DNA DAMAGE AND REPAIR IN HUMAN LYMPHOCYTES: RELATIONSHIP TO AGING AND ALZHEIMER'S DISEASE

STELLINGEN

1

Gezien het lage aantal "achtergrondbreuken" in het DNA van humane witte bloedcellen is het onwaarschijnlijk dat DNA-breuken een rol spelen tijdens de differentiatie van deze cellen.

Johnstone AP en Williams GT (1982) Nature, 300, 368-370

Dit proefschrift

2

DNA-polymerase ß is niet als enige DNA-polymerase betrokken bij het zogenaamde "snelle" herstel van DNA-breuken in humane witte bloedcellen na behandeling met gamma-stralen.

Collins A (1987) Int. J. Radiat. Biol., 51, 971-983

Dit proefschrift

3

Niet elk DNA herstelsysteem vertoont een hogere activiteit in delende cellen in vergelijking tot niet-delende cellen.

Sirover MA (1990) In: Milo GE and Casto BC (Eds.), Transformation of human diploid fibroblasts, CRC Press, Boca Raton, pp. 29-54

Dit proefschrift

4

In sommige patïenten ligt mogelijk een defect DNA-glycosylase ten grondslag aan de ontwikkeling van de familiaire vorm van de ziekte van Alzheimer.

Dit proefschrift

5

De term DNA-herstel impliceert een terugkeer naar een volledig herstelde status die door de meeste DNA-hersteltechnieken niet kan worden aangetoond.

6

Neuropsychologische studies verschaffen meer informatie over de werking van de hersenen dan neuro-anatomische studies.

7

Veroudering is een ziekte en geen aspecifieke degeneratie.

8

De ontwikkeling van een efficiënte markteconomie gaat altijd gepaard met een gelijktijdige groei in wetenschappelijke en technologische kennis, terwijl de omgekeerde situatie niet altijd het geval hoeft te zijn.

9

De bewering dat de werking van het menselijk brein kan worden voorgesteld door een algoritme houdt er geen rekening mee dat elk formeel wiskundig systeem een stelling bevat waarvan het waarheidsgehalte alleen door menselijk inzicht kan worden bepaald.

10

Dromen zijn geen bedrog.

Stellingen behorende bij het proefschrift
"DNA damage and repair in human lymphocytes:
Relationship to aging and Alzheimer's disease"
Michael E.T.I. Boerrigter
22 januari 1992

DNA DAMAGE AND REPAIR IN HUMAN LYMPHOCYTES: RELATIONSHIP TO AGING AND ALZHEIMER'S DISEASE

Proefschrift

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR. L. LEERTOUWER, HOOGLERAAR IN DE FACULTEIT DER GODGELEERDHEID, VOLGENS BESLUIT VAN HET COLLEGE VAN DEKANEN TE VERDEDIGEN OP WOENSDAG 22 JANUARI 1992 TE KLOKKE 15.15 UUR

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Aan mijn ouders

Voor Raymond

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CHAPTER 1
GENERAL INTRODUCTION
DEFECTIVE DNA REPAIR AS A POSSIBLE CAUSE OF ALZHEIMER'S DISEASE: A REVIEW

1.1 Introduction

Alzheimer's disease (AD) is a complex progressive, degenerative brain disorder characterized by an insidious loss of memory and other cognitive functions such as language (aphasia), motor skills (apraxia), and perception (agnosia). The relentless deterioration in AD usually results in death 5-10 years after onset [McKhann et al., 1984]. AD is but one of more than 70 disorders that may produce dementia. Other disorders include vascular disease, brain tumors, multiple strokes, heavy-metal toxicity, manic-depressive disorder, and other neurodegenerative disorders such as Parkinson's disease and Huntington's disease [Katzman, 1986]. However, AD accounts for more than 50% of all dementia cases and is thus the most common cause of a progressive cognitive dysfunction in individuals.

On the basis of the standard methods of examination, which include neurologic, psychiatric and clinical evaluations, together with a detailed medical history, a diagnostic accuracy of about 90% may be achieved [Sulkava et al., 1983; Wade et al., 1987; Joachim et al., 1988]. Recent epidemiological data on the prevalence of clinically diagnosed AD suggest that it affects 5-10% of individuals over 65 years of age and perhaps as many as 40% in those persons over the age of 85 [Evans et al., 1989]. It is becoming increasingly clear that the impact of AD on public health care will be augmented with increasing longevity of the population.

The major morphologic changes characteristic for the AD brain (on which present post-mortem diagnosis is based) include loss of neurons and the presence of neuritic plaques and neurofibrillary tangles. Neuritic plaques consist of a central core of amyloid surrounded by numerous swollen degenerating nerve endings and glial cells. Neurofibrillary tangles are made up of paired helical filaments accumulated within neurone perikarya, often extending into the axon and dendritic processes. Cell loss, neuritic plaques and neurofibrillary tangles occur primarily in the cerebral cortex, hippocampus and amygdala, and in a limited number

of subcortical areas, particularly the basal nucleus, the locus coerulus, the dorsal raphe, and parts of the hypothalamus [Mann, 1985].

Among the best documented abnormalities in AD brain are changes in oxidative metabolism. There is a generalized decrease in cerebral blood flow, oxygen and glucose utilization which can be observed with positron emission tomography [Frackowiak et al., 1981; Foster et al., 1984; Duara et al., 1986]. These changes are most marked in those parts of the brain that are most affected by the AD process and they can, indeed, be correlated with the neuropsychological changes [Foster et al., 1986]. Other radiological imaging techniques which yield information about brain morphology and physiology include computed tomography (CT) and magnetic resonance imaging (MRI). CT is used to assess ventricular volume and cortical density whereas MRI can visualize local physiological alterations in abnormal brain tissue. MRI is thought to be more sensitive than CT in detecting the presence of multiple strokes and in delineating atrophic cortex [Erkinjuntti et al., 1984; for a review, see Hollander et al., 1986]. The brain uses some 20% of oxygen uptake of the body and is extremely sensitive to interruption of oxidative metabolism by decreased availability of oxygen and/or glucose for only a few minutes. Any disorder that leads to defective (brain) oxidative metabolism is likely to impair cellular function and to present itself clinically as a neurological or psychiatric disease. Traumatic head injury with impairment of consciousness, which might conceivably contribute to neuronal loss and damage, has been found to be positively associated with AD [Heyman et al., 1984; Mortimer et al., 1985; Graves et al. 1990; Amaducci et al., 1986; Chandra et al., 1987a; Ferini-Strambi et al., 1987; Shalat et al., 1987] but only the first three found a statistically significant association. Three other epidemiologic studies failed to find a relation between head trauma and AD [Soininen et al., 1982; Chandra et al., 1987b; Ferini-Strambi et al., 1990].

The most consistent finding in neurochemical studies of AD brains is a large reduction of choline acetyltransferase (CAT) activity in cerebral cortex and hippocampus [for reviews, see Perry, 1986; Price, 1986]. This decline is due to selective degeneration of basal forebrain cholinergic neurones projecting from the basal nucleus to cortex, and from the septum and diagonal band of Broca to the hippocampus [Whitehouse et al., 1982; Pioro et al., 1984]. The decreased activity of CAT results in a reduced availability of the

neurotransmitter acetylcholine, thereby impairing neuronal functioning. Clinicopathological comparisons indicate a high degree of correlation between the reduction in CAT activity and, both, numbers of neuritic plaques and scores on cognitive and behavioral rating scales [Perry et al., 1978].

Acetylcholine is, however, not the only neurotransmitter which is present at reduced concentrations. Cortical levels of somatostatin [Davies et al., 1980; Rossor et al., 1980] and somatostatin receptors [Beal et al., 1985] have been reported to be reduced in AD brains. Additionally, serotonin concentrations are markedly reduced but are apparently not related to disease severity [Gottfries et al., 1983; Arai et al., 1984]. A variety of other neurotransmitters, including dopamine, glutamate, gamma-aminobutyric acid, noradrenalin and aspartate and the neuropeptides substance P, cholecystokinin, vasoactive intestinal peptide, neuropeptide Y and neurotensin have not been found to be consistently reduced in brain samples of AD patients [for references, see Lauter, 1985; Hollander et al., 1986; Katzman, 1986].

Both environmental and genetic causes of this disease are mentioned in the literature [Katzman, 1976; Amaducci et al., 1986; Katzman, 1986]. A viral etiology has been proposed because of the similarities between AD-type dementia and Creutzfeldt-Jakob disease, although no definite evidence has been obtained concerning the transmissibility of AD-type dementia [Goudsmit et al., 1980; Masters et al., 1981].

The absence of "classical" viral infection in AD has led to the suggestion that these diseases may belong to the so-called class of "prion diseases". These include Kuru, Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler syndrome (GSS) [for a recent review see Gabizon and Prusiner, 1990]. The term "prion" was introduced to distinguish the transmissible agent from viruses and viroids and stems from proteinaceous infectious particle [Prusiner, 1982]. Studies of the molecular structure of prions in highly purified preparations indicate that they contain one major protein "PrP", which is required for infectivity [McKinley et al., 1983]. PrP is encoded by a chromosomal gene and it represents an abnormal isoform of a highly conserved cellular protein [Gabizon and Prusiner, 1990]. The presence of PrP now distinguishes transmissible dementias from other neurodegenerative diseases such as AD and is regarded as a diagnostic criterium for spongiform encephalopathies. The incidence of prion diseases is thought to be low as compared to AD,

but may be underdiagnosed, as was recently suggested by Collinge et al. [1990], due to clinical mimicry of prion diseases leading to misdiagnosis as AD in the absence of diagnostic features of CJD and GSS. On the other hand, Will estimated that clinical recognition of CJD and GSS is very high [Will, 1990]. Nevertheless, the PrP gene product might play a role in the development of a variety of neurodegenerative diseases, including AD, perhaps by increasing the susceptibility for amyloidosis, i.e. the deposition of amyloid fibrils in the brain. In this respect, the recent development of mice, transgenic for the hamster PrP gene, which display characteristic hamster PrP amyloid plaques and spongiform degeneration of the neuropil, will be of great value in determining the possible influence of "viral (or prion)-induced" amyloidosis in AD [Scott et al., 1989].

An environmental factor which has been proposed to be involved in the etiology of AD is intoxication by aluminum [Nikaido et al., 1972; Crapper et al., 1978; Perl and Brody, 1980; Birchall and Chappall, 1988]. Long-term exposure to aluminum via renal dialysis results in the development of encephalopathy [Wills and Savory, 1983], but the detailed pathological manifestations of AD differ from those of dialysis encephalopathy. Although aluminum has been identified within diseased neurons in brains of AD patients [Perl and Brody, 1980], a causal relationship need not be present since the ability to concentrate metals from their immediate environment might be a secondary characteristic of already diseased tissue.

A major impetus for a putative role of a neurotoxin in age-related dementias has been given by the discovery of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) which is metabolized to 1-methyl-4-phenylpyridine (MPP^+) by brain monoamino oxidase B [Markey et al., 1984]. MPP^+ confers a neurotoxicity to dopaminergic neurons owing to its active uptake by these neurons [Chiba et al., 1985; Javitch et al. 1985]. Exposure to MPP^+ results in clinical manifestations similar to those observed for Parkinson's disease [Ballard et al., 1985].

A selective action of certain neurotoxic agents may also result from differences in the metabolic activation of endogenous and exogenous chemicals due to a differential distribution and/or specificity of mixed function oxidases and multiple forms of cytochrome P-450 [Dhawan et al., 1990]. Interindividual variation in the activation of chemicals, stemming from different genotypes, may render some individuals particularly sensitive to the

pernicious effects of these substances [Autrup, 1990; Lu et al., 1990].

Others have surmised a role for immunopathology in the causation of AD. This was based on the findings that some HLA haplotypes are more prevalent in AD [Henschke et al., 1978; Walford et al., 1980] although this could not be confirmed [Reed et al., 1983]. Some evidence has been obtained for altered distribution of complement factors C3 [Mehne et al., 1976; Giometto et al., 1988] and C4 [Nerl et al., 1982; Hulette and Walford, 1987; Giometto et al., 1988], although this was not confirmed in other studies [Eikelenboom et al., 1984, 1988].

The description of several large families in which the occurrence of the disease is consistent with the pattern of transmission of an autosomal dominant gene (familial AD) justifies the assumption that at least in these cases AD is caused by a genetic defect [Goudsmit et al., 1981; Nee et al., 1983; Foncin et al., 1985]. The occurrence of most AD patients as single, sporadic cases in families, with a disease phenotype indistinguishable from that of familial AD with respect to clinical, pathological and biochemical features, may then reflect the incomplete penetrance of an autosomal dominant gene due to premature deaths of the ancestors or due to the modulation by environmental factors [Davies, 1986].

In the preceding paragraphs several environmental and genetic factors thought to be involved in the etiology of AD were addressed. To date, the causal relationship between these risk factors and the development of AD remains obscure. Possibly, both genetic and environmental factors determine the expression of the AD phenotype in both the genetic cases (familial AD) and the general population (sporadic AD). It is not inconceivable that the expression of a genetic defect is modulated by environmental factors. One particular hypothesis which combines these two aspects assumes the presence of a systemic genetic defect in the repair of chromosomal DNA damage in cells from AD patients. The manifestation of such a defect, i.e. decreased cellular survival as a consequence of the accumulation of DNA damage, will predominantly occur in neuronal cells because of the ubiquitous presence of DNA-damaging agents (e.g. oxygen radicals) in these cells. In the following paragraphs the present knowledge on the genetic basis of AD and its clinical manifestation as a systemic illness are summarized. The relevance of DNA-repair deficiencies to the etiology of AD is discussed within the broader context of aging and age-related diseases, and available data from the literature on an altered DNA-damage

metabolism in AD are presented. Finally, the impact of defective DNA repair as a possible cause of AD on current therapeutic approaches will be discussed.

