Aluminofluoride Complexes in the Etiology of Alzheimer's Disease

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The question of aluminum's relevance to the etiology of Alzheimer's disease cannot yet be adequately answered. The mechanisms of how aluminum could evoke the hallmarks of AD are not known. Reflecting many studies, which utilize aluminofluoride complexes in laboratory investigations, we suggest that these complexes may act as the initial signal stimulating impairment of homeostasis, degeneration, and death of the cells. Aluminum ions in the presence of fluoride can accelerate the aging and impair the functions of the nervous system. In respect to the etiology of Alzheimer's disease, the long-term synergistic action of aluminum ions and fluorides may represent a hidden but serious and powerful risk factor for the development of this devastating threat to human civilization.

Keywords: Alzheimer's disease, Aluminum, Fluoride, Aluminofluoride complexes, G-protein

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List of Abbreviations

$1,4,5\text{-}IP_3$	inositol 1,4,5-trisphosphate
1-IP	inositol 1-phosphate
AD	Alzheimer's disease
AChE	acetylcholinesterase
AlF_x	aluminofluoride complex
AMP	adenosine monophosphate
ApoE	apolipoprotein E
APP	amyloid-precursor protein
ATP	adenosine triphosphate
BBB	blood-brain barrier
$[Ca^{2+}]_i$	cytosolic calcium level

cAMP	cyclic AMP
CMP	cytidine monophosphate
DAG	1,2-diacylglycerol
GAP	guanosine triphosphatase activating protein
GDP	guanosine bisphosphate
G-protein	guanosine triphosphate-binding protein
GTP	guanosine triphosphate
GDP(S)	guanosine 5′-[β thio]diphosphate
GTP(S)	guanosine 5′-[thio]triphosphate
IPs	inositol phosphates
LPA	lysophosphatidic acid
PA	phosphatidic acid
PI	phosphatidylinositol
PIP	phosphatidylinositol 4-phosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PKC	protein kinase C
RBC	red blood cells
TSH	thyroid-stimulating hormone

1 Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder with impairment of cognitive functions and the loss of memory. The number of patients has risen dramatically in the industrialized world as well as in the developing countries. AD is among the most frequent obstacles to healthy aging. The increasing size of the very old population has led to the increasing impacts on the cost of social and health care. In spite of accumulated knowledge of cellular and molecular aspects, it is not known how to arrest or delay specifically the course of this devastating disease. Intense efforts are underway to find a cause or a systemic metabolic disorder, which could be targeted by therapy.

The idea that aluminum is the risk factor for AD has attracted considerable attention from general public as well as from researchers. A higher amount of aluminum was found in the human brain with AD than in brains of age-matched healthy controls [1, 2]. The hypothesis that the accumulation of aluminum in the brain is the cause of this disease has been postulated and discussed very often [3–5]. This suggestion was further supported by a positive correlation between the incidence of AD and concentrations of aluminum in drinking water. Nine out of 13 published epidemiological studies of aluminum drinking water and AD have shown statistically significant positive relations [6]. The study including 3,777 subjects aged 65 years in southwestern France suggests that a concentration of aluminum in drinking water above 0.1 mg per

liter may be a risk factor of dementia and, especially AD [7]. A major problem in the interpretation of these studies is that drinking water only contributes a fraction of the total dietary intake of aluminum. Yokel [8] calculated that drinking water provides only about 1% of normal daily human intake. Only some of the studies that determine aluminum in post-mortem brains, senile plaques and neurofibrillary tangles of patients with AD are consistent with the aluminum hypothesis. The relatively poor sensitivity of some of the techniques available for these analyses could explain the discrepancies [9]. Considerable evidence exists that aluminum may play a role in the etiology and pathogenesis of AD, but whether the link is causal is still open to debate.

Recent fundamental research of the pathogenesis of AD brings evidence that this disease is connected with the alterations in neurotransmission, abnormal polymerization of cytoskeletal protein τ, changes in β-amyloid precursor protein and β-amyloid production, apolipoprotein E accumulation, and alterations in mechanisms of calcium homeostasis. Inflammation and impairment in the action of free oxygen radicals are probably also involved in the pathogenesis of AD [10]. Three genes have been identified that cause the less common early-onset, familial cases of the disease: the β-amyloid precursor protein gene on chromosome 21, the presenilin 1 gene on chromosome 14, and the presenilin 2 gene on chromosome 1. More than 50% of the cases are late-onset and related to the apolipoprotein E gene on chromosome 19 [11].

The mechanisms of how aluminum could evoke the multiple pathophysiological changes in AD are not known. Many authors suggest that aluminum alone is not a cause of AD. Neither the increased content of aluminum in the brain nor environmental studies can explain whether and why aluminum constitutes a risk. Aluminum is therefore regarded as a putative risk factor along with alcohol consumption, stressful life events, and manual occupation. On the other hand, a comprehensive book reflecting the myriad of ways that aluminum is known to impact upon mammalian physiology and the etiology of AD has been published recently [10].

2 Aluminofluoride Complexes

The intensive laboratory research of mechanisms of signal transduction brings numerous experimental data, which could change our understanding and interpretation of aluminum's action at the cell level. Reflecting many laboratory studies we suggest that some of the pathological changes are not raised by aluminum alone, but by the synergistic action of aluminum and fluoride [12].

Fluoride anions have long been known to influence the activity of various enzymes and adenylate cyclase [13]. A breakthrough came later when Sternweis and Gilman [14] demonstrated that fluoride activation of adenylate cyclase depends on the presence of traces of aluminum. This fact had at first been ignored because aluminum was present as a leached-out impurity in millimolar solutions of fluoride in glass. Gilman and coworkers found that the target of the activation was a heterotrimeric G-protein and that the active

stimulatory agent was aluminum fluoride. The soluble aluminofluoride complexes (AlF_x) are formed in water solutions containing fluoride and traces of aluminum [15, 16]. The idea that the aluminofluoride complex acts as a new high affinity analogue of γ-phosphate [16, 17] has been accepted during the last decade and a great number of reports on its use has appeared.

2.1 The Structure of Aluminofluoride Complexes

The aluminofluoride complexes are not permanent and the proportions of species such as AlF_3 and AlF_4^{1-} are still disputed. Phenomenological observations seems to verify that the pH determines the complexation state of AlF_x. Factors other than pH, such as fluoride concentration and the location of positively charged amino acid of the active site of the phosphoryl-transferring enzyme, may cause deviation from the strict pH dependence of AlF_3 versus AlF_4^{1-} in biological systems [18]. In aqueous solutions with a pH of less than 5.5, aluminum exists as the octahedral hexahydrate $Al(H_2O)_6^{3+}$, usually abbreviated to Al^{3+}. At pH values above 6.2 $Al(H_2O)_6^{3+}$ undergoes successive deprotonation, becoming tetrahedral aluminate $Al(OH)_4^{1-}$ [19]. If fluoride is added, the four equatorial water molecules of the hexahydrate are replaced by fluoride to give $AlF_4\ (H_2O)_2^{1-}$. At pH values above 6.2 the tetrahedral aluminate species predominates with a varying composition of hydroxy groups and fluoride.

Chabre and coworkers [16, 17] suggested that AlF_4^{1-} is probably the active species, which mimics the role of the γ-phosphate. According to these authors, it is unlikely that the whole AlF_4^{1-} complex binds with its four fluoride atoms. Most probably, when entering the site, one of the fluoride atoms is released and the tetracoordinated aluminum binds to the oxygen on the β-phosphate [16, 17].

What the high concentration of fluoride in the solution does is to induce the formation of a soluble tetracoordinated state of aluminum, which has the same geometry, size, and coordination as a phosphate. Among many metals tested in the presence of fluoride, Sternweis and Gilman [14] found that only beryllium could substitute for aluminum for the activation of adenylate cyclase. This fact reinforced the assumption that aluminum acts through its tetrahedral phosphate-like complex AlF_4^{1-}, because all beryllium complexes are tetracoordinated [16].

Martin [19] has recalculated the equilibria of AlF_x and contested that AlF_4^{1-} is the major species in the presence of millimolar fluoride at neutral pH and is tetrahedral in aqueous solution. According to his calculations, the predominant species are the neutral complex AlF_3 and the mixed complex $AlF_3(OH)^{1-}$. These complexes should be hexacoordinated, with water molecules occupying the free sites. It is uncertain whether the complex that enters the site is an AlF_3 that becomes tetrahedral by losing its three bound water molecules and contracting a fourth bond with the β-phosphate oxygen, or if it is an already tetrahedral $AlF_3(OH)^{1-}$ that exchanges its hydroxyl for the β-phosphate oxygen or an AlF_4^{1-} that exchanges a fluorine. In earlier reviews Martin had

also admitted that the active species might be simply the aluminum ion Al^{3+} and that fluoride complexation would only be needed to allow the penetration of aluminum across the plasma membrane. Once corrected for these effects, Antonny and Chabre [20] proposed that $AlF_3(OH)^{1-}$ is the main activating species and that the bound form of the complex is tetracoordinated GDP-AlF_3. These complexes can bind to proteins by hydrogen bonds to the fluorine atoms just as to oxygen atoms of a phosphate ion. So can arise an aluminofluoride analogue of pyrophosphate, R-O-PO_2-O-AlF_3, which may be bound at the site for the γ-phosphate. Thus, for instance, in G-protein whose nucleotide site already contains GDP, AlF_x can form hydrogen bonds with the donor groups of the β-phosphate site and bind ionically to the terminal oxygen of the β-phosphate of the GDP. AlF_x was found to be a good analogue of a γ-phosphate for a number of ATP- and GTP-converting enzymes. Enzyme-bound GDP or ADP could therefore form a complex with AlF_x that imitates ATP or GTP in its effect on protein conformation.

Studies of the crystal structures of nucleotide binding proteins complexed with AlF_x show that in the case of G-proteins, myosin-S1, and nitrogenase, AlF_4^{1-} is the active site species, whereas in others metabolic enzymes, e.g., nucleoside diphosphate kinase and uridylate monophosphate kinase [18, 21], AlF_3 is bound. It is not clear whether these differences reflect some differences between proteins or are due to technicalities. Schlichting and Reinstein [18] compared the coordination numbers at different crystallization conditions and suggested that the different coordination numbers originate mainly from the difference in pH at which the enzymes were crystallized. AlF_x occurs as AlF_3 at pH 7.5–8.5 but as AlF_4^{1-} at pH below 7. To minimize the influence of factors other than pH on manifestation of AlF_x, they treated the two uridylate monophosphate kinase-ADP-CMP-AlF_x complexes (this enzyme from *Dictyostelium discoideum* catalyzes the reversible trans-phosphorylation between ATP and CMP) as similarly as possible except for the pH of the crystallization buffer. The bound AlF_x occurred as AlF_3 at pH 8.5 but as AlF_4^{1-} at pH 4.5.

2.2
Aluminofluoride Complexes and G-Proteins

Chabre and coworkers [16, 17] suggested that AlF_4^{1-} mimics the role of the γ-phosphate only if the β-phosphate is present and remains unsubstituted on its oxygen. The effect is more readily seen with G-proteins because GDP is always tightly bound in the nucleotide site of the protein. Low cost and availability of $AlCl_3$ together with NaF has probably contributed to their widespread use as a tool in laboratory studies of G-proteins [12]. These studies brought a great deal of knowledge about the involvement of heterotrimeric G-proteins in cell signaling. The heterotrimeric G-proteins mediate the transfer of information from cell-surface receptors to effector molecules. Heterotrimeric G-proteins thus play a pivotal role in transmembrane signaling processes.

2.2.1
G-Protein-Coupled Receptors

The family of cell-surface receptors that require coupling to G-protein transducers for functional signaling is vast and diverse. These receptors detect inputs, such as photons, odorants, neurotransmitters and hormones, such as dopamine, epinephrine, norepinephrine, serotonin, acetylcholine, glucagon, vasopressin, melatonin, neuropeptides, opioids, excitatory amino acids, prostanoids, purines, growth factors, and morphogens, linking extracellular stimuli to intracellular signaling networks. The isolation of cDNAs for G-protein-coupled receptors enabled a comparison that indicated a shared structural configuration. These receptors consist of a single protein chain that crosses the plasma membrane seven times. The cytosolic C-terminal domain, Asp-Arg-Tyr (339–341) motif at the cytosolic end of helix III, and the loops between the helices III–IV and V–VI are involved in G-protein recognition and binding [22].

