

THE INFLUENCE OF FLUORIDE IONS ON THE VIABILITY, REDUCTION OF NBT, CYTOLYSIS, DEGRANULATION, AND PHAGOCYTOSIS OF HUMAN AND RABBIT NEUTROPHILS

J Bober,^{a,b} E Kucharska,^c J Zawierta,^a Z Machoy,^a D Chlubek,^a K Ciechanowski^a
Szczecin, Poland

Summary: The influence of fluoride ion at concentrations of 10, 20, and 30 mmol/L on various properties of human and rabbit granulocytes was investigated. We found that fluoride ion inhibited phagocytes functions of rabbit granulocytes. It also decreased the viability of rabbit polymorphonuclear leukocytes (PMN) which was connected with increased degranulation. Fluoride ion activated mainly the oxygen-dependent bactericidal system in human neutrophils and the oxygen-independent one in rabbit neutrophils.

Keywords. Fluoride ion, Granulocytes, Human neutrophils, Phagocytosis, Polymorphonuclear leukocytes, Rabbit neutrophils.

INTRODUCTION

The fluoride ion (F^-) at relatively high concentrations is a known inhibitor of some functions in various types of cells. For example, evidence has accumulated¹⁻³ that fluoride inhibits glucose metabolism in human neutrophils (PMN – polymorphonuclear leukocytes). At the same time, sodium fluoride at a concentration of 20 mM leads to a marked rise in oxygen uptake by these cells,⁴ with an accompanying inhibition of lactate production and phagocytic activity.⁵ Calcium ions at concentrations up to 0.3 mM exert a significant influence on human and rabbit PMN, evidenced by a rise in oxygen uptake and superoxide production.⁶ On the other hand, Elferink^{7,8} demonstrated an inhibitory effect of fluoride (in 1 mM Ca^{2+}) on superoxide production by rabbit PMN. Selvaraj⁹ reported that fluoride leads to degranulation of guinea pig PMN, just as observed during phagocytosis. Rabbit neutrophils exposed to fluoride undergo degranulation (reflected by the release of lysozyme and β -glucuronidase), as well as cytolysis evidenced by increased levels in the reaction medium of a cytolytic enzyme – lactate dehydrogenase.⁷

Degranulation of human neutrophils in the presence of fluoride ions has so far not been observed.^{1,3,4,7} The influence of 20 mM fluoride on phagocytosis by neutrophils has been studied using *Staphylococcus aureus*,¹⁰ and it was noticed that phagocytic activity was reduced, without any change in bactericidal (destructive) potency. It was also found that fluoride ions in vitro do not inhibit bactericidal action of amoxicillin and erythromycin on *Staphylococcus aureus*. Silva¹¹ failed to reproduce these results with *S. aureus* and *Str. agalactiae*, probably because the concentrations of fluoride ranged from 1.25 μ M to 1.25 mM, greatly below those normally used to stimulate granulocytes. It is interesting to note, however, that granulocytes lost their phagocytic activity against

^aChair and Department of Biochemistry, Pomeranian Academy of Medicine, Al. Powstańców Wlkp. 72, 70-111 Szczecin, Poland. E-mail: biochem@pam.szczecin.pl (Head: Asst Prof Dariusz Chlubek, MD, PhD). ^bFor correspondence: Joanna Bober, MD, PhD, same address. ^cChair and Department of Microbiology and Immunology, Pomeranian Academy of Medicine, Al. Powstańców Wlkp. 72, 70-111 Szczecin, Poland.

E. coli at a low fluoride concentration of 1.25 mM. In line with the results of Silva, Gabler¹² found that fluoride ions inhibit phagocytosis and intracellular killing of yeast cells. The effect intensified with rising fluoride levels and was independent of opsonization or concentration of calcium ions. Separate experiments revealed that uptake of oil emulsion particles in the presence of fluoride was inhibited.⁴ Stimulation of neutrophils in the presence of nitroblue-tetrazolium (NBT) led to generation of superoxide ions. This was reflected by deposits of dark-blue formazan (NBT reduction product) in 96% of cells.¹³ Contrary to this, Gabler³ found that fluoride (20 mM) inhibited latex-stimulated reduction of NBT by more than 50%. DeChatelet,² however, did not find any significant influence of fluoride on NBT reduction by granulocytes.

In view of the conflicting nature of the above-mentioned results, we decided to carry out additional experiments on the interaction between neutrophils and fluoride ions.