1.2 Alzheimer's disease as a systemic disease

1.2.1 Genetics of Alzheimer's disease

The existence of a genetic basis for at least a portion of AD, termed familial AD (FAD), can be inferred from epidemiologic and molecular genetic studies. Epidemiologic family studies have generally found a familial incidence of 50% among relatives of AD probands compared to a 10% risk in relatives of controls, indicative of the expected segregational pattern of an autosomal dominant mutation with age-dependent expression [for reviews, see Rocca and Amaducci, 1988 and St George-Hyslop et al., 1989]. Although familial aggregation of AD has been shown to occur in these families, most pedigrees did not contain sufficient numbers of affected individuals in multiple generations in order to firmly establish the existence of an autosomal dominant disorder in that specific pedigree. Therefore, the presence of several etiologic subgroups of AD, either of genetic and/or environmental origin, cannot be excluded on the basis of these case control studies.

Some epidemiologic evidence implicates late maternal age at the subject's birth, head injury and thyroid disease as possible risk factors for AD [reviewed by Rocca et al., 1986] but no clear consensus has been reached. A recent population study of more than 5,000 subjects in China reported that increasing age, female gender, and low education were highly significant and independent risk factors for dementia. AD accounted for 65% of the subjects with dementia, while vascular dementia, including multi-infarct dementia, was the second most common type of dementia [Zhang et al., 1990]. Survey studies, which may be better suited to determine the risk of AD in relatives of affected probands, have confirmed only one important risk factor (apart from age) for AD: its tendency to occur repeatedly within certain families. A strong association between incidence in first degree relatives and affected probands was found in cases with an early (< 65 years of age) onset of AD [Heston et al., 1977; Heston et al., 1981; Whalley et al., 1982; Heyman et al., 1983; Amaducci et al., 1986; Sobel et al., 1988; Hofman et al., 1989; Farrer et al., 1990]. In

contrast, no measurable increase in the reported occurrence of dementia in the first degree relatives of AD cases with late onset of illness (after 70 years) has been reported [Heston et al., 1981; Chandra et al., 1987b].

Breitner et al. [1986a, 1986b], using the phenotype amnesia with aphasia and apraxia (*aaa*) as a clinical marker to identify genetic AD, developed a biomathematical model from the empirical age-specific incidence of AD in relatives. According to the model, all cases with the *aaa* phenotype are probably genetically based, but there is also a substantial possibility of genetic illness in virtually all pedigrees without affected relatives. Because the model estimated the mean age of onset in relatives at 83.2 years, most genetically predisposed relatives of AD probands would escape the disease by dying of other causes. Furthermore, age of onset in unselected AD probands does not follow a bimodal distribution which would suggest that two conditions were present [Kay, 1986] although Farrer et al. and Mayeux et al. showed evidence for a bimodal distribution of age-at-onset in 71 kindreds with FAD and 121 unrelated cases, respectively [Mayeux et al., 1985; Farrer et al., 1990]. The bimodal distribution in early- and late-onset FAD in the study by Farrer et al. [1990] was attributed to the occurrence of a proportion of AD caused by a dominant gene, most notably in early-onset cases, whereas co-occurrence of late-onset FAD may be a consequence of shared polygenes or environmental factors.

Another possible way to assess the genetic basis of AD is comparison of concordance rates for monozygotic and dizygotic twins. Although concordant monozygotic and dizygotic twin pairs have been reported [Jarvik et al., 1980; Cook et al., 1981; Embry and Lippmann, 1985; Rocca et al., 1986; Nee et al., 1987], some investigators have also observed widely discordant ages-at-onset [Cook et al., 1979; Embry and Lippmann, 1985; Nee et al., 1987; Creasey et al., 1989]. These data suggest that not all AD results *only* from the effects of a *fully* penetrant autosomal dominant disorder, but that environmental factors may modulate the penetrance and expression of the AD gene(s).

It has been known for a long time that patients with Down's syndrome (DS; trisomy 21) who survive past 40 years develop an AD-like pathology [Mann, 1985]. Several groups have investigated the risk of acquiring AD in relatives of DS cases. In two epidemiologic studies the frequency of DS among relatives of AD patients with early onset appeared to be significantly higher than among controls, or than expected in the general population [Heston

et al., 1981; Heyman et al., 1983] but this could not be confirmed by three other family studies [Whalley et al., 1982; Nee et al., 1983; Berr et al., 1989]. Pathological characteristics in DS patients at middle age (i.e the distribution of neuritic plaques and neurofibrillary tangles and the pattern of atrophy of neuronal systems) are qualitatively the same as those of AD at that age, although slight quantitative differences do occur [Mann, 1988]. However, development of dementia in DS patients after the age of 40 years has still not been confirmed. Although some investigators have described clinical characteristics, which are thought to reflect the development of AD in adult DS patients [Dalton et al., 1974; Wisniewski et al., 1985; Lai and Williams, 1989; Percy et al., 1990], the underlying mental retardation will almost certainly obscure the clinical manifestation of changes due to AD. Nevertheless, considering the similarities between DS and AD, the characterization of neuropathological changes, such as neuritic plaques and neurofibrillary tangles in the brains of DS patients might give us some insight into the early pathological changes in AD.

Genetic linkage analysis using anonymous DNA markers has been applied to locate a genetic region on the long arm of chromosome 21 which cosegregates with the disease phenotype [St George-Hyslop et al., 1987; Goate et al., 1989]. The chromosomal localization of the gene for amyloid ß protein precursor (APP) to a region on chromosome 21 which does not show close linkage with FAD [Van Broeckhoven et al., 1987; Tanzi et al., 1987], excludes this gene as a candidate for the primary genetic defect in some FAD families with early onset and autosomal dominant inheritance and late-onset FAD families. However, Goate et al. recently reported that a mutation within the gene for APP segregates with the disease in two early-onset FAD families [Goate et al., 1991]. This suggests that some cases of hereditary AD could be caused by mutations in the APP gene. Nevertheless, one may speculate that the disease locus in FAD interacts in one way or another with either the APP gene or its product. This could for instance lead to aberrant APP cleavage resulting in the accelerated deposition of amyloid ß protein (AßP) in the brain, as has been suggested by Esch et al. [1990]. An increased transcription of APP in DS, because of an elevated gene dosage, may cause an increased alternative cleavage of APP into AßP leading to the early ß-amyloid deposition in so-called "diffuse" or "preamyloid" plaques. This deposition may precede the development of neurite-containing plaques and neurofibrillary tangles [Giaccone et al., 1989; Rumble et al., 1989] as observed in AD.

The observed widespread deposition of amyloid protein in the brains of non-demented elderly persons [Davies et al., 1988; Rumble et al., 1989] and within the cerebral cortex of certain patients with degenerative conditions such as supranuclear palsy, Huntington's chorea, and Parkinson's disease [Mastaglia et al., 1988; Tan et al., 1988; Mann and Jones, 1990] implies that the process of amyloid deposition within the brain may be a universal characteristic of aging. Nevertheless, the deposition of AßP into extracellular amyloid plaques becomes widely associated with neuronal degeneration and clinical symptomatology only in AD and DS. Tissue-specific distribution of the secreted and the transmembrane form of pre-amyloid proteins has been described in AD [Catteruccia et al., 1990] implicating different posttranslational modifications of these products, e.g. proteolysis, as being responsible for the tissue-specific accumulation of AßP in AD. Other compelling evidence for a role of amyloidogenic AßP deposition in the etiology of AD comes from recent studies on Dutch families which have an autosomal dominant form of hereditary cerebral hemorrhage with amyloidosis (HCHWA-D). Some patients develop cortical AßP plaques similar to the "diffuse" plaques in AD and DS [Duinen et al., 1987]. Linkage analysis has revealed a tight linkage of HCHWA-D to APP indicating that a mutation in the APP gene might be the primary defect in HCHWA-D [Van Broeckhoven et al., 1990]. Indeed, the detection of a point mutation which causes the substitution of a glutamine for glutamic acid only six amino acids downstream from the normal APP cleavage site in two of these Dutch patients implicates this alteration as a possible cause of the alternative, aberrant processing of APP leading to AßP formation [Levy et al., 1990]. Although no such mutations have been detected in AD cases, other genetic mechanisms may mediate the deposition of amyloidogenic AßP in AD. Unfortunately, the lack of sufficient numbers of multi-generational pedigrees with multiple affected members in all generations, together with the absence of more closely linked DNA markers, impedes the assignment of the genetic defect in FAD to a more precise location on chromosome 21. Moreover, the genetic linkage approach is encumbered by inaccuracies in the clinical diagnosis of AD, which is still a diagnosis of exclusion and can only be confirmed at autopsy.

As mentioned above, due to the relatively late onset of the disease in sporadic AD it is practically impossible to exclude a genetic component in these cases. Thus both FAD, characterized by a somewhat younger age of onset, and sporadic AD may be the result of

the same genetic abnormality [Gusella et al., 1984]. However, some studies provide evidence for the apparent existence of a second disease locus in some late-onset FAD cases [Pericak-Vance et al., 1988,1991; Roses et al., 1988; Schellenberg et al., 1988,1991]. Indeed, it is difficult to infer from the high frequency of AD a single dominant gene defect causing all the abnormalities in this disease. Biochemical heterogeneity would thus not be surprising, merely reflecting genetic heterogeneity.

A noteworthy observation is the age-dependent penetrance of the gene(s). The ever increasing incidence of AD with old age suggests pleiotropy of the gene(s) involved in this syndrome. Before and/or during the reproductive period of a species such pleiotropic genes might be advantageous for life but they could gradually exert their harmful effects with the onset of senescence. Harmful genes effective only after the reproductive period has ended, will not be selected against by natural selection as they do not have an effect on the "fitness" of the individual while it matters [Medawar, 1952; Williams, 1957; Charlesworth, 1980]. A genetic defect in AD would exist in all cells whereas the expression and functional consequences of this defect might vary from cell to cell dependent on its particular environment. AD may thus be considered a systemic illness which preferentially becomes manifest in brain cells owing to specific physiological circumstances, e.g. oxygen consumption, which is very high in brain cells [for reviews see Hollander et al., 1986 and Baker et el., 1988 and references cited therein].

1.2.2 Systemic manifestations in Alzheimer's disease

Among hereditary ataxias and a number of neurodegenerative disorders, including Huntington's disease and Down's syndrome, AD has been termed a secondary disorder of energy metabolism [Blass et al., 1988]. Such disorders are thought to be the result of derangements of energy metabolism which are secondary to some other insult but are still critical to the pathophysiology at the cellular level. It should be noted that syndromes with apparently similar clinical or even clinico-pathological phenotypes might arise from distinctively different genotypic abnormalities. Several neuropsychiatric illnesses, including Charcot-Marie-Tooth disease [Harding and Thomas, 1980], schizophrenia [Kennedy et al., 1988; Sherrington et al., 1998] and olivocerebellar ataxia [Jackson et al., 1977; Pedersen et

al., 1980] have multiple genetic etiologies. Conversely, different allelic mutations within a gene can lead to clinically distinct syndromes, e.g. sickle cell disease and ß-thalassemias which ensue from different genetic modifications in the hemoglobin gene [McKusick, 1986] or Duchenne and Becker muscular dystrophy which are caused by different deletions within the dystrophin gene [Brown and Hoffmann, 1988]. With respect to AD, different mutations within one gene or in genes with functionally similar end products might be manifested as both sporadic and familial cases with similar clinico-pathological criteria. In Down's syndrome (DS), the presence of an extra copy of the inferred aberrant gene(s) might be related to the universal occurrence of AD-like histopathology in those DS subjects who survive past the 4th decade of life.

Additionally, limited epidemiologic evidence points to familial aggregation of AD and Parkinson's disease [Amaducci et al., 1986; Hofman et al., 1989]. Parkinson's disease is a neurodegenerative disorder with similar neuropathologic characteristics as AD [Mayeux et al., 1985; Ditter et al., 1987]; it is thought to arise from a combination of oxidative stress generating free radicals and exposure to environmental toxins [Marsden, 1990]. Although far from definite, this is suggestive for a, possibly genetically determined, joint etiology of neurodegenerative diseases such as AD and Parkinson's disease.

Apart from the marked decreases in neurotransmitter availability and reduction of glucose and oxygen metabolism in specific parts of brains of AD patients, numerous abnormalities have been described in peripheral cells. Oxidative metabolism is functionally impaired in fibroblasts in vitro [Peterson and Cotman, 1987; Sims et al., 1987]. Superoxide dismutase (SOD) is encoded on chromosome 21 [Philip et al., 1978] and the presence of three copies of this gene results in an approximately 1.5-fold elevation of SOD activity in red blood cells of DS subjects [Pantelakis et al., 1970; Sinet et al., 1975; Frischer et al., 1981; Percy et al., 1990]. SOD catalyzes the conversion of superoxide radical (O_2.-) to hydrogen peroxide (H_2O_2) and oxygen [Fridovich, 1975]. Subsequently, glutathione peroxidase and catalase, which are present at normal levels in DS patients [Percy et al., 1990], catalyze the breakdown of H_2O_2 to water. The relative buildup of H_2O_2 due to an excess of SOD relative to the peroxidases is thought to be, at least partially, responsible for some of the manifestations of DS. This is supported by a recent report from Norris and Hornsby [1990] who indicated that increased in vitro expression of SOD in normal

differentiated bovine adrenocortical cells caused a cytotoxic effect involving cell death, cell fusion and nuclear fragmentation.

