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FLUORIDE-INDUCED SUPEROXIDE PRODUCTION IN RABBIT POLYMORPHONUCLEAR LEUKOCYTES

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Abstract—Fluoride-induced superoxide (O_2^-) production in rabbit peritoneal polymorphonuclear leukocytes (PMN's) occurs in the absence of extracellular Ca^{2+}. Addition of Ca^{2+} to the medium results in an inhibition of O_2^- production. The pH during the interaction of F^- with PMN's has a strong influence on the O_2^- production. This is not due to the dependence of the O_2^--producing oxidase on pH. A lowering of pH causes an increase of initial O_2^- production and a lowering of the F^- concentration which is required to induce the O_2^- production. Prolonged interaction with F^-, or relatively high F^- concentrations, caused an inhibition of O_2^- production. This inhibitory effect of F^- is also favoured by a low pH. The results are compatible with the view that F^- exerts its activating and inhibitory effect after it has penetrated into the rabbit PMN's.

Exposure of polymorphonuclear leukocytes (PMN's) to fluoride (F^-) results in metabolic activation measured as oxygen uptake, superoxide anion (O_2^-) production and chemiluminescence [1–5]. In peritoneal rabbit PMN's fluoride activates the cell in such a way that subsequent addition of calcium results in extensive exocytosis [6]. This process does not occur in human peripheral PMN's.

It is well known that F^- inhibits glycolysis by interaction with the enzyme enolase [1, 7]. Glycolysis in the PMN plays an important role in functions such as superoxide [8] production and exocytosis [9] and thus the above results present a riddle. The interaction of F^- with PMN's strongly depends on the origin of the leukocytes as is evident from the fact that calcium-dependent degranulation can be induced by F^- in rabbit peritoneal PMN's, but not in human peripheral PMN's. Because such a difference might also play a role in F^- induced O_2^- production, we studied the interaction of sodium fluoride with rabbit peritoneal PMN's, especially with regard to the role of extracellular calcium, and the pH during the interaction.

MATERIALS AND METHODS

PMN's were obtained [10] from rabbits injected intraperitoneally with 200 ml isotonic saline containing 1.5 mg/ml glycogen. After 4 hr the exudate was collected by flushing the peritoneal cavity with isotonic saline containing citrate (0.4 per cent, pH 7.4). Then the cells were briefly (1 min) exposed to 1 mM EDTA, followed by centrifugation and washing with medium. The medium used consisted of 140 mM NaCl, 5 mM KCl, 20 mM Tris–HCl pH 7.2 and 10 mM glucose. In experiments with different pH's another buffer was used; the composition of this buffer was: 120 mM NaCl, 5 mM KCl and 30 mM HEPES of the given pH.

Two experimental procedures were followed: (a) F^- was present during incubation, together with the reagents for O_2^- measurement; thus cytochrome *c* reduction occurred at the pH of the experiment. (b) PMN's (4×10^6 in a total volume of 1 ml, in both procedures) were preincubated with F^-, followed by removal of F^- containing supernatant after centrifugation. Then new medium (pH 7.2), containing cytochrome *c* but no F^-, was added, followed by incubation for 30 min at 37°. The latter procedure results in measurement of residual O_2^- production, but under uniform conditions.

Superoxide dismutase-inhibitable superoxide production was determined according to the methods of Babior *et al.* [11], with minor modifications [12]. 4×10^6 PMN's, in a total volume of 1.0 ml, with 0.1 mM ferricytochrome *c* (Type III, Sigma Chemical Co.) in medium, with additional reagents described, were incubated for 30 min at 37°. (In one experiment for a variable time at 37°). For comparison a mixture of the same composition, but with 20 μg superoxide dismutase per ml, was treated in the same way. After centrifugation the supernatant was assayed spectrophotometrically at 550 nm, and the absorbance values were used to calculate nanomoles of O_2^- produced. Experiments were carried out in triplicate. 1 mM EDTA was included in all experiments during the interaction of F^- with PMN's unless other conditions are specified.

RESULTS

The activity of the oxidase which produces O_2^- is pH dependent [13, 14]. Because this may interfere with our aim to study the influence of pH on F^- interaction with PMN's, we have performed the experiments in two ways: (a) cells were incubated with F^- at the given pH, and O_2^- production was measured simultaneously; and (b) cells were preincubated at the given pH with F^-, then the F^- containing medium was removed, followed by measurement of the residual O_2^- production at pH 7.2. In

Table 1. Effect of extracellular Ca^{2+} and Mg^{2+} on fluoride-induced superoxide production

Additions	O_2^- production (nmoles $O_2^-/5 \times 10^6$) PMN's	
	A*	B†
None	1.3 ± 1.1	78.8 ± 1.4
1 mM EDTA	2.0 ± 0.7	80.6 ± 2.7
1 mM EDTA, 20 mM F^-	80.5 ± 2.2	51.8 ± 0.7
1 mM Ca^{2+}	4.7 ± 0.9	3.8 ± 1.8
1 mM Ca^{2+}, 20 mM F^-	26.7 ± 5.0	14.4 ± 4.4
1 mM Mg^{2+}	4.7 ± 1.5	79.9 ± 3.8
1 mM Mg^{2+}, 20 mM F^-	53.6 ± 6.0	51.9 ± 2.0

* Incubation of PMN's with the reagents indicated, under conditions of O_2^- measurement, for 30 min at 37°.

