Cytotoxicity of fluoride

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I. Effect of sodium fluoride on LS cells.
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II. Fluoride, fluoride resistance and glycolysis in cultured cells.
(Coauthor: Jan K. Hongslo)
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III. Fluoride and energy metabolism in LS cells.
(Coauthor: Jan K. Hongslo)
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IV. On the role of cyclic AMP in the cytotoxic effect of fluoride.
(Coauthors: Jan K. Hongslo and Thoralf Christoffersen)
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V. The effect of fluoride on the cellular uptake and pool of amino acids.
(Coauthor: Jan K. Hongslo)
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VI. Fluoride inhibition of protein and DNA synthesis in cells in vitro.
Acta Pharmacol. Toxicol. 1979, 45, 96 - 101

VII. Effect of sodium fluoride on protein and DNA synthesis, ornithine decarboxylase activity, and polyamine content in LS cells.
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GENERAL INTRODUCTION

All human beings and animals are regularly exposed to fluoride, although in very variable doses. Food and drinking water always contain fluoride (73,92), sometime in excessive amounts. Extended use of fluorine compounds in industry, medicine and dentistry have increased the possible exposure to fluoride. The sources of fluoride intake can be summarized as follows:

1. Caries prophylaxis (77)
   water fluoridation (31)
   fluoride tablets (14, 113)
   tooth paste (117)
   other vehicles (13, 80, 83)
2. Treatment of diseases
   osteoporosis (29, 41, 105)
   otosclerosis (19, 20, 108)
   osteogenesis imperfecta (39)
3. Pollution from industry (30, 109)

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4. Decomposition of fluorine-containing compounds such as anaesthetics (28, 94, 106)
5. Accidental and suicidal events (33)

The effects of fluoride are dependent on the applied dose, ranging from physiological through pharmacalogical to toxicological. Fluoride is now regarded as an essential element for mammals (48, 86), but it also possesses a powerful toxic potential when administered in large doses. The effects of fluoride poisoning may be summarized as follows:

A. Acute (one or a few large doses) (33)
   Digestive effects (vomiting, diarrhoea, haemorrhage)
   Kidney failure (84, 104)
   Neuromuscular effects (paraesthesia, spasms)
   Pulmonary damage
   (Death)
B. Chronic (gradual fluoride accumulation paticularly in the mineralized tissues)
   Skeletal and dental fluorosis (27, 33, 37, 40, 72)
   Biochemical and cytological effects (15, 38, 85, 103)

The mechanism(s) for some of the toxic effects of fluoride are known, e.g. the neuromuscular effects (spasms) are caused by fluoride binding to Ca^{++} in plasma and thereby lowering the Ca^{++} concentration (11, 110). The gastrointestinal effects are due to production of corrosive substances like hydrogen fluoride (HF) and sodium hydrogen fluoride ($NaHF_2$) in the stomach. Some of the other effects (kidney failure, dental fluorisis) are probably caused by the cytotoxic action of fluoride (88, 119). The cytotoxic effect of fluoride has mostly been studied on cells cultured *in vitro*, and a number of effects have been reported following addition of fluoride to different systems of cultured cells.

Growth-inhibition and cell death

Added to mammalian cell cultures, fluoride at and above approx. 1 mM inhibits cell growth and may cause cell death (2, 3, 21, 79, 100). The sensitivity of the cells may vary somewhat depending upon pH (53) and on the cell type (8, 9, 74). Cytotoxic effects can also be detected in tissues exposed to fluroide, both *in vitro* (43, 46) and *in vivo* (17).

Resistance development

By slowly increasing the concentration of fluoride in cell cultures cells resistant to otherwise lethal concentrations of fluoride can be developed (21, 58, 99, 100). The resistance persists also after removal of fluoride (58, 60, 100).

A recent report (79) indicates that development or fluoride resistance may be a mutational event, fluoride resistant cells evolved after treating the cells with a mutagen.

Intracellular fluoride content

The main difference between sensitive and resistant cells is that the latter are able to keep much lower intracellular concentration of fluoride (58, 100). Normally cells keep an intracellular/extracellular fluoride concentration ratio in the range of 0.25–0.4 at pH about 7.4 (32, 58, 100), grossly irrespective of the extracellular fluoride concentration, but pH-dependent, the ratio increases when pH is lowered (53). In fluoride resistant cells, this ratio is kept at $\sim 0 - 0.06$, attributed to an active exclusion of fluoride (58, 100).