Percy et al. [1990] observed that the elevation in mean SOD-1 activity was more pronounced in DS patients without clinical manifestations of AD as compared to matched control individuals or DS patients with manifestations of AD, and concluded that a decreased antioxidant potential in red cells is a contributing factor to the development of AD in severely to profoundly retarded adults with DS.

Zubenko et al. recently reported an increased specific activity of cytochrome c reductase in the superior temporal and entorhinal cortex of AD patients, whereas cerebellum contained normal levels of this enzyme activity. These authors suggested that, since cytochrome c reductase is associated with smooth endoplasmatic reticulum (SER), the increased activity of this enzyme might be the result of an accumulation of SER due to a deficit in the regulation of SER biogenesis or turnover [Zubenko et al., 1990]. Increased membrane fluidity was found to be inherited within families with familial AD consistent with a fully penetrant autosomal dominant trait [Zubenko et al., 1987].

Evidence for a defective Ca^{2+} homeostasis in lymphocytes and fibroblasts of AD patients [Peterson et al., 1985; Peterson and Goldman, 1986; Gibson et al., 1987] has been reported. This might be related to (1) the decreased activity of Ca^{2+}-dependent protein phosphorylation in fibroblasts [Peterson and Cotman, 1987; Van Huynh et al., 1989] and brain homogenates [Cole et al., 1988]; (2) the altered response of AD fibroblasts to drugs that elevate cytosolic free calcium [Peterson et al., 1981]; and (3) the increased activity of calpain I, a Ca^{2+}-dependent neutral protease in AD cortex, as compared to controls [Nilsson et al., 1990]. Specific reductions of Ca^{2+}-binding protein (calbindin-D) gene expression have been observed in the hippocampus, nucleus raphe dorsalis and nucleus basalis of AD patients, but not in the neocortex or corpus striatum [Iacopino and Christakos, 1990]. The inability to bind free Ca^{2+} might render neuronal cells more susceptible to the initiation of Ca^{2+}-mediated cytotoxicity. Increased intracellular Ca^{2+} concentrations result in the dose-dependent release of glutamate from cerebellar granular cells which can trigger cell death by activation of the NMDA receptor [McCaslin and Smith, 1990], especially under energy-limiting conditions [Novelli et al., 1988].

Other, miscellaneous findings include the diminished secretion of cholinergic growth

factor in AD fibroblasts [Kessler, 1986], reduced levels of phosphatidylinositol in temporal cortex of AD brain samples [Stokes and Hawthorne, 1987] and an increased cyclic-AMP production after α-adrenergic stimulation of AD fibroblasts [Malow et al., 1989]. Red blood cells [Miller et al., 1986] and fibroblasts [Mokrasck, 1988] of AD cases display an aberrant transport of choline. Deficits in the correct functioning of components of signal transduction systems (i.e. CAMP, protein kinase C, Ca^{2+}) are thus strongly implicated in the pathogenesis of AD [see also Fowler et al., 1990].

The recent development of an autoradiographic technique capable of imaging phosphoinositide turnover in brain slices may permit the elucidation of the regulation of this second messenger system in normal and AD brain in response to cholinergic stimulation of specific neuronal pathways. For example, cholinergic stimulation affects phosphoinositide turnover in several areas of the hippocampus (CA1, CA3, CA4, and subiculum), whereas glutamanergic effects are only observed in CA3 and the subiculum [Hwang et al., 1990]. Additionally, Mufson et al. [1989] recently described a significant loss of nerve growth factor receptor-immunoreactive neurons in the Ch4 region of the basal forebrain in AD which inversely correlated with the duration of the disease process and may reflect neuron death.

Decreased energy metabolism might also result from mutations in the mitochondrial genome. Mitochondrial DNA mutations could result in a lowered efficiency of mitochondrial enzymes involved in energy metabolism. Mitochondrial encephalomyopathies, i.e. neurological syndromes characterized by proximal muscle weakness, include (1) progressive external ophthalmoplegia, (2) Kearns-Sayre syndrome [Moraes et al., 1989] and (3) Leber's hereditary optic neuropathy [Singh et al., 1989]. These 3 disorders are the consequence of a deletion and a specific point mutation, respectively, in the mitochondrial genome. The continuous generation of oxygen free radicals by the mitochondrial electron-transport chain and a possibly less efficient overall capacity in repairing DNA damage could result in the accumulation of deleterious mutations in mitochondrial DNA during the whole life of an individual [Brown et al., 1979]. This will gradually impair cellular energy metabolism and ultimately manifest itself as an age-related, late-onset degenerative disease in particular tissues of an organism.

Alternatively, a mitochondrial deletion may arise early in embryonic development and

clonally propagate under the influence of mechanisms that control mitochondrial accumulation [Shoubridge et al., 1990]. Evidently, mutant mitochondria will only be propagated if, in some way, the mutations confer a selective advantage on these mitochondria [Wallace, 1989]. Possibly, this might be effected by provoking mitochondrial replication owing to the cells' attempts to compensate for reduced oxidative potential [Grossmann, 1990]. This is possible since all factors for mitochondrial growth and replication are encoded by the nuclear genome and imported from the cytoplasm. The presumptive involvement of mitochondrial mutations in vivo aging is supported by the finding that the senescence mutant ER-3 of *Neurospora crassa* possesses a mixed population of defective and normal-respiratory mitochondria which is associated with both biochemical, genetic and ultrastructural mitochondrial lesions, including decreased growth rate, abnormal levels of cytochromes a, b and c, and decreased surface density of the mitochondrial inner membrane [Niagro and Mishra, 1990]. Additionally, mitochondria from old mammalian cells may be more sensitive to environmental stresses than those from young cells [Miquel et al., 1980], thereby increasing the likelihood of developing such a degenerative disease (e.g. AD) with increasing age.

The numerous abnormalities in both neuronal and peripheral cells of AD patients summarized in the preceding paragraphs suggest that more than one genetic defect is involved in the pathogenesis of AD. It is not inconceivable that the occurrence of one or more defects in AD influences the expression of pleiotropic genes in such a way that age-related changes which occur in healthy persons are augmented in neurodegenerative diseases such as AD.

1.2.3 Aging and Alzheimer's disease

The age-dependent increase of the prevalence of AD may be better understood in the context of some of the proposed evolutionary aspects of the aging process itself. Aging might be visualized as an inadvertent by-product of the process of natural selection, possibly due to the accumulation of specific harmful genes whose time of expression lay beyond the greater part of the lifespan normally encountered in natural populations [Medawar, 1952]. Williams used this notion to emphasize so-called pleiotropic genes which have benificial

effects early in life, but have adverse effects later [Williams, 1957]. This model is widely accepted among evolutionary biologists and has been given mathematical exactitude by Charlesworth [1980]. Although these hypotheses might clarify the importance of age in relation to the force of natural selection and show that selective control over the later portions of the lifespan should gradually diminish, they nevertheless don't explain the possible molecular basis of aging.

It should be realized that in most natural populations, individuals do not survive long enough to show obvious signs of aging. Individuals are subject to predation, disease or starvation. Therefore, the existence of those inaccurate metabolic processes, the harmful effects of which only manifest themselves in the last part of the lifespan of a species, will not be selected against by natural selection and, henceforth, not be eliminated from the population's progeny. Accordingly, age-related diseases such as cancer, artherosclerosis, cerebrovascular accidents, osteoporosis, diabetus mellitus and Alzheimer's disease will only become apparent in those species which outlive the rigors of nature, i.e. individuals of a species that ages.

The degenerative changes which are seen during senescence are ultimately the consequence of an organism's failure to preserve the functional integrity and viability of its organs, tissues and cells due to a decreased accuracy (of the regulation) of macromolecular synthesis. This, in its turn, is caused by the failure to maintain the integrity of key macromolecules such as DNA and RNA, and the inability to replace faulty by correct ones [Orgel, 1963, 1973; Kirkwood et al., 1984].

The evolvement of maintenance and repair processes during evolution is thought to be the effect of natural selection acting on populations in such a way that the progeny of individuals which are more resistant to macromolecular change are selected for and finally will take over the population. Individual organisms incapable of maintaining themselves would be continually eliminated. The selection of advantageous phenotypic changes in the adult may be achieved by increasing the efficiency of some specific enzymes, e.g. those involved in DNA repair. However, the overall metabolic cost invested in mechanisms dedicated to the maintenance of accuracy in macromolecular synthesis is limited. Natural selection will "prefer" suboptimal levels of accuracy in macromolecular synthesis thus ensuring that sufficient metabolic energy is available for maintaining the efficiency of basic

metabolic pathways [Kirkwood et al., 1984; Hastings, 1989].

For example, Bohman et al. [1984] showed that an increased fidelity of protein synthesis in the *str* A ribosome mutant of *Escherichia coli* resulted in a net reduction in growth rate, arising from dissipative energy expenditures associated with changed ribosomal proofreading activity and impaired kinetic efficiency of the translation apparatus. Thus, increased accuracy in macromolecular synthesis may only be realized at the cost of a significant increase in the amount of metabolic energy which is normally reserved for these processes. Consequently, the level of accuracy attained in each cell is expected to be less than the minimum that is necessary for complete fidelity in macromolecular synthesis. From these premises it should be possible to quantify the genetic relationships among traits such as energy storage pool sizes and activities of enzymes involved in energy metabolism and macromolecular maintenance, and begin to determine an association between these traits and components of fitness, e.g. viability and fecundity. A first study has shown significant genetic correlations between storage quantities of several metabolic pools in 83 lines of *Drosophila melanogaster* and several enzymes that were thought a priori to be relevant to these storage pools by their location in the metabolic pathways. Additionally, the data indicated significant correlations between the storage and enzyme activity traits, and viability and fecundity [Clark, 1989]. Genetic co-regulation of longevity and several enzymes involved in the detoxification of oxygen radicals has been found recently in *Neurospora crassa*
[Munkres, 1990] suggesting that the ability to maintain the integrity of the DNA is an important determinant of life span.

From the organism's point of view, actively transcribed genes are the components that are the most vulnerable to changes; once lost, cellular functioning usually will be compromised and cell deterioration is the result. To preserve the integrity of the DNA, the cell has several ingenious mechanisms to its disposal. Firstly, there is the DNA replication-machinery, which is intrinsically very accurate owing to proofreading and the correction of mismatched bases at the replication fork. Secondly, the cell has the ability to repair spontaneously arising DNA damage (depurination, deamination) or damage induced by external agents (alkylating agents, UV radiation etc.). To date, about 50 genes involved in DNA repair have been described, although not in the same species.

Although organisms have developed cellular mechanisms which preserve the integrity of the DNA and the fidelity of its synthesis, these mechanisms will never achieve complete accuracy. The allocation of an optimal amount of metabolic energy (ATP, NAD^+) to DNA-repair mechanisms would compromise viability and fecundity and, consequently, be selected against by natural selection. The inefficiency of DNA-repair processes ultimately will result in an accumulation of defects leading to changes in the expression of genes important for cell survival. An accumulation of DNA damage due to the disinclination of the cell to invest enough energy in mechanisms counteracting the build-up of pernicious DNA damage might thus be considered one of the major causes of aging [Kirkwood, 1977; Kirkwood and Holliday, 1979, Kirkwood et al., 1984]. In the next sections specific attention will be given to DNA in view of its role as the ultimate template of virtually all living organisms and its dominant role in many theories of aging and disease, including AD.

1.3 DNA damage metabolism in Alzheimer's disease

1.3.1 Sensitivity to DNA damage in Alzheimer's disease

Xeroderma pigmentosum (XP) is a hereditary disorder, usually resulting in serious skin abnormalities in light-exposed parts of the body. Cultured skin cells from affected individuals show a significant increase in sister chromatid exchanges [De Weerd-Kastelein et al., 1977] and a marked reduction in cell survival [Cleaver et al., 1968; Friedberg, 1985a] after UV-irradiation. In general, the DNA-repair capacity of XP cells following exposure to UV or treatment with a variety of chemicals was found to be defective [Cleaver et al., 1975; Friedberg et al., 1979]. The observation that some XP patients progressively develop neuronal degenerations [Andrews et al., 1978; Robbins et al., 1983a] has led to studies for other disorders associated with neuropathological abnormalities and hypersensitivity to DNA-damaging agents. These studies showed that there is an increased susceptibility to the killing effects of X-rays in cells from patients with ataxia telangiectasia [Tarone et al., 1984], Friedreich's ataxia [Chamberlain et al., 1982], familial dysautonomia [Scudiero et al., 1981], Huntington's disease [Moshell et al., 1980; Scudiero et al., 1981; McGovern et al., 1982; Imray et al., 1983] and Duchenne muscular dystrophy [Robbins et al., 1984; Tarone

et al., 1984]. In addition, these disorders also display hypersensitivity to radiomimetic chemicals such as N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) [Scudiero et al., 1981; Tarone et al., 1983]. Hypersensitivity to the lethal effects of X-rays and/or MNNG was also observed in cell lines derived from patients with Parkinson's disease [Scudiero et al., 1982a; Robbins et al., 1983b; 1985a], Down's syndrome (DS) [Otsuka et al., 1985] and Alzheimer's disease (AD) [Scudiero et al., 1982a; Robbins et al., 1983b; 1985a; Scudiero et al., 1986]. It appears that the hypersensitivity is specific for the X-ray-type of DNA-damaging agent, as opposed to the UV-radiation type, as was shown by a normal response to UV irradiation in cell lines from patients with ataxia telangiectasia [Moshell et al., 1980; Scudiero et al., 1982b], Huntington's disease [Moshell et al., 1980; Scudiero et al., 1981], Parkinson's disease and AD [Robbins et al., 1985a]. Thus, cell killing in these radiosensitive neurodegenerative syndromes cannot be attributed to a failure to respond normally to DNA-damaging agents in general. Apparently, the radiosensitivity in cultured cells from patients with primary neuronal degeneration, such as AD, could result from one or more defects in cellular pathways responsible for coping with the interaction of cells with the X-ray-type of DNA-damaging agents. In this regard it seems important to consider the possibility that in cells of AD patients a rapid accumulation of damage to the genetic material occurs. In the following sections the evidence for such an accumulation and the presence of a DNA-repair defect in cells of AD patients is reviewed.

