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OXIDATIVE DNA DAMAGE AND APOPTOSIS IN DOWN'S AND ALZHEIMER'S LYMPHOCYTES

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ABBREVIATIONS

AD	Alzheimer's disease
Apaf-1	apoptotic protease-activating factor-1
APP	amyloid precursor protein
Aβ	beta-amyloid peptide
BER	base excision repair
CAT	catalase
Ca^{2+}	calcium ion
Chr	chromosome
CSF	cerebrospinal fluid
CNS	central nervous system
CNT	control
DS	Down's syndrome
DSB	double strand break
EDTA	ethylenediaminetetraacetic acid
EndoIII	endonuclease III
FAD	familial AD
FITC	fluorescein isothiocyanate
Fpg	formamidopyrimidine DNA-glycosylase
GPX	glutathione peroxidase
H_2O_2	hydrogen peroxide
mAb	monoclonal antibody
mtDNA	mitochondrial DNA
MMSE	Mini-Mental State Examination
nDNA	nuclear DNA
NER	nucleotide excision repair
NFT	neurofibrillary tangle
NF-κB	nuclear factor kappa B
$OH^{\bullet}$	hydroxyl radical
OS	oxidative stress
$O_2^{\bullet -}$	superoxide anion
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PE	phycoerythrin
r	correlation coefficient
ROS	reactive oxygen species
SCE	sister chromatid exchange
SD	standard deviation
SEM	standard error of mean
SOD-1	cytoplasmic Cu^{2+}/Zn^{2+} superoxide dismutase
SP	senile plaque
SSB	single strand break
TNF	tumor necrosis factor
UVB	ultraviolet B light
8-OH-dG	8-hydroxy-2'-deoxyguanosine
8-OH-G	8-hydroxyguanosine
21q	long arm of chromosome 21

CONTENTS

1. INTRODUCTION

1.1. ALZHEIMER'S DISEASE

A hundred years after the first description, Alzheimer's disease (AD) is one of the most devastating age-related illnesses of the central nervous system (CNS) leading to dementia, and one of the most disabling and burdensome health conditions worldwide [35]. Today, according to the latest Delphi consensus study, 24-25 million people have dementia and this number will double every 20 years, assuming no changes in mortality and no effective prevention strategies or curative treatments. Of those with dementia, 60% live in developing countries, with this number rising to 71% by 2040. In Eastern Europe, an increase of 84% has been estimated in the number of people with dementia. Nowadays, the incidence of dementia in Hungary is 10% over the age of 65 years, which means approximately 200 000 demented aging patients [126]. Taken together, due to the enhanced life expectancy of the general population, the aging community and thereby the dementia syndromes involved are becoming significant public health, social, economic and political problems all over the world. Therefore, intensive research is needed with the goals of elaborating efficient preventive, diagnostic and therapeutic possibilities via the identification of the risk factors, and an exact knowledge of the pathogenesis and clinical features of AD, one of the most common form of aging dementia (60-70%). The etiology of the disease is not yet known, and no really effective treatment is available.

To date, although the knowledge of the pathophysiological mechanisms implicated in the development of AD is far from complete, several parallel hypotheses have been proposed to explain its etiology, including the oldest cholinergic hypothesis, the oxidative stress (OS) hypothesis, the aberrant cell cycle hypothesis, the most recent "two-hit" (OS and cell cycle abnormalities) hypothesis, the cell death hypothesis, the "amyloid cascade" hypothesis, the inflammation hypothesis, the glutamate excitotoxicity hypothesis, the cholesterol hypothesis and the genetic hypotheses. The "amyloid cascade" hypothesis forms the most comprehensive interpretation, integrating all these theories, but it has some weaknesses, and ample evidence indicates that all the factors mentioned take part in the development of AD.

Three pathological hallmarks of AD are observed at autopsy: (1) extracellular senile (amyloid) plaques (SPs), (2) intracellular neurofibrillary tangles (NFTs) in large numbers in specific areas of the brain, and especially in the neocortex, hippocampus and other subcortical regions which are essential for the cognitive functions [125], and (3) vascular amyloid deposits in the cerebral blood vessels. Subsequent, additional characteristics of AD are deficits in multiple neurotransmitter (cholinergic, noradrenergic and serotonergic) signaling systems and synaptic loss, leading to the cardinal clinical symptoms: a progressive decline in cognition and memory, resulting in dementia associated with behavioral (affective) disturbances [117].

The classical SP in AD consists of a central core that is formed mostly from the deposition of beta-amyloid peptide (Aβ), derived from cleavage of the amyloid precursor protein (APP) by β- and γ-secretases, surrounded by a rim of dystrophic neurites and activated microglia and astrocytes. Neurotoxicity and neuronal death mediated by Aβ have been shown to involve OS, perturbation of the intracellular calcium (Ca^{2+}) homeostasis and the activation of apoptotic cell death pathways [22, 25, 45, 76]. Thus, identification of molecular and cellular mechanisms that predispose neurons to apoptosis in AD may provide defined targets for new treatment strategies for the earlier diagnosis, prevention or amelioration of the course of the disease. The main component of the NFTs is the hyperphosphorylated protein tau, which assembles with neurofilaments into paired helical filaments. Several lines of evidence indicate that Aβ aggregation is important in the etiology of AD, as is tau in its clinical manifestation [30]. Both SPs and NFTs play critical roles in the cytotoxic events causing the neuronal cell death that leads to dementia [23, 145].

The hereditary forms of AD generally have an earlier age of onset than that of the sporadic one. Mutations of the *APP*, presenilin-1 and -2 and apolipoprotein E (epsilon 4) genes seem to be the most important in causing these rare (1-5%), familial AD (FAD) autosomal dominant forms. However, there may be multiple genetic and environmental factors, e.g. OS, that affects the age of onset and disease progression.

1.2. DOWN'S SYNDROME

Down's syndrome (DS) is one of the most common genetic abnormalities in liveborn children (1 in 700-1000), leading to an early mental decline and premature aging. These patients often suffer from other aberrations, e.g. specific cardiac and gastrointestinal congenital malformations, various types of leukemias, cataracts and growth retardation [108]. Moreover, immune disorders such as celiac disease, a thyroid dysfunction and diabetes mellitus are also prevalent. Full trisomy of chromosome 21 (Chr 21) accounts for approximately 95% of the cases, and the remainder involve mosaicism or translocation to Chr 14 or 21. DS can affect many aspects of development, producing a wide and variable set of clinical features in a given person [106]. The excessive synthesis of multiple gene products derived from overexpression of the genes present on Chr 21 is thought to underlie both the dysmorphic features and the pathogenesis of the neurological, immunologic, endocrine and biochemical deviations that are characteristic of DS [103].

A link between the DS phenotype and an increased risk of the development of AD has now been firmly established [12]. A presenile dementia syndrome that is clinically, neuro- and histopathologically similar to AD [14, 47, 63] occurs in almost all those over the age of 40 years with DS. The prevalence of dementia among DS patients is 8% in the age range 35-49, 55% in the age range 50-59, and 75% above the age of 60 years, but the AD neuropathology is present in all of the cases by the age of 40. This may indicate some common pathways and a close relationship between these two disorders.

1.3. THE ROLE OF OXIDATIVE STRESS IN ALZHEIMER'S DISEASE AND DOWN'S SYNDROME

One of the pipeline hypotheses, the "gene dosage effect" hypothesis of DS, holds that the dosage imbalance of a specific individual gene or a small group of genes is responsible for the specific individual DS traits [62, 106]. The presence of an extra copy of Chr 21, or rather the consecutive overexpression of the genes located on it, has been regarded as the central point for the development of the DS phenotype. Overexpression of the encoded proteins leads to overconsumption of their substrates and overproduction

of their metabolic end-products. The best-documented AD-associated gene products of this phenomenon are the APP and the cytoplasmic enzyme Cu^{2+}/Zn^{2+} superoxide dismutase (SOD-1), both of which are responsible for the regulation of reactive oxygen species (ROS) homeostasis [113], which may be a fundamental factor in the development of OS, preceding the signature pathology by decades and leading to neuronal death and disease progression. However, not all proteins whose genes are encoded on this chromosome display overexpression, suggesting that the DS phenotype can not be explained simply by the "gene dosage effect" [15].

The "two-hit" hypothesis for AD recently proposed that either OS or alterations in the regulation of the cell cycle (mitotic signaling) can independently serve as initiators, but both processes are necessary and sufficient to propagate AD pathogenesis [148]. This model described in relation to AD may also have implications in DS and other neurodegenerative diseases. It has been suggested that susceptible neurons (under stress or bearing mutations) devote their compensatory potential to adjusting to current stimuli and thereby lose the capability of further adaptation needed to respond to other insults in the future. In other words, the first insult may leave the neurons very vulnerable to an additional insult. In light of this interpretation, it seems to be that OS alone is a necessary, but not sufficient event leading to AD.

1.3.1. The role of amyloid precursor protein in Alzheimer's disease and Down's syndrome

The *APP* gene, mapped to Chr 21q21.3-22.05, codes for a transmembrane protein expressed in both neurons and astrocytes. Recent years have seen significant advances in the understanding of Aβ pathogenesis and the functional consequences of Aβ accumulation, but there are still large gaps in our knowledge of the pathways involved in Aβ degradation and clearance [48]. Although the function of APP is not entirely clear, it is implicated in the neuroprotection against oxidative insults [52]. The close relationship between DS and AD may be attributed in part to an overdose of the *APP* gene and to the subsequent enhanced production of Aβ. Mutations in the *APP* gene are connected with FAD, and it is hypothesized that the overexpression of APP causing

elevated Aβ(1-40/42) formation is the crucial event leading to the development of the AD pathology in DS [14].

