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Fluoride Inhibition of Polymorphonuclear Leukocytes

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Significant inhibition of PMN metabolic activity by F^- occurred at 0.1, 0.5 and 1.0 mM F^- for O_2^- generation, $1-C^{14}$ CO_2 release labeled glucose and NBT-reduction respectively. This inhibition resulted primarily from F^- suppression of non-oxidative glucose metabolism. As the concentration of F^- in the O_2^- generating system increased beyond 10 mM, a rise in production of O_2^- anion occurred, peaking at 20 mM F^- , followed by a rapid decline.

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Introduction.

Fluoride (F^-) has been used at relatively high concentrations as a metabolic inhibitor of a number of types of cells, including leukocytes.^{1,2} Because of the importance of polymorphonuclear leukocytes (PMNs) in the host-parasite relationship, we decided to study the effect of varying concentrations of F^- on PMN CO_2 release as a measure of glucose metabolism, on nitroblue tetrazolium (NBT) reduction and on superoxide anion (O_2^-) generation. The latter two agents are important components in the microbicidal function of the PMN.

Materials and methods.

Cell isolation. — Informed consent was obtained from all subjects following the guidelines established by our Human Investigation Committee. Blood obtained from volunteers by means of venipuncture was defibrinated by gentle swirling in a flask containing a siliconized steel wool pad. All glassware used in this study was also siliconized. Polymorphonuclear leukocyte-rich isolates were prepared by the Ficol-Hypaque discontinuous gradient method.³ The PMN-

rich collection typically contained 95% neutrophils and three red blood cells per 100 white cells. The leukocytes were washed once by centrifugation in MEM-S and re-suspended in the same media containing 10% heat-inactivated fetal calf serum. Final suspension contained 1×10^7 PMNs per ml of media. Trypan blue exclusion showed cell viability to be better than 99%.

Preparation of activator. — Polystyrene latex particles^a (0.48 μ m) were exhaustively dialyzed in the cold against Krebs Ringer Phosphate buffer (KRP · pH = 7.4), and then the particles were suspended in the buffer containing 1.0 mg of bovine gammaglobulin per ml. Activated PMN systems contained approximately 800 latex particles per leukocyte.

Fluoride. — Analytical grade NaF was dissolved in KRP buffer, such that the amount of F^- ion needed to obtain the desired concentration of anion in the reaction mixture was contained in 0.1 ml of this solution.

Reaction Systems

Glucose metabolism. — $1-C^{14}$, $6-C^{14}$ and uniformly labeled glucose^b were prepared in KRP buffer such that the 1.11×10^5 dpm of labeled substrate was contained in each ml of 10 mM glucose solution. CO_2 release experiments were carried out in double-armed Warburg flasks at 37°C. The main compartment contained 3×10^6 cells, 0.2 ml of autologous serum, 1.0 ml of 10 mM glucose and enough KRP buffer to bring the volume in this compartment to 1.9 ml. When inhibitor was present, 0.1 ml of the F^- solution replaced an equivalent amount of buffer. The center well contained a filter paper fan saturated with 0.1 ml of 1.0 N KOH. Following gassing for 2 minutes with 100% O_2 (bubbled through a 1.0 N KOH solution), the flasks were pre-incubated

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^aDow Chemical

^bNew England Nuclear

for five minutes, and then either 0.1 ml of latex particles or KRP buffer, contained in one of the side arms, was tipped into the main compartment. All systems were allowed to run for 60 minutes, and then the reactions were stopped by tipping into the main compartment 0.2 ml of 3 N HCl, contained in a second side arm. The acid also helped drive dissolved CO₂ out of solution. The flasks were incubated for an additional 15 minutes, and then the content of the center well was quantitatively transferred to a scintillation vial, to which was added 10 ml of Insta-Gel.^c After equilibration, the vials were counted in a liquid scintillation counter, and the efficiency calculated by the channels-ratio method. Results were recorded in terms of DPM per 5 × 10⁶ cells per 60 minutes.