2.2.2
G-Proteins

The members of heterotrimeric G-protein family are composed of a α-(39–52 kDa), a β-(35–36 kDa), and a γ-(8–10 kDa) subunit. The G-protein α-subunit binds to GDP in the inactive state. The agonist receptor binding facilitates the exchange of GDP for GTP. The activated α-subunit dissociates from β- and γ-subunits to interact with effector enzymes on the cytosolic side. Low K_M GTPase intrinsic activity of the α-subunit hydrolyzes GTP to GDP to end the cycle activation [23]. Simultaneously, a conformational change occurs, leading to an increased GTPase activity of the α-subunit. On washing off the agonist, the GTP-bound form converts to the GDP-bound form, which is dependent on the rate of GTPase activity. GTP hydrolysis drives the conformational change and is responsible for interrupting the interaction with the effector molecule. As G-proteins are activated when they go from the GDP-bound to the GTP-bound state, it was assumed that the AlF_x occupies the γ-phosphate-binding site on the protein and together with bound GDP makes the G-protein act as if it has bound GTP. Chabre [17] concluded that AlF_x does not seem to form an analogue of the transition state since the structural change locks the site and prevents the dissociation of the analogue. The determination of the three-dimensional structures of heterotrimeric G-proteins bound to GDP and AlF_x in 1994 unexpectedly demonstrated that they mimic the transition state of the phosphoryl-transfer reaction rather than the ground state. This is because aluminum is bound to four fluoride ligands in a square-planar coordination with two oxygen ligands at the apical positions of the resulting octahedron [21]. One oxygen ligand is a β-phosphate oxygen, whereas the other is the oxygen from water believed to represent the attacking nucleophile of the hydrolysis reaction. Invariant arginine and glutamine residues, which are required for GTP hydrolysis but not for its binding, stabilize the AlF_x binding to GDP. In addition, a new class of GTPase-activating proteins (GAPs) has

been discovered to modulate the rate of G-protein-mediated effects. Biochemical evidence showed that GAPs (also called regulators of G-protein signaling) bind with higher affinity to $G \cdot GDP \cdot AlF_x$ complex of G α-subunit than to the triphosphate state of G-protein. GAPs also stabilize the GTPase active conformation of G-proteins. These observations further support that AlF_x stabilizes the transition state [24].

The observation that AlF_x complexes activate G-proteins has been therefore useful for the study of the mechanism of G-protein activation, for understanding the biochemical mechanism of GTP hydrolysis, and for the elucidation of three-dimensional structures of several GTPases, including the discovery of the GTPase-activating proteins.

Although it was initially believed that AlF_4^{1-} only interacts with Gα-subunits, recent studies have revealed that a small G-protein Ras can interact stably with AlF_3 in the presence of RasGAP [24]. The proto-oncogene product Ras is a component of intracellular signaling pathways involved in cell growth and division. It has a very low intrinsic GTPase reaction rate that is stimulated 105-fold by RasGAPs that down-regulate the accumulation of $Ras \cdot GTP$. The determination of the structure of a complex between RasGAP and $Ras \cdot GDP$ in the presence of aluminum ions and fluoride [21, 24] shows that AlF_3 forms a pentagonal bipyramid, with the fluorides forming the trigonal base with two apical oxygen ligands. Similar studies demonstrated that several classes of small GTPases can stable interact with their respective GAPs in the presence of AlF_3, suggesting that the aluminofluoride complex could bind to a wide variety of GTPases. These observations demonstrate that the GTP hydrolysis mechanism is similar for both small GTPases and Gα-subunits.

In most systems, the α-subunit determines the specificity of interaction with receptor and effector molecules. Molecular cloning has revealed a large molecular diversity within the subunits of G-protein. During the last decade, the number of known mammalian G-protein α-subunits grew rapidly to comprise more than 20 individual proteins. There are 5 G-protein β-subunits and at least 12 γ-subunits [25]. Small monomeric G-proteins were found in eukaryotes from yeast to human. They constitute a superfamily consisting of more than 100 members, classified into at least five families: the Ras, Rho, Rab, Sar1/Arf, and Ran. The Ras family regulates gene expression, the Rho family regulates cytoskeletal reorganization and gene expression, the Rab and Sar1/Arf families regulate vesicle trafficking, and the Ran family regulates nucleocytoplasmic transport and microtubule organization. Many upstream regulators and downstream effectors of small G-proteins have been isolated, and their modes of activation and action have gradually been elucidated [26].

2.2.3 *G-Protein-Coupled Effector Enzymes*

Adenylyl cyclase is the effector enzyme that synthesizes cyclic adenosine monophosphate (cAMP) from ATP. Cyclic AMP functions as a second messenger to relay extracellular signals to intracellular enzymes such as protein kinase A. Adenylyl cyclases are integral membrane proteins that

consist of two bundles of six transmembrane segments. Two catalytic domains extend as loops into the cytoplasm. There are at least nine isoforms of adenylyl cyclase, based on cloning of full-length cDNAs. Early studies indicated that cyclase activity was regulated primarily by interactions with α-subunits of heterotrimeric G-proteins. Binding of a stimulatory α-subunit (G_s) enhanced activity while binding of an inhibitory α-subunit (G_i) inhibited adenylyl cyclase activity [23, 26]. More recently, it has become clear that cyclase activity is regulated by multiple effectors, which include not only the α-subunits of G_s and G_i proteins, but also the G-proteins $\beta\gamma$-subunits and the protein kinase C. Five of the adenylyl cyclases known are regulated by calcium [27].

Phospholipase C (PLC) catalyzes the phosphodiesteric cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) into two moieties, inositol 1,4,5-trisphosphate (1,4,5-IP_3) and 1,2-diacylglycerol (DAG) [27, 28]. 1,4,5-IP_3 is a second messenger that controls many cellular processes by generating intracellular calcium signals. The other second messenger DAG is responsible for activating a protein kinase C (PKC), which catalyzes protein phosphorylation. PIP_2 is the main substrate for PLC. Many isoforms of PLC have been identified, based on their purification and characterization, and upon the established amino acid sequence. The differing functions of the various PLC families are not yet fully known. Some isoforms are regulated by G-proteins and can, in turn, regulate these by serving as GTPase-activating G-proteins (e.g., PLCβ1). The $G\alpha_{q/11}$-subunit activates β-isoforms of PLC. Others are regulated by tyrosine-kinases (e.g., PLC γ) [28].

Tyrosine kinase receptors generate 1,4,5-IP_3 and DAG by interacting directly with PLCγ. PLCγ contains two SH_2 domains and one SH_3 domain. The SH_2 domain of PLCγ1 recognizes and binds to a specific phosphotyrosine residue. Once PLCγ1 is phosphorylated, it starts to hydrolyze PIP_2. Signal transduction using tyrosine kinase receptors is an energy-consuming process: ATP is utilized for receptor phosphorylation and for PLCγ phosphorylation. The resynthesis of phosphoinositides also requires ATP for the phosphorylation of phosphatidylinositol [27].

Phospholipase D (PLD) is now known to be also involved in receptor-effector cascade [29]. Activation of PLD occurs concurrently with PLC activation. This enzyme is stimulated by receptors having tyrosine kinase activity. PLD preferentially hydrolyzes phosphatidylcholine to yield phosphatidic acid (PA) and choline. PA is hydrolyzed by PA phosphohydrolase to produce DAG. DAG might be in turn phosphorylated by DAG kinase to yield PA. PA may also serve as a potential lipid messenger, or it undergoes attack by phospholipase A_2 (PLA_2) to yield lysophosphatidic acid (LPA). LPA has been described as the lipid mediator, which acts through a specific G-protein-coupled receptor [30]. When LPA is added, PLC is activated and adenylate cyclase is inhibited through G_i. LPA stimulates PLD activity and also induces arachidonate release, probably as a result of PLA_2 activation. PLA_2 may generate arachidonic acid, which goes on to form prostaglandins, thromboxanes and lysophospholipids. PLA_2 was found in the cytosol of non-stimulated cells. Ca^{2+} binding in the lipid domain and phosphorylation enhances the

translocation of PLA_2 to the plasma membrane surface. It has been suggested that G-proteins also mediate the activation of PLA_2.

The evidence is compelling that ion channels are direct G-protein effectors. K^+ channels are activated by G_a and inhibited by G_i, whereas the opening of L-type Ca^{2+} channels is regulated by G_s or G_o protein [31]. Studies on G-protein-ion channel membrane interactions have shown that G-proteins may have several membrane targets, including more than one type of ionic channels.

2.3 Aluminofluoride Complexes in Laboratory Investigations

Fluoride activation in the presence of trace amounts of aluminum has been often used as evidence for involvement of a G-protein in a system. A great number of reports on their use has appeared, with far-reaching consequences for our understanding of fundamental living processes. Laboratory investigations during the past decade bring evidence that aluminofluoride complexes influence all cells and tissues of the human body with powerful pharmacological efficacy [12]. It has been demonstrated that aluminum ions in the presence of millimolar fluoride may clone or potentiate the action of numerous extracellular signals. The principle of amplification of the initial signal during its conversion into functional response has become a widely accepted tenet in cell physiology (Fig. 1).

AlF_x therefore affects the levels of second messenger molecules and cytosolic calcium with extensive effects on cell metabolism [12, 32]. Biological signaling pathways interact with one another to form complex networks. The question as to how cells manage to make the right responses at the right times has yet to be fully understood. The discoveries of receptor diversity, numerous isoforms of G-proteins and effector molecules broaden enormously the possibilities of interactions of signal transduction events. Yet, it seems that we shall not probably find any physiological process, which is not potentially influenced by AlF_x. Science has already accumulated evidence how diverse biological problems can be investigated using the small inorganic molecule AlF_x [12, 21, 32]. It has led to the advance in our understanding of living processes, the common denominator of which is the transfer of a phosphoryl group. The phosphate-analogue model of AlF_x action was accepted for G-proteins but it was later extended to all enzymes that bind phosphate or nucleoside-polyphosphate. Phosphoryl-transfer reactions are involved in processes such as cell signaling, energy transduction, regulation of cell metabolism, cytoskeletal protein assembly, growth, cell differentiation, aging, and apoptosis. Considering that these reactions are fundamental for nearly all biological systems, we can understand the choice of aluminum fluoride as the biological molecule of the month March 1997 [21]. In view of the ubiquity of phosphate in cell metabolism, a phosphoryl-transfer transition state analogue might represent a useful tool for laboratory investigations, but also a strong potential danger for living organisms including humans. We should try to understand how the simple signal could lead to multiple events resulting into a pathophysiological state.

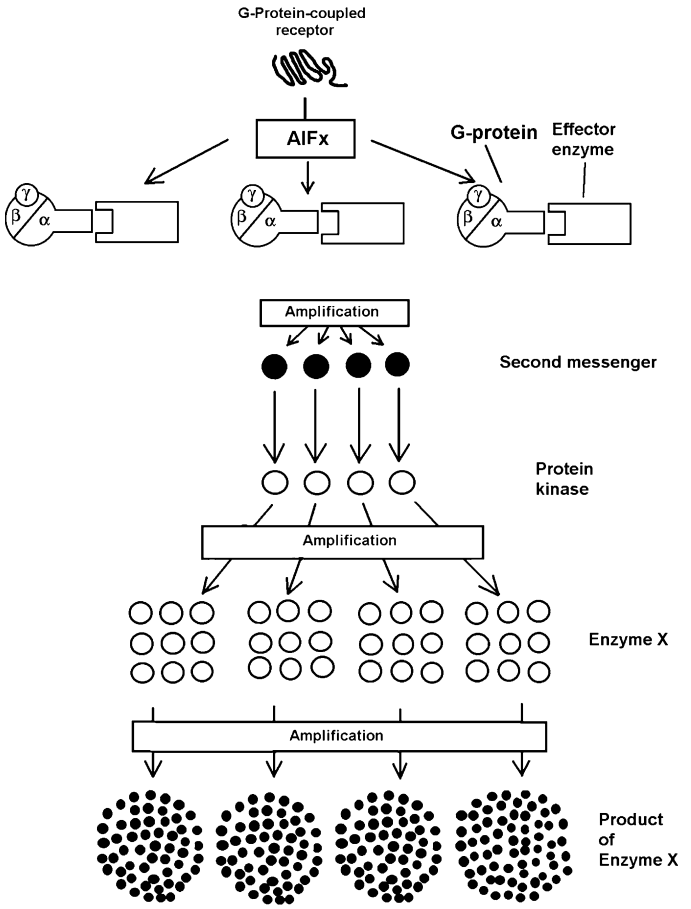


Fig. 1. AlF_x acts as the messenger of a false information. Its message is greatly amplified during the conversion into the functional response of a cell. The second messenger molecule could be cAMP, 1,4,5-IP_3, and DAG. Moreover, AlF_x can participate as the analogue in the phosphoryl-transfer reactions involved in the signaling cascade

3
Reevaluation of the Role of Aluminofluoride Complexes in the Etiology of Alzheimer's Disease (AD)

The scientific world and the pharmaceutical companies are seeking one reaction, one enzyme, one substrate or one gene, which could be targeted by therapy. The biochemical approach is based on the hypothesis that the causative disorder of AD may lurk at the level of cell physiology, altering various brain processes, such as the recognition, memory, language, and visuospatial skills. The recent fundamental research of pathogenesis of AD has brought evidence that this disease is connected with the alterations in neurotransmission, β-amyloid production, plaque formation, and cytoskeletal

abnormalities in brain tissue [5, 10]. An alteration in calcium homeostasis could affect most of these AD-related deficiencies. A disturbance in signal transduction between brain cells could underlie the above-mentioned AD-related pathophysiological alterations. G-proteins and transfer of phosphate groups are the most important regulatory factors in these processes.