According to the "amyloid cascade" hypothesis, the formation of Aβ requires proteolytic cleavage of the large, type-1 integral membrane-spanning glycoprotein APP by secretases. APP can be processed into several different biologically active compounds, e.g. the soluble, secreted form (sAPP), derived from cleavage by α- and γ-secretases, which has been shown to have neurotrophic activities, and the longer aggregating forms, of which Aβ(1-42) is the most toxic. Aβ itself could function as a neurotrophic factor for undifferentiated/differentiating neurons at low concentrations, but it could be neurotoxic to mature neurons at higher concentrations [143]. Aβ begins to accumulate in DS in childhood (as young as 8 years) and its amount appears to rise progressively with increasing age [68]. In younger DS individuals (under 30 years), Aβ accumulation is manifested by diffuse deposits that are not related with neuritic degeneration and consist of the toxic and less soluble peptide Aβ(1-42). Since SOD-1 immunoreactivity has been reported to be absent from early diffuse plaques, it has been presumed that an elevated SOD-1 level may not be necessary in the initial pathologic events leading to SP formation [14]. Despite the fact of extensive extracellular Aβ accumulation in the brain in both DS and AD, inconsistent associations have been published between Aβ and dementia severity [24], pointing to the significance of some other (genetic or environmental) factors predisposing to dementia in DS. Between the ages of 35 and 45 years, the rate of Aβ-associated neuropathology accelerates [138], together with the NFTs (consisting mainly of intracellular deposits of microtubule-associated protein tau) and neuroinflammation [70]. In line with these findings, autopsy studies have demonstrated SPs and NFTs in the brains of all older DS patients, with some individuals showing a much earlier onset [68]. It has been suggested that an apoptotic mechanism may be involved in the process of ultimate neuronal death in DS [111].

Aβ is neurotoxic directly by inducing OS, and indirectly by activating microglia [133, 134]. The binding with the receptors of advanced glycation end-products (RAGEs/AGEs) increases the intracellular Ca^{2+} concentration, giving rise to apoptotic cell death [145]; this is related to an elevation in lipid peroxidation [13] and

can be stopped by catalase (CAT) and free radical scavengers. These results are in accordance with the notion that the alterations in the mitochondrial energy metabolism may be in the background of this process as a major endogenous source of ROS that contribute to neuronal apoptosis, leading to a chronic state of increased vulnerability to oxidative injury [10]. Energy depletion and OS can also induce amyloidogenic changes in APP processing [83], suggesting a potential link between a mitochondrial dysfunction, OS and Aβ production, as has been described in AD and DS brains [10, 51]. Indeed, a reduced level of mitochondrial complex V (ATP synthase) β-chain protein has been noted in the DS cortex [61], indicating that the mitochondrial function impairment and OS are closely associated with DS. Despite the presence of enhanced neuronal apoptosis being refuted, unaltered levels of Fas, caspase-3, Bcl-2 and annexins (I, II, V and VI), proteins known to be associated with apoptotic cell death, have been reported in fetal DS brains [34].

In both AD and DS, alterations in APP metabolism have also been noted in the periphery, e.g. in the platelets [29], lymphocytes [98] and fibroblasts [131]. A systemic change in the APP metabolism was suggested by 2-3-fold enhanced plasma concentrations of both Aβ(1-40) and Aβ(1-42) in DS [130], which are in harmony with the findings that elevated APP contents have been revealed in both AD and DS lymphocytes [98], and an enhanced proportion of mRNA encoding for the three major APP transcripts (APP770+751/695) in DS fibroblasts [42].

1.3.2. The role of superoxide dismutase-1 in Alzheimer's disease and Down's syndrome

Oxygen is necessary for life, but paradoxically, by-products of its metabolism produce ROS, including free radicals (superoxide ($O_2^{\bullet-}$) and hydroxyl ($OH^{\bullet}$) species) and other molecules, such as hydrogen peroxide (H_2O_2) and peroxynitrite, which have the ability to become highly toxic to cells [3]. The latter molecules can lead to the generation of free radicals through various chemical reactions. Thus, via the Fenton reaction, in the presence of reduced metal ions (e.g. ferrous or copper) as catalysts, H_2O_2 can be converted to $OH^{\bullet}$. Peroxynitrite, formed by the reaction of nitric oxide with $O_2^{\bullet-}$, is a highly reactive molecule that also breaks down to form $OH^{\bullet}$. Together, ROS can

interact with cellular components such as proteins, lipids and DNA [128], and ultimately cause cell death.

OS is defined as the lack of balance between the generation and removal of ROS, which may play a significant part in the pathogenesis of both DS and AD [9, 44, 60, 114]. SOD-1 is thought to have a major role in the first line of antioxidant defense by catalyzing the transformation of $O_2^{\bullet-}$ to molecular oxygen (O_2) and H_2O_2, which can be converted by CAT and glutathione peroxidase (GPX) to water. The triplication of Chr 21, on which the *SOD-1* gene is localized (mapping in 21q22.1), leads to an imbalance in the ratio of SOD-1 to CAT and GPX, resulting in the accumulation of H_2O_2.

A recent observation that SOD-1 forms proteinaceous aggregates that are related to SPs in AD brains for the first time implicated the involvement of oxidative damage to SOD-1 in the AD pathogenesis [16], confirming the close connection between DS and AD. An increased SOD-1 activity in the cerebrospinal fluid (CSF) with aging has been reported in AD, suggesting a possible compensatory process, secondary to the increased OS, with time [96]. The same adaptive, OS-activated responses were hypothesized in both AD and DS neurons [85, 147], which reduce the oxidative injuries by inducing apoptosis or protective mechanisms and ensure that the neurons do not rapidly succumb to OS. It has been suggested that Aβ deposition and tau hyperphosphorylation function as compensatory responses and downstream adaptations.

Not only the CNS, but all DS tissues display an altered SOD-1/GPX activity ratio [27]. SOD-1 is found at levels approximately 50% higher than normal in a variety of DS cells and tissues, including erythrocytes, neutrophils, B and T lymphocytes and fibroblasts [88, 101, 118], which may affect the gene expression by altering the binding and/or availability of transcription factors, e.g. nuclear factor kappa B (NF-κB) [27] and the activator protein (AP)-1 [1], to DNA. This is in harmony with the recent observation on AD lymphoblasts that serum addition induced an increase in NF-κB-DNA-binding activity [28]. NF-κB opposes apoptosis through the induction of antiapoptotic Bcl-2 family members and some inhibitors of apoptosis proteins. Indeed, inhibition of NF-κB activation produces a corresponding increase in apoptosis, indicating that the balance of cell viability versus cell death is maintained by the degree of NF-κB activation.

1.3.3. APOPTOSIS IN ALZHEIMER'S DISEASE AND DOWN'S SYNDROME

Cell death can occur by either of two specific cellular mechanisms, necrosis or apoptosis, with different characteristic morphological and biochemical features. The former ("accidental" cell death) is a pathological process which appears when cells are exposed to a serious physical or chemical insult, e.g. hypothermia and hypoxia, and results in a significant inflammatory response. In contrast, apoptosis, also known as "programmed" cell death, is a complex, conserved and tightly regulated physiological event that takes place continuously throughout life in the whole organism, and may occur in different varieties, e.g. "paratosis" or "abortosis" [136]. It helps to eliminate unnecessary and aged or damaged cells and maintains tissue homeostasis [30]. Recent accumulating evidence strongly suggests that apoptosis may additionally contribute to neuronal death in a variety of neurodegenerative disorders [75]. During apoptosis, the cell body shrinks, the plasma membrane blebs and the nuclear DNA (nDNA) condenses and fragments, while the organelle integrity is preserved until late stages of the process. Exposure of acidic phospholipids on the cell membrane is a signal for the ultimate recognition and phagocytotic uptake of cells undergoing apoptosis, ensuring that no inflammatory response is elicited in the neighboring tissue. *In vitro*, the apoptotic bodies and remaining cell fragments swell and finally lyse; this terminal phase has been termed "secondary necrosis".

Several apoptosis-inducing factors have been identified, e.g. OS, DNA damage, a disturbance in Ca^{2+} homeostasis, ceramides, the withdrawal of growth factors and infections, but the exact mechanism of apoptosis, i.e. the cascade of events starting from the detection of the signal at the cell surface to the events that occur in the nucleus, has not yet been clarified [110]. The occurrence of apoptosis involves relatively few pathways that converge on activation of the caspase (cysteine aspartate-specific protease) cascade [38]. Generally, caspases are found in relatively large amounts as inactive precursors/proenzymes (procaspases) within the cytoplasm and they undergo processing to form activated heterotetramers upon proteolysis. The central players in the apoptotic program are the upstream initiator caspases (8, 9 and 10); these activate other caspases, called effector caspases (3, 6 and 7), which are important regulators of postmitotic homeostasis. The latter, downstream, executioner caspases are responsible for the majority of the intracellular caspase-induced cleavages that result in cell death.

Two principal pathways are well known with respect to the activation of caspases: the cell surface death receptor (members of the tumor necrosis factor (TNF) receptor superfamily, e.g. TNF-α- and Fas-receptor) pathway and the mitochondrial pathway. In the former, the activation of caspase-8 is the critical event that transmits the death signal, and in the latter, caspase activation is triggered by the formation of an apoptotic protease-activating factor-1 (Apaf-1)/cytochrome *c* complex that is fully functional in recruiting and activating procaspase-9, and creating the "apoptosome" (Fig. 1).

On the one hand, the initiator caspase-8 directly activates caspase-3, and on the other hand, it cleaves one of the proapoptotic members (Bax, Bad and Bcl-x_S) of the Bcl-2 family, Bid, giving rise to the promotion of apoptosis: Bid translocates to the mitochondrial membrane, where it interacts with the one of the antiapoptotic members of this protein family, Bcl-2. These events result in the formation of a mitochondrial transition pore and the release of cytochrome *c* into the cytoplasm [57]. Subsequently, caspase-9 cleaves and activates downstream caspase-3, finally resulting in the occurrence of apoptosis.

The proto-oncogene Bcl-2 protects cells from the apoptotic consequences of oxidative damage: its overexpression blocks the lipid peroxidation that accompanies apoptotic cell death. The balance of pro- and antiapototic members of the Bcl-2 protein family determines whether apoptosis is promoted or prevented.