NBT-reduction. — A modification of the NBT-reduction assay of Baehner and Nathan⁴ was used. The reaction systems consisted of centrifuge tubes containing 0.1 ml of autologous serum, 0.5 ml of 0.1%NBT^d (in KRP buffer containing 10 mM glucose), 0.1 ml of latex particles or KRP buffer and 0.1 ml F⁻ or an equivalent amount of buffer. Both the reaction tubes and cell suspensions were pre-incubated for five minutes at 37°C. At time zero, 0.2 ml of the PMN suspension was added to each reaction tube. The tubes were briefly vibrated and then incubated for 15 minutes. Reactions were inhibited by addition of 1.0 ml of cold 1.0 N HCl, and the tubes were centrifuged for 30 minutes at 675 xg and 3°C. Resulting supernatants were aspirated off the cell packs, and the cells were re-suspended in 4.0 ml of pyridine.^e In order to insure that all of the reduced NBT was in solution, the tubes were heated in a boiling water bath until no sign of cell pack remained (about two hours). The tubes were recentrifuged for 15 minutes to settle cell fragments, and the amount of reduced NBT in the supernatant was determined in a Coleman 125 spectrophotometer at a wave length of 565 nm against a pyridine blank. Results were recorded as ΔOD per 2.5 × 10⁶ cells per 15 minutes.

Superoxide Anion Generation

A modification of the system of Weening, Wever and Roos⁵ was used, which measures O₂⁻ generation by its reduction of cytochrome c.^f Reaction systems consisted of 0.1 ml autologous serum, 0.5 ml of a cytochrome c solution (2 mg cytochrome c per ml of KRP buffer containing 10 mM glucose), 0.1 ml of F⁻ or buffer. Activation of PMNs was achieved by addition of 0.1 ml of latex particles to the reaction mixture, while resting cell systems received an equivalent amount of buffer. The reaction mixtures and cell suspensions were pre-incubated separately for five minutes at 37°C, and, at time zero, 0.2 ml of the cell suspension was added to each reaction tube. The tubes were briefly vibrated and allowed to continue incubating for 15 minutes. The reactions were stopped by the addition of 0.1 ml of 13 mM n-ethyl maleimide^g and the tubes were centrifuged for 30 minutes at 675 xg and 3°C. In order to insure complete removal of all latex particles remaining in suspension, the supernate of each tube was filtered through 0.2 μ Millipore filters. Blanks were handled in an identical manner, except the inhibitor was added prior to the cells. Five-tenths of a ml of the filtered supernate was diluted with 1.6 ml of KRP buffer. The amount of reduced cytochrome c formed was determined as the difference in the optical density at 550 and 600 nm and using an absorbance coefficient of 21.1 mM⁻¹cm⁻¹. Specificity of cytochrome c reduction was checked by reaction mixtures containing 10 μg superoxide dismutase^h in addition to appropriate compounds and stimuli. Addition of superoxide dismutase inhibited cytochrome c reduction by better than 95%, indicating that the overwhelming reduction of cytochrome c resulted from O₂⁻ generation by PMNs. We also found the F⁻, at any of the test concentrations, had no effect on superoxide dismutase activity.

Presentation of Data

Because of fairly large variations in activities of cells obtained from different donors, we expressed results in terms of

^cPackard Instrument Company

^dSigma grade III

^eBaker Chemical

^fSigma-type VI, horse heart

^gSigma

^hSigma — type 1, bovine blood

relative activities (R.A.), rather than actual metabolic values. The relative activity is the ratio of the metabolic value in the presence of F⁻ divided by its value in the absence of this ion. Each point on a curve represents at least six experimental runs consisting of duplicate samples. Statistical comparisons of data were done, using the Student's *t* test. Significant difference between groups was set at $P < 0.05$.

Results.