† PMN's were preincubated with 20 mM F^- for 20 min at 37°, then the cells were centrifuged and the supernatant was discarded. Next the pretreated cells were incubated with the reagents indicated under conditions of O_2^- measurement, and residual O_2^- production during 30 min at 37° was measured.

The values given are the mean values of three determinations ± S.D.

the latter procedure the O_2^-, produced during interaction with F^-, is not measured.

Table 1 shows that fluoride induces O_2^- production in rabbit PMN's in the absence of extracellular Ca^{2+}. In fact extracellular Ca^{2+} strongly inhibits the F^--induced O_2^- production; in contrast Mg^{2+} has a relatively small effect. This effect of Mg^{2+} is only observed when all reagents are added together (column A), and not with the residual O_2^- production (column B). The nature of this is not clear. The inhibitory effect of Ca^{2+} is more pronounced on the residual O_2^- production of F^--pretreated cells, as compared to cells treated with F^- and Ca^{2+} together in the medium. Addition of fluoride to fluoride-pretreated cells results in a diminished O_2^- production.

The effect of incubation time at different pH on F^--induced O_2^- production is given in Fig. 1. At pH 7.2 O_2^- production starts after a short lag time, and O_2^- production levels off at long incubation time. At pH 5.8 there is a stronger O_2^- production at low incubation times, but after 10 min O_2^- production has stopped completely. These effects can be seen more clearly in Fig. 2, giving the effect of a variable preincubation time with F^-, at different pH, on subsequent residual O_2^- production. A short preincubation time (2 min) at low pH (5.8) results in a strong residual O_2^- production, whereas at a preincubation time of 10 min residual O_2^- production is no longer observed. Both the rising and descending part of the curve shift to longer preincubation times for higher pH's.

Fluoride-induced O_2^- production is dependent on F^- concentration, as is shown in Fig. 3, and also depending on the pH. At low pH a smaller concentration of F^- induces O_2^- production. High fluoride concentrations may cause decrease of O_2^- production. At low pH this inhibitory effect is apparent at relatively low concentration of F^-, whereas at pH 7.8 the inhibitory effect is not observed even with 30 mM F^-. With cells preincubated with a variable F^- concentration at different pH's, after which residual O_2^- production is measured, we obtained qualitatively similar results (Fig. 4). Both the activating and the inhibiting effects of F^- shift to lower concentrations when the pH is lowered.

DISCUSSION

In a previous study [6] we have shown that F^- stimulates Ca^{2+}-induced exocytosis in rabbit PMN's, but not in human PMN's. Apparently these two types of cells differ significantly with regard to their ability to perform exocytosis. With regard to F^--

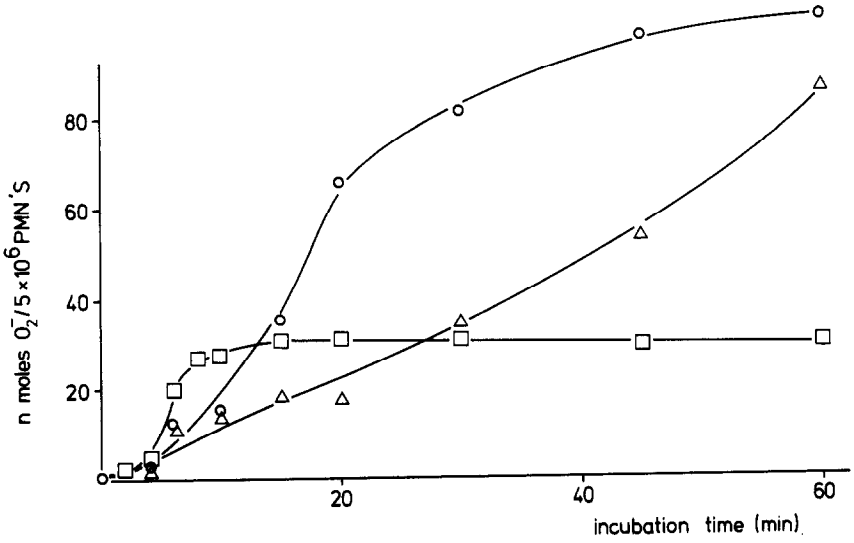

Fig. 1. Superoxide production as a function of incubation time, at different pH. Composition of buffer during incubation: 120 mM NaCl, 5 mM KCl, 30 mM HEPES of given pH; 20 mM F^-. □ pH 5.8; ○ pH 7.2; △ pH 7.8. Each point represents the mean value of three experiments.