Ultraviolet (UV) radiation (wavelength band: 100-400 nm), and in particular UVB with a wavelength range in the interval 290-320 nm, is one of the most important environmental factors affecting humans [65]. A crucial event following exposure of the skin to UVB light is the formation of sunburn cells within the epidermis; these are in fact severely UVB-damaged cells which bear the risk of becoming malignant and which therefore undergo apoptosis in favor of the surrounding tissue. Although the majority of studies were performed with epidermal cell lines and primary keratinocytes, evidence exists that this concept also holds true for other cell types. UVB-mediated apoptosis is a highly complex process involved in a variety of signaling pathways, including DNA damage, cell death receptor activation and the generation of ROS. To exert its biological influences, UV radiation has to be absorbed by a cellular chromophore that transduces the energy into a biochemical signal. In this respect, genomic DNA has been regarded as

being the main target for UVB since the absorption maximum matches the action spectrum of biological UVB effects (the DNA absorption peak is at 260 nm). The DNA damage seems to predominate (see Section 1.3.4.2), but UVB can also influence molecular targets, via either a direct and ligand-independent activation of membrane-bound cell death receptors or the mitochondrial apoptotic pathway. UVB is known to be a potent inducer of ROS that initiate cellular damage and apoptosis: changes in the structure of the inner mitochondrial membrane result in a loss of the membrane potential and subsequent cytochrome *c* and ROS release into the cytoplasm [65].

Apoptosis is a potentially important event for the initiation of both AD and DS and progression of the neuronal loss [4, 7, 56], implicating a disturbed Ca^{2+} homeostasis, a mitochondrial dysfunction and a caspase activation in OS. Additionally, ample evidence of augmented apoptosis has been reported not only in the brain, but also in the periphery, e.g. in the lymphocytes [33, 112], cells which at the same time represent the main compartment of the immune system. A higher concentration of 8-hydroxy-2'-deoxyguanosine (8-OH-dG), a marker of oxidative damage to the DNA, has been demonstrated in AD lymphocytes [79], reflecting cell injury. Mitogen-induced cultured T cells from AD patients have revealed that elevated levels of caspase activation [123] and intracellular Ca^{2+} are prerequisites for apoptosis [122], supporting the theory of the hypersensitivity of these cells to apoptotic stimuli. Furthermore, Aβ, the major neurotoxic protein component of SPs, is able to promote apoptotic death in lymphocytes *in vitro* via an OS mechanism [132]. In DS, an altered free radical metabolism and an impaired mitochondrial function are linked to neuronal degeneration [4, 11] that may be associated with both mental retardation and AD pathology in these patients. In addition to the overexpression of the above-mentioned *APP* and *SOD-1* genes located on Chr 21, the Ets-2 family of transcription factors is also involved in multiple cellular processes, including the activation of signaling cascades [49]. Elevated levels of Ets-2 predispose to apoptosis via a p53-dependent mitochondrial pathway and an activation of caspase-3 in fibroblasts overexpressing Ets-2, in primary neuronal cultures from *ETS-2* transgenic mice and in both DS and AD cortical neurons, supporting the involvement of a dysfunction of the mitochondria and

increased apoptosis in DS and AD neuropathology [49, 109, 140]. The tumor suppressor gene *p53* has a critical role in the regulation of the cell cycle: it is required for G_1/S arrest, allowing the cell to accomplish DNA repair prior to DNA synthesis, thereby reducing the DNA mutation rate.

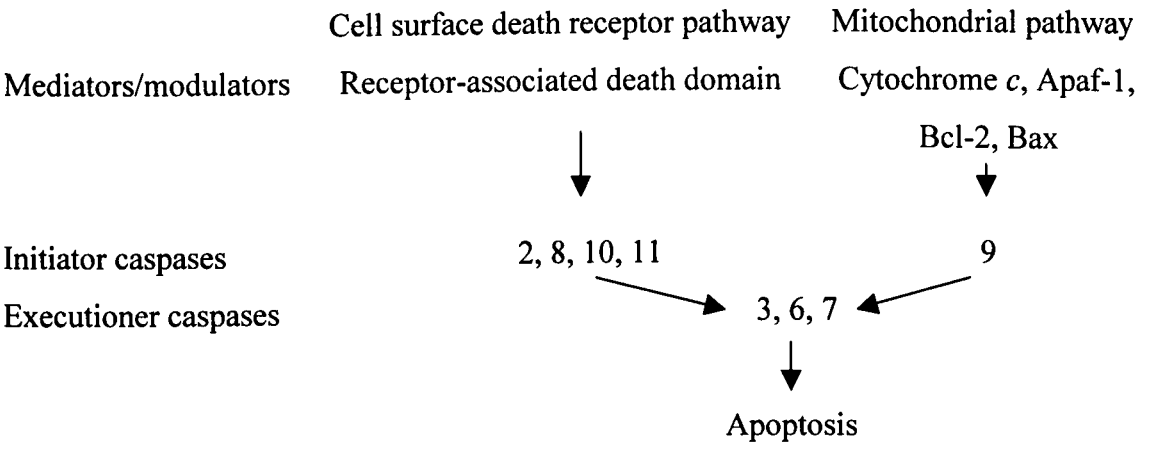


Fig. 1. Molecules implicated in the two main signaling pathways of apoptosis.

1.3.4. Oxidative stress-induced DNA damage and repair in Alzheimer's disease and Down's syndrome

1.3.4.1. **DNA damage in Alzheimer's disease and Down's syndrome**

ROS-mediated oxidative injury can result in DNA modifications, including base alterations, single (SSBs) and double strand breaks (DSBs), sister chromatid exchanges (SCEs) and DNA-protein crosslinks [119]. The contributions of free radical-mediated DNA cleavage, ongoing or incomplete DNA repair processes or endonuclease cleavage as part of an apoptotic cascade to the generation of DNA strand breaks are unknown [26], but recent evidence suggests that they may have fundamental and complex roles in both AD and DS progression.

DNA strand breaks can be detected by the alkaline elution assay at the tissue level, or by terminal deoxynucleotidyl transferase-mediated *in situ* end-labeling or comet assay at a single cell level (see Section 2.2.2.2). SCE, the exchange of homologous stretches of DNA between sister chromatids, normally occurs in cells

during mitosis or cell division, but when the DNA is damaged by genotoxic agents, the rate of SCE increases.

Mainly $OH^{\bullet}$ plays a major role in the formation of oxidative DNA damage. Since the copper ion (see Section 1: Fenton reaction) participates in the attachment of DNA to the nuclear matrix, it is likely that $OH^{\bullet}$ is formed in close proximity to the DNA target [18], generating strand breaks with various sugar modifications and simple abasic sites. Free radicals can damage all four DNA bases, and therefore methods have been developed for the detection of all these modified bases. One of the best-characterized, 8-OH-dG, is the most predominant marker of *in vivo* oxidative DNA damage resulting from $OH^{\bullet}$ attack on deoxyguanosine.

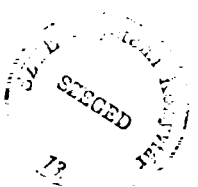
Indeed, a higher incidence of SSBs, DSBs and alkali-labile DNA lesions has been detected in the AD cerebral cortex [2, 89, 120]. On the other hand, both increased and unaltered 8-OH-dG levels have been described in AD brain nucleus samples [39, 116, 127]. In contrast, there was not found to be a significant elevation in the 8-OH-dG content of postmortem aged DS brain tissues [116], questioning the involvement of OS in the DNA damage leading to neuronal apoptosis in DS. A complex study has reported augmented degrees of some other (8-OH-adenine, 8-OH-guanine and thymine glycol), but not all oxidized bases in the AD cerebral cortex [73]. The amounts of multiple oxidized bases of both nDNA and mitochondrial DNA (mtDNA) were greater in the frontal, parietal and temporal lobes in AD as compared with the controls [137]; and, as expected, DNA from the temporal lobe displayed the most oxidative damage, whereas the cerebellum was only slightly affected, since the cerebellum is poor in Aβ deposition. The 8-hydroxyguanosine (8-OH-G) (the main oxidation product of RNA) immunoreactivity was demonstrated to be enhanced in young DS patients, whereas the Aβ burden was elevated only after the age of 30 years; the increased level of oxidative damage therefore appears prior to the onset of Aβ deposition in DS brains [93]. This observation is in harmony with the presumption of the presence of *in vivo* OS and suggests that chronic oxidative injury constitutes a risk factor for subsequent neurodegeneration in aged DS patients [11]. Likewise, a systematic examination of the spatiotemporal link between the extents of 8-OH-dG, 8-OH-G and the hallmark AD lesions at early AD stages indicated that markers of OS are present in those susceptible

neurons without NFTs [94], confirming the primacy of OS. On the other hand, neurons containing NFTs showed a decrease in relative 8-OH-G level as compared with neurons free of NFTs, and the same relationship was noted between 8-OH-G immunoreactivity and Aβ deposition, similarly to the findings in DS [93]. These observations also point to the oxidative damage being an early event in the pathogenesis of both AD and DS, and to the fact that it decreases with disease progression and increased histopathology, suggesting compensatory intracellular mechanisms that reduce OS [92]. In both AD and DS brains, this pattern of oxidation to RNA coincided with that of a protein oxidation marker (3-nitrotyrosine) which may reflect global levels of OS.

In the single published comet assay study on lymphocytes from newborns with DS, a significantly elevated number of strand breaks was reported [74]. SCE studies on both AD and DS lymphocytes and fibroblasts have exhibited an increased or unchanged frequency following different types of exposure [40, 41, 80, 129, 141]. Likewise, both enhanced and unaltered levels have been described as concerns SSBs in resting AD lymphocytes and leukocytes [82, 87]. An elevated level of 8-OH-dG in the intact DNA and a decreased concentration of its free repair product were demonstrated in the CSF [71] and lymphocytes [78, 79] of AD individuals. In line with these observations, enhanced amounts of oxidized purines were described in AD lymphocytes [59, 87] and leukocytes [82], supporting the existence of a heightened OS *in vivo*. The higher level of urine 8-OH-dG found in DS [46] may also be in agreement with the observed premature aging and the enhanced incidence of dysfunctions (e.g. infections, cancer and cataracts) and early AD-like changes in DS.