Glucose metabolism. — The principal pathway for glucose metabolism in the PMN was found to be by way of the non-oxidative pathway rather than the oxidative (Table 1). Ratios of 1-C¹⁴CO₂ DPM/6-C¹⁴CO₂ DPM of 9.68 and 15.7 for resting and activated cell, respectively, were found and are quite similar to those reported by Sbarra and Karnovsky.¹ Addition of latex particles resulted in a very modest increase of 6-C¹⁴CO₂ release (~60 DPM), but caused a marked enhancement of 1-C¹⁴CO₂ production. Addition of 10 mM cyanide to reaction systems did not inhibit either 1-C¹⁴ or 6-C¹⁴ CO₂ emission. Fluoride inhibited 1-C¹⁴CO₂ release from glucose in a dose related fashion, while generation of 6-C¹⁴CO₂ rose slightly, but consistently, with increasing concentrations of F⁻ ion (Table 2).

Fig. 1 gives a graphic representation of a series of experiments on the effect of F⁻ dose on CO₂ release by stimulated PMNs using uniformly labeled glucose as substrate. Significant inhibition of glucose metabolism was first noted at 0.5 mM anion (relative activity = 0.93). Fifty percent inhibition occurred at approximately 8.0 mM F⁻. At 100 mM F⁻, a slight amount

TABLE 2
EFFECT OF FLUORIDE ON CO₂ RELEASE BY ACTIVATED PMNS FROM 1-C¹⁴ AND 6-C¹⁴ GLUCOSE

F ⁻ concentration mM	Labeled carbon	
	1-C ¹⁴ (CO ₂ DPM/5×10 ⁶ PMNs/10 min.)	6-C ¹⁴
0	1810 ± 211*	53 ± 21
10	514 ± 67	60 ± 12
20	341 ± 16	62 ± 4
30	324 ± 21	68 ± 10

Each assay was run in duplicate. The data represent the results from 3 experiments. F⁻ was added to the cell suspension 10 minutes prior to transfer of PMNs to reaction flask. The reaction mixture also contained enough F⁻ to bring the final concentration in the reacting system to the proper level.

*Standard error.

of glucose metabolism was still detectable (R.A. = 0.06). The general shape of F⁻ dose/CO₂ release curve was the same for unstimulated cells, however, shifted somewhat to the right. Significant inhibition of glucose metabolism by unstimulated cells did not occur until a F⁻ ion concentration of approximately 10 mM was reached (R.A. = 0.85) and 50% inhibition occurred at 18 mM anion.

The effect of F⁻ on the viability of the PMN was studied. PMNs incubated for 2 hours at 37°C in systems containing up to 20 mM F⁻ anion retained greater than 94% viability as measured by trypan blue exclusion.

NBT-reduction. — The general shape of the F⁻ dose/NBT-reduction curve (Fig. 1) was similar to that developed for glucose metabolism, but shifted to the right. Signifi-

TABLE 1
SOURCE OF CO₂ ARISING FROM GLUCOSE METABOLISM BY PMNS

Labeled Carbon	Unstimulated (CO ₂ DPM/5×10 ⁶ PMNs/60 min.)	Stimulated	Stimulation Index Stim./Unstim.
1-C ¹⁴	242 ± 25*	1370 ± 118	5.66
6-C ¹⁴	25 ± 7	82 ± 6	3.2
Ratio 1-C ¹⁴ /6-C ¹⁴ CO ₂ DPM	9.68	15.7	

Each assay was run in duplicate. The data represent 3 experiments.

*Standard error.