3.1 Cholinergic Transmission and AlF_x

The cholinergic hypothesis suggests that the reduction of cholinergic neurotransmission could explain the most important cognitive deficit in AD [33]. The basal forebrain nuclei that give rise to cholinergic fibers show cell degeneration in brains of AD patients. The 60–70% depletion of acetylcholinesterase and choline acetyltransferase in the cortex and in the limbic structures of patients with AD is one of the most consistent and profound changes as early as one year after onset of the disease [34, 35]. The cortex of patients shows decreased rates of acetylcholine synthesis and choline uptake. Aluminum has been found to inhibit choline transport in isolated rat brain nerve endings and reduce neuronal acetyltransferase activity [5]. It is therefore hypothesized that raised brain aluminum levels in AD may contribute to the cholinergic neuronal deficits in AD. Therefore, it was thought that the study of cholinergic lesions could explain the most important cognitive deficits and help in the discovery of a rational therapy. The alterations of nicotinic and muscarinic cholinergic receptors in brains of patients with AD were found [36].

Among different drug strategies, the cholinergic approach has gained great interest. There are two basic principles of cholinergic therapy: first, stimulation of various cholinergic receptors with selective agonists; second, reduction of acetylcholine hydrolysis by cholinesterase inhibitors. Cholinesterases are today the targeting point for the most used group of AD drugs.

3.1.1 *Cholinergic Receptors*

Molecular biology shows the diversity of acetylcholine receptors. Twelve genes were found for nicotinic cholinergic receptors; the synthesis of muscarinic receptors is coded by five separate genes [37]. Different subtypes of these receptors have different physiological and pharmacological characteristics. Muscarinic cholinergic receptors m1, m3, and m5 stimulate the generation of cAMP, but in the case of m1 such stimulation is secondary via the activation of phospholipase C, production of inositol 1,4,5 trisphosphate and calcium mobilization. These receptors also stimulate the activation of PLA_2, PLD, and tyrosine kinase, and activate voltage-gated calcium channels. On the other hand, muscarinic receptors m2 and m4 inhibit the activity of adenylate cyclase, calcium influx, and the activity of PLA_2. The cholinergic receptors in the brain are mostly muscarinic and their activation has been connected with the cognitive function. At postsynaptic muscarinic receptor sites acetylcholine acts by reducing potassium conductance making the cholinoreceptive neuron

more susceptible to other excitatory inputs [38]. The discovery of different muscarinic receptor subtypes has offered new opportunities to develop specific inhibitors of the m1 postsynaptic receptor subtypes. However, the therapeutic strategy of direct pharmacological activation of muscarinic receptors is somehow questionable since the additional effects could be derived from muscarinic receptor antagonism. It is unclear, e.g., whether treatment should aim to enhance or diminish glutamatergic transmission, since the positive modulation could facilitate learning while increasing the glutamate function may enhance excitoxicity and neuronal death.

The decreased number of nicotinic acetylcholine receptors in the cortex and hippocampus associated with AD and the beneficial effects of nicotine observed in AD patients suggest the important role played by nicotinic receptors [39]. Presynaptic nicotinic receptors have a key role in the regulation of release of many transmitters such as acetylcholine, glutamate, and serotonin. Indeed, AD involves more than cholinergic deficiency. In brains of patients with AD several other transmitters are depleted. Attempts to intervene in acetylcholine receptors pharmacologically may thus present us with large problems. For example, agonists of muscarinic receptors stimulate the formation of β-amyloid whose presence in the membranes induces the uncoupling of muscarinic receptors from phospholipase C. Stimulation of muscarinic acetylcholine receptors leads to phosphorylation of β-amyloid precursor protein, promoting the plaque formation.

Similar interactions may occur in the presence of the small molecule AlF_x. The acetylcholine receptors were not thought of as G-protein receptors, but the use of fluoride plus aluminum salts contributed to the discovery of very complicated regulatory and modulatory relationships in their action [31].

The use of AlF_4^{1-} in the investigation of canine cerebral cortex [40] led to the discovery of a previously unrecognized signaling pathway in the brain. These experiments demonstrated that muscarinic acetylcholine receptor-G-protein uses PLD as the effector enzyme. AlF_4^{1-} caused a two- to three-fold increase in breakdown of phosphatidylcholine and rapid accumulation of choline and phosphatidic acid was observed.

Binding studies of muscarinic-receptor agonists suggested that mechanisms of G-protein-receptor coupling or uncoupling might be disturbed in AD [41]. Wallace and Claro [42] reported the increased sensitivity of PLC to GTP(S) during AD. Also the impairment of carbachol-stimulated low K_m GTPase activity in AD basal ganglia was observed. However, this area warrants further study. In view of the role of both heterotrimeric as well as small G-proteins, the aluminofluoride complex may act as a bullet, which could evoke the marked disturbances in complex networks in the brain.

3.1.2 *Acetylcholinesterase*

Detailed studies of the molecular structure of acetylcholinesterase (AChE) have shown that this enzyme exists in many molecular forms [43]. There are globular (G) and asymmetric classes, which are further subdivided. The

globular forms predominate in mammalian brain. Most brain AChE consist of assemblies of four identical 77 kDa catalytic subunits linked by disulfide bridges (G_4). The brain contains a small amount of the monomeric G_1 form with a small amount of G_2 form. The only AChE molecular form found in erythrocytes is globular dimer G_2. The enzyme is anchored in the membrane by a glycosylated phosphatidylinositol. Despite this multiplicity, all cholinesterase molecular forms have equivalent catalytic activities, but some inhibitors affect them differentially. The decrease of presynaptically bound G_4 and the increase of the soluble G_1 a G_2 form was observed in the brains of aging rats [38]. Similar changes were found in the frontal and parietal cortex, in the hippocampus and cholinergic projection nuclei in brains of AD patients.

Platt and coworkers [44] applied histochemical techniques to identify changes in AChE distribution induced by intracerebroventricular aluminum injections (5.4 μg in 5.5 μL, daily over a period of 5 successive days) in the adult rat brain after survival periods of either 1 or 6 weeks. Damage of the cingulate bundle in aluminum-treated animals led to a severe anterograde degeneration of cholinergic terminals in the cortex and hippocampus, as indicated by AChE labeling. Immunoreactivity of astrocytes and phagocytic microglia revealed a greater inflammatory response in aluminum-injected animals compared to controls. These data suggest that the enhancement of inflammation and the interference with cholinergic projections may contribute to pathological processes in AD.

Therapeutic application of AChE inhibitors requires the need to monitor the activity of this enzyme on the periphery. Since the brain is the target organ for all potential cholinergic drugs, any peripheral measures can provide important information about a compound efficacy and mechanism of action. Such studies are relatively non-invasive and simple. The reliable correlation between peripheral and central cholinesterase inhibition in humans depend on many factors, clearly vary from drug to drug, and require detailed pharmacokinetic studies.

3.1.2.1
The Effect of AlF_x on AChE Activity in Human Red Blood Cells

We studied the effect of 20 μM $AlCl_3$ and 0.01–1 mmol L^{-1} NaF on the AChE activity in erythrocytes of AD patients, age-matched healthy subjects, and young healthy controls. The AD patients were chosen on the level of clinical diagnosis "probably Alzheimer's disease" according to DSM IV, NINCDS/ADRDA criteria, Hachinski Ischemic Score, GBS scale, and MMSE. No subject of this study had concurrent somatic illness or a neurological or psychiatric disorder. The study was approved by the Ethic Committee of the Prague Psychiatric Center, and before admission to the study a written informed consent was obtained from all subjects. While 20 μM $AlCl_3$ increases the AChE activity in RBC in all groups of investigated subjects to 158–285%, 1 mM NaF and 20 μM $AlCl_3$ inhibit the AChE activity in intact RBC as well as in hemolyzate in all groups of investigated subjects (Table 1).

Table 1. The effect of 1 mM NaF + 20 μM $AlCl_3$ on the acetylcholinesterase activity (AChE) in freshly prepared intact RBC and in hemolysate of patients with AD (mean age 72.5 ± 5.1 years), age-matched healthy controls (AM-HS) (72.1 ± 1.6 years), and the group of young healthy subjects (YS) (35.9 ± 8.5 years). Whole venous blood samples were drawn from each subject after overnight fasting., always at 07:30 AM. Red blood cells (RBC) were isolated from the blood of patients with AD, AM-HS, and YS by centrifugation [68]. RBC AChE activity was evaluated in intact freshly prepared RBC or hemolyzate following the spectrophotometric method [45] with modifications. Buffer was Tris-HCl, pH 7.5 in the solution of 154 mmol L^{-1} NaCl, acetylthiocholine iodide was a substrate. Measurement of enzymatic activity was performed in fluorimeter polystyrene cuvettes for 3 min (UV/VIS spectrophotometer Shimadzu, Japan). The effects of 1 mmol L^{-1} NaF in the presence of 20 μmol L^{-1} $AlCl_3$ were measured. Data are expressed in percentage of the AChE activity in the absence of aluminum and fluoride ions. No differences between the AChE activity were found between the investigated groups

Subjects	AD	AM-HS	YS
N	23	16	16
Intact RBC	16.3 ± 5.5	18.9 ± 4.2	17.1 ± 2.3
Hemolyzate	19.8 ± 3.5	21.4 ± 4.6	26.0 ± 6.3

While 20 μmol L^{-1} $AlCl_3$ increased the AChE activity in RBC in all groups of investigated subjects to 158–285%, in the presence of 1 mmol L^{-1} NaF aluminum inhibited the AChE activity in intact RBC as well as in hemolyzate in all groups of investigated subjects (Table 1). The inhibition induced by AlF_x was dependent on the concentration of fluoride in the cuvette (Fig. 2). The activation of AChE activity by 3.7 μmol L^{-1} Al^{3+} in the human RBC membranes and by 1–100 μmol L^{-1} Al^{3+} in bovine brain was observed by Zatta et al. [46].

We could hardly accept these results as the discovery of a new available AChE inhibitor. Therapeutic strategies to affect one enzyme pharmacologically may be followed by various side effects, since AChE is present in many tissues. For example, the initial studies of the effect of tacrine showed its hepatotoxicity. The AD patients with two $\varepsilon 4$ alleles of the ApoE gene do not respond to such a therapy and have higher AChE activity in cerebrospinal fluid than controls and AD patients with one or no $\varepsilon 4$ [47]. The therapeutic use of AChE inhibitors directed the research on AD to the study the catalytic function of this enzyme. However, the presence of AChE in the RBC plasma membrane does not seem to be connected with the processes of neurotransmission. In connection with the pathogenesis of AD the non-catalytic and non-specific role of AChE is under discussion. AChE co-localizes with β-amyloid deposits of AD brains [48]. AChE accelerates β-amyloid formation and it may therefore act as a pathological chaperone inducing a conformational transition of the β-amyloid protein. This action of AChE was not affected by edrophonium, an active site inhibitor, but it was affected by propidium, a peripheral anionic binding site ligand. Moreover, AChE associated with plaques, tangles and β-amyloid angiopathies possesses different enzymatic properties, and, quite possibly, is of a different source as compared with the enzyme associated with

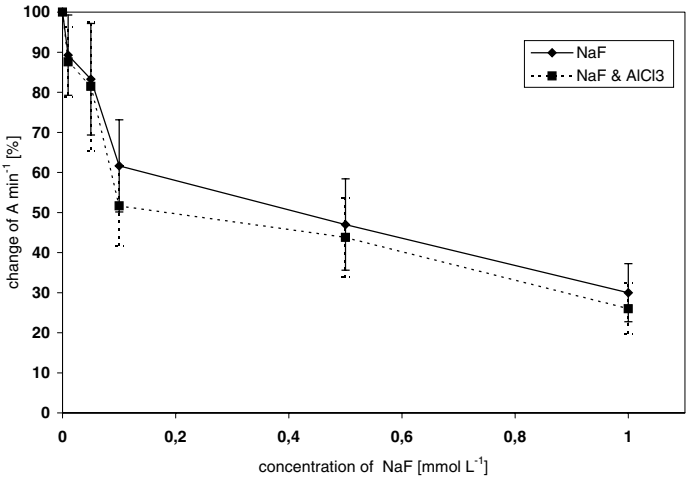
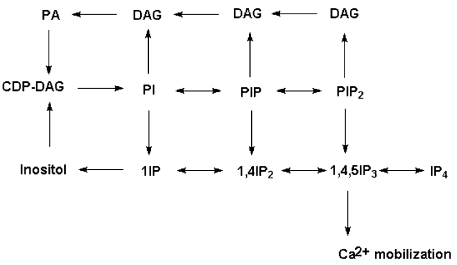


Fig. 2. The effect of various concentrations of NaF (–♦–) or NaF in the presence of 20 μmol L^{-1} $AlCl_3$ (–■–) on the AChE activity in hemolyzate from RBC of young healthy subjects. Data are expressed in percentage of the AChE activity in the absence of aluminum and fluoride ions. n = 8–16 measurements

normal neurons and axons [48]. It can thus participate directly not only in the processing of β-amyloid protein but also by causing aberrant growth and development of neurons. The results of intensive research of AChE as a therapeutic target provide us with many parts of a puzzle and lead us to postulate many further questions. Is the degeneration of cholinergic neurons and the reduction of acetylcholine the primary change or is it evoked by the action of soluble AChE? Why are there so many forms of AChE? Can erythrocytes release AChE into the brain? Some hypotheses suggest that the pathogenesis of AD may be connected with a systemic cholinesterase abnormality or with damage to the blood-brain barrier, allowing AChE to leak from the brain to the plasma.