1.3.4.2. **The repair of oxidative stress-induced DNA damage**

Three fundamental cellular responses to DNA damage have been identified in living cells: reversal, excision and tolerance. The latter practically does not involve a DNA repair mechanism, because it does not include the removal of the damage even it often leads to permanent mutations. The reversal of damage in DNA is the most direct mode of DNA repair, with a number of advantages, e.g. only a single gene product is required, it is relatively error-free because of the high degree of specificity, and it is more rapid than multistep biochemical pathways such as excision repair. However, the



types of DNA damage that are repaired by direct reversal are limited. The most general DNA repair mode observed in nature is one in which damaged, mispaired or inappropriate bases are excised from the genome and replaced by the normal nucleotide sequence and chemistry, including base excision repair (BER) (Fig. 2), nucleotide excision repair (NER) and mismatch repair.

The DNA damage induced by OS may be repaired by a variety of pathways, but particularly by BER (Fig. 2) [72]. Failure to repair oxidatively modified bases in DNA may lead to the accumulation of damage and result in a decay in normal cell functions, which could contribute to the aging process and age-related disorders [72]. Although the BER pathway is the main repair mechanism for the handling of oxidative DNA damage, the finding that the expressions of NER enzymes (DNA helicases) and their mRNA levels were higher in the brains of both DS and AD individuals [50] may indicate ongoing oxidative DNA damage. Ionizing radiation, which is considered to cause chromosomal instability, did not affect the DNA synthesis in DS fibroblasts [6], whereas the normal repair of SSBs and DSBs was seen in both fibroblasts [121] and lymphocytes [67]. Moreover, DS lymphocytes displayed a lower DNA repair efficiency and an accelerated decline in DNA repair capacity with age that is one of the probable reasons for the premature aging [104].

The mtDNA is 10 times more vulnerable to oxidative damage *in vivo* as compared with nDNA, and an enhanced ROS concentration is known to give rise to damage in it. Indeed, in agreement with these observations, DS fibroblasts displayed a reduction in their mtDNA repair ability [32], and AD and other age-related neurodegenerative disorders have been demonstrated to be associated with mutations in mtDNA [51].

Some researchers have detected decreased UV light-induced DNA repair in DS fibroblasts [105], leukocytes [66] and lymphocytes [104], while others have not [144]. UVB introduces preferably two types of DNA damage, cyclobutane pyrimidine dimers and 6-4 photoproducts, these latter lesions being removed by NER. Upon UVB irradiation of the cells, gene *p53* was found to be upregulated proportionally to the amount of cyclobutane pyrimidine dimers introduced into the genomic DNA [65]. The cells have evolved complex signaling pathways to arrest the progression of the cell cycle

in the presence of DNA damage, providing increased time for repair (and tolerance) mechanisms to operate, resulting in a reduced apoptotic rate [37]. Finally, when the burden of genomic insult is too large to be effectively met by the various responses, the cells are able to initiate apoptosis, eliminating themselves from a population that otherwise might suffer serious pathological consequences. Thus, the nDNA damage plays a crucial role in the mediation of UVB-induced apoptosis; when the DNA repair enzymes are inadequate to fix the damage, apoptosis is the most effective defense mechanism against DNA damage and mutation.

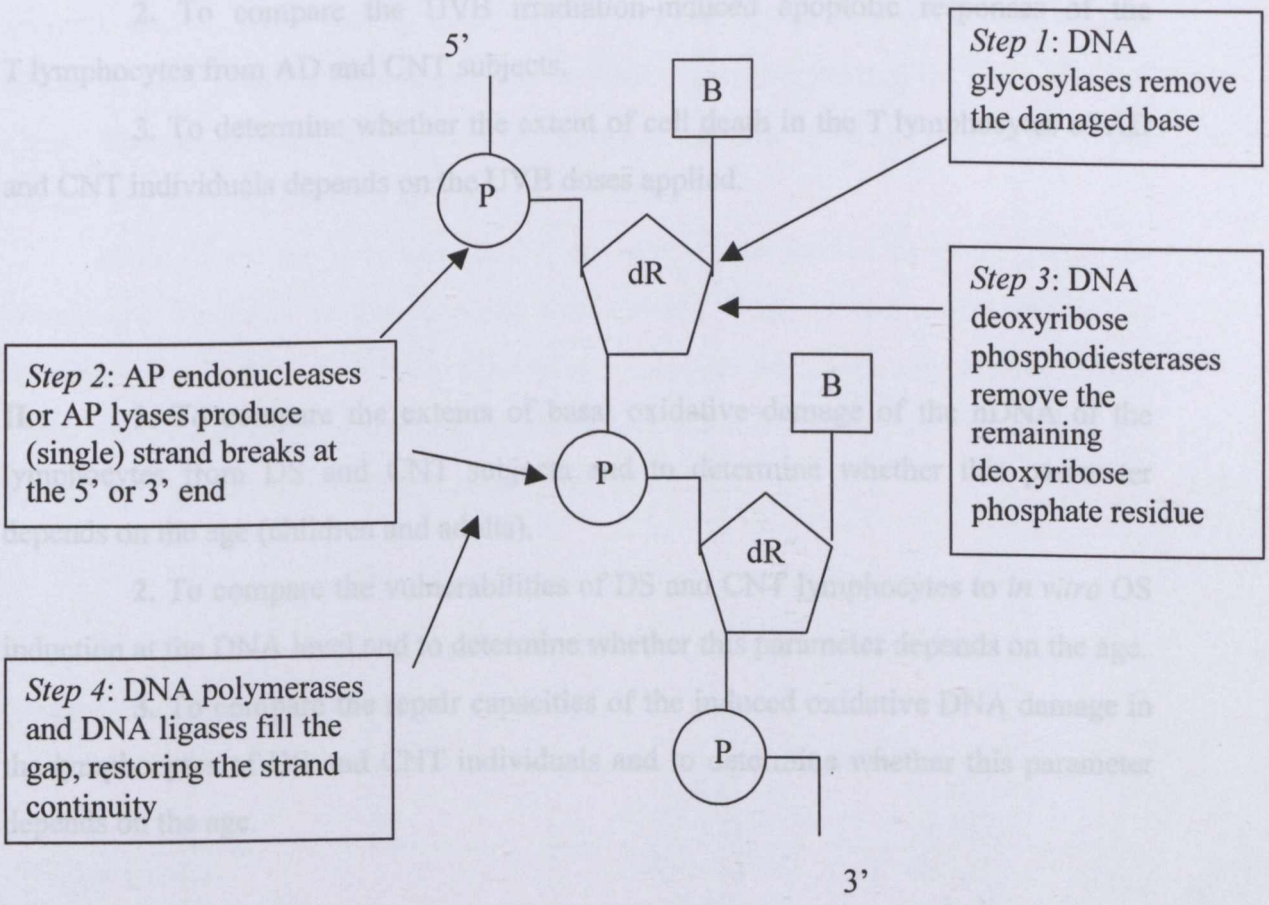


Fig. 2. Schematic representation of BER, correcting most of the oxidative DNA damage. For simplicity, only the relevant DNA strand is shown.

AP, abasic site; B, base (adenine, thymine, cytosine or guanine); dR, deoxyribose; P, phosphate

1. 4. AIMS

I. **1.** To determine whether UVB irradiation can induce apoptosis in T lymphocytes from AD and CNT individuals.

2. To compare the UVB irradiation-induced apoptotic responses of the T lymphocytes from AD and CNT subjects.

3. To determine whether the extent of cell death in the T lymphocytes of AD and CNT individuals depends on the UVB doses applied.

II. **1.** To compare the extents of basal oxidative damage of the nDNA of the lymphocytes from DS and CNT subjects and to determine whether this parameter depends on the age (children and adults).

2. To compare the vulnerabilities of DS and CNT lymphocytes to *in vitro* OS induction at the DNA level and to determine whether this parameter depends on the age.

3. To compare the repair capacities of the induced oxidative DNA damage in the lymphocytes of DS and CNT individuals and to determine whether this parameter depends on the age.

2. SUBJECTS AND METHODS

2.1. SUBJECTS

2.1.1. ALZHEIMER'S DISEASE PATIENTS AND THEIR CORRESPONDING CONTROLS

Thirty-four subjects participated in this project. Twenty-two patients with mild AD were selected according to the DSM-IV (Diagnostic and Statistical Manual of Mental Disorders 4th edition) and the NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke, and Alzheimer's Disease and Related Disorders Association) criteria [77]. All of them were probable, late-onset, sporadic cases. Twelve healthy control (CNT) individuals of similar age, but without dementia, were selected from among the spouses of patients visiting the local Memory Clinic. The age, gender and Mini-Mental State Examination (MMSE) scores [36, 54] of the AD and the CNT subjects are presented in Table 1.

None of the subjects were taking any medication known to interact with the lymphocyte functions or the apoptotic mechanisms. None of them were alcohol or nicotine users. Their routine blood laboratory parameters were within the normal ranges. There was no indication of any kind of immune disorder, severe infection or malignancy in their past history or in their present physical examination.

Table 1. Clinical data on the AD patients and their corresponding CNT probands (mean ± standard deviation (SD))

	CNT (n=12)	AD (n=22)
Age (years)	70 ± 7.1	75 ± 7.2
Age range (years)	63-86	61-82
Gender (male/female)	1/11	4/18
Age of dementia (AD) onset (years)	-	69 ± 2.3
MMSE score (maximum 30 points)	29 ± 1.2	21 ± 2.0*

*p=0.000 (p<0.0001)

2.1.2. DOWN' SYNDROME PATIENTS AND THEIR CORRESPONDING CONTROLS

Fifty subjects participated in this study. The DS group consisted of 25 patients living either in communities or in institutions. They were divided into two main age categories: children (<18 years) and adults (≥18 years) (Table 2). The clinical diagnosis of DS was confirmed by chromosome analysis: all the DS subjects demonstrated trisomy of Chr 21. Seven DS individuals (3 children, 4 adults) had chronic medical problems, e.g. heart defects, diabetes mellitus, renal insufficiency or gastrointestinal disorders. Patients with other diseases that were current or in their history or who had participated in the last 6 months in antilymphocyte, anti-inflammatory or antioxidant medication known to interfere with the lymphocyte number and/or function were excluded. The routine blood laboratory parameters, differential and total leukocyte counts and urinalysis results were within the normal ranges. Six DS subjects were taking low doses of psychotropic medication, i.e. carbamazepine, clonazepam, diazepam or sodium valproate, but there were no differences between these treated and the untreated patients as concerns the parameters examined in this work.