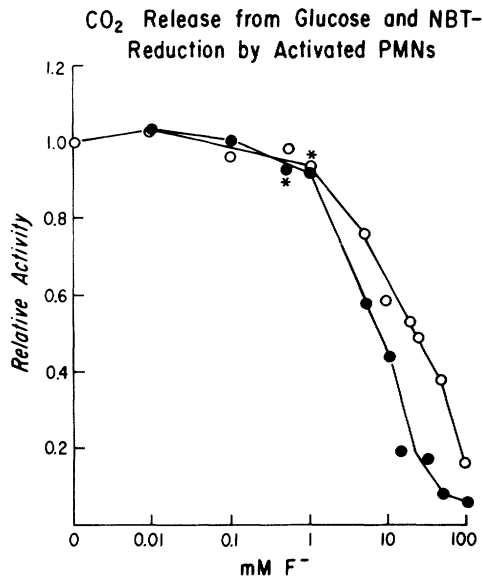


Fig. 1—The effect of F^- on release of C^{14} CO_2 from uniformly labeled glucose (—●—) and NBT-reduction (—○—) by activated PMNs. Each point represents the average of at least six runs. *From this F^- concentration and higher, significant inhibition occurred ($P < 0.05$). Uninhibited systems gave CO_2 release values of 1950–4460 dpm/ 5×10^6 PMNs/60 minutes, while ΔOD values for NBT-reduction ranged from 0.073 to 0.135 OD units/ 2.5×10^6 PMNs/15 minutes.

cant inhibition of NBT-reduction was obtained at a F^- concentration of 1.0 mM (R.A. = 0.93). Fifty percent inhibition was not reached until a F^- ion level of around 23 mM was obtained. At 50 mM anion, the R.A. for NBT-reduction is still quite high (0.38). In contrast, the very low NBT-reducing activity of unstimulated PMNs made it difficult to obtain statistically significant differences between relative activities of systems containing low levels of F^- ion. We were, however, able to establish that 50% inhibition of NBT-reduction by unstimulated cells occurs at an anion concentration in excess of 30 mM.

Superoxide anion generation. — Generation of O_2^- , as measured by cytochrome c reduction, was much more sensitive to low levels of F^- than either NBT reduction or glucose metabolism, as measured by CO_2 release (Fig. 2). Statistically significant inhibition of O_2^- production by activated

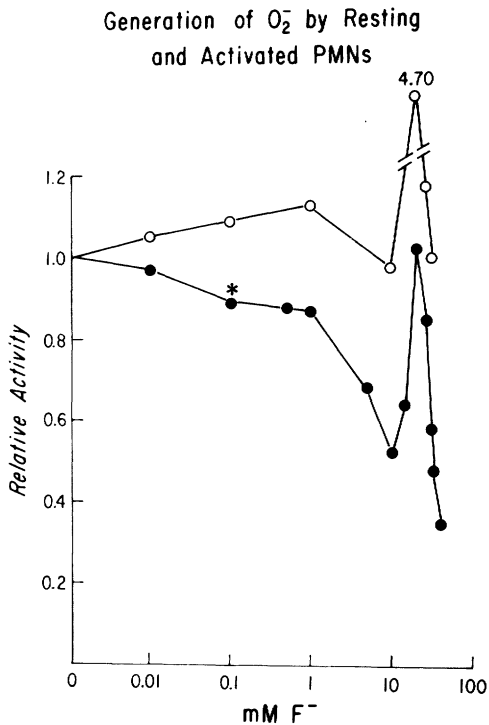


Fig. 2—The effect of F^- on generation of O_2^- by resting (—○—) and activated (—●—) PMNs. Each point represents the average of at least 6 runs. *From this concentration and higher, significant inhibition occurred ($P < 0.05$). Uninhibited systems gave O_2^- generation values of 30–76 and 181–381 nm cytochrome c reduced/ 5×10^6 PMNs/15 minutes for resting and latex stimulated cells respectively.

PMNs was noted at 0.1 mM anion (R.A. = 0.89) and 50% inhibition achieved at 10 mM F^- . Beyond this concentration of anion, F^- markedly enhances O_2^- generation. In the case of activated PMNs, doubling the F^- concentration from 10 to 20 mM results in an approximate doubling of O_2^- production, as measured by reduction of cytochrome c. At 20 mM F^- , the rate of O_2^- generation is roughly the same as it is in the absence of the anion. As the concentration of F^- in the system increases beyond 20 mM, another inhibition phase is entered, so that by 35 mM F^- ion, a second 50% inhibition level is reached. Though not shown on the curves, we increased the concentration of F^- in the systems by 10 mM increments up to 100

mm F⁻ and were unable to elicit another enhancement of O₂⁻ generation.