3.2
Phosphoinositide Signaling System

During the last decade, a new phosphoinositide signaling system has been postulated [27, 28]. A great amount of experimental evidence supports the general concept that phosphatidylinositol 4,5-bisphosphate (PIP_2) from the plasma membrane is hydrolyzed and yields inositol 1,4,5-trisphosphate (1,4,5-IP_3) and diacylglycerol (DAG) upon receptor stimulation (Scheme 1). Both products of this hydrolysis catalyzed by phospholipase C (PLC) have second messenger roles. 1,4,5-IP_3 binds to a receptor in membranes of the endoplasmic reticulum, which results in a release of Ca^{2+} into the cytosol. The 1,4,5-IP_3 receptor comprises an 1,4,5-IP_3-gated Ca^{2+}-channel. DAG activates protein kinase C (PKC). The coupling between the receptor and PLC is



Scheme 1. The hydrolysis and resynthesis of phosphoinositides. Hydrolysis of phosphatidylinositol (PI), phosphatidylinositol 4-phosphate (PIP), or phosphatidylinositol 4,5-bisphosphate (PIP_2) is catalyzed by PLC in the plasma membrane. The resynthesis of phosphoinositides proceeds in the endoplasmic reticulum

mediated by G-proteins. In this "dual" second messenger hypothesis, 1,4,5-IP_3 is the link between PIP_2 and Ca^{2+}.

Numerous laboratory results demonstrate that micromolar $AlCl_3$ in the presence of millimolar fluoride affects the phosphoinositide turnover, levels of inositol phosphates, and cytosolic free calcium ions in various cells and tissues [32]. Aluminofluoride complexes affect G-proteins involved in the phosphoinositide signaling system [49]. The resynthesis of phosphoinositides and the generation of inositol phosphates requires ATP for phosphorylation. AlF_x might thus also affect the phosphoionositide metabolism as an analogue for a γ-phosphate for ATP-converting enzymes. Fluoride salts in the presents of aluminum ions have been used to stimulate phosphoinositide hydrolysis in laboratory experiments *in vitro*. The ability of AlF_x to mimic the effects of Ca^{2+}-mobilizing hormones suggests that the coupling of hormone receptors to phosphoinositide breaks down through G-proteins [49]. Phosphoinositides might have multiple roles in the cell membranes. For example, phosphatidylinositol serves not only as the parent phospholipid for PIP_2 synthesis and the substrate for PLC, but also as the source of arachidonic acid for prostaglandin synthesis [49]. Glycosylphosphatidylinositol serves as lipid anchor of many proteins such as AChE and cytoskeletal proteins [43, 49]. Information on the possible biological significance of this anchoring principle is just starting to emerge, but the implication of inositol lipids in the organization of cytoskeletal proteins can be suggested. Changes in cell-membrane phosphoinositide content appear to result in impaired neurotransmitter-triggered signal transduction related to dysfunction in the coupling of G-proteins to their receptors or effectors.

3.2.1
The Effect of AlF_x on the Phosphoinositide Signaling System in the Brain

Phosphoinositides were shown to turn over rapidly in brain and participate in many processes of neurotransmission. The phosphoinositide signaling system is involved in the activation of muscarinic acetylcholine receptors. Early

studies on the role of phosphatidylinositol in signal transduction [50] demonstrated that the activation of muscarinic cholinergic receptors resulted in a net loss of phosphatidylinositol (PI). This observation was explained by the initial phosphodiesteratic breakdown of PI, liberating DAG, which was in turn rapidly rephosphorylated in the presence of ATP to yield phosphatidic acid. The involvement of phosphoinositides was later demonstrated for dopamine, γ-aminobutyric acid and 5-hydroxytryptamine (serotonin) receptors in slices from the guinea pig brain [51]. The discovery of a phosphoinositide signaling pathway focused the attention on the role of PIP_2 in agonist-dependent responses [52].

The hypothesis that the stimulated turnover of PIP_2 can reflect the increased receptor activation in pathogenic neurons has been postulated [53]. It has been suspected that a change in the amount or in the turnover of inositol lipids can represent a change in the receptor activation since PI or PIP_2 is the target of PLC after receptor stimulation.

Chandler and Crews [54] described the formation of inositol phosphates in rat cerebral cortical synaptoneurosomes stimulated by AlF_4^{1-} in sodium-free medium. It was not inhibited by the PKC activator phorbolester, which is known to exert a negative feedback control on agonist-stimulated phosphoinositide metabolism. In experiments with brain cortex membranes, Candura et al. [55] observed that NaF mimicked the action of GTP(S) in stimulating phosphoinositide turnover and generation of inositol phosphates. A much greater hydrolysis of phosphoinositides was observed when $AlCl_3$ and NaF were present together, supporting the concept that AlF_4^{1-} is the active stimulatory species. Coincubation of submaximal concentration of GTP(S) with AlF_4^{1-} did not result in an additive stimulation of phosphoinositide hydrolysis. Paradoxically, $AlCl_3$-induced phosphoinositide hydrolysis was potentiated by coincubation with both GDP(S) and phorbolester. While in hepatocytes AlF_4^{1-} was found to stimulate phosphoinositide metabolism in a manner similar to GTP(S) and sensitive to GDP(S) [56], it seems that AlF_4^{1-} stimulation in brain cortex membranes occurs through a different mechanism [55]. Accumulation of inositol phosphates in the suprachiasmatic nuclei region of rat hypothalamus over a 40 min incubation with AlF_4^{1-} was observed [57].

The tremendous possibilities of potential molecular interactions of aluminum, fluoride and aluminofluoride complexes probably exist in the brain. However, it is evident that interventions of aluminofluoride complexes into the nerve regulations could have severe physiological and pathophysiological consequences.

3.2.2
Changes in Phosphoinositide Signaling System in the Brains of AD Patients

The changes in the proportions of various isozymes of PLC with their different specificity to phosphoinositides indicate that the rate of hydrolysis of inositol lipids may be altered in patients with AD. In the normal human brain matter, PLC-β was the main type present. In AD brains, the amount of PLC-β was

significantly reduced in the membranous fraction of the temporal cortical tissues. PLC-δ1 was abnormally accumulated in neurofibrillary tangles, the neurites surrounding senile plaque cores, and neuropil threads in AD brains [58]. The finding that antigenic determinants for PLC-δ1 and PLC-γ are shared by several filamentous neuronal inclusions occurring in diverse neurologic disorders may reflect a close association between the phosphoinositide signaling system and cytoskeletal components. In this connection, the aberrant PLC-δ1 accumulation is a common metabolic defect underlying the formation of these inclusions. The total PLC activity was significantly reduced also in platelets of AD patients. These results indicate that a PLC abnormality is present in non-neural tissues as well as in the brains of AD patients. On the other hand, no significant differences in PI-specific PLC activity were found in post-mortem brains between control and AD patients [59]. However, data obtained from the analysis of post-mortem brains of AD patients may have been affected by the long and variable post-mortem delay, because phosphoinositides are rapidly degraded post-mortem. With respect to changes in phosphoinositide metabolism in AD, there is growing evidence that specific changes in this signaling system could be implicated in the development of all characteristic AD lesions.

3.3
The Effect of Aluminum and Fluoride on Calcium Homeostasis

Calcium has been recognized for a long time as an important regulator of many physiological processes. Extensive experimental evidence, accumulated during the last decade, has demonstrated the highly diversified mechanisms in the control of calcium homeostasis. Numerous recent papers have documented that the cytosolic Ca^{2+} level oscillates when cells are exposed to agonists that stimulate hydrolysis of inositol lipids [28]. It also seems, that each cell has its own specific "calcium fingerprint". The calcium transients may serve as an important mechanism encoding information about stimulus intensity and activating selectively different Ca-dependent pathways. It has been demonstrated in various cells and tissues, such as hepatocytes, thrombocytes, fibroblast, osteoclasts, neurons, brain, and many others, that aluminofluoride complexes might impair the calcium homeostasis [32]. The mechanism of the effect is not fully known at this point, but it seems that the changes in cytosolic Ca^{2+} level are mostly due to the alterations of the phosphoinositide signaling system.

3.3.1
Alterations in Cytosolic Ca^{2+} Level in AD

The calcium hypothesis of brain aging and AD states that long-term, slightly elevated cytosolic Ca^{2+} levels and/or disturbances in Ca^{2+} homeostasis are the cellular mechanisms underlying neuronal aging [60, 61]. However, the precise details on the processes, which may lead to destabilization of calcium homeostasis in aging brain, are not known. Although many experimental data show the alteration of various Ca^{2+} regulatory mechanisms during aging and

AD, the heterogeneity of types of neuronal preparations and experimental techniques does not allow full confirmation of the Ca^{2+} hypothesis [62, 63].

Alteration in calcium homeostasis could affect most of the AD-related deficiencies. On the other hand, it is not clear whether changes in cytosolic calcium concentration ($[Ca^{2+}]_i$) are the result or the cause of pathogenic effects. Most of evidence regarding the changes of calcium homeostasis in aged neurons demonstrates an increase in resting cytosolic Ca^{2+}, and, after stimulation, a prolongation of Ca^{2+} recovery towards the resting concentration [63].

It is difficult to get brain cells of AD patients while they are alive for laboratory studies. Therefore, various peripheral cells are used for parallel investigations of alterations in brain cells. The biochemical approach using peripheral cells is based on the assumption that the pathological disorder may lurk at the level of cell physiology and may be common for some peripheral cells and neurons. Measurements of $[Ca^{2+}]_i$ in peripheral human cells during aging and from AD patients bring controversial results. No age-related alterations in $[Ca^{2+}]_i$ were reported in human lymphocytes, granulocytes, and neutrophils, while basal $[Ca^{2+}]_i$ was increased in lymphocytes of AD patients [64]. Increased superficial binding of Ca^{2+} and lower $[Ca^{2+}]_i$ were found in fibroblasts from aged and AD donors when compared to cells from young donors. Comparison of the activation-induced rise in $[Ca^{2+}]_i$ revealed parallel age-related changes in the different cell-types investigated. In agreement with these findings, the abnormality of various intracellular stores in cultured fibroblasts from AD patients was reported [65]. It is evident that a number of alterations in calcium homeostasis have been found in peripheral cells of patients with AD.

All these above mentioned controversial findings could not solely be attributed to methodological variations among different laboratories, as age-matched controls and AD patients were examined in the same conditions. However, the AD patients undergoing examinations are in different stages of the disease and, unfortunately, they are not well defined in some studies. Ripova et al. [64] measured the $[Ca^{2+}]_i$ in platelets isolated from the blood of AD patients in early stages in comparison with the groups of healthy young and age-matched controls and found a significantly decreased $[Ca^{2+}]_i$ in platelets of patients with AD. Our data also suggest that the buffering capacity is disturbed in platelets of patients with AD. Any stimulation of extracellular Ca^{2+} influx results in increased cytosolic Ca^{2+}. Our experimental data show that platelets from AD patients have impaired compensatory mechanisms, resulting in movement of $[Ca^{2+}]_i$ towards the harmful levels. Such a disturbance was not observed in platelets from healthy age-matched donors [64]. We could therefore suggest that the alterations in cytosolic calcium regulatory mechanisms are not due to aging but might rather be connected with some pathogenic change such as the presence of the β-amyloid protein. In fact, one of the postulated mechanisms of the β-amyloid toxicity seems to involve a calcium disturbance accompanied by enhanced vulnerability to excitoxic stimuli. It has been recently demonstrated that the β-amyloid proteins form Ca^{2+} channels across cell membranes [66]. We propose the possibility that AD might be associated with a generalized systemic defect in

calcium homeostasis, which is not limited only to the brain neurons. Calcium overload may be detrimental to neurons, resulting in degeneration and death of brain cells in the late stages of AD.