1.3.2 Spontaneous and induced chromosomal abnormalities in Alzheimer's disease

Several studies which examined chromosomal changes in AD patient-derived blood cells have provided contradictory results as to whether AD is associated with an increase in chromosomal abnormalities, either numerical or structural or both. Using G-banding, Brun et al. [1978] did not find significant chromosomal changes in ten patients. In contrast, one of the first cytogenetic studies concerned with chromosomal abnormalities in blood cells from AD patients reported the presence of acentric chromosome fragments [Bergener and Jungklaass, 1970]. This was subsequently confirmed by Nordenson et al. [1980] who also observed an increased frequency of chromosome fragments in ten cases with sporadic AD, as compared with matched control subjects. Structural abnormalities, including dicentric

chromosomes and translocations, were also found by Moorhead et al. [1983] and Fischman et al. [1984]. Aneuploidy changes were reported to be increased in blood cells from AD patients [Nielsen, 1970; Ward et al., 1973; Buckton et al., 1983], but these findings could not be confirmed in several other studies [Mark et al., 1973; White et al., 1981; Moorhead et al., 1983]. Additionally, hypoploidy was observed to be increased with age, but no differences between AD and matched controls could be found [Martin et al., 1981].

Another, more sensitive cytogenetic method used in the investigation of chemical and physical influences on chromosomal reciprocal exchanges is the sister chromatid exchange (SCE) analysis. The increased SCE frequency in cells treated with DNA-damaging agents has led to the suggestion that quantitation of SCEs may be a sensitive indicator that DNA has been damaged [Perry et al., 1975]. Small increases in the SCE frequency in AD blood cells following in vitro exposure to mitomycin C were reported by Fischman et al. [1984] and Morimoto et al. [1984]. However, other studies failed to detect a significant difference between the AD and control group in either the mean baseline SCE frequency or the SCE response induced by mitomycin C [Sulkava et al., 1979; Das, 1986; Matsuyama et al., 1988]. Das also reported that the SCE frequency in AD cells was not increased as compared to matched control subjects, after in vitro exposure to the alkylating agent methyl methanesulfonate.

The increased incidence of spontaneous and induced chromosomal abnormalities in cells of AD patients, which needs to be confirmed however, may be the result of an underlying defect in one or more DNA repair mechanisms. The variability of the results may reflect diagnostic differences, different methodologies, small sample sizes and genetic heterogeneity of AD, but also a normal interindividual variability present as such in the general population.

1.3.3 Single-strand breaks and chromatin structure in Alzheimer's disease

In addition to the studies which examined chromosomal abnormalities in AD cells, the putative existence of a DNA-repair defect in cells from AD patients has been assessed directly by measuring the amount of chromosomal DNA damage, e.g. single-strand breaks (SSB). Nucleoid sedimentation has been employed to show that neuronal and glial cells from

AD patients contain more DNA breaks as compared to cells from age-matched controls [Bachelard et al., 1986]. However, these results can be challenged on the basis of findings suggesting that nucleoid sedimentation is unreliable for the detection of SSB since changes in the amount or type of DNA-bound ligands in vivo such as RNA and protein may affect the estimation of the amount of SSB present [Boerrigter et al., 1989; Jackson et al., 1989; Jostes et al., 1989; Hartwig, 1990]. This is also indicated by studies which found alterations in the chromatin structure of AD cells [Crapper et al., 1979; Lewis et al., 1981; Crapper-McLachlan et al., 1984; Lukiw et al., 1990] which were thought to result from an increased condensation of the DNA. Clearly, an increased condensation of DNA in neuronal cells from AD patients is not easily reconcilable with the surmised presence of more DNA breaks in such cells as compared to cells from matched controls.

In contrast to nucleoid sedimentation, the technique of alkaline elution as developed by Kohn et al. [1976] may detect SSB more directly, since it is not based on changes in DNA tertiary structure but on the degree of unwinding of the two strands in the DNA double helix under specified conditions, which is determined by the number of SSB present. Using this technique, Mullaart et al. [1990a] quantitated the amount of "spontaneous" DNA breaks in brain cells from AD patients. Their results indicated an at least twofold increase in the level of DNA breaks in both the occipital and frontal cortex of AD patients as compared to control subjects. According to the authors this augmented level of SSB was not a result of neuronal degeneration [Mullaart et al., 1990a], although the possibility that the observed SSB were the result rather than the cause of AD could not be excluded. An interesting finding was that the cerebral cortex of a Parkinson's patient did not contain elevated levels of DNA damage, whereas another patient having the neuropathological signs of both Parkinson and AD had almost the same amount of DNA breaks in cerebral cortex samples as the clinically diagnosed AD patients.

1.3.4 DNA repair in Alzheimer's disease

Early studies showed that the degree of neurological degeneration in xeroderma pigmentosum (XP) patients was paralleled by the extent to which their cells, cultured in vitro, were oversensitive to UV irradiation [Andrews et al., 1976, 1978; Robbins et al.,

1983a]. This and the observed hypersensitivity to DNA-damaging agents in a number of neurodegenerative disorders lend support to the theory that unrepaired DNA damage in neuronal DNA, as a consequence of a defect in one or more DNA-repair pathways, leads to impaired cellular functioning and ultimately to cell death [Andrews et al., 1978; Robbins et al., 1983b].

Apart from its possible involvement in disorders with neuropathological abnormalities, the accumulation of DNA damage has attracted much interest with respect to "normal" aging. Throughout an individual's lifespan, different kinds of DNA lesions will be induced, the type and/or site of formation of which are not equally deleterious to cellular functioning and survival per se. Conceivably, during an individual's lifespan those DNA damages which are the most detrimental to cellular functioning and survival could be selectively repaired as is seen, e.g., for transcribed DNA sequences [Mellon et al., 1986; Bohr et al., 1989; Mellon and Hanawalt, 1989]. Other DNA lesions, including DNA-base damages and mutations, might accumulate in the genome due to the "unwillingness" of the cell to commit sufficient metabolic resources to the repair pathways responsible for removing these DNA lesions. In this context, the investment of metabolic resources in specific DNA-repair pathways should be conceived as a specific pattern of preferentially repaired classes of DNA sequences (e.g. transcribed genes). Although these repair mechanisms would be expected to have developed in those species that have undergone a significant increase in their life expectancy during their recent evolutionary development, such as primates and in particular humans, the inherent inaccuracy of these processes would only postpone senescence and the appearance of age-related diseases such as AD.

It has been hypothesized that the gradual accumulation of deleterious DNA lesions in target genes would result in the slow deterioration of essential gene functioning until cell death occurs [for recent reviews see Tice and Setlow, 1985; Hanawalt, 1987; Mullaart et al., 1990b]. A large proportion of such DNA damage would be caused by endogenous cellular metabolites. One class of the most obvious DNA-damaging agents that occur as a result of metabolism, are free radicals. In biological systems, oxygen can readily accept single-electron transfers to form highly reactive oxygen species [for a review see Kanofsky, 1989]. Apart from protein oxidation or lipid peroxidation, these oxygen free radicals are able to cause strand breaks in DNA [Birnboim et al., 1985] and to induce a variety of DNA

modifications [Adelman et al., 1988].

In order to be protected from the pernicious effects of persisting DNA damage, mammalian cells have several defense mechanisms to their use. Specific enzymes such as SOD and catalase convert the oxygen radicals to a less reactive form. In addition, peptides, particular those with a sulfhydryl group, e.g. glutathione, may react directly with the oxygen species, thereby preventing their reaction with the vulnerable cellular constituents [Balin et al., 1989]. Small DNA adducts, such as induced by alkylating agents and ionizing radiation, may be removed from the DNA by the concerted actions of a variety of DNA-repair enzymes [Friedberg, 1985a]. Specific DNA-glycosylases recognize some forms of base damage (e.g. alkylations at certain positions in the DNA base) and catalyze the excision of the base, leaving apurinic or apyrimidinic (AP) sites in the DNA. An AP endonuclease then incises the DNA near the AP site. Subsequently, the strand containing the incision is degraded locally whereby the AP site is removed. The resulting gap can be filled by the action of a DNA polymerase which inserts the correct complementary nucleotides. When the last nucleotide has been inserted the DNA strand continuity is restored by the action of a DNA ligase.

In spite of the presence of DNA-repair mechanisms, a certain fraction of the lesions in the DNA will remain unrepaired [Mullaart et al, 1989; Boerrigter et al., 1991b]. Although most essential genes are believed to be repaired more proficiently than others within the same cells [Hanawalt, 1987], the inability to repair certain DNA adducts could significantly influence the rate of aging and the development of a variety of disorders. Apart from XP, there is evidence for altered responses to DNA damage in cells of patients suffering from ataxia telangiectasia, Bloom's syndrome, Cockayne syndrome and Fanconi's anemia [Friedberg, 1985a]. Specifically, the genetic defect in Cockayne syndrome recently was found to be associated with a defect in the repair of transcriptionally active DNA in fibroblasts irradiated with UV [Venema et al., 1990].

Cells with a compromised DNA-repair machinery would suffer to a greater extent than repair proficient cells, because the damage persists. In particular, postmitotic cells such as neurons and parenchymal liver cells are thought to accumulate a greater amount of DNA damage during their lifespan as compared to proliferating cells [Gensler and Bernstein, 1981; Vijg and Knook, 1987]. Such an accumulation may result from the absence of specific

repair enzymes in these postmitotic cells. It has been observed that during neuronal development in the rat there is a rapid decrease in the activity of uracil DNA-glycosylase which might impair the removal of this mispairing base from the DNA of postmitotic neurons [Focher et al., 1990]. Since neuronal DNA in vivo is constantly being damaged by endogenous DNA-damaging metabolites, the inability to repair certain DNA lesions could result in premature neuronal death. This theory as originally proposed by Robbins et al. [1985b] has evoked several groups, including our own, to study DNA damage and its repair in relation to AD.

Even though the characteristic pathological changes in AD occur predominantly in brain cells, the recognition of AD as a systemic illness has led to the suggestion that a putative DNA-repair defect may also become manifest in other cells such as lymphocytes and fibroblasts after in vitro treatment with DNA-damaging agents of the X-ray-type. By using the alkaline filter elution assay, which is based on the principle of increased elution of DNA fragments through a filter with an increasing number of DNA breaks present, it was shown that fibroblast cell lines from AD patients do repair a significantly smaller amount of strand breaks as compared to matched controls, after in vitro treatment with either N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) [Li and Kaminskas, 1985] or methyl methanesulfonate (MMS) [Robison et al., 1987]. Treatment of AD fibroblasts with UV radiation resulted in a repair response comparable to that found in cells from control subjects [Robison et al., 1987]. These results agreed well with the cell survival data in which X-rays and the alkylating agent MNNG, but not UV radiation, were shown to elicit an increased cell killing in AD cells. Using the same fibroblast cell lines, however, Kinsella et al. found comparable levels of DNA repair after exposure to MNNG [Kinsella et al., 1987a] or MMS [Kinsella et al., 1987b]. These authors reasoned that it would be unlikely to detect any defect in DNA repair with the alkaline elution assay because this method measures only a small fraction of the damage produced by alkylating agents such as MNNG and MMS. This was indeed shown earlier by Fornace et al. [Fornace et al., 1986]. It still remains to be seen whether the lesions detected by the alkaline elution assay are relevant to cell survival. From our own studies using freshly isolated peripheral blood lymphocytes (PBL) from apparently normal young donors it appeared that a decreased repair capacity observed after in vitro exposure to N-ethyl-N-nitrosourea (ENU) correlated with a lower survival of cells as

measured by ^{3}H-incorporation in phytohemagglutinin (PHA)-stimulated cells [Boerrigter et al., 1991b].

Using the alkaline elution assay, Jones et al. [1989] observed a DNA-repair defect in six lymphoblastoid cell lines from familial AD (FAD) patients after in vitro treatment with MNNG or MMS. The primary adduct induced by MMS and MNNG is N7-methylguanine (N7-meG) which accounts for 87% and 66% of the total DNA methylation, respectively [Hemminki, 1983]. Thus, it is quite possible that a defective repair of N7-meG is responsible for the decreased repair capacity of AD lymphoblastoid cell lines as observed by alkaline elution. Although N7-meG is not by itself a harmful lesion, it can undergo a rearrangement to yield a ring-opened imidazole form. The ring-opened N7-meG, or formamidopyrimidine is a block to DNA synthesis in vitro [Boiteux et al., 1983]; thus, if not repaired, it is a lethal lesion for the cell.

Another adduct that can be induced by MMS and MNNG is O^6-methylguanine (O^6-meG). This adduct is removed stochastically by a so-called "suicide" protein, the O^6-methylguanine transferase (O^6-mGT) which transfers the methyl group to a cysteine residue within the protein leaving the guanine intact. This particular lesion is not alkali-labile and its removal does not generate SSB as intermediates. As such, O^6-meG is not detected by the alkaline elution and could therefore also be responsible for a decreased cell survival when repaired to a lesser degree in AD patient-derived cells. However, studies by Edwards et al. [1989] and Jones et al. [1989] have shown that lymphoblastoid cells from AD patients and matched controls contain comparable levels of O^6-mGT activity. Similarly, non-transformed exponentially growing T lymphocytes of AD patients are not different in their O^6-mGT activity, when compared to normal age-matched controls [Bartlett and Robison, 1990].