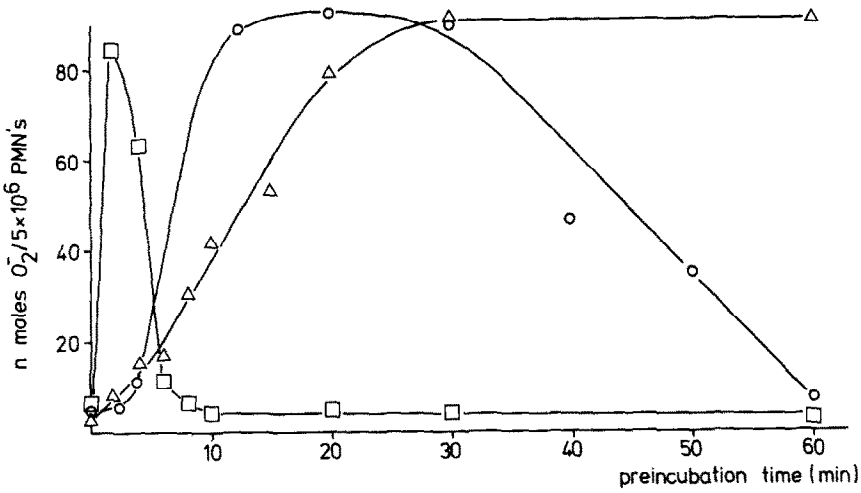

Fig. 2. Effect of preincubation time, and pH during preincubation on the subsequent residual O_2^- production. Cells were preincubated with 20 mM F^- for the time indicated. Then the F^- containing medium was removed and cytochrome *c* containing medium (pH 7.2) was added, followed by incubation for 30 min at 37°. □ preincubation at pH 5.8; ○ preincubation at pH 7.2; △ preincubation at pH 7.8. Composition of buffer during preincubation: 120 mM NaCl, 5 mM KCl, 30 mM HEPES of given pH, 20 mM F^-.

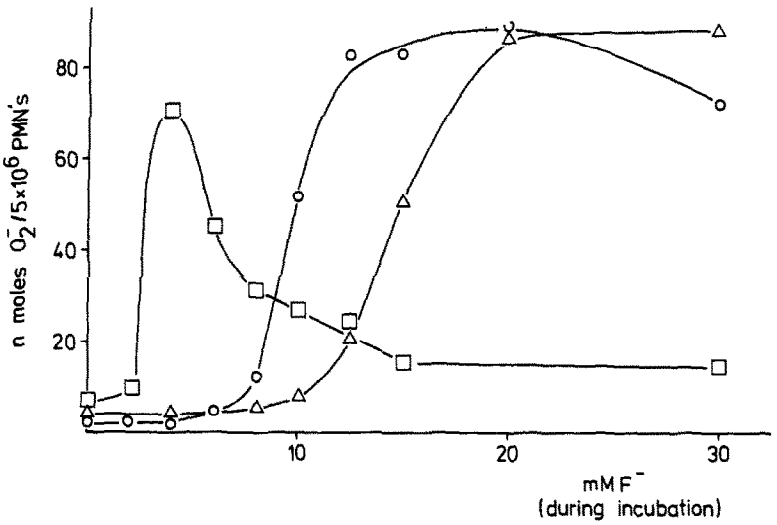

Fig. 3. Effect of pH and F^- concentration during incubation on O_2^- production. Composition of buffer during incubation: 120 mM NaCl, 5 mM KCl, 30 mM HEPES of given pH and a variable F^- concentration. □ pH 5.8; ○ pH 7.2; △ pH 7.8.

induced O_2^- production there is also an important difference between human peripheral and rabbit peritoneal PMN's. Curnutte *et al.* [5] have shown that F^- can induce O_2^- production in human PMN's, and that for maximal O_2^- production the presence of extracellular calcium was required.

Our experiments with rabbit peritoneal PMN's show that maximal O_2^- production is obtained in the absence of extracellular calcium. The addition of calcium to the medium results in a strong inhibition of O_2^- production.

In some way the effect of F^- on O_2^- production resembles that of phorbol myristate acetate, which is also capable to provoke O_2^- production in the absence of extracellular calcium [15]. In rabbit PMN's the chemotactic peptide formyl-methionyl-leucyl-phenylalanine induces significant O_2^- production in the absence of extracellular calcium, but here the presence of extracellular calcium enhances O_2^- production [15, 16]. It remains, however, to be determined, whether intracellular calcium is required for F^--induced O_2^- production.