3.3.2
The Effect of AlF_x on Calcium Homeostasis

Aluminum in micromolar concentrations was found to inhibit calcium pumping in endoplasmic reticulum. The Ca^{2+} ATPase activity of rat brain and cerebellum was remarkably reduced and mitochondria showed increased Ca^{2+} release in the presence of exactly estimated 50 μmol L^{-1} Al^{3+} [67]. Aluminum was found to be an important disrupter of intracellular calcium homeostasis, interfering also with the mitochondrial Ca^{2+} pump, as well as activating an Na^{+}-K^{+} ATPase – the antiport mechanism of ion exchange in the plasma membrane, which regulates the Ca^{2+}-Na^{+} antiporter exchange [67].

We observed that the incubation of platelets or red blood cells in the presence of AlF_x evoked changes in the phosphoinositide signaling system and a significant decrease of platelet $[Ca^{2+}]_i$ [68, 64]. This response was not different in platelets isolated from the blood of AD patients as compared with healthy age-matched subjects or young healthy subjects.

Chen and Penington [69] tested two hypotheses concerning the actions of AlF_4^{1-} in the inhibitory effect of G-proteins on calcium channel activity. It has been mentioned above that Ca^{2+} channels are also regulated by G-protein [31]. Earlier studies using intracellular fluoride, presumably made up in glass containing Al^{3+}, found that high-threshold L-type Ca^{2+} currents are eliminated by this intracellular solution. L-type current components show very slow inactivation. Consequently, inhibition of a large L-type component of Ca^{2+} current would increase the relative amplitude of a rapidly inactivating component when using a fluoride-containing recording solution. Chen and Penington [69] suggested that there can be a competition between the receptor and AlF_4^{1-}-stimulated G-protein activity, resulting in the short-term removal of tonic G-protein stimulation by AlF_4^{1-} in the dorsal raphe neurons. Serotonergic dorsal raphe neurons have a small L-type Ca^{2+} current and its abolition by fluoride is not very apparent. They also occasionally observed an increase in the rate of voltage-dependent Ca^{2+} current inactivation. They investigated whether the interaction occurs at the level of the G-protein, or at the level of the interaction of the Gα-subunit with Ca^{2+} channels. The main findings of these very extensive study were that intracellular AlF_4^{1-} caused approximately one-third of the maximum tonic stimulation of the G-protein coupled to Ca^{2+} channels of dorsal raphe neurons, consistent with a $G \cdot GDP \cdot AlF_4^{1-}$ complex resulting in a mimicry of the $G \cdot GTP$ complex (G-release).

3.4
The Effect of AlF_x on Changes of Cytoskeletal Proteins

AD is commonly characterized as the neurodegenerative disorder with the occurrence of senile plaques and neurofibrillary tangles. Neurofibrillar protein

aggregates in patient brains contain abnormally phosphorylated protein τ [70]. Since phosphorylation is the regulatory mechanism of cytoskeletal protein assembly and function, it can be suggested that aluminofluoride complexes might induce important pathophysiological changes. The τ protein regulate the dynamics of the microtubule network, especially that involved in the axonal transport and neuronal plasticity. Tau belongs to a family of developmentally regulated isoforms generated by alternative splicing and phosphorylation. This generates several τ variants that interact with tubulin and other proteins. Therefore, τ might be influenced by many physiological regulations. In AD all six adult isoforms of τ can become maximally phosphorylated and can, rather than binding to microtubules, bind to each other, destabilizing the neuronal cytoskeleton [70].

The cytoskeletal network is responsible for the mechanical properties of the cell that modulate functions such as cell shape, locomotion, cytokinesis, and translocation of organelles. Experimental evidence suggests that the cytoskeleton also provides connections between cellular structures and presents a large surface area for interactions of various proteins and signaling molecules. Modulation of the cytoskeletal network may influence cell signaling, ion channels and intracellular calcium levels. Cytoskeleton is thus essential for regulation of cellular functions, cell integrity, and viability.

Microtubules of all eukaryotic cells are unbranched cylinders built up from the microtubule protein tubulin that occurs in two related forms (α- and β-tubulin). Microtubule functions are based on their capacity to polymerize and to depolymerize. Tubulin is a GTP-binding protein. The binding of GTP is required for microtubule polymerization, whereas the hydrolysis of GTP is required for microtubule depolymerization. It has been reported that AlF_4^{1-} inhibits tubulin depolymerization [16, 17, 71]. AlF_x seems able to mimic the terminal phosphate only after its dissociation from the nucleotide but before its release from the protein. The aluminofluoride complex can bind to the tubulin molecule in GDP β-phosphate and mimic the GDP+P intermediate state. Their binding affinity is three-fold higher than that of phosphate. AlF_x may in this way impair the polymerization-depolymerization cycle of tubulin. The effects of Ca^{2+}, 1,4,5-IP_3, and AlF_x on the organization of spectrin in human red blood cell membrane skeletons were studied in the past in our laboratory [68]. We demonstrated that the phosphoinositide signaling system plays an important role in the shape maintenance and organization of the cytoskeletal network. The formation of holes, disorganization of the spectrin network, and shape changes in RBC were seen after 5 min of incubation with AlF_x. Here we show the changes in tubulin structure after 5 min incubation with AlF_x (Fig. 3).

The breakdown or disorganization of the membrane skeleton can be followed by the release of some membrane-bound enzymes, such as AChE [43] or amyloid precursor protein (APP), which are crucial for the pathologic changes in the brains of AD patients. AlF_x might affect the structure and function of cytoskeletal proteins by several routes [68]. It can activate various G-proteins and protein kinases, act as the analogue of GTP in the assembly-disassembly cycle, and affect the binding of cytoskeletal proteins to

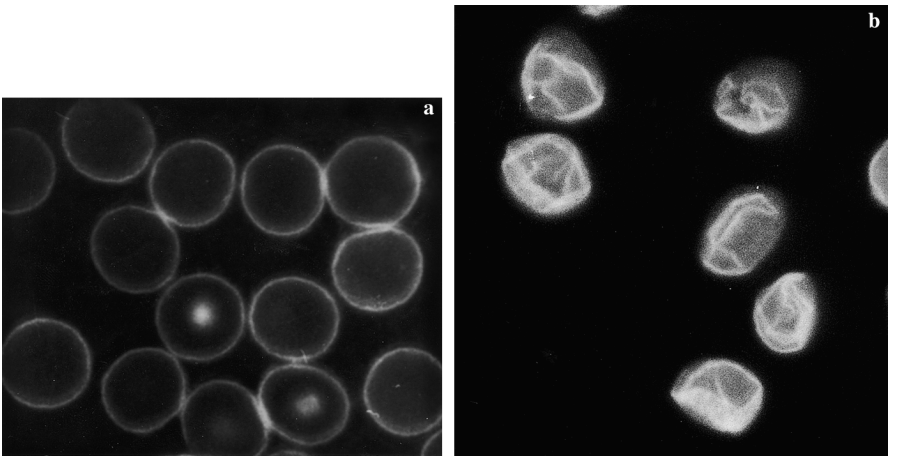


Fig. 3. Visualization of tubulin structures was carried out by indirect fluorescence, using monoclonal antibody TU-01 against α-subunit of tubulin. The visualization of tubulin was done in cooperation with Prof. Dr. J. Palecek from the Faculty of Science, Charles University, Prague. By using 0.25% glutaraldehyde-1% Triton-X-100 fixation, we were able to detect the presence of tubulin as a diffusable fluorescence staining with a ring underlies the RBC plasma membrane (Fig. 3, **a**). The effect of 1 mmol L^{-1}M NaF and 20 μmol L^{-1} $AlCl_3$ is shown in Fig 3, **b**. Tubulin aggregated in an irregular shape inside the cells. Neither NaF nor $AlCl_3$ evoked the tubulin response to the extent observed with their combined action

membrane. It can affect the interactions between various cytoskeletal proteins. It has been documented that AlF_x might affect the intracellular transport of vesicles with APP [72]. The splitting of APP is regulated by phosphorylation. Activation of protein kinase C increases the splitting of APP [73].

3.5
The Effect of AlF_x on ATPases

Lunardi et al. [74] reported the inhibition of mitochondrial ATP synthase in the presence of AlF_4^{1-}. This enzyme couples the hydrolysis of ATP or its synthesis from bound ADP and phosphate. The terminal phosphate undergoes a sequence of coordination changes through which it is bound or released. ATP generation in mitochondria requires the association of the F_1 subunit with F_0 transmembrane subunit transporting protons. The binding of ADP and P_i in a catalytic site of F_1 triggers conformational changes, which lock both of them into the site and induce the formation of pyrophosphate bonds by eliminating a water molecule [17].

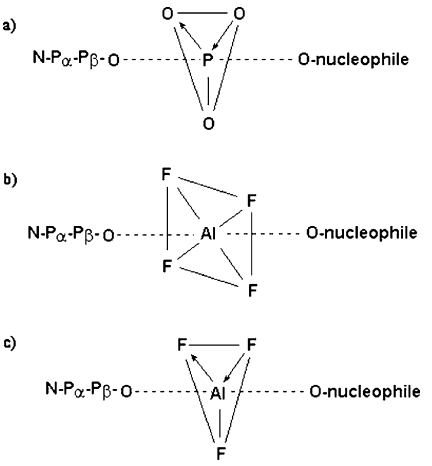
In the presence of ATP, isolated F_1 functions as an ATPase. The binding of AlF_4^{1-} into the catalytic site inhibits the ATPase activity. This inhibition is not reversed by elution of fluoride from solution or by addition of strong chelators of aluminum. Lunardi et al. [74] observed that no significant release

of the complex occurred over a period of days. AlF_4^{1-} also inhibits P-type cation-transport ATPases [75]. NaF plus $AlCl_3$ inhibits completely and reversibly the activity of the purified Na^+-K^+-ATPase and of the purified plasmalemmal Ca^{2+}-Mg-ATPase. An inhibition of the activity of the sarcoplasmic reticulum Ca^{2+}-Mg-ATPase was also observed. Due to the fact that AlF_4^{1-} still inhibits ATPases, in the presence of GDP(S), and guanosine 5-[$\beta\gamma$-imido] triphosphate does not inhibit the ATPases, inhibition due to the activation of G-protein is unlikely. Aluminum alone at micromolar concentrations was found to inhibit Ca^{2+}-ATPase and Na^+-K^+-ATPase of rat brain and cerebellum [67].

The ATPase pathway thus must go through a pentacoordinated transition state for the γ-phosphate. The analogy with beryllium bonding that is strictly tetrahedral led Chabre to suggestion that AlF_x cannot follow this route [17]. Wittinghofer describes in his article [21] an admirable methodological and intellectual approach, which led to further understanding of the mechanism of phosphoryl- transfer reactions using AlF_x and proved that AlF_x acts as the transition state analogue. The analogy with nitrogenase suggests a way in which ATP hydrolysis and a subsequent conformational change would break the interaction between the ATPase and the electron transfer system [21]. The structures of the residue that interact with AlF_x have been determined for some enzymes and protein complexes, which contain a P-loop motif found in many ATP- and GTP-binding proteins.

The most important of the protein residues seems to be a lysine, which contacts a β-phosphate oxygen of ADP, and an aspartic acid, which is in a suitable location to serve as a potential general base for activation of water. An important element of the conformational change is a DxxGD motif, the glycine of which is involved in a main-chain interaction to a fluoride [21]. The second aspartic acid is homologous to the invariant glutamine in GTP-binding proteins. In the case of uridylate monophosphate kinase it was arginine 93, which interacted more strongly with one of the fluorides of AlF_3. Mutagenesis studies indicated that substitution of this arginine results in a 100- to 1000-fold reduced catalytic rate [18].

Structures of transition state analogue complexes of phosphoryl-transferring enzymes can be used to deduce whether the mechanism of the phosphoryl-transfer reaction is mostly dissociative, with a tetrahedral metaphosphate-like intermediate, or associative, with a pentavalent phosphorus (Scheme 2). Since the two mechanisms differ in charge distribution and bond order of the transition state, the location of positively charged amino acids and the distance of the AlF_x to the leaving and attacking groups are crucial. The structures of transition state mimics show that the distances between aluminum and the leaving group and nucleophilic oxygen are intermediate between van der Waals and covalent bonds [18], suggesting a mostly associative character. According to Wittinghofer [21] the fact that, in most cases, positively charged groups contact the phosphoryl group to be transferred is an even stronger argument for an associative mechanism, since positive charges contacting the phosphoryl group would be anticatalytic in the case of a dissociative mechanism.



Scheme 2. Conformational changes of the γ-phosphate in **a**) phosphoryl-transfer reaction transition state K), and various species of AlF_x: **b**) AlF_4^{1-}, **c**) $AlCl_3$. Dotted lines indicate that the degree of bond making and bond breaking determines whether the transition is more dissociative, with a metaphosphate-like intermediate, or associative, with a pentavalent intermediate. Charges have been omitted for clarity. N = adenosine or guanosine. According to [16, 17, 21]

3.6
Complexity in Signaling Systems on the Cell Level

Signaling via the large family of G-proteins can lead to many cellular responses ranging from regulation of intracellular messengers to gene transcription. Synergistic cross-talk interactions between G-coupled receptors have an important role in fine-tuning signals from multiple signaling pathways. At present we have no conceptual framework, which could allow us to integrate all knowledge about the regulatory mechanisms in processes of neurotransmission. Nevertheless, the widespread use of Al^{3+} and fluoride salts in laboratory investigations has led to a jump in our understanding of the complexity of signal transduction processes.