The CNT group comprised 25 gender- and age-matched healthy individuals. Their mean age, age range and gender distribution were comparable to those in the DS group (14 males, 11 females (p=0.78); age: 23 ± 12.1 (mean ± SD) years (p=0.96); age range: 3-57 years). The CNT subjects were also divided into two age categories: <18 and >18 years (Table 2). They had neither active medical problems nor any personal or family history of neurological or psychiatric disorders, alcohol or drug abuse, severe infection or malignancy, as determined via clinical interviews and physical examinations. None of the adult CNT individuals met the clinical criteria for dementia syndrome or mild cognitive impairment, and they were not taking any preventive medication (antioxidant or vitamin treatment). All of the CNT subjects yielded normal results as regards the routine blood chemistry, differential and total leukocyte counts and urinalyses.

Table 2. Clinical data on the DS patients and their corresponding CNT probands overall and in the two age categories (mean ± SD)

	CNT			DS		
	Total (n=25)	Children (n=7)	Adults (n=18)	Total (n=25)	Children (n=7)	Adults (n=18)
Age (years)	23 ± 12.1	8 ± 4.2	29 ± 8.6	23 ± 12.8	8 ± 3.6	29 ± 10.0
Age range (years)	3-57	3-15	21-57	4-58	4-14	18-58
Gender (male/female)	14/11	3/4	11/7	13/12	3/4	10/8

2.2. METHODS APPLIED

2.2.1. MEASUREMENT OF APOPTOTIC DAMAGE IN ALZHEIMER'S LYMPHOCYTES

2.2.1.1. **Isolation and *in vitro* ultraviolet B irradiation of lymphocytes**

Venous blood samples were collected between 9 and 11 a.m. Peripheral blood mononuclear cells (PBMCs: granulocytes, monocytes and lymphocytes) were prepared by density gradient centrifugation from EDTA (ethylenediaminetetraacetic acid)-anticoagulated blood. The freshly drawn, whole blood was diluted 1:1 in phosphate-buffered saline (PBS). The mononuclear cell interface was collected over Ficoll (type 400) (Sigma, Germany) (1:1=Ficoll solution : blood in PBS) after centrifugation at 400 g for 30 min at room temperature. The PBMCs were washed three times in PBS (300 g, 10 min). The cells in uncovered 24-well tissue culture plates (Nunclon, Denmark) were irradiated at a final concentration of 10^6 cells/well in PBS at 308 nm with a XeCl (xenon chloride) UVB-laser apparatus (Photomedex Incorporation, USA). The exposure doses applied were 100, 200 or 300 mJ/cm^2, as previously reported [53, 91]. The frequency of the impulses was 200 Hz and their energy was 3 mJ, with a pulse width of 30 nanoseconds. The PBMCs were washed once (300 g, 10 min) after the irradiation and resuspended in a complete RPMI 1640 medium (Gibco, Scotland) supplemented with 10% AB-positive heat-inactivated human serum (Sigma, Germany), 2 mM L-glutamine and antibiotics (0.1 mg/ml streptomycin and 100 IU/ml penicillin) (Gibco, Scotland). The cells were cultured for 20 h at 37 °C in the presence of 5% CO_2.

2.2.1.2. **Flow cytometry measurements**

The PBMCs were stained with a 10-µl anti-human CD3-FITC (fluorescein isothiocyanate) monoclonal antibody (mAb) (Dako, Denmark) cell suspension (10^6 cells in 100 µl of PBS) for 30 min at 4 °C. Samples were then washed twice with PBS containing 2% bovine serum albumin. The cells were fixed in 2% paraformaldehyde for 30 min at room temperature, and permeabilized for 15 min on ice in 0.01% saponin in PBS supplemented with 1% fetal bovine serum and 0.02% sodium azide. Later, 20 µl of Apo2.7-PE (phycoerythrin) mAb (Immunotech, France) and 80 µl of PBS were added, and the liquid was left to stand for 15 min at room temperature. Apo2.7-PE mAb binds to a 38 kDa mitochondrial membrane protein which appears on cells undergoing apoptosis [146]. To exclude false-positive reactions, the CNT samples were stained with isotype-matched anti-human IgG_1-PE mAb. Finally, the cells were resuspended in 250 µl of PBS and analyzed by flow cytometry. The percentage of Apo2.7-PE- and at the same time CD3-FITC-positive PBMCs, in other words apoptotic T cells, as compared with the total number of T lymphocytes was acquired on a FACStar Plus (Becton Dickinson, USA) flow cytometer, using CellQuest software (Becton Dickinson, USA).

2.2.2. MEASUREMENT OF OXIDATIVE DNA DAMAGE IN DOWN'S LYMPHOCYTES

2.2.2.1. **Isolation and *in vitro* oxidative stress induction of lymphocytes**

EDTA-anticoagulated venous blood samples were collected between 7 and 9 a.m. At the same time, qualitative blood tests were performed in order to exclude acute diseases. Lymphocytes were immediately prepared from 1 ml of freshly drawn, whole blood with Histopaque 1077 (Sigma, Germany). The interface was collected after centrifugation at 200 g for 15 min at room temperature. The cells were then washed twice in PBS (200 g, 5 min). The order of the yields was 10^6 cells/ml blood.

One-third of the separated lymphocytes remained untreated, and were used to detect the basal, spontaneous DNA damage levels. The remainder were treated with 100 µM H_2O_2 in PBS for 5 min on ice in order to induce oxidative DNA damage, which can reflect the antioxidant status of the cells [17]. The lymphocytes were washed three times in PBS, and half of the treated cells were then resuspended in 1 ml of RPMI 1640

medium (Gibco, Scotland) supplemented with 2 mM L-glutamine and antibiotics (0.1 mg/ml streptomycin and 100 IU/ml penicillin) (Gibco, Scotland) and incubated at 37 °C for 1 h in the presence of 5% CO_2, as previously described [19, 87], to allow the process of DNA repair. A 1-h time interval proved appropriate for the correcting processes to take place in both DS and CNT samples during our previous optimization of the method, and there was no sign of better removal during incubation for 2, 3 or 4 h, as found in previous publications [19, 21]. It did not appear advisable to choose a too long repair period, because the DNA repair of isolated lymphocytes is not highly efficient [20], and atmospheric oxygen could induce oxidative damage, possibly leading to artifacts [18].

2.2.2.2. **Comet assay**

The comet assay, also known as single-cell gel electrophoresis, is suitable for the quantification of genomic damage at the individual cell level and for the specific detection of DNA SSBs, alkali-labile sites, DNA-DNA or DNA-protein cross-linking, and DNA adducts and SSBs associated with incomplete excision repair sites [81]. Damage to nucleic acids caused by ROS includes base modifications, SSBs and DSBs if the SSBs are in close proximity [26]. The combination of the alkaline comet assay with the digestion of DNA by two lesion-specific (excision repair) endonucleases provides information about specific oxidative damage involving endonuclease III (EndoIII) [531] and formamidopyrimidine DNA-glycosylase (Fpg) [8]. These enzymes recognize and cut oxidized pyrimidines and purines, respectively, and convert these damaged sites into SSBs, producing fragments which migrate toward the anode during electrophoresis, forming the tail of the comet in the assay.

The modified alkaline comet assay [20, 87] was carried out according to the following protocol. Two sets of microscope slides were prepared for each experimental point and the average values were used for statistical analysis. Slides were spread with 160 µl of 1% normal-melting agarose (Sigma, Germany) in PBS, covered with a coverslip, and left at 4 °C to solidify. Centrifuged lymphocytes were suspended with 70 µl of 0.75% low-melting agarose (Sigma, Germany) in PBS at 37 °C, rapidly dropped onto the first agarose layer, covered with a coverslip, and left at 4 °C for 5 min

to solidify. After removal of the coverslips, the slides were incubated for 1 h in freshly prepared, cold lysis buffer (2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris, pH 10, 1% Triton X-100 added fresh) to remove cellular proteins. This treatment leaves residual nuclei embedded in the gel and makes the DNA susceptible to the following lesion-specific endonuclease treatment.

After lysis, the slides were washed three times for 5 min with enzyme buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/ml bovine serum albumin, pH 8) at 4 °C for 5 min. 50 µl of enzyme buffer (used as control) or buffer containing EndoIII (dilution: 1:10 000) (Biolabs, New England) or Fpg (dilution: 1:3 000) (Biolabs, New England) was dropped onto the slides, which were next covered with a coverslip. The slides were then placed into a moist box and incubated at 37 °C for 45 min.

After incubation, the coverslips were removed and the slides were placed into an ice-cooled horizontal gel electrophoresis tank filled with freshly made 4 °C alkaline buffer (0.3 M NaOH, 1 mM EDTA, pH 13) and left in this solution for 20 min to allow DNA unwinding. The DNA fragments were then separated by electrophoresis for 20 min at 25 V and 300 mA. After electrophoresis, the slides were flooded with three changes of neutralization buffer (0.4 M Tris, pH 7.5) at 4 °C for 5 min, and drained before staining with ethidium bromide (20 µg/ml).

Finally, the slides (each containing a minimum of 10^4 cells) were viewed under coverslips, using a fluorescence microscope (Axioskop 2 MOT, Zeiss) equipped with a digital camera (Axiocam, Zeiss). Observations were performed at a magnification of 400× (Axiovision 3.1 photo program (Zeiss)). 100 randomly selected cells from each replicate slide were evaluated by using Komet 5.0 video image analysis software (Kinetic Imaging Ltd., Liverpool, UK). Results are reported as the percentage of DNA in the comet tail for each cell ("tail DNA %"), which is indicative of the extent of DNA damage, expressed as the mean of the sum of the "tail DNA %" ± SEM (standard error of mean). The comet assay has long been known to give high SD values (evidence of the high interindividual variation) [64, 74, 139]. Accordingly, all our data are expressed as means ± SEM.