Fluoride slightly, but consistently, elevated O₂⁻ production by resting PMNs up to 1.0 mM (Fig. 2). Even at 10 mM F⁻ ion, the relative O₂⁻ generating activity of the resting cell was approximately the same as that of systems in the absence of F⁻. When the F⁻ ion concentration was increased from 10 to 20 mM, there was a marked increase in O₂⁻ production, so that at 20 mM ion the rate of O₂⁻ generation by 4½ times the rate of its production by resting cells in the absence of F⁻. In fact, at 20 mM F⁻, O₂⁻ generation by resting cells approached that of activated PMNs in the absence of F⁻. As is the case with activated PMNs there was a rather sharp O₂⁻ production maximum at 20 mM F⁻, followed by a rapid drop in O₂⁻ generation by unstimulated cells as the concentration of F⁻ in the media was increased. Incremental addition of F⁻ to reaction systems up to 100 mM F⁻ ion revealed no additional enhancement of O₂⁻ generation by unstimulated cells.

Discussion.

The principal role of the PMN in the host-parasite relationship is the phagocytosis and killing of invading micro-organisms. Energy required for phagocytosis is derived from the catabolism of carbohydrate in the glycolytic pathway, while reducing equivalents needed for its microbicidal function are primarily generated through the hexose monophosphate shunt.⁶ Apparently, mitochondrial metabolism plays little or no role in either PMN functions. This lack of mitochondrial participation is borne out by the 1-C¹⁴/6-C¹⁴CO₂ ratios of 9.68 and 15.7 obtained in this study for unstimulated and latex activated cells, respectively. Activation of PMNs enhanced 1-C¹⁴CO₂ release by 1370 DPM, while causing only a modest 57 DPM rise in 6-C¹⁴CO₂ production. Clearly, PMN activation enhances the flow of glucose through non-oxidative rather than oxidative pathways. Further support is the fact that cyanide, a known inhibitor of respiratory enzymes, had no effect on CO₂ release in our system. Fluoride, on the other hand, an inhibitor of glycolysis, suppressed 1-C¹⁴CO₂ release in a dose-related fashion,

while 6-C¹⁴CO₂ production rose consistently (albeit only slightly) with increasing F⁻ concentrations. These data indicate that F⁻ suppresses non-oxidative glucose metabolism but does not inhibit glucose uptake into the cell. If F⁻ suppressed glucose uptake (as is seen with certain types of bacteria), both 1-C¹⁴ and 6-C¹⁴CO₂ production should have decreased proportionately.

It was considered possible that suppression of PMN function by F⁻ resulted from a generalized toxic effect of the anion on the cell with a resultant loss of viability. That this is not the case was shown by the fact that incubation of PMNs in media containing up to 20 mM F⁻ for two hours did not cause any significant loss of cell viability as measured by trypan blue exclusion.

Statistically significant ($P < 0.05$) inhibition of product formation was first noted at 0.1, 0.5, and 1.0 mM F⁻ anion for O₂⁻ generation, CO₂ release and NBT-reduction, respectively. Part of this difference in sensitivity to F⁻ may be due to the sensitivities of the specific assay procedures. It is also likely that differences exist in the F⁻ sensitivities of enzymes associated with specific product formation. The initial or low level sensitivity of PMN leukocytes to F⁻ appears to lie within the range of initial sensitivities to the same ion as reported for spleen cells (2-3 mM),⁷ murine leukemic lymphoblasts (0.26-1.0) mM,⁸ and HeLa cells (1.05-1.25 mM).⁹ The concentration of F⁻ required to inhibit the PMN functions measured in this study was considerably greater than that found in the plasma of persons living in water fluoridated communities (2-5 × 10⁻³ mM). Fifty percent inhibition of CO₂ release, O₂⁻ generation and NBT-reduction by latex activated PMNs was found to occur at 8, 10, and 23 mM F⁻ ion respectively. Thus, it is unlikely that water fluoridation has any effect on the ability of PMNs to resist bacterial invasion, since a F⁻ ion concentration greater than 1000 times that found in the plasma of persons living in a fluoridated area was required to suppress PMN function by 50%.