We could approach the complex integrative view by the detailed study of the action of one small molecule, namely AlF_x. We could also judge its role in the etiology and pathogenesis of AD on the cell level. The knowledge from studies of the phosphoinositide signaling system and Ca^{2+} homeostasis could help our understanding of the multifactorial nature of AD.

3.6.1
The State of PPI Signaling System Depends on the Physiological State of the Cell

In our opinion, the final functional response after the generation of the first second messenger molecules of 1,4,5-IP_3 depends on the physiological state of the cell. The healthy cell maintains its content of ATP, Ca^{2+} homeostasis,

and keeps the ability to maintain the equilibrium between the breakdown of PIP_2 and the phosphorylation of PI. Calcium mobilization proceeds as a transient peak or as a short wave oscillation. The physiological functional response proceeds and second messenger molecules – 1,4,5-IP_3 and DAG – undergo metabolic transformation. The generation and degradation of second messengers is regulated by the mechanism of a negative feedback. AlF_x may induce the state of sustained activation characterized by increased levels of 1,4,5-IP_3, DAG, and cytosolic Ca^{2+} [32]. A disorder in calcium homeostasis or sustained G-protein activation might induce an amplification cascade regulated by a positive feedback. In such a case the breakdown of PIP_2 increases, DAG increases the affinity of protein kinase C, phosphorylation of numerous proteins including cytoskeletal proteins is enhanced, 1,4,5-IP_3 releases Ca^{2+} from intracellular pools, Ca^{2+} channels are opened, etc. Such a state of sustained activation consumes great amounts of energy. If the cell does not produce enough energy, the content of inositol lipids in the plasma membrane declines. At the same time, the level of cytosolic Ca^{2+} is increased, because mechanisms of calcium homeostasis, such as the activity of the Ca^{2+} ATPase, are disturbed. The generation of second messenger molecules is still regulated by positive feedback in such conditions. This may lead to disturbances of cell homeostatic mechanisms, cell damage, or apoptosis. Experimental observations support the view that AlF_x may evoke the state of sustained activation with the stimulation of G-protein. The impairment of ATP synthesis can lead to energy depletion of the cell.

3.6.2
Interactions in Signaling Networks

Moreover, the phosphoinositide signaling system interacts with many other transducing systems. The interactions between this system and the cAMP generating system have been well documented [27, 49, 76]. Different subtypes of cholinergic receptors also reveal multiple interactions. Many reactions proceed simultaneously or are overlapping. A biochemical link between tyrosine kinases and the phosphoinositide signaling pathway has been also suspected. It is possible that PI kinase may be a substrate for tyrosine kinase and, as well, that PLC may be regulated by tyrosine kinase-catalyzed phosphorylation. The release of arachidonate and subsequent synthesis of eicosanoids appear to be stimulated by the same receptors that control phosphoinositide hydrolysis. Experimental evidence suggests that protein kinase C may be involved in PLA_2 activation. There are multiple pathways involved in the phosphoinositide cascade that has been implicated in arachidonate release. On the other hand, arachidonate and eicosanoids may modulate PLC activity and the metabolic turnover of phosphoinositide. Prostaglandin E_2 and prostacyclin inhibit phosphoinositide breakdown and generation of inositol phosphates [76].

3.6.3
Complexity as Observed in the Experiments in vitro

Shafer and coworkers [77] compared the effect of various concentrations of $AlCl_3$ and 5–30 mmol L^{-1} NaF on muscarinic, adrenergic, and metabotropic receptor-stimulated phosphoinositide hydrolysis in hippocampal and cortical slices from rat brain. Their detailed study reveals the multiple possibilities of interventions into the phosphoinositide cascade and, simultaneously, the difficulties in the interpretations of results *in vitro*. In agreement with many others, Shafer and coworkers found that NaF stimulates inositol phosphate (IPs) accumulation as well as the cholinergic agonist carbachol, the adrenergic agonist norepinephrine, and the glutaminergic agonist quisqualate. The higher concentrations of $AlCl_3$ (0.5 mmol L^{-1}) inhibited IPs accumulation stimulated by agonists and that stimulated by NaF. They concluded that AlF_4^{1-} is not responsible for the effects of $AlCl_3$ on receptor-stimulated IPs accumulation. There was a biphasic effect of $AlCl_3$ on agonist-stimulated IPs accumulation. The authors are aware that trace amounts of aluminum plus NaF stimulate the accumulation of IPs. The observed effects of the high concentrations of aluminum ions suggest their direct effect on G-proteins or PLC activity. However, the estimation of the total amount of IPs cannot reflect the hydrolysis of PIP_2, the major target of PLC after the receptor stimulation. The level of inositol 1-phosphate (1-IP) is about 30 times higher than that of 1,4,5-IP_3. The role of 1-IP in the cell physiology is not known, but some studies have suggested that changes in the level of inositol 1-phosphate in the brain are in parallel with excitation and inhibition [49]. It is very likely the increased Ca^{2+} cytosolic level leads to the shift of phospholipase C activity to PI instead of PIP_2. Further studies of the effect of AlF_x on the phospholipase C isoforms are therefore warranted.

The highly expert and detailed paper of Chen and Penington [69] could also demonstrate the complexity of the cellular system *in vitro*. AlF_4^{1-} placed intracellulary in a patch pipette, activated a G-protein, resulting in a "tonic" inhibition of the Ca^{2+} current of isolated serotonergic neurons of the rat dorsal raphe nucleus. Serotonin also inhibits the Ca^{2+} current of these cells. After external bath application to and quick removal of serotonin from an AlF_4^{1-} containing cell, there was a reversal or transient disinhibition of the inhibitory effect of AlF_4^{1-} on the Ca^{2+} current. A short predepolarization of the membrane potential to +70 mV, a condition that is known to reverse G-protein-mediated inhibition, reversed the inhibitory effect of AlF_4^{1-} on the Ca^{2+} current and brought the Ca^{2+} current to the same level as that seen at the peak of the transient disinhibition current. With AlF_4^{1-} in the pipette, the transient disinhibition phenomenon could be eliminated by lowering the pipette MgATP, or by totally chelating Al^{3+} in the pipette. In the presence of AlF_4^{1-}, but with either lowered MgATP or extreme efforts to eliminate pipette Al^{3+}, the rate of recovery from serotonin on wash was slowed, a condition opposite to that where a transient disinhibition occurred. The putative complex of AlF_4^{1-}-bound G-protein appeared to free Gα-subunits, mimicking the effect on Ca^{2+} channels of the G·GTP complex. The ON-rate of the

inhibition of Ca^{2+} current, after a depolarizing pulse, by α-subunits released by AlF_4^{1-} in the pipette was significantly slower than that of the agonist-activated G-protein. The OFF-rate of the AlF_4^{1-}-mediated inhibition in response to a depolarizing pulse, a measure of the affinity of the free G α-subunit for the Ca^{2+} channel, was slightly slower than that of the agonist stimulated G-protein. In summary, agonist application temporarily reversed the effects of AlF_4^{1-}, making it a complementary tool to GTP-γ-S for the study of G-protein interactions. Based on the literature, Chen and Penington suggest a fairly parsimonious explanation of the sequence of events occurring after agonist application to a dorsal raphe cell containing AlF_4^{1-}. They propose that after several applications of serotonin, some G-proteins are in the basal state and some are activated by AlF_4^{1-}. This should produce only partial channel inhibition, allowing further inhibition by serotonin. This example is a large simplification of experiments and discussion in the above-mentioned study.

3.6.4
The Unifying Hypothesis?

A new unifying hypothesis of AD, which explains the mechanism whereby aluminum could induce, amplify, and orchestrate a cascade of neurotoxic events characteristic of AD, has been proposed by Exley [5, 10]. He has identified a mechanism through which a truly chronic exposure to aluminum would bring about subtle and persistent changes in neurotransmission, which, in time, could instigate the cascade of events known collectively as AD. This mechanism involves the potentiation of the activities of neurotransmitters by the action of aluminum-ATP at ATP receptors in the brain. There is growing evidence that ATP plays an important role not only as the universal donor of energy but also as an important multi-functional signaling molecule. In the brain, ATP acts upon ionotropic (P2X) and metabotropic (P2Y) receptors to optimize the activities of neurotransmitters including acetylcholine, glutamate, and γ-aminobutyric acid. Two characteristics make ATP a particularly interesting neurotransmitter. After release, ATP is rapidly degraded to adenosine, which can also act as a neurotransmitter and modulate the transmitter release at presynaptic terminals. Also, ATP ionotropic receptors are highly permeable to calcium ions and, unlike NMDA receptors, are also functional at negative potentials. Therefore, ATP receptors may represent a new route for synaptically gated calcium entry into dorsal horn neurons at the cell resting potential and could be involved in the phenomena of synaptic plasticity and central sensitization, and in modulating synaptic transmission under particular conditions, such as during inflammation. The extracellular action of ATP is mediated via release of intracellular ATP. In the brain, ATP is secreted by neurons, glia, and endothelial cells. The changes in the extracellular concentration of ATP could thus evoke various and multiple cascades of events. Of great importance in the Exley hypothesis is the fact that a very subtle change in the extracellular ATP concentration could evoke a very complex network of events in the brain. Aluminum is bound by ATP to form a complex, which is orders of magnitude more stable than that with magnesium

[5]. Exley elaborated the complex theory by describing how a physiologically significant concentration of aluminum could induce various pathophysiological changes. According to our hypothesis, it seems that fluoride could further potentiate the action of aluminum. AlF_x evokes the effects via G-proteins probably at very low concentrations, even under such situations when the actual content of aluminum in the brain is not increased. It seems that scientists still need more significant data to be sure about the causal relationship between the action of a small ions and molecules, such as Al^{3+} and AlF_x, and disturbances of human health. Science has already accumulated evidence about their detrimental effects on the whole organism.

4
The Holistic Complexity

AD is probably an example of a multifactorial disease whose pathophysiology cannot be understood on the basis of one altered molecule or gene. The interpretation of laboratory investigations using isolated animal and human cells or tissues on the intact human organism could be discussed. However, many ecological and clinical studies bring evidence about the detrimental effects of the synergistic action of aluminum and fluoride ions in humans. Several processes are implicated in the uptake and distribution of aluminum and fluoride in tissues and organs of the human body. The severity and the development of the symptoms depend on a person's age, genetic background, nutrition status, kidney function, and many other factors. It is evident that the dietary intake of aluminum and fluoride varies all over the world. It is not surprising that various ecological studies from different geographical locations might bring controversial or less significant results. In epidemiological studies many additional limitations and variables may occur. The history of the use of fluoride in the prevention of caries provides the example that the evaluation and interpretation of obtained evidence is, to some extent, subjective. Individual susceptibility to AD will be as relevant as the truly chronic exposure to an increased load of aluminum and fluoride from the environment and food chain. It might be difficult to decide whether aluminum and fluoride are the main responsible factors for reported risks or benefits. How many subjects shall we need to prove their hidden danger?

4.1
Aluminum and Fluoride in the Environment

It is generally believed that exposure to aluminum is mainly through oral intake, and the major sources are drinking water, residues in foods, cooking utensils, food, and beverage packaging. There are very few data on the oral bioavailability of aluminum from food. An excellent comprehensive review about oral bioavailability of aluminum from drinking water and food has been published recently by Yokel [8]. According to his conclusion, the major source of aluminum absorption daily is food, not water. Exposure is also

possible from aluminum-containing medications (e.g., antacids and buffered aspirins), parenteral solutions, and vaccinations containing aluminum adjuvant. The daily intake of aluminum from medications can greatly exceed that from the diet and water. Occupational exposure to aluminum occurs in the refining of the primary metal and in secondary industries that use aluminum products.

Fluorine was not produced by industry until the 2nd half of the 20th century and its amount in the water sources and food was negligible. The development of fluorine chemistry was followed by its increasing consumption in military, pharmaceutical, and electronic industry. During the last 50 years, fluorides have been widely used in tooth decay prevention. Thanks to the reduction of dental caries observed both in children and adults due to the increased fluoride intake, fluoride supplementation of different food sources, oral cosmetics, and prophylactic dental devices has begun. Fluoridation of water supplies and industrial fertilizers increase the amount of this element in agricultural products and food chains. Fluoride comes from fluoridated water, from crops grown with fluoridated water, from medicines, dental products, pesticides, fertilizers, and fuels [78]. It may thus be justifiably postulated that the fluoride intake may be in some individuals supraoptimal and, together with the increased intake of aluminum may exceed their tolerable limits. The contents of fluoride salts in drinks and food (www.bruha.com/fluoride) contribute significantly to the total dietary intake and exceed the recommended daily dose (2 mg per day).