Effect on enzymes and cellular energy metabolism

Fluoride is an enzyme inhibitor, with effect on several enzymes. The best known

example is enolase (112, 120) which, in the presence of 5 mM phosphate, was competitively inhibited by fluoride concentrations exceeding 0.03 mM. In the absence of phosphate much higher fluoride concentration was needed to achieve inhibition, which also was changed to a noncompetitive type (24). Acid phosphatase (112), ATPase (82, 87, 91), succinic dehydrogenase (111) and cholinesterase (23) also belong to the enzymes inhibited by fluoride.

The mechanism(s) for fluoride inhibition of enzymes have not been fully clarified. In a review, Wiseman (126) divided enzymes inhibited by fluoride into four categories: 1) Enzymes requiring metal ions and whose inhibition is enhanced by phosphate, 2) enzymes requiring divalent metal ions, 3) enzymes with no demonstrated requirement for metal ions, and 4) enzymes containing trivalent metal ions.

In intact cells, fluoride has been reported to inhibit glycolysis and lactate production, although marked discrepancies can be found. Generally, relatively high fluoride concentrations (>4 – 10 mM) have been used to achieve effect, and it seems that the effect was influenced by several factors such as: The pH and the growth phase of the cells (53), the medium (61), the cell type, erythrocytes seemed more sensitive than other cells (47), and the concentration of phosphate (90). The cellular ATP level has been reported to be influenced by fluoride by some workers (36, 47, 64), but others have found little or no effect (22, 65).

Inhibition of protein synthesis

Fluoride inhibits protein synthesis both in cell homogenates (116) and in intact cells (25), associated with a progressive dissociation of polyribosomes to 80S ribosomes (10, 81). The effect is reversible, the inhibition is lifted after the removal of the fluoride by washing (81). The mechanism for the inhibition is rather well known and can be divided into two: The intitiation of new peptide chains is impaired due to inhibition of the addition of the 60S ribosomal subunit in the formation of the complete ribosome (45, 54). The second effect is on the dissociation of the 80S ribosome to 40S and 60S subunits after completion of the peptide chain (25, 55). The peptide chain elongation process, after initiation, seems nearly unaffected by fluoride (45, 54).

Effect on adenylate cyclase

The «second messenger» action of adenosine 3', 5'-monophosphate (cAMP) is well known. Fluoride increases the production of cAMP in homogenates from most tissues of multicellular organisms, by stimulating the activity of the enzyme adenylate cyclase (66). Bacterial adenylate cyclase is, however, not stimulated by fluoride (66, 114). The mechanism by which fluoride stimulates adenylate cyclase activity, is not fully understood, but some reports (26, 107) suggest that adenylate cyclase exists in two forms: an inhibited phospho- and an activated dephospho-form. The stimulation found by addition of fluoride should then be due to inhibition of the phosphorylation resulting in an increased proportion of the active dephospho-adenylate cyclase.

Increased cAMP concentration after fluoride treatment has been reported in intact cells (5, 12, 44, 64, 125), and also in several organs of animals given low doses of fluoride (4). However, these reports are unconfirmed or even contradicted (1, 35, 63, 66, 96), and several questions concerning fluoride-induced stimulation of adenylate cyclase in intact cells are still unsettled.

Membrane functions

Membrane bound ATPase is generally accepted as a participant in the active

transport of sodium and potassium over the cell membrane (97, 124). The ATPase, especially in erythrocytes, is sensitive to fluoride; detectable inhibition starts at about 1 mM of F^- (87, 91). For erythrocytes it has been reported that 1 mM fluoride can influence the potassium gradient over the cell membrane (6), although others achieved effect only at much greater fluoride concentrations (76). In nuclear cells cultured *in vitro*, the intracellular sodium and potassium concentrations seem less sensitive to fluoride, effect is reported only at supralethal concentrations (3.7 mM) (101). Also transport and intracellular concentrations of amino acids are reduced by fluoride (52, 78), although discrepancies exist abouth the lowest effective fluoride concentration (7, 67).

The barrier function of the cellular membrane seems to be affected by fluoride only in very high concentrations (56, 57).

Contradictory results have been presented concerning the effect of fluoride on glucose transport, ranging from inhibition (18, 93) to marked stimulation (50).

Cytomorphological changes have been reported for cells exposed to fluoride both *in vivo* and *in vitro*. In rats given one or a few high doses of fluoride, the ameloblasts showed vacuolization and intravacuolar mineralization, together with swelling of mitochondria and the endoplasmatic reticulum (69, 70, 71, 88, 119). Alteration of mitochondria has also been described for cells *in vitro* (118). Contradictory results have emerged concerning possible cytogenetic effect of fluoride (62, 68, 75).