The studies concerning DNA repair in cells from AD patients have used actively dividing cells (fibroblast or lymphoblastoid cell lines) which allowed radioactive labelling of the cells in order to detect the DNA. This may not be representative for the in vivo situation as the repair capacity of a certain cell population can be influenced by its proliferative status [Boerrigter et al., 1991a; Boerrigter and Vijg, 1991a]. The contradictory results on the presence of a decreased DNA repair in peripheral cells from AD patients may have resulted from differences in culture conditions, treatment with the alkylating agents or other experimental conditions during the alkaline elution itself. Therefore, our group has applied a

modified, highly sensitive alkaline elution assay, in which the DNA can be quantitated spectrofluorometrically, to measure the repair capacity of freshly isolated peripheral blood lymphocytes [Stout and Becker, 1982] after in vitro exposure to ENU. When using this method, the average extent of ENU-induced DNA repair was found to be significantly lower in PBL from familial AD patients, whereas the average repair capacity of PBL from sporadic AD patients did not differ significantly from the average ENU repair capacity observed in cells from matched normal control subjects [Boerrigter et al., 1991c; Chapter 7]. Although repair seems not to be defective in cells from sporadic AD patients, our findings do not exclude alterations in the fidelity of DNA repair which may be caused by perturbations of intracellular deoxyribonucleotide triphosphate (dNTP) pools [Mattano et al., 1990] or inherently faulty DNA-repair enzymes. In all studies on DNA repair a great variability among individuals was observed. In our studies, this interindividual variation was consistently present in PBL from young [Boerrigter et al., 1991b] and old [unpublished results] control subjects over a period of 3-12 months. However, preliminary results indicate that PBL from AD patients display a greater variability with respect to ENU repair capacity [unpublished results].

Apparently, the greater sensitivity of AD fibroblast and lymphoblastoid cell lines to the cell-killing effects of alkylating agents might be a consequence of a defective removal of certain adducts induced by these DNA-damaging agents. Robison et al. also reported a decreased level of unscheduled DNA synthesis (UDS) in MMS-treated fibroblast cell lines from AD patients [Robison et al., 1987]. UDS is measured as the incorporation of [^{3}H]thymidine ([^{3}H]TdR) in DNA of cells exposed to DNA-damaging agents.

With respect to ionizing radiation, Smith et al. [1987] detected a decrease in UDS after gamma-irradiation in unstimulated PBL with age but not in PBL from AD patients as compared to PBL from age-matched control subjects. However, when PBL were stimulated with the mitogen PHA, the incorporation of [^{3}H]TdR in non-irradiated cells was found to be significantly lower in AD cells as compared to cells from age-matched controls [Miller et al., 1981; Singh et al., 1987; Smith et al., 1989]. As was found for unstimulated PBL, PHA-stimulated cells from AD patients did not differ in their response to gamma-irradiation as measured by [^{3}H]TdR-incorporation [Smith et al., 1989].

The relevance of altered cellular responses of AD cells to DNA-damaging agents is

confounded by the recognition of variations among individuals, which may be either acquired or inherited. Interindividual variations in DNA repair have been noted for a diversity of DNA-damaging agents [for reviews see Setlow, 1983; Vahakangas et al., 1984; Harris, 1989], including ionizing radiation and alkylating agents. The bearing of a lower DNA-repair capacity to a human disease state such as AD might be complicated by several factors including i) variations associated with the degree or duration of the disease, and ii) the relevance of the observed repair deficiency in terms of cellular functioning and survival. These factors should be evaluated thoroughly before variations in DNA repair can be regarded as relevant to the development of a certain disease. Particularly, the identification of the DNA damage for which cells of AD patients possibly display a defective repair is crucial to our understanding of how a defect in one or more repair pathways might influence the pathogenesis of AD, and whether apparently normal subjects with a low repair capacity might be at an increased risk of developing AD.

1.4 Therapeutic implications

1.4.1 Amelioration of neurotransmitter deficits

Current therapeutic approaches to AD focus on the amelioration of the neurotransmitter deficits. With regard to acetylcholine, oral administration of choline [Boyd et al., 1977; Etienne et al., 1978a; Thal et al., 1981;] or lecithin [Etienne et al., 1978b; Brinkman et al., 1982; Weintraub et al., 1983], a natural dietary source of choline, did not produce clinically significant improvements in memory or other cognitive functions. Administration of the cholinesterase (ChE) inhibitors physostigmine [Davis et al., 1979, 1982, 1983; Thal et al., 1983] or tetrahydroaminoacridine (THA) [Davies et al., 1989; Chatellier and Lacomblez, 1990; Gauthier et al., 1990] have generally not shown any particular marked and/or consistent effects although Summers et al. [1986] have reported a significant improvement in clinical and psychological assessments. However, THA carries a high risk of causing a potentially lethal hepatitis [Ames et al., 1988] and its use as a treatment for AD cannot be recommended. Central cholinergic stimulation by physostigmine did not increase regional cerebral blood flow as evidenced by single photon emission computerized tomography in

occipital, frontal, and posterior and anterior parietotemporal cortex in AD patients, as compared to controls [Geaney et al., 1990]. Small, but statistically significant improvements in Alzheimer Disease Assessment Scale scores were recently reported in AD patients participating in an open trial with the long-acting ChE inhibitor metrifonate [Becker et al., 1990]. Metrifonate has several advantages over physostigmine and THA such as a longer half-life and, consequently, longer duration of action and less severe side-effects, but the clinical usefulness of metrifonate should be evaluated in phase III clinical trials before any conclusions regarding its therapeutic use in AD can be established. The widespread administration of Hydergine®, a combination of ergyloid mesylates which is the only medication for AD approved by the Food and Drug Administration of the United States of America, does not seem to be warranted by recent results of Thompson et al. [1990] who showed Hydergine to be ineffective in the clinical treatment of AD.

Currently, a range of new ChE inhibitors is being developed but the value of these various drugs in the symptomatic therapy of AD remains to be evaluated [Pomponi et al., 1990]. Intracerebroventricular infusion of bethanechol, a direct muscarinic agonist, led to only small increases in cognitive function as assessed by the Mini-Mental State Test; in view of the major surgical risks involved, this treatment was reprobated [Harbaugh et al., 1989]. Moreover, intraventricular administration of bethanechol can induce parkinsonism in patients with AD, possibly due to the functional antagonism between the cholinergic and dopaminergic systems [Fox et al., 1989].

Although increasing the availability of neurotransmitters in AD seems to be the most logical course, the results obtained so far are rather disappointing. Moreover, treatments designed to increase neurotransmitter levels are likely to be at best palliative and will probably not prohibit the relentless progressive neuronal degeneration which underlies the neurotransmitter deficiencies. In all probability, only those patients with minimal physiological losses of neurones will profit to some degree from such therapies, but inexorably the disease process will emerge anew and proceed into its final stage. A recent study by Flynn et al. [1991] suggested that the failure of cholinergic replacement therapies might be related to a defective coupling of the M1 muscarinic receptor subtype to its respective G protein resulting in a defect in signal transduction. Since the M1 muscarinic receptor is the predominant postsynaptic cholinergic receptor site in the cerebral cortex and

the hippocampus [Araujo et al., 1988; Mash et al., 1985, 1988] this suggests that the administration of direct-acting M1-selective muscarinic agonists will not be clinically beneficial in AD [Flynn et al., 1991].

Graft implantation of new brain cells in order to replenish the decreased neurotransmitter levels has been tried in Parkinson's disease with grafts from adrenal or embryonic origin, which were implanted into the caudate and putamen of these patients [Williams et al., 1990]. Although such studies reported only small and non-persistent changes in mental and motor function, such a treatment is not viable for AD in view of the widespread neuronal degeneration. Potential use of nerve growth factor (NGF) to stave off further neuronal degeneration has been discussed [Phelps et al., 1989] when studies indicated that treatment with NGF can prevent injury-induced degeneration of cholinergic neurones and may improve cognitive function in rats with memory impairments [for references see Phelps et al., 1989; Everall and Kerwin, 1990]. So far, no direct evidence supports a primary involvement of NGF in the pathogenesis of AD, although, in contrast to putative therapeutic effects of NGF, some evidence suggests that neurotrophic factors may in fact be contributory to the pathology of AD by promoting excessive neuronal growth and inhibiting the formation of stable neuronal networks [Butcher et al., 1989].

Ideally, therapeutic agents for AD or, for that matter, any other age-related disease, should be modelled to prevent further progression into the final stages of the diseases or, if possible, to protect the individual from acquiring the disease process. Obviously, development of biological and/or genetic markers for AD for the identification of individuals in the population which are at risk of developing AD would facilitate the early administration of potentially protective agents at a time, if possible, before any deterioration of cognitive functions would become apparent. Moreover, these agents ideally should correct the actual defect which ultimately causes the neuronal degeneration and not merely ameliorate one of its consequences, such as decreased availability of acetylcholine in the brain.

1.4.2 Enhancement of metabolic energy levels

Based on the possibility that AD is based on defects in some aspects of DNA damage

metabolism one can envisage several therapeutic strategies. First, attempts can be made to prevent the accumulation of those DNA damages implicated in AD, e.g. single-strand breaks and alkyl base damages. This approach will be complicated by the present lack of understanding of the type of damages that are actually accumulating. Moreover, it will be extremely difficult to intervene with DNA-damage induction itself. A second possibility is to enhance the ability of cells to remove the pernicious DNA damages. Although it should be possible to increase the activity of specific repair enzymes, this will be inherently more difficult than boosting DNA-repair capacity in a more general way. The latter possibility could be approached by augmenting the levels of metabolic energy necessary for DNA-repair processes by effecting a long-term increase in the efficiency of energy production.

L-Carnitine, which acts as an essential transmitochondrial carrier of acetyl and long-chain acyl groups to be used in fatty acid oxidation [Bremer, 1983], is one substance which may be applied as a therapeutic agent in putative secondary disorders of energy metabolism, e.g. AD. L-Carnitine has been used in the treatment of a number of neurological disorders [Seccombe et al., 1982; Rebouche and Engel, 1983; Coulter, 1984; Stumpf et al., 1985], but also in several animal model disorders, including cerebral ischemia [Shug et al., 1980] and other metabolic encephalopathies [Kim et al., 1984; Costell et al., 1987]. L-Carnitine normalizes the increased α-adrenergic stimulated cAMP production and the decreased pH of AD fibroblasts [Malow et al., 1989]. It also delays the killing of cultured hepatocytes by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [Snyder et al., 1990], a human neurotoxin that induces neuropathological, and neurochemical changes, very similar to those of Parkinson's disease, and causes a clinical illness resembling Parkinson's disease itself [Ballard et al., 1985]. L-Carnitine also increases the mitogen-induced proliferation of peripheral blood lymphocytes, particularly in cells from old subjects [Monti et al., 1989]. Furthermore, an acetyl analogue of L-carnitine is being used in Europe to treat senile patients with a moderate degree of cognitive impairment, and its use has shown a moderate improvement in some behavioral profiles [Bonavita, 1986; Passeri et al., 1988; Battistin et al., 1989; Rai et al., 1990; Sinforiani et al., 1990]. However, these clinical trials do not allow any definite conclusions yet. Acetyl-L-carnitine appears to improve cognitive functions in rats [Angelucci et al., 1986; Drago et al., 1986] and stimulates spontaneous release of acetylcholine in striatum and hippocampus of freely moving rats [Imperato et al., 1989]. Reduction of the

age-related decrease in NGF-binding to hippocampus and basal forebrain [Angelucci et al., 1988] and of the age-dependent loss of glucocorticoid receptors in rat hippocampus [Patacchioli et al., 1989] have also been reported. Moreover, acetyl-L-carnitine can improve the age-related decrease in levels of heart- and brain mRNA [Gadaleta et al., 1990]. However, until more is known about the underlying cause of AD, the possible effectiveness of treatment with acetyl-L-carnitine remains tentative at best.

1.5 Recapitulation and discussion

The phenomenon we envisage as aging may find its ultimate cause in the persistence of alleles which enhance early fitness-components at the expense of later survival. Because the deleterious effects of such alleles will only become apparent during the later part of an individual's lifespan, it is not possible for natural selection to select against these alleles. A decreasing influence of intrinsically imperfect "protective" factors, e.g. DNA repair, together with a concurrent accumulative effect of environmental factors (e.g., DNA-damaging agents) would lead to the gradual accumulation of defects impairing, not abolishing, cellular functioning. This will result in the expression of a phenotypic effect only late in life.

Normal brain aging, benign senescent forgetfulness, and AD can thus be seen as a continuum, which may reflect a single underlying process, e.g. impaired energy metabolism [Mann et al., 1985; Brayne and Calloway, 1988] or the expression of DNA-repair defects [Robbins et al., 1983b]. In this respect, FAD could result from mutant, dominant genes, the penetrance and expression of which would be modulated by environmental factors. Sporadic AD (SAD), on the other hand, would predominately be caused by interaction with these environmental factors, possibly endogenous or exogeneous DNA-damaging agents or, alternatively, an infectious agent. Evidence for environmental modulation of AD expression may be found in the observed widely discordant ages-at-onset of AD in monozygotic and dizygotic twins but also in the observed bimodality of ages-at-onset within FAD and between FAD and SAD. Interestingly, peripheral lymphocytes from FAD patients, but not from SAD patients, display a decreased mean percentage of DNA repair following exposure to alkylating agents [Jones et al., 1989, Boerrigter et al., 1991c].