The quantity of fluoride and aluminum in living environment tends to increase, causing their increase in human food chains and resulting in their increased load to the human body. Several studies reported a high content of fluoride and aluminum in all tested Chinese, Indian, and herbal teas [79, 80]. Tea is the second most-consumed beverage after water in the world. That the aluminum present in tea is indeed resorbed in the simultaneous presence of fluoride was demonstrated in healthy male volunteers after drinking equal volumes (1.2 L) of tea, coffee, or tap water on separate days. In every case the amount of the urinary excreted aluminum increased on the day when tea was taken [81]. The results indicated that tea consumption must be considered in any assessment of the total dietary intake of aluminum in human beings. A significant fact to be taken into account is that the increasing fluoride and aluminum load may be cumulative and health threatening. The acid rains, fluoridation of water supplies, the use of fluoride and aluminum in industry as well as medicine started the era of supplementation of human bodies with these ions as never before in the history of the human race.

4.2 Aluminum and Fluoride in the Human Blood and Body Fluids

The small Al^{3+} cation with a high charge does not exist in the free form under physiological conditions. In mildly acid intracellular conditions, aluminum is bound to phosphate. Aluminum forms soluble salts with some acids, for example, with citric acid [82], or complexes with some compounds, for

example maltol [80]. It seems that the oral bioavailability of aluminum is very low, about 0.3% [8]. The oral aluminum bioavailability was not found to be significantly different between young and aged subjects [83]. The proximal intestine appears to be the primary site of aluminum absorption. The putative mechanisms of absorption include diffusion, carrier- and vesicular-mediated transport as well as paracellular diffusion between the mucosal cells in the gastrointestinal tract.

Calcium channels are suggested to mediate intestinal aluminum absorption [84]. Aluminum distributes unequally to all tissues. The values given for the aluminum levels in the human body vary widely. Under normal conditions the aluminum concentration in blood is 5–10 μmol L^{-1} [14, 85]. The mean aluminum concentration in human milk was 23.4 ± 9.6 μg L^{-1} and did not differ significantly between colostrum, intermediate-stage, and mature-stage milk [86].

The estimation of fluoride concentration in human body fluids has been widely performed. However, data are expressed in various units. Kissa [87] reported that the mean plasma fluoride level in healthy subjects is between 20 to 60 μg L^{-1} (1–3 μmol L^{-1}). Others [88, 89] present L-1 plasma fluoride concentrations in the range of 38.8–88.6 mol. The average fluoride concentration in plasma of 8–16-year-old students from Winterthur (Switzerland) was 12.7 ± 3.8 μg L^{-1} (0.65 μmol L^{-1}) [90]. Human breast milk (samples were collected from 57 lactating mothers) contains 0.019 ± 0.004 ppm of fluoride [91].

The accepted therapeutic range of the serum fluoride levels has been suggested to be 5–10 μmol L^{-1} and the "therapeutic window" 95–100 μg L^{-1} (5 μmol L^{-1}) is not assumed to be overtly cytotoxic [92].

The normal concentration of fluoride in saliva is about 1 μmol L^{-1}, which is somewhat less than that in plasma. The concentration of fluoride in whole saliva mirrored the fluoride concentration in plasma, but at a lower level [93].

The Swiss study [90] reports the urinary fluoride concentrations estimated in 8–16-year-old students from various areas. The fluoride concentrations in urine were higher in Basel and Davos (0.62 ± 0.35 mg L^{-1}; 0.61 ± 0.42 mg L^{-1}) (32 μmol L^{-1}) than in Berne and Winterthur (0.46 ± 0.42 mg L^{-1}; 0.50 ± 0.31 mg L^{-1}) (24 μmol L^{-1}). A relatively high natural fluoride content (0.3 mg L^{-1} to 16 μmol L^{-1}) in the drinking water explained the difference in urine fluoride concentration between students from Davos and Berne + Winterthur with domestic salt fluoridation.

The increased serum and urinary fluoride concentrations of workers exposed to hydrofluoric acid in the working environment were reported [94]. There was a linear relationship between mean serum and urinary fluoride concentrations and hydrofluoric concentration in the workplace. Mean fluoride concentrations of 82.3 μg L^{-1} (4.5 μmol L^{-1} in serum and 4 mg L^{-1} (210 μmol L^{-1}) in urine were found at an atmospheric HF concentration of 3 ppm.

The individual fluoride exposure and the corresponding body fluid levels were studied in 41 workers in an aluminum plant in Sweden. During the shift (8 h) personal air samplings were performed, plasma fluoride levels and urine fluoride excretion determined. The average total fluoride exposure was

0.91 mg m^{-3} of which 34% was gaseous fluoride. The mean fluoride plasma level before the shift was 23 μg L^{-1} (1.2 μmol L^{-1}) and increased on average to 48 ng/ml (range 14–151 μg L^{-1}) at the end of the shift [95]. There was a high correlation between fluoride renal clearance and urinary flow. It seems therefore that a high fluid intake during the shift increases the capacity of the kidney to excrete fluoride and decrease the levels of fluoride in the body.

The increase of fluoride concentration in the blood has been reported in hemodialysed patients. Arnow et al. [96] reported that the serum concentrations of fluoride in the sick patients were markedly increased to as high as 716 μmol L^{-1}.

4.3
The Action of Aluminum Ions and Fluoride in the Whole Organism

It is known that aluminum binds to fluoride in the diet [97]. The natural barrier systems and various physiological ligands for aluminum were efficient buffers preventing the increased intake of this metal in natural conditions. Most aluminum ingested leaves the intestines without resorption and only a small percentage of it reaches the blood stream [8, 98]. In the blood, aluminum is bound to transferrin and to albumin [8] and transported for renal elimination. Transport of aluminum from extracellular fluid to blood is believed to be mediated by a monocarboxylate transporter [5]. It is implied that the transport mechanism uses citrate as its vehicle. Measurements of uptake of radioactive soluble aluminum (^{26}Al) from stomachs of rats showed that trace amounts of ^{26}Al directly entered the brain [99]. Despite the extensive use of aluminofluoride complexes in experiments *in vitro*, the mechanism of their transport across the plasma membrane was not studied in detail. However, their effect on numerous intracellular reactions and functions was observed. Aluminum has been found in various human tissues in concentrations between 1–6 μg g^{-1} dry weight [85]. Higher concentrations were found in bone and skin. Concentrations almost 10 times higher were seen in the lungs, probably due to deposition of particles from inhaled atmospheric dust [8, 85].

4.3.1
Endothelial Cells and Blood Vessels

Endothelial cells and the kidneys play probably an important role in the long-term homeostasis as well as in the development of AD. Endothelial cells synthesize and release vasorelaxant and vasoconstrictor substances. The vasoconstrictor mediators, endothelins, were isolated, purified, sequenced, and cloned [100]. The human endothelin receptor has seven membrane helices and is apparently G-protein-coupled. Endothelin-induced smooth muscle contraction involves the following processes: activation of PLC, PIP_2 hydrolysis, generation of 1,4,5-IP_3, accumulation of DAG, mobilization of intracellular Ca^{2+} with facilitation of Ca^{2+} influx, activation of PKC, activation of PLA_2 and arachidonic acid release, activation of PLD, and stimulation of Na^+-H^+

exchange. It can be therefore suggested that AlF_x would influence markedly the action of endothelins.

Endothelial cells produce prostacyclins in response to ATP. AlF_4^{1-} enhanced the stimulatory effect of ATP on prostacyclin release [101]. A dose- and time-dependent generation of inositol phosphates, release of arachidonic acid, and the production of prostacyclin (PGI_2) in human umbilical vein endothelial cells was described [102]. Stimulation of arachidonic acid release and prostacyclin production by AlF_4^{1-} was described in cultured endothelial cells from rabbit coronary microvessels [103].

ATP stimulates the synthesis of platelet-activating factor, which has an important function in mediating the adherence of inflammatory cells to the endothelium. The addition of AlF_4^{1-} to endothelial cells resulted in the production of platelet-activating factor with a maximal effect within 20–60 min of exposure. AlF_4^{1-} also augmented the production of platelet-activating factor, which occurs in response to a hormonal agonist [104]. In addition, submaximal concentrations of AlF_4^{1-} converted an ineffective hormonal agonist to a maximally effective agonist.

Boonen and De Mey [105] investigated G-proteins' involvement in the resistance of arterial smooth muscle to contractile agonists. AlF_4^{1-} induced contractions dependent on the presence of Ca^{2+}. Increased PIP_2 breakdown and the generation of inositol phosphates after the action of AlF_4^{1-} was observed [106] in permeabilized vascular smooth muscle.

NaF evoked contractions by stimulating L-type Ca^{2+} channels in rabbit femoral arteries, while AlF_4^{1-} stimulated PLC, which produced additional muscle activation [107].

The modern view of the mammalian blood-brain barrier (BBB) has shifted from a purely anatomic concept to a more physiological and dynamic definition. Morphologically, a lining of specialized endothelial cells forms the BBB. Specialized regions of intercellular contact (tight junctions) that prevent leakage of blood-borne substances into the brain parenchyma characterize the cells of the intraluminal portion of brain capillaries. The failure of the structural integrity and function of the BBB plays a pivotal role in the pathogenesis of many diseases of the central nervous system. The presence of fluoride caused more aluminum to cross the blood-brain barrier and be deposited in the brain of rat [108]. Varner and coworkers studied alterations in the nervous system resulting from chronic administration of AlF_3 or equivalent levels of fluoride in the form of sodium fluoride. Twenty-seven adult male Long-Evans rats were administered one of three treatments for 52 weeks. The aluminum levels in samples of brain and kidney were higher in both the AlF_3 and NaF groups relative to controls. The effects of the two treatments on cerebrovascular and neuronal integrity were qualitatively and quantitatively different. Aluminum-induced neural degeneration in rats is greatly enhanced when the animals were fed low doses of fluoride. Animals develop both symptoms and brain lesions that are similar to those found in AD [108].

The kidney is both a source of endothelin generation and an important target organ of this peptide.

4.3.2 *The Kidney*

The effects of aluminofluoride complexes on the kidney were studied using glomerular mesangial cells, proximal tubular cells, and inner medullar collecting tubule cells of rat kidney.

Bradykinin elicits a complex response in the renal glomerulus, including reduction in the glomerular capillary ultrafiltration coefficient. The effects of bradykinin and vasopressin on calcium mobilization in renal glomerular mesangial cells of rat were studied using the calcium-sensitive fluorescent probe Indo 1 [109]. Both hormones were found to cause a transient concentration-dependent rise in the cytosolic Ca^{2+} concentration followed by a sustained secondary rise. AlF_4^{1-} induced a transient rise of cytosolic Ca^{2+}, which was abrogated by prior exposure of the cells to pertussis toxin. In contrast, the responses to bradykinin and vasopressin were unaffected by pertussis toxin.

The proximal tubule is responsible for the reabsorption of the major part of the glomerular ultrafiltrate. Proximal cells possess a brush border on their luminal surface. This membrane contains intrinsic transporting proteins like ion channels and ionic pumps. Hormones regulate the transport of ions and the involvement of adenylate cyclase has been well documented. AlF_4^{1-} was the most effective stimulator of adenylate cyclase. A 9.3-fold peak increase in activity was observed in the basolateral membranes after its action. On the other hand, brush border membranes showed only a small increase [110]. The activity of amiloride-sensitive Na^+-H^+ exchanger is regulated by cAMP-dependent protein kinase. Incubation of vesicles, as prepared from renal brush border membranes of rat in the presence of 10 mmol L^{-1} NaF and 10 μmol L^{-1} $AlCl_3$, resulted in a significant inhibition of amiloride-sensitive ^{22}Na uptake [111]. Incorporation of GTP(S) also resulted in significantly reduced ^{22}Na uptake. cAMP-dependent protein kinase activity, strongly stimulated by exogenously added cAMP in these vesicle preparations, was not stimulated by GTP(S). These findings suggest that the amiloride-sensitive Na^+-H^+ exchanger could be regulated by G-proteins, independently of adenylate cyclase and cAMP-dependent protein kinase.