In this study, we compared the influence of fluoride ions on the viability and some functions, especially phagocytosis, of human and rabbit neutrophils. Phagocytosis was studied directly by measuring the uptake and breakdown by human or rabbit neutrophils, of sheep erythrocytes labeled with ⁵¹Cr, and indirectly with the nitroblue-tetrazolium test, or by following cytolytic and degranulation activities.

MATERIALS AND METHODS

Granulocytes were obtained from healthy males, aged 20 to 40 years (blood donors), and from rabbits of the Giant Belgian strain. Blood was anticoagulated using 1:4 ACD (acid-citrate-dextrose) for human and 1:1 for rabbit blood obtained by cardiac puncture. Cells were incubated at 37° C with varying concentrations of fluoride ions (from NaF).

Granulocytes were separated from other cells using dextran sedimentation. The resulting suspension contained 85-95% granulocytes. Cells were suspended in Hank's buffered solution (HBSS). All steps were carried out using siliconized glass or plastic test tubes. Glass was covered by silicon lubricant which has anti-adhesive properties for cells. The percentage of viable granulocytes in the cell suspension was determined based on staining of dead cells with trypan blue.

The nitroblue-tetrazolium test (NBT) was performed as follows.¹⁴ Granulocytes were suspended in HBSS and were activated by sodium fluoride at a final F⁻ concentration of 10, 20 or 30 mM. Next, 0.2% NBT was added and the cells were incubated at 37° C for 30 min. Smears were fixed for 3-5 min in methanol and stained for 13 min with Giemsa reagent. For each smear, 200 cells were evaluated microscopically.

In a separate experiment, granulocytes were stimulated with latex (100 particles per granulocyte), and reduction of NBT was followed in the absence and presence of fluoride ions. NBT is a pigment soluble in water. Intracellularly, it is reduced to formazan after engulfment and produces insoluble blue crystals.

Percent of neutrophils with blue deposits inside was examined with use of light microscope. Ability to produce formazan allows evaluation of oxygen metabolism of phagocytes and phagocytic properties.

The extent of cytolysis and degranulation⁷ was determined as follows. Neutrophils were suspended in HBSS and split into two aliquots. One aliquot was sonicated (four times for 30 s, with 1-min intervals), the other was supplemented with 0.5 M NaF to a final concentration of 10 or 20 mM. The suspensions were next incubated at 37° C for 10 or 30 min and centrifuged. Activities of lactate dehydrogenase (EC.1.1.1.27), acid phosphatase (EC.3.1.3.2), and alkaline phosphatase (EC.3.1.3.1) were measured in the supernatant. The activity in the supernatant from the sonicated aliquot represented total activity in PMN, while the activity in the NaF supernatant corresponded to the extent of cytolysis (release of cytosolic lactate dehydrogenase) and degranulation (release of granular phosphatases).

The activity of lactate dehydrogenase (LDH) was measured using a spectrophotometric method¹⁵ at a wavelength of 340 nm. Acid and alkaline phosphatase activities were measured using a colorimeter and p-nitrophenyl phosphate as substrate. The substrate is hydrolyzed by both phosphatases, giving i.a. p-nitrophenol with a strong absorbance at 405 nm. Phagocytic activity and degradation of phagocytosed antigen by granulocytes were assessed with a radioisotope method¹⁶ and ⁵¹Cr-labeled sheep erythrocytes.

RESULTS AND COMMENTS

The percentage of viable cells after isolation from blood reached 96% for human PMN and 93.4% for rabbit granulocytes. Human PMN after incubation in 30 mM NaF for 50 min showed no significant change in the percentage of viable cells (94.8%, i.e. a loss of only 1.2%). The loss of viable cells without NaF was 0.8%. Unlike human PMN, rabbit granulocytes after incubation with fluoride ions showed a gradual loss of viability, proportional to time and fluoride concentration. In the absence of fluoride, the percentage of viable cells fell by 1.2%. After incubation for 50 min with 30 mM NaF the percentage of viable cells fell by 14.1%, from 92.2 to 78.1%. Loss of viability for each concentration of F⁻ was a linear function of time (Table 1).

Table 1. Viability of human and rabbit neutrophils in the presence of fluoride, depending on F⁻ concentration and time of incubation

[F ⁻] mM	Incubation time [min]					
	Human PMN % viable cells			Rabbit PMN % viable cells		
0	10	30	50	10	30	50
0	96.0 ± 0.82	95.8 ± 0.75	95.2 ± 0.80	93.4 ± 1.03	92.8 ± 0.85	92.2 ± 0.96
10	96.0 ± 0.79	95.8 ± 0.75	95.0 ± 0.72	90.0 ± 0.93	86.6 ± 0.98	84.5 ± 1.00
20	95.8 ± 1.00	95.4 ± 0.86	94.9 ± 0.89	88.2 ± 1.10	85.2 ± 0.99	82.0 ± 1.05
30	95.6 ± 1.02	95.0 ± 0.92	94.8 ± 0.89	85.0 ± 0.92	80.0 ± 1.15	78.1 ± 1.10

Values are means ± SD, n=9.