Although a plausible candidate gene for AD, with an as yet unknown function, was recently identified on chromosome 21 [St George-Hyslop, 1987; Goate et al., 1989], the development of a genetic marker for AD is impeded by the unprecise location of the disease locus. Moreover, the apparent existence of a second disease locus in some late-onset FAD cases [Pericak-Vance et al., 1988, 1991; Roses et al., 1988; Schellenberg et al., 1988,1991] currently complicates the use of any genetic marker in the identification of individuals at risk of developing AD.

On the other hand, considering the possibility that a genetic defect in AD is expressed and can be detected in cells other than neurons, one might be able to develop a biological marker for AD. The observed sensitivity of AD cells to gamma-rays and alkylating agents and the reduced capacity of AD cells to remove DNA damage induced by alkylating agents argues for the possibility of applying a decreased DNA repair as a phenotypic, biological, marker for AD. This would require the ability to distinguish between normal, healthy subjects and AD patients and/or subjects at risk on the basis of a defective DNA repair in peripheral cells. Whether this is possible will depend on how well the defect can be characterized. This means that the DNA-repair criterion to be applied should be carefully selected, with sufficient knowledge of the possible influence of internal or external factors and of the variation among healthy individuals, before the attention is turned to the AD patients.

1.6 Introduction to the experimental work

Based on the above, it can be hypothesized that in some cases brain aging, and more specifically late-onset neurodegenerative disorders like Alzheimer's disease (AD), find their ultimate cause in some specific defect in DNA repair leading to DNA-damage accumulation in the brain. To test this hypothesis it is necessary to assess whether or not comparatively high levels of DNA damage are present in the brains of AD patients. Mullaart et al. [1990a], recently demonstrated that this indeed appears to be case. On the basis of this finding and literature data [Li and Kaminskas, 1985; Robison et al., 1987; Jones et al., 1989] suggesting that DNA damage is removed more slowly from cultured cells of AD patients as compared to cells from normal individuals, the present study on DNA-damage induction and removal in freshly isolated PBL was initiated.

A variety of methods are available for the evaluation of DNA damage and its repair. However, most techniques only measure a putative endpoint of DNA damage, e.g. micronuclei, sister chromatid exchanges or decreased survival, or require a relative high dose of the DNA-damaging agent for accurate quantitation of the amount of DNA lesions induced. Other methods rely on the detection of radioactively labeled DNA-bound metabolites of DNA-damaging agents or on the incorporation of radioactive DNA precursors during excision repair ("unscheduled DNA synthesis"). These methods are not well suited for the intended studies with blood lymphocytes. On the other hand, some other techniques are available that allow a sensitive assessment of DNA lesions induced by low doses of different types of non-radioactive DNA-damaging agents. These methods include alkaline unwinding, alkaline filter elution and nucleoid sedimentation. Results obtained by the last mentioned technique, which measures alterations in the sedimentation of nucleoids through sucrose gradients, are, however, inherently more difficult to interpret than those obtained by alkaline unwinding or alkaline filter elution (see also paragraph 1.3.3). Alkaline unwinding and alkaline filter elution are based on the alkali-induced unwinding of the double-stranded DNA, the extent of which is dependent on the number of single-strand breaks (SSB) present. The present study, therefore, employed the sensitive alkaline filter elution technique for the assessment of the induction of SSB and their subsequent disappearance in freshly isolated PBL treated with ionizing radiation or alkylating agents.

The present study is concerned with the characterization of the induction and subsequent disappearance of DNA damage in freshly isolated peripheral blood lymphocytes exposed to gamma-rays or alkylating agents. For the reasons discussed above, the attention was focussed on DNA lesions that can be detected as strand breaks. After proper characterization of the processes of induction and repair in PBL from healthy persons, the question was addressed whether PBL from patients with AD display an impaired ability to remove this type of DNA damage.

Firstly, we compared the number of SSB present in untreated and mitogen-stimulated PBL in order to assess if possible variations in <u>in vivo</u> lymphocyte activation might influence the background number of SSB present, which could potentially interfere with DNA-damage induction and quantitation of its repair [Boerrigter et al., 1989; Chapter 2].

The influence of mitogen-stimulation of the cells on the induction and repair of SSB in

PBL of normal donors was investigated with cells exposed to gamma-rays or methyl methanesulfonate (MMS). These studies are presented in Chapters 3 [Boerrigter and Vijg, 1991a] and 4, respectively [Boerrigter et al., 1991a]. In the MMS experiments the effects of cell-cycle differences were also studied on cultured fibroblasts from normal healthy donors; confluent and actively dividing cells were compared as to the removal of MMS-induced SSB [Boerrigter et al., 1991a; Chapter 4].

Apart from the methylating agent MMS, which predominantly alkylates nitrogen sites in DNA bases, we examined the induction of SSB and their repair in PBL exposed to an ethylating agent, ENU, which effectively induces alkylation damage on oxygen sites [Hemminki, 1983; Boerrigter et al., 1991b; Chapter 5]. Evidence for interindividual variation in ENU-induced SSB repair in quiescent PBL from normal, healthy donors was found, making this agent a suitable candidate for use as test agent in assessing AD patients. The possible causes of the observed variability in the repair of ENU-induced SSB are discussed in Chapter 6, where the results are presented of a study in which B- and T-lymphocytes were investigated separately [Boerrigter and Vijg, 1991b].

Finally, the hypothesis was tested that AD is associated with a decreased repair of alkylating-agent-induced SSB in freshly isolated PBL of these patients. The results obtained indicate that a decreased ENU-induced DNA repair is related to the familial, but not the sporadic, form of AD [Boerrigter et al., 1991c; Chapter 7].

CHAPTER 2

QUIESCENT HUMAN PERIPHERAL BLOOD LYMPHOCYTES DO NOT CONTAIN A SIZABLE AMOUNT OF PRE-EXISTENT DNA SINGLE-STRAND BREAKS[1]

2.1 Introduction

The activation of human peripheral blood lymphocytes (PBL) is thought to be accompanied with the joining of preexistent DNA breaks in quiescent PBL [Johnstone and Williams, 1982; Carson et al., 1986]. Analysis of the rate of sedimentation of nucleoids, i.e. supercoiled DNA that has lost most of the nuclear protein, showed that such nucleoids from phytohaemagglutinin (PHA)-stimulated PBL sedimented faster through the neutral sucrose density gradients than those derived from quiescent lymphocytes [Johnstone and Williams, 1982]. This increased sedimentation rate was interpreted as the effect of rejoining of single-strand breaks (SSB), present in the DNA of resting cells. The change in sedimentation rate was smaller when inhibitors of ADP-ribosyl transferase (ADPRT) were included in the culture medium of activated lymphocytes [Johnstone and Williams, 1982]. Because ADPRT is involved in increasing the activity of DNA ligase II [Creissen and Shall, 1982] this effect was thought to be indicative of an obligatory role of DNA ligation during lymphocyte activation [Johnstone and Williams, 1982].

Nucleoid sedimentation has been used extensively for studying the effect of radiation or carcinogen treatment on cellular DNA [Cook and Brazell, 1975, 1976; Farzaneh et al., 1982; Romagna et al., 1985]. As compared to nucleoid sedimentation, the technique of alkaline elution as developed by Kohn et al. [Kohn et al., 1976] may measure SSB more directly, since it is not based on changes in DNA tertiary structure but on the degree of unwinding under specified conditions, which is determined by the number of SSB present.

This work demonstrates that according to alkaline elution results unstimulated PBL do not contain a sizable number of SSB, or alkali-labile sites, at the time of isolation. Stimulation with PHA did not affect the level of detectable sites. These results are discussed

[1]Parts of this chapter were published previously by Boerrigter et al., 1989.

in relation to the observed changes in nucleoid sedimentation rate of stimulated PBL as compared to resting cells.

2.2 Materials and Methods

2.2.1 Cell isolation and culture

Human peripheral blood was collected from healthy volunteers not taking medication. Lymphocytes were isolated using Ficoll-Paque (Pharmacia) gradients [Boyum, 1968], washed twice in RPMI 1640 medium (Flow Laboratories) plus 2% fetal calf serum (FCS). All steps were performed at 4°C. Lymphocytes that were stimulated with PHA (Welcome Foundation Ltd), were resuspended at a final concentration of 2 x 10^6 cells/ml in RPMI 1640 medium plus 10% FCS supplemented with 2 mM glutamine and antibiotics. Mitogen stimulation with PHA (10 μg/ml) was for 3 days at 37°C.

2.2.2 Detection of DNA strand breaks

The technique of alkaline filter elution was used to measure SSB [Kohn et al., 1976]. Cells were resuspended in cold phosphate-buffered saline (PBS) at a concentration of 2 x 10^6 cells/ml; 0.8 - 1 x 10^6 cells were added to 0.5 ml sarkosyl buffer (0.2% sarkosyl, 2 M NaCl and 0.02 M EDTA, pH 10.0) layered onto a 25-mm diameter polycarbonate membrane filter (Nuclepore; pore size 5 μm). This lysis solution was removed by passage through the filter by gravity and replaced by an SDS lysis buffer (0.5% SDS, 0.01 M NaCl, 0.01 M Tris, 0.01 M EDTA, pH 8.0, and proteinase K (Merck; 0.5 mg/ml)). After a 1-h lysis period at 20°C the solution was removed by gravity and the DNA on the filter was washed twice with 5 ml 0.02 M EDTA, pH 10.0. The DNA was eluted through the filter with 0.06 M NaOH, 0.02 M EDTA, pH 12.6 (calculated), at 0.03 ml/min. Loading and lysing of the cells, as well as the elution of the DNA were performed under subdued lighting in order to minimize artificial induction of strand breaks [Bradley et al., 1978]. Six 4.5-ml fractions were collected. After the elution, the filter with the residual DNA was transferred to a glass vial and, after addition of 4.5 ml elution buffer, irradiated with 100 Gy of ^{60}Co-γ-rays (Gamma-

cell 100, Atomic Energy of Canada; dose rate 6 Gy/min) in order to release the DNA. The six eluate fractions and the irradiated membrane fraction were neutralized with 0.8 ml 4 M NaCl, 0.6 M NaOH, 1.0 M NaH_2PO_4, which further contained Hoechst 33258 dye (0.5 mg/l). The fluorescence of the Hoechst/DNA complex was measured at 430 nm (filters 3-73, 4-76 and 5-58) by excitation at 370 nm (filters 7-54 and 7-60) in a Pye Unicam LC-FL detector. The elution result was plotted as the log percent of DNA remaining on the filter as a function of time. Mean slopes of the linear part of elution curves were used to calculate the number of SSB plus alkali-labile sites, by calibration with mean elution curves of cells exposed to 4 Gy ^{60}Co-γ-radiation (Gamma-cell 100, Atomic Energy of Canada), which were assayed in the same experiment. Four Gy γ-radiation introduce approximately 4000 SSB per diploid genome [Van der Schans et al., 1982]. The amount of SSB in unirradiated cells was obtained by using the formula

$$4000 \times \frac{\text{slope of the elution curve of unirradiated cells}}{\text{slope of the elution curve of 4 Gy } \gamma\text{-irradiated cells}}$$

In all experiments the mean slope was based on at least triplicate determinations.

2.3 Results

The alkaline elution technique was applied to untreated PBL and to cells exposed to various doses of ^{60}Co-γ-radiation. Fig. 2.1 shows typical elution profiles. On the semilogarithmic plots the slope of the initial part of the curve increases linearly with increasing dose. This indicates that the rate of elution (slope) is proportional to the number of SSB induced by γ-radiation within the range 0-8 Gy. It is clear that under these conditions low numbers of SSB or sites in DNA that turn into SSB in alkali can readily be quantitated. With respect to unirradiated lymphocytes, almost all the DNA is retained on the filter which shows that only few SSB are present or formed.

The absence of a sizable amount of SSB in freshly isolated quiescent PBL was confirmed with cell samples from other donors. The amount of SSB per cell (mean $\pm$ SD)

was 345 (± 114) (Table 2.1). The observed variation in the level of SSB could result from methodological factors or reflect a biological variation, that is, a small but different level of preexistent SSB in resting PBL from different donors.

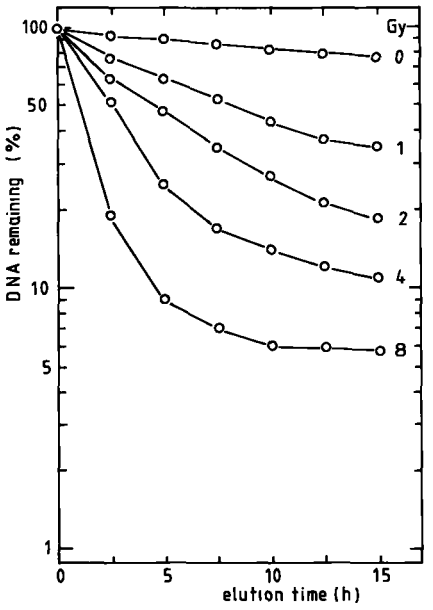

Fig. 2.1: Alkaline elution of DNA through membrane filters. Elution curves for freshly isolated PBL either untreated or exposed to various doses of γ-radiation. Elution was plotted as the log percent of DNA remaining on the filter as a function of time. The initial slopes of the various curves are: 0 Gy: 0.015; 1 Gy: 0.08; 2 Gy: 0.13; 4 Gy: 0.22; 8 Gy: 0.50.

