 15 s pulses at 75 W with a 45 s cooling period between pulses), using the above instrument, fitted with a microtip. The sonicated mixture thus obtained was used as a source of the O_2^--forming enzyme as in [18,19].

The O_2^--forming enzyme activity of the solubilized fraction was assayed at 22°C by measuring a difference spectrum which developed between a pair of reaction mixtures containing the enzyme together with cytochrome *c* incubated under identical conditions, except that one contained 30 μg superoxide dismutase and the other did not. The reaction mixture used contained the enzyme, 80 nmol ferricytochrome *c*, 0.1 mM NADPH, 0.1 mM FAD and 0.1 mM NaN_3 in 1.0 ml 12.5 mM potassium phosphate buffer (pH 7.4). The amount of O_2^- formed was calculated from the difference spectrum at 550 nm measured by the use of a Hitachi double beam recording spectrophotometer, model 200-20, as described for the measurement of O_2^- production by intact leukocytes.

2.4. *Materials*

Protein concentration of solubilized O_2^--forming enzyme preparations was determined by the protein assay reagent (Bio Rad Labs), using bovine IgG as a standard protein. Zymosan, equine ferricytochrome *c*, FAD, NADPH, NADH and bovine superoxide dismutase were purchased from Sigma Chemical Co.

3. Results

As observed with human leukocytes [7], guinea pig peritoneal polymorphonuclear leukocytes also promoted O_2^- production estimated by superoxide dismutase-inhibitable cytochrome *c* reduction, when exposed to C3b-zymosan (table 1). The enhancement

Table 1
Inhibition of O_2^- production (nmol/6 × 10^6 cells) from leukocytes with Dip-F

Dip-F (mM)	Stimulated cells	Non-stimulated cells
0	56.2	0.2
2	27.6	0.3
10	0.1	0.4

The leukocytes were incubated with varying concentrations of Dip-F for 15 min at 37°C and then stimulated or not stimulated with C3b-zymosan. The amounts of O_2^- produced from the stimulated or non-stimulated cells were determined by measuring the superoxide dismutase-inhibitable cytochrome *c* reduction at 37°C for 15 min. The cytochrome *c* reduction in each reaction mixture containing 30 μg superoxide dismutase also was measured as control

of O_2^- production was found to be markedly inhibited with Dip-F, as in the case of stimulation with antigen–antibody complexes or concanavalin A [12]; incubation of leukocytes with 10 mM Dip-F caused complete inhibition of O_2^- production. Both Dip-F and 2-propanol used as a solvent of the inhibitor at each concentration added did not significantly influence the cell viability, as estimated by a trypan blue exclusion test [12,21]. Hence the effect of Dip-F on O_2^- production may be caused by direct inhibition of the activation process of a certain O_2^--forming enzyme with C3b-zymosan.

To study this point further, the effect of Dip-F on the O_2^--forming enzyme was determined by measuring the enzyme activity. For this purpose, the enzyme was solubilized with Triton X-100 from a particulate fraction of sonicated leukocytes and its activity was assayed by measuring superoxide dismutase-inhibitable cytochrome *c* reduction, as in [18,19]. When the solubilized fraction was prepared from the leukocytes stimulated with C3b-zymosan for 15 min at 37°C, superoxide dismutase-inhibitable cytochrome *c* reduction attributable to O_2^- formation was found to proceed.

To confirm that the measured rate of O_2^- formation was an accurate reflection of the amount of the O_2^--forming enzyme embedded in the particles, we therefore conducted a series of experiments. Table 2 shows the effect of omission of individual components of the reaction mixture. The O_2^- formation observed was dependent on the presence of both NADPH and a heat-labile factor, probably NAD(P)H oxidase, in the solubilized fraction. The O_2^--forming rate increased

Table 2
Requirement for O_2^--dependent cytochrome *c* reduction by solubilized preparations

Conditions	O_2^- production (nmol . 5 min^{-1} . mg $protein^{-1}$)
Complete reaction mixture	12.3
−FAD	5.4
−NADPH	2.2
−NADPH, + 0.1 mM NADH	9.0
−Cytochrome *c*	0.0
Heated fraction	0.0