NBT reduction did not depend on the concentration of fluoride ions but on the presence (not concentration) of calcium ions. NaF was next replaced by latex particles, routinely used to study oxidative metabolism of PMN. NBT reduction by human PMN did not differ significantly when stimulated by latex or fluoride. Furthermore, fluoride was without effect on latex-stimulated NBT reduction. The same pattern was observed with rabbit granulocytes (Table 2).

Table 2. Influence of fluoride on the reduction of nitroblue-tetrazolium by human and rabbit neutrophils, as measured by the percentage of cells containing formazan deposits

Ca ⁺⁺ [mM]	F ⁻ [mM]				F ⁻ [mM]			
	0	10	20	30	0	10	20	30
	% cells with formazan deposits							
	Human PMN				Rabbit PMN			
0	10 ± 4	9 ± 2	10 ± 3	9 ± 4	7 ± 2	8 ± 3	7 ± 2	7 ± 1
latex	23 ± 4	33 ± 2	28 ± 3	30 ± 2	16 ± 3	18 ± 2	17 ± 2	20 ± 4
0.2	12 ± 4	35 ± 3	31 ± 2	35 ± 2	8 ± 2	19 ± 4	22 ± 2	28 ± 6
0.2 and latex	24 ± 2	36 ± 2	40 ± 2	37 ± 7	14 ± 3	19 ± 3	18 ± 2	25 ± 3
0.3	12 ± 3	28 ± 3	28 ± 2	38 ± 6	9 ± 3	17 ± 5	24 ± 3	28 ± 4
0.3 and latex	27 ± 1	32 ± 3	33 ± 3	37 ± 2	17 ± 2	13 ± 2	20 ± 4	24 ± 5

Values are means ± SD, n=9.

Degranulation of human granulocytes in the absence (resting) or presence of varying concentrations of calcium and fluoride ions did not differ significantly (Table 3), quite unlike rabbit granulocytes (Table 4). Enzyme activities were measured after 10 or 30 min incubation periods. Fluoride alone (without calcium) was ineffective in releasing either cytosolic (LDH) or granular (AP and AcP) activities. However, activity in the supernatant increased in parallel with rising concentrations of Ca⁺⁺ and F⁻.

Table 3. Influence of fluoride on cytolysis and degranulation of human neutrophils, as measured by the percentage of enzyme activity in the supernatant to the total activity, being 40 nmol lactate/min/10⁶ cells for lactate dehydrogenase, 3.5 µmol/min/10⁶ cells for alkaline phosphatase and 0.83 µmol/min/10⁶ cells for acid phosphatase. Incubation time - 30 min

Ca ⁺⁺ [mM]	F ⁻ [mM]	LDH activity % total activity	AP activity % total activity	AcP activity % total activity
0	0	3.5 ± 0.3	2.1 ± 0.14	3.1 ± 0.21
0.2	10	4.2 ± 0.7	0.85 ± 0.08	2.95 ± 0.18
0.2	20	4.3 ± 0.6	2.83 ± 0.21	1.85 ± 0.09
0.3	10	3.8 ± 0.5	1.95 ± 0.32	2.05 ± 0.11
0.3	20	2.9 ± 0.8	2.14 ± 0.19	2.28 ± 0.15

Values are means ± SD, n=9.

Peak activities of all three enzymes (LDH, AP, and AcP) were noted for 0.3 mM calcium and 20 mM fluoride. The results did not change significantly when the incubation time was increased from 10 to 30 min.

Table 4. Influence of fluoride on cytolysis and degranulation of rabbit neutrophils, as measured by the percentage of enzyme activity in the supernatant in relation to the total activity, being 32 nmol lactate/min/10⁶ cells for lactate dehydrogenase, 6.14 μmol/min/10⁶ cells for alkaline phosphatase and 4.12 μmol/min/10⁶ cells for acid phosphatase