To determine the methodological variation in the amount of SSB detected in quiescent PBL, cells were isolated from blood samples collected repeatedly from two donors, which were analysed for SSB with the alkaline elution technique. The level of SSB per cell was 456 (± 163) for donor N18 and 358 (± 115) for donor N01 (Table 2.1). The coefficient of variation ranged from 10-57% for cells isolated from different donors as compared to 1-50% and 10-59 % for lymphocytes obtained from sequential blood samples of donor N18 and donor N01, respectively. The difference between the coefficients of variation for different donors on the one hand and for the same donor on the other is small, indicating

Table 2.1 SSB AND ALKALI-LABILE SITES IN HUMAN WHITE BLOOD CELLS.

	n	mean	SD	range
quiescent[a)]	16	345	114	148 - 476
quiescent[b)]	9	456	163	226 - 691
quiescent[c)]	6	358	115	148 - 436
PHA-stimulated[d)]	3	519	165	378 - 700

The number of SSB plus alkali-labile sites in DNA was determined by alkaline elution of DNA isolated from freshly isolated PBL. Lymphocytes from donors N18[b)] and N01[c)] were analysed repeatedly over a period of 1 year, with intervals of 5 weeks.

[a)]different donors; one blood sample per person

[b)]donor N18

[c)]donor N01

[d)]lymphocytes from 3 different donors were stimulated with PHA (10 μg/ml; 37 °C) and analysed at the third day of stimulation. Activation of PBL was 40-50 fold as analyzed by the incorporation [^{3}H]thymidine. Control cultures without mitogen incorporated (mean ± SD) 1,200 (± 558) c.p.m., whereas PHA-stimulated cells incorporated 61,149 (± 2,563) c.p.m. when treated with [^{3}H]thymidine during the last 16 hr of culture.

that the variation in the sample populations is about the same. This suggests that the observed variation in the level of SSB stems from methodological variation only, possibly due to background breaks induced during isolation of the PBL.

2.4 Discussion

In contrast to the supposed disappearance of SSB in PBL after mitogen stimulation, as concluded from the increased rate of sedimentation of nucleoids [Johnstone and Williams, 1982], we found no differences in the amount of SSB after stimulation of PBL with PHA as compared to freshly isolated cells. The level of SSB per cell was 519 (± 165), not significantly different from the values for resting PBL (Table 2.1). Similar low levels of SSB in quiescent and PHA-stimulated PBL were reported recently by Jostes et al [Jostes et al., 1989]. In addition, SSB do not appear to play a significant role in the differentiation of Friend erythroleukemia cells [Wintersberger and Mudrak, 1982] as originally proposed

based on findings obtained by alkaline sucrose gradients and sedimentation of folded genomes [Scher and Friend, 1978].

The increased rate of sedimentation in sucrose density gradients of nucleoids from PHA-stimulated PBL has been interpreted as the rejoining of SSB present in the DNA of resting lymphocytes. However, in view of the discrepancy with the results obtained with alkaline elution, this interpretation may be questioned, since the nucleoid sedimentation is less direct with regard to the detection of SSB than alkaline unwinding or alkaline sucrose gradient sedimentation [Charles and Cleaver, 1982]. Indeed, recent evidence indicates that an additional long-range folding of the genome, leading to loops of smaller mean size, is an early process during mitogenic stimulation of PBL. This altered DNA folding pattern might conceivably account for the increased sedimentation of nucleoids from PHA-stimulated PBL. A second discrepancy may be relevant in this respect: human skin and embryonic fibroblasts show comparable levels of repair replication after UV-irradiation, but they differ in the rate at which their nucleoids recover normal sedimentation behavior [Charles and Cleaver, 1982]. Another aspect involves the role of ADPRT inhibitors, such as 3-aminobenzamide (3AB). The presence of 3AB during PHA-stimulation of PBL is thought to inhibit ADPRT, thus indirectly inhibiting DNA ligase [Creissen and Shall, 1982], and therefore leading to an increased number of SSB as compared to untreated PHA-stimulated PBL. However, using the alkaline filter elution Jostes et al. could not detect any differences in the elution profiles of PHA-stimulated PBL cultured either in the absence or presence of 2 mM 3AB [Jostes et al., 1989]. In accordance with this is the observation that the presence of 3AB does not influence the proliferative capability of PHA-stimulated PBL [Jostes et al., 1989; Marini et al., 1989].

Considering the structure of the nucleoid and the fact that quiescent PBL do not contain a high level of preexistent strand breaks, the observed differences in sedimentation rate of nucleoids isolated from quiescent and PHA-stimulated lymphocytes, may be explained by differences in compactness of the nucleoids. In this regard it is not inconceivable that nucleoids isolated from PHA-stimulated PBL have a more compact supercoiled structure resulting in an increased rate of sedimentation through neutral sucrose gradients. Changes in the amount or type of DNA-bound ligands in vivo such as RNA and protein may affect the superhelical density of nucleoid DNA [Cook et al., 1976]. Moreover,

nucleoid sedimentation depends critically on the structure of the "nucleoid cage" which is made up by the supercoiled DNA on which cytoskeletal elements have collapsed during lysis of the cells on top of the sucrose density gradients. In fact, changes in the structure of the nucleoid cage may affect the rate of sedimentation far more dramatically than the presence of SSB [Jackson et al., 1989]. Although a role of SSB in mouse splenic lymphocytes, as reported by Greer and Kaplan [Greer and Kaplan, 1983, 1984, 1986] cannot be disclaimed it is deemed unlikely that quiescent human PBL contain a high number of preexistent SSB which are ligated following a mitogenic stimulus.

Nucleoid sedimentation is a very sensitive method to detect small changes in the number of SSB [Cook and Brazell, 1976]. However, alterations in the rate of nucleoid sedimentation can arise not only from changes in the amount of strand breaks but also from changes in DNA supercoiling. Given the high number of artifacts [Weniger, 1982] and the sometimes difficult interpretation of the results [Charles and Cleaver, 1982; Weniger, 1982] it is preferable to use other, equally sensitive, techniques such as the alkaline filter elution for direct determinations of DNA damage and study of its repair.

CHAPTER 3

SINGLE-STRAND BREAK DISAPPEARANCE IN QUIESCENT AND PHYTOHEMAGGLUTININ-STIMULATED HUMAN PERIPHERAL BLOOD LYMPHOCYTES EXPOSED TO A SINGLE, LOW DOSE OF GAMMA-RADIATION[1]

3.1 Introduction

Several lines of evidence indicate that the ability of mammalian cells to remove DNA damage is positively correlated with cellular proliferative capacity. Peripheral blood lymphocytes (PBL) are an easily obtainable human tissue which can be efficiently stimulated to divide in vitro. Excision repair in human PBL following exposure to a variety of DNA-damaging agents has been found to increase after a mitogenic stimulus [for a review, see Sirover, 1990]. In particular, Lavin and Kidson reported a 20-fold increase in the amount of radioactively labelled DNA precursors incorporated, as a measure of "unscheduled DNA synthesis" (UDS), after phytohemagglutinin (PHA)-stimulation of PBL and exposure to extremely high (50-650 Gy) doses of γ-radiation [Lavin and Kidson, 1977]. However, UDS provides at best an indirect measurement of DNA repair following exposure to a DNA-damaging agent. It is often not possible to assess early steps in DNA repair due to the low amount of radioactivity built into the repair patches at these early time points. Additionally, the predominant lesions which can be detected as single-strand breaks (SSB) in the DNA of γ-irradiated cells are so-called "nucleotide gaps" which result from the loss of at least one nucleotide [Henner et al., 1982]. Determination of the repair of nucleotide gaps is not possible with UDS because these lesions do not require the incorporation of large numbers of nucleotides for their repair. Other techniques such as alkaline filter elution [Kohn et al., 1976] allow the sensitive quantitation of both nucleotide gaps and excision repair acting on small base damages which is detectable as an accumulation of SSB in the presence of the excision repair inhibitor 1-ß-D-arabinofuranosylcytosine (araC). A possible mechanism by which the presence of araC leads to the accumulation of SSB during DNA repair involves

[1]Parts of this chapter were published previously by Boerrigter and Vijg, 1991a.

incorporation of araC into DNA thereby forming an inadequate primer terminus, followed by a slow or absent rate of addition of the next nucleotide [Clarkson and Mitchell, 1983].

In general, the fast removal of γ-ray-induced SSB is thought to be mediated by an araC-insensitive DNA polymerase, i.e. ß, whereas the slower removal of base damages presumably involves to some extent other, araC-sensitive DNA polymerases [Collins, 1987]. However, we have recently demonstrated that in PBL the accumulation of a significant number of araC-associated SSB after exposure to a low dose of an alkylating agent already occurs during the first 15 min of DNA repair incubation [Boerrigter et al., 1991a, 1991b]. This suggests that base excision repair is in fact very rapid. It is therefore not inconceivable that with a sensitive technique such as tha alkaline filter elution it might be possible to visualize the occurrence of γ-ray-induced base excision repair in PBL involving an araC-sensitive DNA polymerase shortly after γ-irradiation.

In the present study we have investigated the early phases of γ-ray-induced DNA repair in relation to proliferative capacity of the lymphocyte population under study. AraC was used to estimate the number of excision repair events acting on small non-alkali-labile base damages. The results obtained show that the rate of disappearance of "real" SSB is increased approximately 2-fold in PHA-stimulated PBL as compared to quiescent cells. Furthermore, PHA-stimulated PBL from two of three donors studied displayed an increased amount of excision repair measured as araC-induced SSB during the first 15 min of repair incubation. PHA-stimulated PBL from the third donor did not differ from their quiescent counterparts with respect to the rate of excision repair. At 1 h after irradiation, both quiescent and PHA-stimulated PBL from all three subjects had accumulated approximately 1300 araC-associated SSB suggesting that the total number of excision repair events over this time period is not different for PHA-stimulated PBL, as compared to quiescent cells.

3.2 Materials and Methods

3.2.1 Lymphocyte preparation

Human peripheral blood was collected from healthy young volunteers not taking medication. Lymphocytes were isolated using Ficoll-Paque (Pharmacia, Sweden) gradients [Boyum, 1968], and washed twice in RPMI 1640 medium (Flow Laboratories, UK) plus 2% fetal calf serum (FCS). All steps were performed at 4°C. Lymphocytes that were stimulated with PHA (Wellcome Ltd., UK) were resuspended at a final concentration of $2x10^6$ cells/ml in RPMI 1640 medium plus 10% FCS supplemented with 2 mM glutamine and antibiotics. Stimulation with PHA (10 μg/ml) was for 3 days at 37°C.

3.2.2 Cell treatments

For treatment with ^{60}Co-γ-rays, PBL suspensions (3 x 10^6 cells/ml) were irradiated at 4°C in RPMI 1640 medium supplemented with 5% FCS, 2 mM glutamine and 20 mM Hepes, with or without 0.1 mM of the excision repair inhibitor araC, in a Gamma-cell 100 (Atomic Energy of Canada Ltd.) at a dose rate of 6 Gy/min. After irradiation the cells were centrifuged and resuspended in RPMI 1640 medium plus 10% FCS and 2 mM glutamine. Then they were either used in the alkaline filter elution or incubated at 37°C for various time periods for repair studies. The excision-repair inhibitor araC (Sigma, St. Louis, MO) was dissolved in phosphate buffered saline and added 30 min before irradiation. AraC is believed to interfere with gap filling by chain-termination [Robichaud and Fram, 1987]. Because in pilot experiments no additional increase in SSB was observed when araC was used in combination with 2 mM hydroxyurea, the latter was excluded from subsequent experiments.

3.2.3 Alkaline filter elution assay

SSB were measured by using a modification of the alkaline filter elution method [Kohn et al., 1976] suitable for analyzing non-radioactively labelled cells [Stout and Becker, 1982]. In the procedure followed, DNA was quantitated spectrofluorometrically with Hoechst 33258 [Mullaart et al., 1990a]. All steps were performed under subdued lighting in order to minimize artificial induction of SSB. The results were plotted as the log percent of DNA remaining on the filter as a function of elution time. Mean slopes of the linear initial part of elution curves were used to quantitate the SSB. At 4 Gy γ-radiation approximately 4000 SSB per diploid genome are introduced. This was derived from DNA molecular weight distributions after ultracentrifugation through high salt gradients calibrated with DNAs of bacteriophages with known molecular weight [Van der Schans et al., 1982]. Absolute numbers of SSB were calculated by comparison of the elution curves of the treated cells with calibration curves obtained with cells exposed to 4 Gy ^{60}Co-γ-radiation only. Consequently, 4000 times the ratio of the experimental slope to the calibration slope yielded the number of SSB. In all experiments, mean slopes were based on at least triplicate determinations on each sample and standardized by subtracting mean slopes of untreated control cells assayed simultaneously.