The experiments of F^- with rabbit PMN's demonstrate that a complete separation of exocytosis and O_2^- production is possible. Exocytosis only occurs when extracellular calcium is present, whereas O_2^- production is maximal without extracellular calcium.

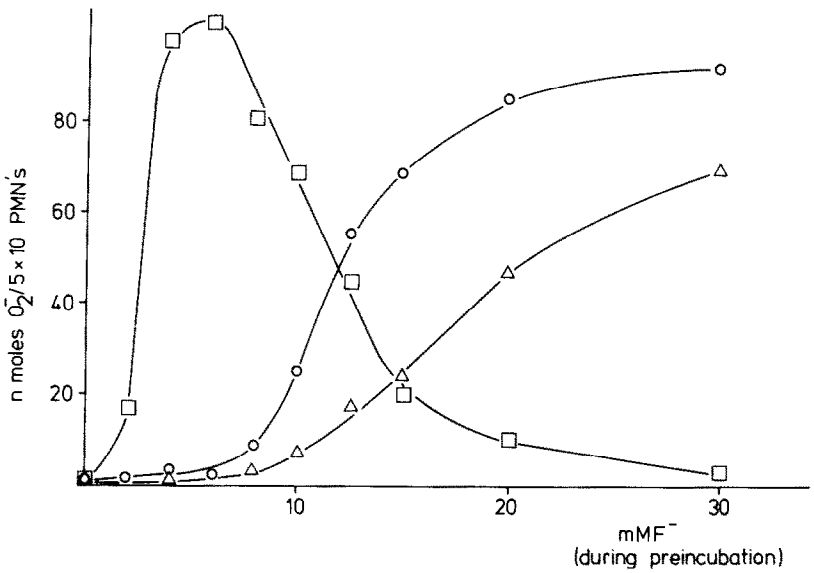

Fig. 4. Effect of pH and F^- concentration during preincubation on subsequent residual O_2^- production. After preincubation with given F^- concentration at the pH indicated, F^- containing medium was removed, followed by the addition of cytochrome *c* containing medium (pH 7.2) and incubation for 30 min at 37°. pH during preincubation: □ pH 5.8; ○ pH 7.2; △ pH 7.8. Composition of buffer during preincubation: 120 mM NaCl, 5 mM KCl, 30 mM HEPES of given pH and a variable F^- concentration.

A comparison of the results of experiments with direct measurement of O_2^- at the pH of the experiment, and the experiments where O_2^- was measured after removal of F^- at pH 7.2, leads to the following conclusions. Firstly, there is still a strong O_2^- production for a considerable time after removal of extracellular F^-. Secondly, the effect of pH on O_2^- production is qualitatively the same in both types of experiments. Hence the effect of pH is, at least for the main part not due to interference with the activity of the O_2^- producing oxidase, but to another phenomenon, namely to the pH-dependence of F^- penetration into the cells.

Fluoride-induced O_2^- production strongly depends on the pH during the interaction of F^- with the PMN's. Initial O_2^- production is most rapid at pH 5.8 and slowest at pH 7.8. Prolonged interaction with F^- results in a decrease of O_2^- production. This inhibition also depends on pH in the same way: a low pH favours a rapid decrease of O_2^- production.

The results support the hypothesis that F^- exerts its effects after it has passed the membrane. Fluoride passes the membrane as HF, which is a weak acid ($K_a = 3.5 \times 10^{-4}$) [17]. Lowering of the pH favours the free acid form and thus facilitates passage. The observed concentration dependence of F^- supports this hypothesis.

Fluoride appears to have two effects: a stimulatory effect, and later, again dependent on the pH, an inhibitory effect. Because at that time a higher intracellular F^- concentration is attained in the cell, it seems likely that the inhibitory effect occurs at a higher intracellular F^- concentration than the stimulatory effect. This inhibitory effect may be associated with the inhibition of glycolysis by F^-.

Glycolysis as source of energy is necessary for O_2^- production. The stimulatory effect of F^- is found at a lower F^- concentration than the inhibitory effect, since stimulation occurs first, and is later followed by inhibition. The pH dependence of stimulation and inhibition indicates that both phenomena are compatible with the assumption that fluoride must enter the cell before an effect can be observed.

An implication of the above mentioned theory is that the O_2^--generating system in rabbit PMN's, is either present intracellularly or as a membrane constituent which can be activated from the inner side of the membrane. This hypothesis can explain why O_2^- production proceeds after removal of extracellular fluoride: after entering the cells intracellular fluoride continues its effect on the oxidase. The alternative explanation suggested earlier [6], namely that F^- reacts with a membrane constituent in a time and concentration dependent way, appears less plausible on the basis of our observations.

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