AIMS OF THE PRESENT WORK

This investigation was carried out in order to explore the cytotoxicity of fluoride, in a search for the primary target for fluoride in cells and thus the mechanism(s) for its cytotoxic effect. Cells cultured *in vitro* were chosen, because this method represents a simple, well controlled and adaptable test system for testing cellular responses to applied agents. The particular cell strain used was LS cells, because these cells are easy to culture, they grow in suspension, they were in culture in the laboratory when the work was initiated, and they responded to fluoride similarly to other cells (Paper I). The following variables were studied:

1. The sensitivity of the cells: The lowest fluoride concentration causing
   a) growth inhibition
   b) cell death.
2. Development of fluoride resistant cells and the mechanism(s) for this.
3. Correlation between the cytotoxic effect of fluoride and possible effects on glycolysis, energy metabolism, protein and DNA synthesis, and transport and levels of amino acids.
4. Fluoride effect on the cyclic AMP level in intact cells.
5. Fluoride influence on DNA synthesis *per se* at low concentrations.

GENERAL SUMMARY

Cultures of LS cells, a L929 (mouse fibroblasts) derived strain adapted to grow in suspension culture, showed reduced growth rate in the presence of 1.3 mM sodium fluoride. At higher fluoride concentrations (> 2 mM) cell death occurred, and fluoride concentrations above 3–3.5 mM completely extincted the culture. If fluoride exposure below this concentration was continued, the cells resumed normal growth rate after a lag period, i.e. the cells had become resistant to this fluoride concentration. By stepwise increasing the fluoride concentration, the cells could be made resistant to high fluoride concentrations (Paper I).

The intracellular concentration of fluoride in sensitive cells was 30–40% of the extracellular concentration, whereas the fluoride resistant cells maintained an intracellular fluoride level near to zero (Paper II). This is the main difference between the sensitive and fluoride resistant cells, and it probably represents the mechanism for fluoride resistance.

The glucose consumption and lactate production were not affected by 6 mM NaF in cells incubated in culture medium, but the lactate production was slightly inhibited at and above 3 mM NaF when the cells were incubated in Krebs-Ringer phosphate buffer supplemented with glucose (Paper II and III). The ATP level in the cells was not reduced even by 12 mM NaF (Paper III).

Fluoride did not stimulate the cyclic AMP level in LS cells (Paper IV), indicating no fluoride stimulation of adenylate cyclase in intact cells.

Protein and DNA synthesis were inhibited by fluoride in concentrations similar to those causing growth inhibition (Paper VI). Protein synthesis was more rapidly inhibited than DNA synthesis was, although their eventual inhibition reached approximately the same level (Paper IV and VII). The activity of the enzyme ornithine decarboxylase (which catalyzes the initial step in the synthesis of polyamines) fell during exposure to fluoride, probably due to inhibition of its synthesis. The intracellular levels of polyamines did not change during the 10 hour observation period (Paper VII).

The inhibition of protein and DNA synthesis was not caused by lower uptake of precursors in the cells, although the level of some amino acids was lowered by fluoride. However, in the fluoride resistant cells growing at the same rate as the normal cells, the level of some amino acids was also lowered (Paper V and VI).

DNA synthesis in isolated nuclei from both fluoride sensitive and fluoride resistant cells was inhibited by fluoride, but taking into account the lower intracellular than extracellular level of fluoride, the DNA synthesis inhibition at moderate fluoride concentrations (< 4–5 mM) seemed to be secondary to the inhibition of protein synthesis (Paper VIII).

GENERAL DISCUSSION

Sodium fluoride at and above approx. 1 mM is cytotoxic to cells cultured *in vitro* (2, 21, 79, 93), although certain cell strains may exhibit slightly different sensitivity (74). LS cells (a L 929 (mouse fibroblasts) derived strain adapted to suspension culture) showed comparable sensitivity to fluoride; concentrations at and above 1.3 mM acted growth-inhibiting. The effect was clearly concentration dependent, at 2.4 mM most cells died, and more than 3 mM killed the culture (Paper I).

Fluoride concentrations of similar magnitude (1.5–2.6 mM) have been found in urine from humans receiving methoxyflurane anaethesia, one of its metabolites is fluoride ions (28, 121). Methoxyflurane is in several cases reported to give renal failure, attributed to the toxic effect of the released fluoride (95, 104, 115).

By continued exposure of the cultured cells to sub-lethal fluoride concentrations, the cell growth assumed normal rate after a lag period, the length of which depended on the fluoride concentration. By stepwise increasing the fluoride concentration fluoride resistant cells developed in this way (Paper I). Several cell types can develop fluoride resistance (21, 79, 100), also cells with genetic biochemical markers without loss of these (59).