Strand breaks generated by the enzymes applied are added to the SSBs (and alkali-labile sites), and thus the values obtained for enzyme-treated slides include the

amounts of the given oxidized base together with the SSBs. Since unrepaired, residual damage is detectable only with the comet assay, the amount of repaired damage can be calculated as the difference from the stress-induced value (repaired damage = stress-induced damage – residual damage).

2.3. STATISTICAL ANALYSIS

In the apoptosis study, SPSS 9.0 for Windows was used for statistical analysis. Two-way ANOVA (analysis of variance) was applied to determine the differences between the AD and CNT groups and the data relating to the various UVB doses. The relationships between the age, the severity of dementia and the degree of apoptosis were determined via the Pearson correlation.

Statistica for Windows 6.1 was used for statistical analysis in the oxidative DNA damage investigation. First, a three-way ANOVA was used, but since the effect of gender and the three-way interaction were not statistically significant, a two-way ANOVA was applied to determine the differences between groups (DS or CNT) and the dependence on age (i.e. the age categories of children and adults). The non-significant interactions of the groups and the age categories (Table 4) indicated that the extent of given DNA damage did not depend on the age categories. This means that the differences between the DS and CNT groups were independent of age. In these cases, therefore, further pairwise comparisons according to age categories were performed only for completeness (Tables 5A and B). Additionally, some significant differences concerning the overall groups could no longer be detected because of the subsequent small size of the sample. When the interactions were significant (or close to the level of significance) (Table 4), the differences between the DS and CNT groups were tested separately in children (Table 5A) and adults (Table 5B) by performing pairwise comparisons on estimated marginal means with the LSD method. The relationships between age and the degree of DNA damage were determined via the Pearson correlation. A level $p<0.05$ was considered to be statistically significant.

3. RESULTS

3.1. APOPTOSIS IN ALZHEIMER'S LYMPHOCYTES

Lymphocytes from CNT and AD subjects which were cultured for 20 h without UVB irradiation underwent apoptosis to only a minimal degree (less than 4%) (Table 3). This spontaneous apoptotic death was considered to be the baseline. In this non-irradiated state, no significant difference was observed between the two groups (p=0.84) as regards cell death.

In both groups, the rate of UVB-induced cell death was enhanced to an extent depending on the intensity of the exposure (Table 3). However, the AD lymphocytes underwent apoptosis to a significantly (p<0.0001) lesser degree than did the CNT lymphocytes at each dose. Flow cytometric histograms representing results on one AD and one CNT proband are shown in Fig. 3. Within both groups, there were significant differences between the apoptotic values at the UVB intensities of 100 and 200 mJ/cm^2 (p_{CNT}=0.001, p_{AD}=0.0001), but no differences were found between the 200 and 300 mJ/cm^2 doses (p_{CNT}=0.978, p_{AD}=0.158). The differences in the apoptotic values between the two groups were dependent on the dose applied ($p_{interaction}$<0.0001).

There was no significant correlation between the degree of apoptosis and the age of the subjects (correlation coefficient (r)=0.059 [p=0.569]). A significant, but weak negative relationship was found between the apoptotic values and the severity of dementia (r of MMSE scores=0.202 [p<0.05]). There was an approximately 22% inter-experimental variation in the apoptotic values (data not shown).

Table 3. Degree of UVB-induced apoptosis in AD patients and their corresponding CNT probands (mean ± SD)

Dose of irradiation (mJ/cm^2)	CNT (n=12) Apoptosis (% of T cells)	AD (n=22) Apoptosis (% of T cells)	Significance (CNT versus AD) p value
0	3.14 ± 1.70	3.81 ± 1.82	0.840
100	55.52 ± 9.42	35.04 ± 8.68	0.0001
200	69.40 ± 6.87	51.76 ± 9.48	0.0001
300	69.51 ± 9.23	55.81 ± 9.76	0.0001

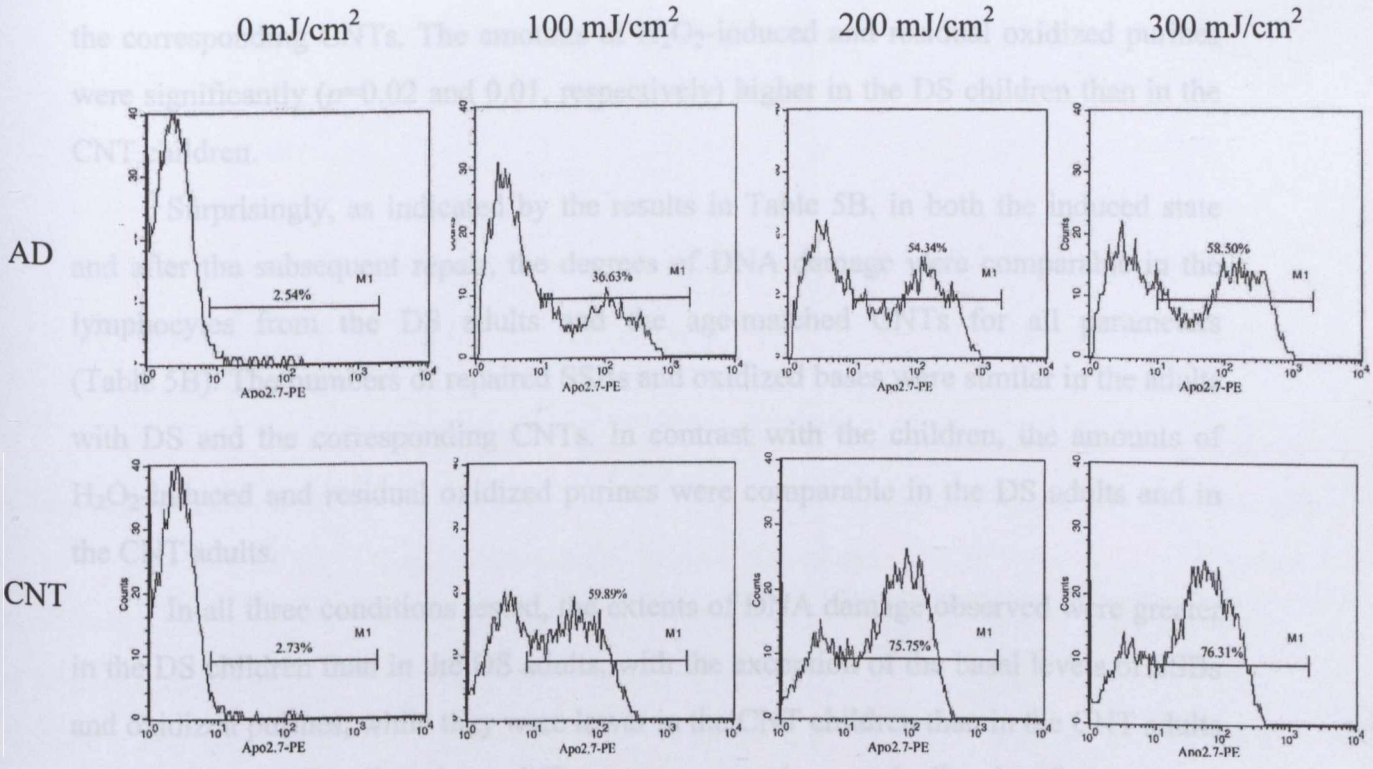


Fig. 3. Representative profiles of apoptotic T lymphocytes of an AD and a CNT proband, assessed by flow cytometry. All of the histograms show only CD3-positive cells. The percentage of apoptotic T lymphocytes can be seen in the M1 region (compared to the total number of T lymphocytes).

3.2. Oxidative DNA damage in Down's lymphocytes

In the routine hematological tests, no clinically significant differences were found between the DS and CNT groups as concerns the parameters examined, e.g. the differential and total numbers of lymphocytes.

In the basal state, as revealed in Table 4, with the exception of the number of SSBs, significantly and age-independently (i.e. the interactions of the groups (DS or CNT) and the age categories were non-significant) elevated numbers of SSBs and oxidized bases (pyrimidines and purines) were demonstrated in the nDNA of the lymphocytes in the overall DS group as compared with the healthy CNT group.

In both the stress-induced state and after the repair period, pairwise comparisons demonstrated markedly elevated DNA injury levels in the DS children with respect to the age- and gender-matched CNTs as concerns all the parameters examined (Table 5A, Fig. 4). The levels of repaired SSBs and oxidized bases were similar in DS children and

the corresponding CNTs. The amounts of H_2O_2-induced and residual oxidized purines were significantly (p=0.02 and 0.01, respectively) higher in the DS children than in the CNT children.

Surprisingly, as indicated by the results in Table 5B, in both the induced state and after the subsequent repair, the degrees of DNA damage were comparable in the lymphocytes from the DS adults and the age-matched CNTs for all parameters (Table 5B). The numbers of repaired SSBs and oxidized bases were similar in the adults with DS and the corresponding CNTs. In contrast with the children, the amounts of H_2O_2-induced and residual oxidized purines were comparable in the DS adults and in the CNT adults.

In all three conditions tested, the extents of DNA damage observed were greater in the DS children than in the DS adults, with the exception of the basal levels of SSBs and oxidized purines, while they were lower in the CNT children than in the CNT adults (Tables 5A and B). But these differences were only marginally significant or not significant (p=0.15-0.93 and 0.06-0.26, respectively), with the exception of the amounts of residual SSBs and oxidized purines in the CNT group (p=0.04), probably because of the relatively small number of children who participated. The extents of repaired SSBs and oxidized bases were similar in all four categories. Further data analysis failed to reveal significant differences between the children and adults in either the DS or the CNT groups as regards oxidized bases.

The effect of gender was not found to be statistically significant (p=0.59). The extent of oxidative DNA damage of the lymphocytes did not correlate with the age of the subjects for any of the conditions and parameters used ($|r|$=0.008-0.16 [p=0.27-0.96]).