Ca ⁺⁺ [mM]	F ⁻ [mM]	LDH activity % total activity	AP activity % total activity	AcP activity % total activity
<i>Incubation time - 10 min</i>				
0	0	4.7 ± 0.9	2.6 ± 0.3	5.1 ± 0.9
0	20	5.4 ± 0.6	2.7 ± 0.6	5.5 ± 0.7
0.2	10	14.2 ± 0.8	8.3 ± 0.7	12.2 ± 1.0
0.2	20	24.5 ± 1.4	18.5 ± 1.1	17.2 ± 1.3
0.3	10	28.2 ± 1.7	19.2 ± 1.0	24.5 ± 1.8
0.3	20	43.3 ± 2.5	24.2 ± 1.3	32.1 ± 2.0
<i>Incubation time - 30 min</i>				
0	0	4.8 ± 1.2	2.6 ± 0.3	5.2 ± 1.0
0	20	5.6 ± 0.7	2.7 ± 0.4	5.5 ± 0.9
0.2	10	16.3 ± 3.5	9.2 ± 1.2	15.2 ± 0.8
0.2	20	28.2 ± 4.2	20.5 ± 2.5	19.5 ± 1.3
0.3	10	30.8 ± 5.3	25.3 ± 1.2	28.0 ± 2.5
0.3	20	45.2 ± 6.0	28.6 ± 3.2	34.2 ± 2.8

Values are means ± SD, n=9.

Table 5. Phagocytic activity of human and rabbit PMN in the presence of fluoride (see text for details)

Ca ⁺⁺ [mM]	F ⁻ [mM]	Human PMN		Rabbit PMN	
		antigen uptake [%]	antigen break- down [%]	antigen uptake [%]	antigen break- down [%]
0	0	3.63 ± 0.320	1.85 ± 0.112	4.12 ± 0.321	2.98 ± 0.198
0.3	0	3.25 ± 0.258	1.92 ± 0.102	3.90 ± 0.294	2.75 ± 0.145
0.3	10	1.97 ± 0.094	1.00 ± 0.088	3.00 ± 0.225	2.14 ± 0.116
0.3	20	0.99 ± 0.074	0.49 ± 0.035	2.38 ± 0.167	1.79 ± 0.072
0.3	30	0.63 ± 0.058	0.26 ± 0.009	1.15 ± 0.082	0.95 ± 0.083

Values are means ± SD, n=6 (human PMN) and n=9 (rabbit PMN).

Fluoride ion exerts a significant influence on the ability of granulocytes to phagocytize and destroy antigen (Table 5). Rabbit granulocytes in the absence of fluoride ingest 1.2 times and destroy 1.6 times more antigen than their human counterparts. The addition of 30 mM fluoride reduced antigen uptake from 3.63 to 0.63% for human and from 4.12 to 1.15% for rabbit granulocytes. Destruction was reduced to a greater extent in human (6 times) than in rabbit

granulocytes (3 times). The ability of granulocytes to phagocytize and destroy antigen decreased with increasing concentrations of fluoride ions.

DISCUSSION

The NBT test is an important laboratory method for the evaluation of oxidative metabolism in granulocytes.¹⁴ Our present results indicate that fluoride ions at concentrations ranging from 10 to 30 mM are just as effective as latex particles in stimulating NBT reduction. However, calcium ions must be present in the incubation medium at a concentration of 0.2 or 0.3 mM. Fluoride did not affect latex-stimulated reduction of NBT. There are conflicting reports in the literature concerning the influence of fluoride on NBT reduction. One study⁴ found that 20 mM fluoride led to 90% reduction of NBT. The same concentration greatly inhibited latex-stimulated NBT reduction³ or was without effect.²

Fluoride ions are quite potent in inhibiting one of the basic functions of granulocytes – phagocytosis. This applies to ingestion of bacteria, yeast cells and latex particles alike.^{4,11,12} We have used antigen in the form of sheep erythrocytes labeled with ⁵¹Cr and have confirmed that ingestion of antigen and subsequent destruction were markedly reduced.

The influence of fluoride ions on viability of human and rabbit neutrophils is reflected by their ability to undergo degranulation in the presence of F⁻. Human granulocytes do not exhibit any significant loss in viability when exposed to fluoride, nor do they undergo degranulation. This finding is in agreement with other authors.^{1,17} On the other hand, rabbit granulocytes show gradual loss of viability depending on incubation time and fluoride concentration. This is reflected by the release of cytoplasmic (LDH) and granular (AP and AcP) enzymes to the incubation medium. Degranulation of rabbit PMN, evidenced by release of LDH, has been observed by Elferink.^{7,8} Taking into account the relative sensitivity of animal vs. human neutrophils to degranulation, and a much lower oxygen uptake,¹³ with lower production of superoxide ions¹⁸ by the former, one may conclude that the chief mechanism used by human PMN against bacteria is oxygen-dependent. Rabbit granulocytes contain large amounts of cationic proteins,¹⁹ and in the excited state generate low quantities of active forms of oxygen.¹⁸ Therefore their basic mechanism of action is the formation of phagolysosomes and secretion into this structure of granular content, a mechanism independent of oxygen.