Resistance development *in vitro* is probably based on selection of appropriate mutants (79), but whether these are preexisting in the cell population or evolve during the development period is not clear. Cell cultures are resistant to

specific concentrations of fluoride above which fluoride is toxic to the culture, although it later may adopt resistance to this higher fluoride concentration. Whether the resistance development involves a multistep mutational event (several mutations) or different types of single step mutations is not clear. The mechanism for fluoride resistance is the ability of the resistant cells to maintain a low intracellular concentration of fluoride (Paper II), probably due to active exclusion of fluoride from the intracellular milieu (100).

The ratio between the intracellular and extracellular fluoride concentrations in normal LS cells was estimated to approximately 0.4 at pH 7.4 (Paper II), which is consistent with other reports (32, 100). This ratio is also shown to be dependent on pH (53, 102). The mechanism accounting for the maintenance of this ratio is probably that only nonionic hydrogen fluoride (HF, $pK_a = 3.45$) is penetrating the cellular membrane (102, 122, 123), together with a lower, more constant intracellular than extracellular pH (34, 51). The fluoride distribution ratio is thus probably determined by the diffusion equilibrium of HF (102). The increased ratio at low pH is due to a decreased difference between the intracellular and extracellular pH. Another mechanism must be responsible for the very low ratio found in fluoride resistant cells (Paper II, 100), a much too low intracellular pH would be required if the only mechanism operating was the diffusion equilibrium of HF.

In the search for the mechanism(s) for the cytotoxic effect of fluoride, cellular processes that might be affected by fluoride in the same concentration range and to similar degree as inhibition of growth were investigated. Most of these candidate processes were affected only by fluoride concentrations much higher than the lowest cytotoxic concentration, or not at all.

The cytotoxic effect of fluoride has usually been attributed to inhibitory effect on the cellular metabolism and in particular the glycolytic pathway by effect on enolase, and fluoride has been applied as an experimental tool in cells where inhibition of glycolysis was desired (16). Paper II and III show, consistent with results obtained by Carlson & Suttie (22), that fluoride may act cytotoxic without having any effect on the glycolysis or the ATP level, indicating that this is not the mechanism for the cytotoxic effect of fluoride. The apparent discrepancies between these results and the fluoride sensitivity of enolase is probably due to the lower fluoride concentration inside the cells than extracellularly.

Adenylate cyclase in homogenates is stimulated by fluoride, and cAMP increasing agents are growth inhibiting (89, Paper IV). Increased cAMP levels have also been reported in intact cells after treatment with fluoride (5, 12, 44, 64, 125). Increased cellular level of cAMP due to fluoride stimulation of adenylate cyclase might thus be a mechanism for the growth inhibiting effect. This seems unlikely however, as fluoride did not induce a physiologically significant increase in the level of cAMP in intact cells (Paper IV). This result is in agreement with other reports (1, 35, 63, 66, 96). The reason for the differing results obtained in measuring the cAMP level in fluoride exposed intact cells is obscure. Possible contamination of the intact cell system with cell fragments (i.e. homogenates), may be at least part of the reason.

Effects on amino acid transport or intracellular levels of amino acids were found only at supralethal fluoride concentrations, indicating that such effects are probably of minor importance for the cytotoxic effect (Paper V).

The only cellular parameters that were affected by fluoride concentrations showing growth inhibitory effect, were protein synthesis, DNA synthesis and the

activity of ornithine decarboxylase (ODC), the initial enzyme in the polyamine synthesis pathway (Paper VI and VII). All these functions were inhibited to the same degree as the growth inhibition, with similar lowest effective concentration and concentration – effect relationship, and with increasing effect at low pH, such as the cytotoxic effect (53). The effects had rapid onsets as they were clearly detectable within 20–30 minutes. The protein synthesis, however, was inhibited even sooner.

ODC has a short half-life, about 1 hour (98), and it seems as the decrease of ODC-activity in the presence of fluoride may be caused by the reduction in protein synthesis, hence reducing its *de novo* synthesis.

Inhibitors of cellular protein synthesis also reduce the DNA synthesis (42, 49), probably as a result of their effect on the protein synthesis. It is thus possible that the inhibitory effect of fluoride on DNA synthesis is a result of its inhibition of protein synthesis. Fluoride inhibited DNA synthesis directly in isolated nuclei (Paper VIII), but only at higher fluoride concentrations that can be expected intracellularly when DNA synthesis is inhibited to a similar degree in intact cells.

It is thus conceivable, consistant with the results of Mankovitz et al. (79), that protein synthesis is the primary cellular target for fluoride, and that the main mechanism for the cytotoxic effect is inhibition of protein synthesis. Other effects observed rapidly and at fairly low fluoride concentrations such as inhibition of DNA synthesis and decreased ODC activity are probably results of this. These parameters may, however, be affected *per se* at high fluoride concentrations.

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