Table 4. Degree of oxidative DNA damage in lymphocytes of CNT and DS individuals (children and adults together)

Conditions examined	CNT (n=25) % tail DNA values	DS (n=25) % tail DNA values	Significance (CNT versus DS) p^a value	Significance of interaction p^b value
Basal SSBs	8.7 ± 0.4	9.1 ± 0.6	*0.42*	0.53
Basal SSBs + EndoIII sites	9.8 ± 0.5	11.2 ± 0.6	*0.04*	0.37
Basal SSBs + Fpg sites	11.2 ± 0.5	13.2 ± 0.7	*0.02*	0.66
Induced SSBs	26.0 ± 1.6	29.0 ± 1.2	0.04	*0.07*
Induced SSBs + EndoIII sites	29.0 ± 1.6	33.0 ± 1.4	0.02	*0.06*
Induced SSBs + Fpg sites	32.3 ± 1.6	37.5 ± 1.8	0.007	*0.07*
Residual SSBs (after repair)	19.0 ± 1.2	21.9 ± 1.1	0.02	*0.06*
Residual SSBs + EndoIII sites	21.0 ± 1.2	23.6 ± 1.2	0.02	*0.02*
Residual SSBs + Fpg sites	25.3 ± 1.3	29.7 ± 1.4	0.004	*0.02*

[a]Two-way ANOVA

[b]Interaction of the groups (CNT and DS) and the age categories (children and adults)

The statistically meaningful p values are given in italics; the others are presented for completeness (cf. Section 2.3).

$p<0.05$ was considered statistically significant

DNA damage values are expressed as means ± SEM

Table 5. Degree of oxidative DNA damage in lymphocytes of CNT and DS children (A) and adults (B).

Pairwise comparisons were performed on estimated marginal means with the LSD method

A

Conditions examined	% tail DNA values in children		Significance
	CNT	DS	(CNT versus DS)
	(n=7)	(n=7)	*p* value
Basal SSBs	8.4 ± 0.5	9.6 ± 1.4	0.40
Basal SSBs + EndoIII sites	9.4 ± 0.4	11.9 ± 1.4	0.09
Basal SSBs + Fpg sites	10.5 ± 0.3	13.1 ± 1.5	0.12
Induced SSBs	23.0 ± 1.2	31.7 ± 2.3	*0.02*
Induced SSBs + EndoIII sites	25.3 ± 1.2	35.7 ± 2.2	*0.01*
Induced SSBs + Fpg sites	28.3 ± 1.8	40.7 ± 2.6	*0.008*
Residual SSBs (after repair)	16.0 ± 1.3	24.0 ± 2.1	*0.01*
Residual SSBs + EndoIII sites	17.5 ± 1.3	26.3 ± 2.1	*0.007*
Residual SSBs + Fpg sites	21.0 ± 1.5	32.6 ± 2.3	*0.002*

B

Conditions examined	% tail DNA values in adults		Significance
	CNT	DS	(CNT versus DS)
	(n=18)	(n=18)	*p* value
Basal SSBs	8.8 ± 0.6	9.0 ± 0.6	0.87
Basal SSBs + EndoIII sites	10.0 ± 0.6	11.0 ± 1.0	0.29
Basal SSBs + Fpg sites	11.5 ± 0.7	13.3 ± 1.2	0.08
Induced SSBs	27.2 ± 2.1	27.9 ± 1.4	*0.76*
Induced SSBs + EndoIII sites	30.5 ± 2.1	31.9 ± 1.7	*0.58*
Induced SSBs + Fpg sites	33.9 ± 2.1	36.3 ± 2.2	*0.40*
Residual SSBs (after repair)	20.2 ± 1.5	21.0 ± 1.4	*0.59*
Residual SSBs + EndoIII sites	22.4 ± 1.5	22.6 ± 1.4	*0.91*
Residual SSBs + Fpg sites	26.9 ± 1.5	28.6 ± 1.7	*0.62*

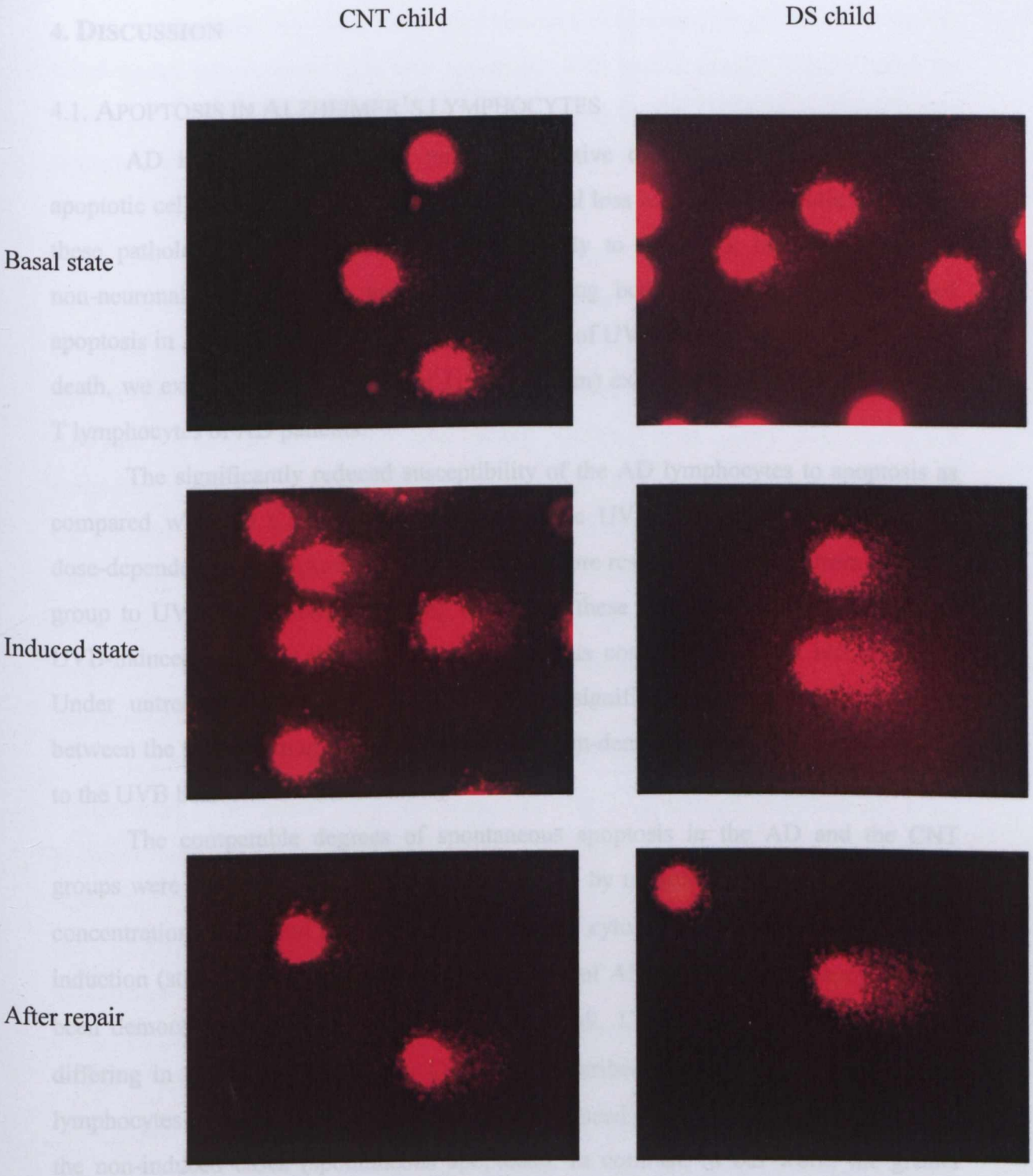


Fig. 4. Representative images of the extents of SSBs of lymphocytes from a CNT and a DS child before and after the addition of H_2O_2, and the following repair, observed by means of comet assay method

4. DISCUSSION

4.1. APOPTOSIS IN ALZHEIMER'S LYMPHOCYTES

AD is the most common neurodegenerative disorder of the elderly where apoptotic cell death may play a role in the neuronal loss leading to dementia. However, these pathological processes are not limited only to the CNS, but also appear in non-neuronal tissues. Since there is an emerging body of evidence of increased apoptosis in AD in the periphery and of the ability of UVB light to trigger apoptotic cell death, we examined the influence of UVB (308 nm) exposure on the peripheral blood T lymphocytes of AD patients.

The significantly reduced susceptibility of the AD lymphocytes to apoptosis as compared with the CNTs in consequence of the UVB treatment was found to be dose-dependent. T cells from AD subjects were more resistant than those from the CNT group to UVB irradiation, suggesting either that these cells responded abnormally to UVB-induced stress or that the AD-related events could render them less sensitive. Under untreated, non-irradiated conditions, no significant difference was observed between the patient group and the age-matched, non-demented CNTs in their sensitivity to the UVB beam.

The comparable degrees of spontaneous apoptosis in the AD and the CNT groups were similar to the results obtained earlier by measuring the intracellular Ca^{2+} concentrations [122] and the *in vitro* production of cytokines [69]. However, following induction (stimulation by mitogens), the tendency of AD lymphocytes to apoptosis has been demonstrated to be significantly elevated [69, 122]. Previous studies, although differing in methodology [33, 79, 112, 122], described an increased vulnerability of lymphocytes to death in AD, both/either in the induced (by apoptotic stimuli) and/or in the non-induced cases (spontaneous apoptosis). In contrast, in our work, the greater sensitivity to the apoptotic stimulus was detected in the CNTs, and not in the AD group. Under the non-induced conditions, the two groups displayed the same levels of susceptibility. Accordingly, the dose-dependent difference in vulnerability of the AD and the CNT lymphocytes could be the explanation of our findings. The hypothesis that the immune system in AD may be in an "activated state" [69] seems to be in harmony with this assumption, postulating the presence of a protective mechanism.

Interestingly, UV light could give rise to a decreased rate of cell death in AD lymphocytes, an observation not in agreement with earlier results. Others noted no significant differences in the numbers of chromatid breaks [100] or in the viability ratios [107]. These were reported in the lymphocytes of DS patients too [97]. AD fibroblasts also exhibited a UV-induced, unscheduled DNA synthesis and removal of pyrimidine dimers [10, 19] as compared with those from normal subjects. A possible interpretation of these differences may be methodological. In our experiments, a longer UV light wavelength (308 nm) and higher doses (100, 200 or 300 mJ/cm^2) were applied than in the experiments of the above authors (254 nm; 12 J/m^2 (=1.2 mJ/cm^2) and 0.08 J/m^2). Furthermore, all of them except Robbins et al. [107] investigated the apoptotic signals at molecular levels, whereas we examined the appearance of apoptosis. On the other hand, Robbins et al. [107] and Otsuka et al. [97] employed Epstein-Barr virus-transformed peripheral blood B lymphocytes, which are generally considered to be more resistant to apoptosis. However, the parameters applied in our work have previously been demonstrated to be efficient in inducing apoptosis in Huntington's lymphocytes [53].