In summary it can be stated that fluoride at concentrations ranging from 10 to 30 mM is an inhibitor of phagocytosis and activates an oxygen-dependent bactericidal system in human granulocytes and an oxygen-independent one in rabbit granulocytes.

REFERENCES

- 1 Clark RA. Neutrophil iodination induced by fluoride: implications for degranulation and metabolism activation. *Blood* 1981;57:913-21.
- 2 DeChatelet LR, Campbell TL, Westrick MA, Shirley PS. Effects of fluoride on the oxygen metabolism of human neutrophils. *Biochem Med* 1981;25:106-13.

- 3 Gabler WL, Leong PA. Fluoride inhibition of polymorphonuclear leukocytes. *J Dent Res* 1979;58:1933-9.
- 4 Curnutte JT, Babior BM, Karnovsky ML. Fluoride-mediated activation of the respiratory burst in human neutrophils. *J Clin Invest* 1979;63:637-47.
- 5 Sbarra AJ, Karnovsky ML. The biochemical basis of phagocytosis. I. Metabolic changes during the ingestion of particles by polymorphonuclear leukocytes. *J Biol Chem* 1959;234:1355-62.
- 6 Bober J. The influence of fluoride ion on the oxygen metabolism and functions of human and rabbit neutrophils [dissertation]. Szczecin: Pomeranian Medical University; 1992. [in Polish].
- 7 Elferink JGR, Alsbach EJJ, Riemersman JC. The interaction of fluoride with rabbit polymorphonuclear leukocytes: induction of exocytosis and cytolysis. *Biochem Pharmacol* 1980;29:3051-7.
- 8 Elferink JGR. Fluoride-induced superoxide production in rabbit polymorphonuclear leukocytes. *Biochem Pharmacol* 1981;30:1981-5.
- 9 Selvaraj RJ, Sbarra AJ. Relationship of glycolytic and oxidative metabolism to particle entry and destruction in phagocytosing cells. *Nature* 1966;211:1272-6.
- 10 Anderson R, Joone G, van Rensburg CE. An in vitro investigation of the intracellular bioactivity of amoxicillin, clindamycin, and erythromycin for *Staphylococcus aureus*. *J Infect Dis* 1986;153:593-600.
- 11 Silva ID, Jain NC. Effects of glycolytic and cytoskeletal inhibitors on phagocytic and nitroblue tetrazolium reductive activities of bovine neutrophils. *Am J Vet Res* 1989;50:1175-9.
- 12 Gabler WL, Hunter N. Inhibition of human neutrophil phagocytosis and intracellular killing of yeast cells by fluoride. *Arch Oral Biol* 1987;32:363-6.
- 13 Bober J, Kucharska E, Zawierta J, Ciechanowski K, Machoy Z. Fluoride ion as a neutrophile oxide metabolism – the influence of fluoride ion on the oxygen uptake. In: Machoy Z, Ogonski T, Samujlo D, editors. *Metabolism of fluorine '99. Fluorine in toxicology, medicine and environment protection*. Szczecin: Pomeranian Medical University 1999, p. 95-98 [in Polish].
- 14 Pawelski S, editor. *Diagnostyka laboratoryjna w hematologii*. 2nd ed. Warszawa: PZWL, 1983.
- 15 Absolom DR. Basic methods for the study of phagocytosis. *Methods Enzymol* 1986;132:95-179.
- 16 Kucharska E. Influence of biostymine of some immunological reactions [dissertation]. Szczecin: Pomeranian Medical University; 1979 [in Polish].
- 17 Prince CR. Superoxide production by neutrophils. *TIBS* 1987;12:8-9.
- 18 Bober J, Zawierta J, Kucharska E, Ciechanowski K, Machoy Z. The influence of fluoride ion on the generation of superoxide anion by human and rabbit neutrophils. In: Machoy Z, Ogonski T, Samujlo D, editors. *Metabolism of fluorine '99. Fluorine in toxicology, medicine and environment protection*. Szczecin: Pomeranian Medical University 1999;88-94 [in Polish].
- 19 Zeya HJ, Spitznagel JK. Cationic protein of polymorphonuclear leukocyte lysosomes. I. Reduction of antimicrobial and enzymatic activities. *J Bacteriol* 1966;91:750-4.