The reactions were performed as in section 2, using the solubilized fraction from the leukocytes stimulated with C3b-zymosan for 15 min at 37°C. Heated fraction was prepared by heating for 30 min at 70°C

Table 3
Inhibitory effect of Dip-F on activation of the O_2^--forming enzyme with C3b-zymosan

Preparations from leukocytes	O_2^- production (nmol . 5 min^{-1} . mg $protein^{-1}$)
Stimulated in the abcence of Dip-F	20.4
Stimulated in the presence of Dip-F	4.5
Non-stimulated in the absence of Dip-F	7.0
Non-stimulated in the presence of Dip-F	7.6

The solubilized fractions containing the O_2^--forming enzyme were prepared from the leukocytes stimulated or not stimulated with C3b-zymosan in either the presence or absence of 10 mM Dip-F, and the O_2^--dependent cytochrome *c* reducing activity was measured as in section 2

on the addition of FAD, as in the case of NAD(P)H oxidase of human neutrophils [18,19]. The O_2^- formation was also observed when NADPH was replaced by NADH, but NADH was less effective as electron donor than NADPH. The O_2^--forming rate did not decline during the 15 min incubation period and was proportional to protein concentration, when determined under the optimum condition thus established.

On the basis of the above results, we attempted to study the effects of stimulation of leukocytes with C3b-zymosan and of the presence of Dip-F during the stimulation on the O_2^--forming enzyme activity. Table 3 shows that the stimulation with C3b-zymosan increased the enzyme activity in the solubilized fraction. Furthermore, the presence of 10 mM Dip-F was found to abolish the increase in the activity by stimulation with C3b-zymosan. On the contrary, 10 mM Dip-F did not influence the activity of solubilized enzyme at all. These results demonstrate that the inhibitory effect of Dip-F on the enhancement of O_2^- production with C3b-zymosan is caused by direct inhibition of the activation process of the O_2^--forming enzyme initiated by the stimulus.

4. Discussion

In [18,19] the NAD(P)H oxidase of human neutrophils responsible for O_2^- formation was reported solubilized by Triton X-100 treatment of a particulate O_2^--forming system obtained from C3b-zymosan-activated cells, though only little O_2^--forming activity was detected in the solubilized fraction from non-activated cells. The O_2^--forming enzyme, probably NAD(P)H oxidase, of guinea pig polymorphonuclear leukocytes was found to be similarly solubilized and also to be activated by stimulation with C3b-zymosan.

In [9–11] the O_2^- production stimulated from human leukocytes was inhibited by substrates of chymotrypsin. Dip-F inhibited the O_2^- production from guinea pig polymorphonuclear leukocytes stimulated with antigen–antibody complexes and concanavalin A [12] though the inhibitor did not influence the binding reactions of these stimuli to the cell surface of leukocytes. These results suggest the involvement of a certain serine protease in the triggering mechanism of various stimuli, including C3b-zymosan, leading to the activation of an O_2^--forming enzyme, probably NAD(P)H oxidase. In fact, the measurements of the O_2^--forming enzyme activity here demonstrate that a certain Dip-F-sensitive protease is essential for the activation process of NAD(P)H oxidase.

The serine protease may play the role of:

(1) The NAD(P)H oxidase may exist as a precursor and be altered to an active enzyme by limited proteolysis by the serine protease, when leukocytes are stimulated with appropriate stimuli;
(2) The NAD(P)H oxidase may be incorporated into the cell membrane by perturbation of the molecular organization of the membrane which may be caused by appropriate stimuli, and this process may be mediated by the serine protease.

To solve which role may be operative, further purifica-

tion and identification of the NAD(P)H oxidase as well as demonstration of existence of its precursor should be attempted. In addition, studies on localization of the serine protease in leukocyte cells and also its activity whereby the NAD(P)H oxidase may be made activated or operative will be required for elucidation of the mechanism of leukocytes to enhance O_2^- production when exposed to various stimuli.

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