One might expect that the PMN might be rather sensitive to F⁻, since it is so dependent upon non-oxidative glucose metabolism to meet its metabolic demands, particularly upon activation. This does not

appear to be the case. Part of the explanation may be related to the penetration of F^- into the cytoplasm of the PMN. Drescher and Suttie¹⁰ reported an intracellular/extracellular distribution ratio of F^- for HeLa cells of 0.27-0.37. Ratios were determined after 60 minutes incubation in F^- containing media. If a similar distribution ratio holds for PMNs, the concentration of F^- in the cytoplasm would be approximately 1/3 that in the media. Two key enzymes associated with glycolysis, phosphoglycerdehyde dehydrogenase and enolase are approximately 50% inhibited at 4 and 2- F^- ion, respectively.¹¹ Thus, the potential intracellular concentration of anion at 50% inhibition of PMN function in our system is within the range of F^- ion needed for a similar degree of inhibition of these two key enzymes. It is also possible that the intracellular concentration of F^- never reached 1/3 of that present in the media. Most studies of cell function/ F^- ion concentration have been run on cells that could be incubated in F^- containing media for prolonged periods of time prior to assaying for a specific function. This is not possible with PMNs, since their activities decay rather rapidly following harvesting, and it is necessary to perform assays at a relatively short (~4 hours) and consistent time interval following bleeding.

Several years ago, Sbarra and Karnovsky¹ found that oxygen uptake by guinea pig granulocytes was significantly enhanced in the presence of 20 mM F^- . More recently, Curnutte and Babior¹² reported that 20 mM F^- exerted a powerful stimulatory effect on the generation of O_2^- by PMNs prepared for human blood samples. They did not report a F^-/O_2^- generation profile on their PMNs. In the present study, we found that enhancement of O_2^- generation begins with F^- concentrations above 10 mM and reaches a maximum of 20 mM F^- . At that concentration of F^- , both stimulated and unstimulated PMNs generate O_2^- anion at a rate equivalent to that found for latex-activated cells in the absence of F^- . The marked enhancement of O_2^- generation did not lead to increased CO_2 release or enhanced NBT-reduction. If the reducing equivalents needed for the increased O_2^- production were being generated primarily by the hexose monophosphate shunt, a

substantial rise in $1-C^{14}CO_2$ release should have occurred. As stated previously, no increase in CO_2 release was found. It is also difficult to imagine that these reducing equivalents were derived from glycolysis, since abundant evidence exists for the inhibitory effect of F^- on the Embden-Meyerhoff pathway. Moreover, we found that in the presence of 5 mM 2-deoxyglucose, a potent inhibitor of glycolysis, 20 mM F^- is still capable of increasing O_2^- generation to a level similar to that seen for activated PMNs in the absence of 2-deoxyglucose and F^- . This suggests the possibility that at 20 mM F^- some substrate other than glucose is supplying reducing equivalents for PMN O_2^- generation. We are intensively pursuing this interesting problem in our laboratory.

Conclusions.

The metabolic demands of PMNs are met primarily by the flow of glucose through non-oxidative pathways. Fluoride inhibition of PMN phagocytosis and microbicidal activity results principally from suppression of these pathways. PMNs seem to be less sensitive to F^- inhibition than several other cell types. There is no evidence that the level of F^- found in the plasma of persons living in a fluoridated community could cause inhibition of any of the PMN functions tested. Enhancement of O_2^- generation at 20 mM F^- ion concentration does not seem to stem from increased non-oxidative metabolism of glucose.

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