UVB light can cause apoptosis via various cellular signaling pathways. The predominant cause seems to be the induction of nDNA damage. Additionally, UVB exposure could activate cell surface death receptors and components of the signal transduction system. Moreover, UVB irradiation can induce the formation of ROS. All these pathways trigger the apoptotic program [65]. The DNA damage may be due to the upregulation of proapoptotic molecules such as p53, and the downregulation of antiapoptotic molecules such as Bcl-2, which is continuously produced in all peripheral T cells to provide resistance and to ensure survival. The expression of Bcl-2 was found to be higher in AD lymphocytes than in those of patients with multi-infarct dementia, which also correlates with the low dexamethasone sensitivity of AD lymphocytes [90]. Since this steroid drug has anti-inflammatory and immunosuppressive effects that are similar to those of UVB, this observation could be a possible explanation of our results, supporting the role of Bcl-2 in the UVB-induced DNA damage and in the presumption that the T cells of AD patients may become more resistant to stress.

Taken together, we have demonstrated a dose-dependent, reduced tendency of AD T lymphocytes to UVB light-induced apoptosis, suggesting at least two alternatives for the mechanism of cell death. Firstly, if our results reflect a deficit in apoptotic cell death pathways, then the decreased vulnerability must be regarded as an abnormal and harmful response of these cells to stress, leading to chronic inflammatory processes. Secondly, the lower sensitivity to UVB exposure could be considered a better response as a consequence of the increased activity of the immune system. This may be in agreement with the ongoing inflammatory events found in AD.

Although the method applied is rapid and reliable, it is expensive to use in the daily clinical practice, and the results observed did not promote our knowledge concerning the prevention or diagnosis of AD. Further experiments at a molecular level may be valuable to clarify these capabilities of the lymphocytes and hence their possible significance in the antiapoptotic treatment or immunotherapy of AD.

4.2. OXIDATIVE DNA DAMAGE IN DOWN'S LYMPHOCYTES

Since, lymphocytes from individuals with AD exhibited an enhanced degree of oxidative DNA damage [59, 82, 87], and DS serves as a unique natural human model with which to examine the potential role of ROS in the early development of pathobiochemical alterations leading to AD, we investigated the degree of oxidative nDNA damage in lymphocytes from children and adults with DS.

The lymphocytes from the DS children displayed enhanced DNA damage following OS induction as compared with the corresponding CNTs, whereas no difference was found between the DS adults and the age-matched CNTs. This result may reflect an age-dependent sensitivity of lymphocytes to *in vitro* H_2O_2-induced OS in DS, indicating that the lymphocytes are more vulnerable to OS in DS children than in CNT children. Moreover, our data demonstrated age-independent elevated levels of oxidative DNA damage of the DS lymphocytes in the basal state (Table 4), which may serve as further evidence of the presence of increased *in vivo* endogenous OS in the periphery, confirming that the OS is manifested throughout the whole DS organism, from the brain to the blood or urine [58, 88, 95, 101, 150]. This observation is similar to earlier ones on AD leukocytes [59, 82, 87], reinforcing the relationship between these

two disorders. The repair of the induced oxidative damage was similar in the DS and the CNT individuals, pointing to an age-independent repair capacity and a plausible difference between DS and AD as regards the rate of repair of oxidative DNA damage.

In the basal state, the numbers of SSBs and oxidized bases were found to be heightened in the DNA of the lymphocytes from the overall DS group as compared with the overall CNT group, with the exception of the number of SSBs, regardless of age (Table 4). To the best of our knowledge, a significantly elevated number of strand breaks has been reported in only a single comet assay study on lymphocytes from newborns with DS [74]. The discrepancy between the observations is probably due to methodological differences. Although the relationship between CNS and peripheral oxidative processes is poorly understood [84], the higher level of oxidative damage obtained here in DS lymphocytes in the resting condition (Table 4) may perhaps support the existence of enhanced systemic OS *in vivo*. The fact that there were no differences between the children and adults with DS as concerns the degrees of DNA injuries in these basal states may reflect a similar *in vivo* oxidative status of their lymphocytes.

Significantly enhanced levels of oxidative DNA damage were detected as regards all the parameters examined, and particularly the oxidized purines themselves, in both the OS-induced state and after the repair period, in the children with DS as compared with the age- and gender-matched CNTs (Table 5A). The extents of DNA damage were similar in the DS adults and the CNT adults (Table 5B), suggesting that the lymphocytes were more vulnerable to OS in the DS children than in the age-matched CNT group, although the *in vivo* milieu is considerably more complex than the *in vitro* conditions currently investigated.

The comparisons between the children and the adults failed to reveal marked differences, with the exception of the amounts of residual SSBs and oxidized purines in the CNT group, possibly because of the small number of children who participated in this cohort. Thus, our findings may indicate that young trisomic lymphocytes are more sensitive than older ones to OS. In harmony with this assumption, the susceptibility of DS lymphocytes to X-ray exposure, measured via chromosome aberrations, has been suggested to decrease with age [124]. Another possible interpretation of the lower sensitivity of the lymphocytes from the DS adults to OS may be that these cells have become more resistant, i.e. they are "survivor" lymphocytes selected during aging,

explaining why these DS patients could live to adulthood. The cause of this finding may be that the proportion of patients with chronic medical problems in the present cohort was much higher in the children (3/7) than in the adults (4/18).

The levels of repaired SSBs and oxidized bases were the same in the different age categories, which may point to the similar and age-independent repair capacity of such damage in DS and CNT subjects. A defective NER pathway was earlier noted in DS lymphocytes [104], but BER, the main repair mechanism for the handling of oxidative DNA damage [115], has not been studied previously. In line with reported observations [82, 87], in all the states examined, purines seem to be more susceptible than pyrimidines to oxidative damage, and their repair is slower [55].

The results here on DS subjects in part resemble those previously found for AD lymphocytes or leukocytes [59, 82, 87]. This similarity may be explained by the elevated APP content of these cells, which may correspond to generalized alterations in the expression or processing of APP, supposing a novel determinant for the timing of AD onset [98]. Likewise, as measured by the production of pro-inflammatory cytokines, there is a partial impairment in lymphocyte functions in both aged DS patients and severely demented AD individuals [99]. Moreover, a decreased mitotic index of DS and AD lymphocytes in the presence of exogenous glutamine points to a lymphocyte dysfunction [102]. A reduced intracellular Ca^{2+} response of mitogen-induced T cells from AD and older DS individuals may strengthen the possibility of a close connection between DS and AD [43].

Taken together, the genomic DNA of the lymphocytes from DS patients has been found to exhibit a damaged oxidative status, regardless of age, providing further evidence of enhanced endogenous OS in DS and thereby strengthening the relevance of antioxidant therapy of this disorder. Moreover, an inverse relationship has been demonstrated between age and the sensitivity to OS in the case of DS lymphocytes, but their DNA repair capacity remains intact. These preliminary observations may not contribute to prevention strategies, simply because of the reverse manner and the similarity, respectively. Furthermore, although the comet assay is a beneficial method at an individual cell level, it is perhaps too expensive and time-consuming to become a routine diagnostic procedure.

4.3. LIMITATIONS

The major weakness of both studies is the small sizes of the cohorts examined.

A further limitation may be the under-representation of male subjects among both the AD patients and the CNTs. Despite this, the results seem to be robust. Additionally, to the best of our knowledge, the importance of gender has not been revealed as concerns apoptosis.

Our observations on DS patients have some further weaknesses:

The main limitation is the small number of children involved.

Moreover, we did not look at different subsets of lymphocytes and their ratios in the groups examined. The metabolic and immune activities of the cells were not checked. It was earlier demonstrated that the CD4+ T lymphocytes are the fraction with the highest induced level of spontaneous genetic damage [86]. Since the extent of DNA fragmentation can be increased during the cell subtype isolation procedure, which is time-consuming and the results of which are prone to artifacts [81], we decided not to subfractionate the cells in our experiment.

Furthermore, we did not assess dementia-related neuropsychological parameters in our adult DS patients, and we therefore cannot determine any connections as regards the existence and degree of dementia, for example. Most studies have shown that the onset of clinical dementia (if it develops [149]) occurs between the ages of 40 and 60 years [70] (mean age 56 years [135]), but only one participant fell within this interval in the present cohort.

Finally, the comet assay procedure allows only an overall picture of DNA oxidation, and we looked at nDNA, which is less extensively oxidized than mtDNA [142].

5. SUMMARY

I. **1.** It has been found that UVB radiation could induce apoptosis in T lymphocytes from AD and CNT individuals, confirming the observations of previous papers.

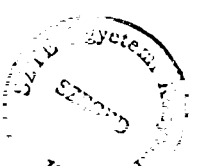
2. We have demonstrated a decreased level of apoptosis in T lymphocytes from AD patients to UVB light induction relative to CNT subjects, indicating either a defective apoptotic pathway or a better response of these cells to stress.

3. It has been reported that the extent of cell death was dose-dependent in both the AD and CNT lymphocytes: larger doses triggered greater degrees of apoptosis, with the exception of the largest dose applied.

II. **1.** Our data demonstrated elevated levels of basal oxidative damage of nDNA of lymphocytes from the DS subjects as compared with the CNTs, regardless of age, providing further evidence of the presence of increased *in vivo* endogenous OS in the periphery in DS.

2. The lymphocytes from the DS children exhibited elevated DNA damage following OS induction as compared with the corresponding CNTs, whereas no difference was found between the DS adults and the age-matched CNTs, reflecting an age-dependent sensitivity of the lymphocytes to *in vitro* H_2O_2-induced OS in DS.

3. The repair of the induced oxidative damage was similar in all the DS and the CNT individuals, pointing to an age- and disease-independent repair capacity.



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8. APPENDIX