These data document that the ion transporting processes are affected by AlF_4^{1-} in kidney tubular cells. AlF_4^{1-} stimulates adenylate cyclase, inhibits amiloride-sensitive Na^+-H^+ exchange regulated by cAMP-dependent protein kinase, enhances epidermal growth factor-stimulated prostaglandin production, and mimics vasopressin and bradykinin induced Ca^{2+} mobilization. It is suggested that AlF_4^{1-} can affect the activity of many other ion channels and enzymes in the kidney. Borke and Whitford [112] examined the effect of chronic fluoride ingestion on ATP-dependent ^{45}Ca uptake by rat kidney membrane vesicles to characterize the mechanism by which fluoride alters calcium transport in the kidney. Twenty weaning female Sprague-Dawley rats were raised on low-fluoride (0.9 mg L^{-1}), semi-purified diet with a Ca^{2+} concentration of 400 mg/100 g diet. Rats were divided into four groups and were fed ad libitum deionized water containing fluoride at concentrations of

0, 10, 50, or 150 mg L^{-1} added as NaF for six weeks. This consumption produced plasma fluoride levels of <0.4, 2, 7, or 35 μmol L^{-1}, respectively. ATP-dependent ^{45}Ca uptake was significantly lower in the 150 mg FL^{-1} exposure group than in the controls. Slot blot analysis of kidney homogenates with specific Ca^{2+}-pump antibodies showed significantly less endoplasmic reticulum Ca^{2+}-pump protein and plasma membrane Ca^{2+}-pump protein in all treatment groups than controls [112]. Both Ca^{2+}-pumps are transport molecules of great importance in the regulation of Ca^{2+} homeostasis. The authors suggest that chronic, high fluoride ingestion producing high plasma fluoride levels may occur in humans and may affect calcium homeostasis.

4.3.3
The Endocrine Glands

The endocrine glands such as the thyroid, the pituitary gland, and the pineal gland, are extremely sensitive to fluoride and accumulate aluminum.

4.3.3.1
Thyroid

Fluoride effects on the thyroid can be observed on many different levels. Of particular importance relating to G-protein activation is the ability of AlF_x to clone the role of thyroid-stimulating hormone (TSH). Allgeier with coworkers [113] demonstrated that TSH led to the activation of two forms of Gs (Gs short and Gs long) as well as of Gq and G11, demonstrating that signaling pathways induced by TSH already bifurcate in the course of the receptor-G-protein interaction and concluded that TSH activation is mediated by Gq/11 and Gs, respectively.

Fluoride is used in laboratory animals specifically to substitute for TSH. There is a direct dose-response relationship with iodine: the higher the fluoride intake the lower is the iodine in the system. The synergistic action of the thyroid on fluoride toxicity has been reported since 1940. The major areas of iodine deficiency are identical to endemic fluorosis areas. The comparison of fluoride toxicity symptoms and symptoms of thyroid disorders has been reviewed by Schuld (www.bruha.com/fluoride/html/symptoms-hypo-f.htm). The functional changes of the hypophysis-thyroid gland system caused by disorders of the regulatory chain and fluorine impact on thyroid hormones metabolism at the level of target cells were also reported. Regarding the crucial role of the thyroid in regulation of growth, development, and metabolism of many tissues, AlF_x might influence the proper function of the entire human body, including mental abilities.

4.3.3.2
Pineal Gland and Melatonin

Knowledge of the pineal gland's function in animal and human organisms has been collected during the last thirty years. The experiments and observations

point to an unbelievable number of functions regulated by the pineal gland. The physiological response depends on age, sex, physiological condition of the animal, time of day and season. The human pineal gland is a very small oval organ (it weighs 150 mg) connected to the surface of the 3rd brain ventricle. The pineal gland originates from the nerve tissue of the mesencephalon in the embryonic state. The brain and the pineal gland have a nerve and circulatory connection. The pineal gland is highly vascularized with no hematoencephalic barrier and has a very high perfusion rate of blood, second only to the kidney.

Melatonin is the chief pineal hormone. It is formed from its precursor, serotonin. The concentration of serotonin in the pineal gland is about 10 times higher than in other organs. Production of serotonin and melatonin is cyclical and is influenced by light. Production of serotonin increases during the day, while production of melatonin increases during the night. The pineal gland secretes melatonin into the blood and other body fluids. Measurable quantities of melatonin have been found in the cerebrospinal fluid, saliva, fluid of the eye, seminal fluid, breast milk, and in amniotic fluid. AlF_x was used to indicate that the effects of melatonin are mediated by heterotrimeric G-protein [114].

From the evidence accumulated so far, it seems likely that the cAMP signal transduction pathway will be a major effector of a stimulatory signal to the pars tuberalis, which can be regulated by melatonin [115]. The effect of aluminum as AlF_4^{1-} has been studied on inositol phosphate accumulation, calcium mobilization, and cyclic AMP production in ovine pars tuberalis cells [116]. In the presence of 10 mmol L^{-1} LiCl, AlF_4^{1-} stimulated the net accumulation of inositol phosphates over a 40-min incubation. Lithium is a known inhibitor of phosphatases in the inositol phosphate-recycling pathway. The results show the existence of a lithium-sensitive phosphoinositide signaling.

Consistent with these findings, AlF_4^{1-} increased intracellular calcium; although this response was attenuated in calcium-depleted medium, indicating that the calcium response comprises both intracellular and extracellular components. AlF_4^{1-} blocked the increase in cyclic AMP stimulation by 1 micromolar forskolin, being as effective as melatonin, achieving approximately 90% inhibition. The authors proposed that these results are consistent with the interpretation that AlF_4^{1-} activates many G-protein-mediated responses, and thus imply that the inhibitory pathway for cyclic AMP predominates over the stimulatory arm.

The pineal gland produces various other hormones and mediators such as dopamine, norepinephrine, glutamic acid, γ-aminobutyric acid, and neuropeptides. Physiologists speculate that the activity of the pineal gland is regulated primarily by light signals: the pineal gland is considered to be a transducer of photoperiodical information. Light signals, which are registered in the eyes, go to the pineal gland via nerves of the suprachiasmatic nuclei of the hypothalamus. This structure functions as a biological clock. Suprachiasmatic nuclei in the hypothalamus are the oscillator, which gives biological rhythms and synchronizes the physiological activities of the organism. Nadakavukaren with coworkers [117] observed AlF_4^{1-} stimulated accumulation of inositol phosphates within the suprachiasmatic region of the rat hypothalamus. Melatonin is responsible for regulating numerous life processes, including aging. The highest

levels of melatonin are generated in young children. Luke [118] found very high levels of fluoride (9000 ppm) in the calcium hydroxyapatite crystals produced by the human pineal gland (she analyzed 11 cadavers). These levels are at, or higher than, the fluoride levels in the bones of people suffering from skeletal fluorosis. In animals (Mongolian gerbils) treated with fluoride, Luke found that melatonin production was decreased.

4.3.4
Aluminum- and Fluoride-Induced Apoptosis

The programmed cell death – apoptosis – has been explained as a defective cell cycle resulting in cell death instead of cell division. Apoptosis is implicated in the pathology of many diseases. AD is connected with a progressive neuronal degeneration and death of cholinergic neurons. Some authors demonstrated that aluminum could induce the apoptotic degeneration of rat hippocampal neurons and astrocytes [119]. After 8–12 days exposure, aluminum ($1\ \text{mmol}\ L^{-1}$) caused strong changes in the morphology of astrocytes including shrinkage of cell bodies and retraction of processes. Aluminum-induced degeneration of astrocytes involved the DNA fragmentation, characteristic of apoptosis. Astrocytes may regulate the expression or function of proteins in the neuronal cytoskeleton. The high concentration of aluminum used in these experiments may be rather unlikely to occur in vivo, since the concentrations of aluminum in human blood and tissues are usually very low. However, the mechanisms for the exclusion of aluminum from the human organism may be impaired during aging and the exposure to aluminum may be long-term. A higher amount of aluminum was found in the human brain with AD than in brains of age-matched healthy controls [2]. The presence of fluoride ions results in alterations in neuronal and cerebrovascular integrity and a greater level of aluminum in the kidney and brain [108].

Loweth et al. [120] showed that fluoride induces apoptosis in clonal pancreatic β cells and in the cells of normal rat islets of Langerhans. The process may reflect the formation of AlF_4^{1-} since it was inhibited by the aluminum chelator desferrioxamine. Recent studies provide evidence that apoptosis of pancreatic β cells is important in the early etiology of diabetes mellitus. Treating thymus lobe cells with aluminofluoride complexes also provoked apoptosis of a wider range of thymocyte subtypes [121] with an accumulation of inositol phosphates. The responses to aluminofluoride complexes were not prevented by inhibitors of tyrosine kinases, suggesting that unidentified G-proteins which couple to phospholipase C activation may also be capable of initiating apoptosis by a route independent of the T cell receptor.

4.4
Evidence about the Neurotoxic Effects of Aluminum plus Fluoride in Humans

Most of the ill effects caused by fluoride were first recognized among workers in aluminum factories, where fluoride and aluminum are present in high concentrations. Observation of industrial fluorosis and bronchial-related

disorders in such workers have been reported since the 1930s. Psychiatric disturbances were also reported in aluminum smelter workers. The study of persons living near an enamel factory reports a distinct decline in mental activity, poorer memory, inability to coordinate thoughts, and reduced ability to write [122].

Elevated aluminum levels have been implicated as the cause of dialysis encephalopathy or dementia in renal failure patients undergoing long-term hemodialysis [85]. Some patients used aluminum-containing medications. Moreover, patients with renal failure cannot remove aluminum from the blood. Dialysis dementia can arise after three to seven years of hemodialysis treatment. Speech disorders precede dementia and convulsions. Since many hemodialysis units rely on systems to purify fluoridated tap water, it is likely that many patients are being exposed inadvertently to increased concentrations of fluoride and aluminum. Increased serum fluoride concentration and fluoride intoxication have been also observed in chronic hemodialysis patients. Arnow et al. [96] reported that 12 of 15 patients receiving dialysis treatment in one room became acutely ill, with multiple non-specific symptoms and fatal ventricular fibrillation. Death was associated with longer hemodialysis time and increased age compared with other patients who became ill.

Since fluoride has been used in the prevention of tooth decay for over 50 years, many studies reporting and evaluating the risks and adverse effects of fluoride on the human organism were published during the same period [78, 122, 123]. Reduction of children's intelligence, various psychiatric symptoms in adults, such as memory impairment, and difficulties with concentration and thinking were reported [78]. Aluminum, the most abundant metal of the earth's lithosphere, is everywhere. Vaccines, allergy skin tests, 25% human serum albumin, baby skin creams, baby diaper wipes, and antacids, which are frequently given to infants, are extremely high in aluminum. For adults, the accumulation continues from suntan lotion, cookware, and aluminum cans and skin moisturizers. Such items as deodorants, vaginal douches and baby wipe not only have high aluminum content, but also are applied to areas where there is a far greater tendency to absorption through the skin.

The synergistic action of aluminum ions with fluoride may be the underlying mechanism of the observed neurotoxic effects of fluoride. Chronic exposure of humans to AlF_x begins in the fetus. Elevated fluoride content was found in embryonic brain tissues obtained from required abortions in areas where fluorosis was prevalent [78, 124]. These studies showed poor differentiation of brain nerve cells and delayed brain development. High fluoride exposure appears to weaken mental function among children, as well as adults [125, 126].

The increasing content of fluorides and aluminum in food chains has raised the possibility that the near future will supply us with much more data about the neurotoxic effects of aluminum plus fluoride on humans.

5 Conclusions

Any endeavor to think about the possible action of aluminofluoride complexes could provide a lesson which could draw us nearer to understanding the multiple nature of the etiology and pathogenesis of AD, providing a better chance of prevention of this most devastating brain disease. The search for AD prevention probably waits for a change in the concepts and strategy of scientific research from the reductionistic approach, which supplies us with the parts of the puzzle, to the integration of these parts into a multidimensional and non-linear whole. No one can predict what happens in the human body after a truly chronic exposure to an increased content of aluminum and fluoride in body fluids and in various tissues.

The aluminum concentration in human blood is comparable with that used in the laboratory investigations. The fluoride concentration in human blood is still many times lower than that used in the laboratory. It is difficult to predict the actual concentrations of aluminum and fluoride in intracellular compartments. But the increase of serum concentrations of fluoride in chronic hemodialysis patients to 756 μmol L^{-1} resulted in illness or death.

Recently, we can witness many discussions about the benefits and risks of the fluoride supplementation. Also the question of aluminum toxicity in men has been discussed. The understanding of the mechanisms of their synergistic action could allow us to explain numerous observations about the effects of increased load of fluoride and aluminum in the environment and to reevaluate their widespread use. Understanding the role of phosphate and G-proteins in cell signaling forces us to accept the fact that aluminum in the environment, water, and food chains followed by fluoride ions could evoke various and multiple pathological symptoms.

G-proteins take part in an enormous variety of biological signaling systems, helping control almost all important life processes. The origins of many human diseases are in the functioning (and malfunctioning) of signaling components [25]. Pharmacologists estimate that up to 60% of all medicines used today exert their effects through G-protein signaling pathways [127]. However, it is evident that processes of signal transmission amplify a signal from a molecule giving the false information, namely AlF_x. In light of the published findings, the long-term action of AlF_x represents a hidden but serious and powerful risk factor for the development of AD. The awareness of the health risks of increasing load of aluminum ions and fluoride as a new ecotoxicological phenomenon would contribute significantly to the decline of risks of intelligence decreases in children and adults, and many other severe disorders in the 21st century.

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