3.3 Results

The slope of the initial part of elution curves obtained with DNA from γ-irradiated PBL increases linearly with increasing dose within the dose range 0-8 Gy [Boerrigter et al., 1989]. Thus, the slope of these elution curves can be taken as an accurate representation of the number of SSB present in the DNA of γ-irradiated cells. The slope of the elution curve of DNA from PBL exposed to 4 Gy γ-radiation without repair incubation represents the presence of 4000 SSB per cell, calibrated as described under Materials and Methods. Figure 3.1 depicts representative elution curves of DNA from quiescent PBL exposed to 4 Gy γ-radiation and subsequently incubated in fresh medium for different repair periods. Following a single exposure of quiescent PBL to 4 Gy γ-rays, the number of SSB declines rapidly and approximates the number of SSB

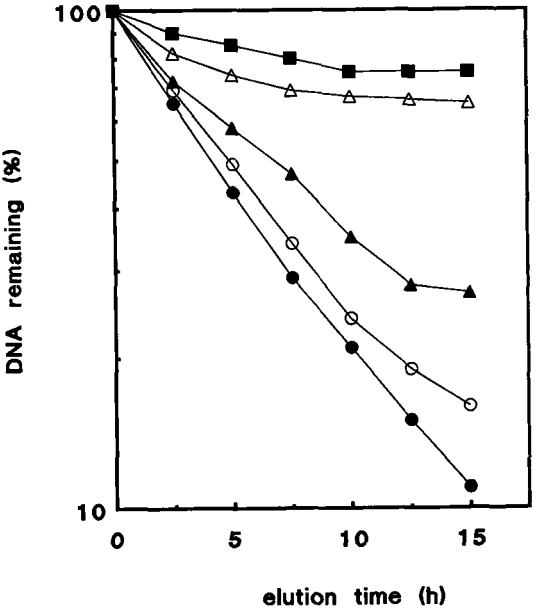

Fig. 3.1: Disappearance of γ-ray-induced SSB. Mean elution curves of untreated quiescent PBL (■) and quiescent PBL exposed to 4 Gy of γ-rays and assayed for SSB directly after irradiation (●), and after repair incubations of 5 min (○), 15 min (▲), or 1 h (△).

found in untreated PBL already at 1 h after irradiation (Figure 3.1).

In order to assess the effect of a mitogenic stimulus on the disappearance of γ-radiation-induced SSB, we compared elution curves of DNA from quiescent and PHA-stimulated PBL which were irradiated with 4 Gy of γ-rays. The number of SSB induced by 4 Gy of γ-rays was not significantly different between quiescent and PHA-stimulated PBL as was observed from the identical slopes of the elution curves obtained with DNA from these two cell populations directly after irradiation (results not shown). The results presented in Figure 3.2, obtained with PBL from three different subjects, indicate that in quiescent PBL the percentage SSB disappearance at 5 min after irradiation is 40.4 ± 8.4% (mean ± SD), whereas in PHA-stimulated cells 71.3 ± 6.8% of the induced SSB had disappeared at this time ($P < 0.05$, one-sided unpaired t-test). At 1 h after irradiation SSB disappearance was almost complete in both quiescent and in PHA-stimulated PBL (Figure 3.2).

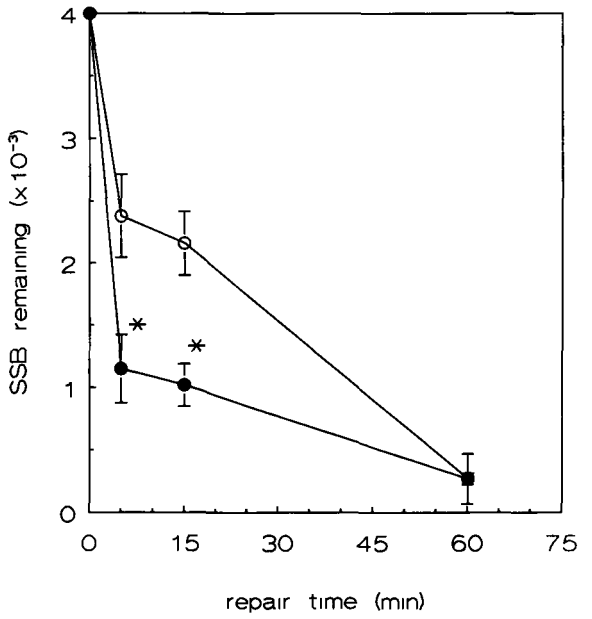

Fig. 3.2: Disappearance of γ-ray-induced SSB. Quiescent (o) and PHA-stimulated (•) PBL were irradiated with 4 Gy of γ-rays and incubated for the indicated repair periods. The number of SSB was calculated as described in Materials and Methods. The number of SSB in treated cells was corrected for the number of SSB present in untreated control cells. Data points represent the mean of three experiments with PBL from three different subjects. Bars indicate standard deviation. *Significantly different at $P < 0.05$ from quiescent PBL.

To quantitate the number of excision repair events during the first h of repair incubation after γ-irradiation, SSB were determined in quiescent and PHA-stimulated PBL in both the absence and presence of the excision repair inhibitor araC (Figure 3.3). Clearly, PHA-stimulated PBL from two subjects accumulated a higher amount of araC-associated SSB during the first 15 min of repair incubation, as compared to their quiescent counterparts (Figures 3.3A-B and 3.3C-D). Quiescent and PHA-stimulated PBL from the third donor did not differ in the amount of araC-sites accumulated (Figure 3.3E-F). At 1 h after irradiation there was no significant difference in the number of araC-associated SSB between quiescent and PHA-stimulated PBL from all three donors; both cell populations accumulated approximately 1300 SSB within this repair period. Table 3.1 summarizes the number of araC-associated SSB detected at the indicated repair times for quiescent and PHA-stimulated

PBL of the three donors studied.

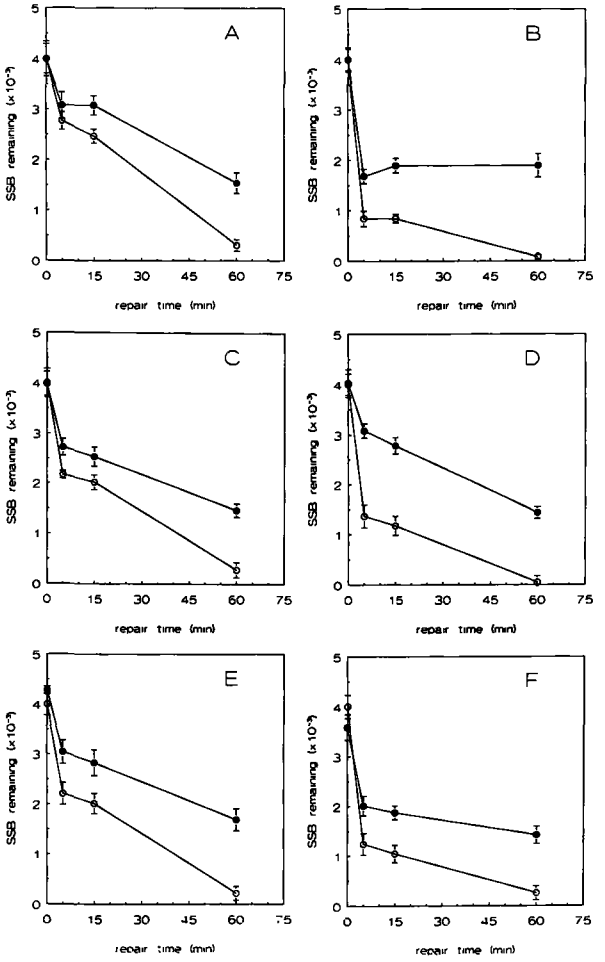

Fig. 3.3: Accumulation of araC-associated SSB. Quiescent (panels A, C and E) and PHA-stimulated (panels B, D and F) PBL from three different subjects were irradiated with 4 Gy of γ-rays and incubated for the indicated repair periods. Subject 1: panels A and B; subject 2: panels C and D; subject 3: panels E and F. The number of araC-associated SSB was calculated from the difference in the number of SSB detected in PBL which were incubated in the absence (o) or presence (•) of araC. The number of SSB in treated cells was corrected for the number of SSB present in untreated control cells as described in Materials and Methods. Data points are the mean of three elution curves. Bars indicate SD of three experiments.

Table 3.1 ACCUMULATION OF ARAC-ASSOCIATED SSB

donor	growth state	repair time (min)		
		5	15	60
2164-03	quiescent	308 ± 316	615 ± 236	1230 ± 376
	proliferating	842 ± 208[a]	1053 ± 169[a]	1390 ± 236
1167-09	quiescent	553 ± 187	514 ± 313	1181 ± 202
	proliferating	1717 ± 358[b]	1608 ± 250[b]	1388 ± 203
1166-03	quiescent	836 ± 324	816 ± 327	1472 ± 259
	proliferating	769 ± 293	828 ± 224	1163 ± 212

Values represent the mean (± SD) number of araC-associated SSB as calculated from the difference in the number of SSB detected in cultures incubated in the absence or presence of araC during the indicated repair periods. [a]Significantly different from the number of araC-associated SSB in quiescent PBL from the same donor at $P < 0.05$ (one-tailed unpaired t-test). [b]Significantly different from the number of araC-associated SSB in quiescent PBL from the same donor at $P < 0.005$. Other comparisons did not show any statistically significant difference.

3.4 Discussion

We have used the alkaline elution technique to study the early phases of γ-radiation-induced DNA repair in quiescent and PHA-stimulated human PBL. The use of the excision repair inhibitor araC made it possible to distinguish between mere SSB rejoining and excision repair events. The removal of γ-radiation-induced SSB is apparently influenced by the proliferative state of the lymphocyte population under study (Figure 3.2). For the three different donors, mitogen-stimulation of PBL resulted in approximately the same increase in SSB repair, as is evidenced by the relatively low standard deviation when the results obtained with PBL from the three subjects were averaged (Figure 3.2).

In contrast to the rapid disappearance of "real" SSB (nucleotide gaps and transient SSB that appear as a result of incision), the removal of small base damages by base excision

repair mechanisms is, in general, thought to occur after 1-2 hr of repair incubation [Collins, 1987]. However, the occurrence of araC-associated SSB indicates that a significant number of γ-ray-induced base damages is removed within 1 h in both quiescent and PHA-stimulated PBL by excision repair. The rate of accumulation of araC-associated SSB, i.e. the rate of excision repair, was increased in PHA-stimulated PBL from two of the three different donors (Figure 3.3); in a third donor no increase in araC-associated SSB was observed indicating interindividual variation in this form of repair. In a previous study we reported on the occurrence of interindividual variation in the excision repair of small base damages in quiescent PBL exposed to the alkylating agent N-ethyl-N-nitrosourea [Boerrigter et al., 1991b]. The present results suggest that quiescent PBL from different donors remove approximately the same percentage γ-ray-induced base damages via base excision repair mechanisms, whereas PHA-stimulated PBL from these donors apparently differ in their capacity to increase the rate of these repair pathways following mitogenic stimulation. Lymphocyte cultures from all three donors incorporated approximately 40,000 cpm when incubated in the presence of 10 μCi of [^{3}H]thymidine during the last 16 h of culture. Hence, differences in repair are probably not attributable to differences in the activation state of the lymphocyte population (results not shown).

Incorporation of araC into DNA leads to the formation of an inadequate primer terminus for further chain elongation [Clarkson and Mitchell, 1983]. Direct inhibition of DNA polymerases is probably much less significant. The disappearance of nucleotide gaps is not expected to be inhibited by araC to any large extent because rejoining of these particular SSB is thought to be mediated by DNA polymerase ß [Bryant and Blöcher, 1982; Iliakis et al., 1982] and it does not require a prolonged action of the DNA polymerase during which araC may get incorporated. Although a previous study by Cleaver indicated that a fraction (± 30%) of araC-associated SSB are eventually ligated within 2.5 h [Cleaver, 1983] this is not expected to influence the number of araC-associated SSB detected over the short repair periods used in the present study. Because DNA polymerase ß is resistant to araCTP [Lynch et al., 1976; Yosida et al., 1977] the accumulation of araC-associated SSB represents the inhibition of repair processes dependent on DNA polymerase α, δ or ε. Which type of DNA polymerases is actually inhibited by araC during γ-ray-induced base excision repair cannot be determined from the present data. Recent evidence suggests that one of the two classes of

DNA polymerase δ is involved in UV-induced excision repair [Dresler and Frattini, 1986; Dresler and Kimbro, 1987; Nishida et al., 1988]. Based on its ability to become highly processive in the absence of the auxiliary protein of DNA polymerase δ (proliferating cell nuclear antigen) it has recently been renamed DNA polymerase ϵ [Burgers et al., 1990]. It is not inconceivable that DNA polymerase ϵ is also involved in excision repair after γ-irradiation.

In any case, our results clearly show that base excision repair acting on γ-radiation-induced base damages is very rapid and apparently mediated to some extent by and araC-sensitive DNA polymerase, possible DNA polymerase ϵ. This does not exclude, however, a role for DNA polymerase ß in the excision repair of small base damages as was previously postulated by Collins [Collins, 1987] and Cleaver [Cleaver, 1983]. Assuming that the ratio between base damages and SSB is approximately 0.5 [Van Loon et al., 1991], this would indicate that about 1300 of the 2000 induced base damages are removed within 1 h after irradiation via excision repair processes dependent on an araC-sensitive DNA polymerase. It should be realized, however, that we have no indication whether the araC-associated SSB detected in the present study represent the same base damages as observed by Van Loon et al., or whether this previous study might have detected all base damages induced by γ-irradiation [Van Loon et al., 1991]. Nevertheless, our present results do show that at least 1300 γ-ray-induced base damages are removed within 1 h after γ-irradiation.

Which of the several types of base damages induced by ionizing radiation [see Téoule, 1987] are actually removed via excision repair pathways cannot be concluded from our present results. Human cells are known to remove 5',6'-dihydroxydihydrothymine [Mattern et al., 1975] and a further type of uncharacterized base damage(s), recognized as sites in the DNA sensitive to a γ-endonuclease activity in extracts of Micrococcus luteus [Paterson and Setlow, 1972; Van Loon et al., 1991].

The absence of any difference in the total amount of excision repair between quiescent and stimulated PBL is seemingly at variance with earlier data from Lavin and Kidson who observed a 20-fold increase in excision repair in PHA-stimulated PBL [Lavin and Kidson, 1977]. However, these authors quantitated UDS in the presence of HU at 5 days of PHA-stimulation after exposure to extremely high doses (50-650 Gy) of γ-radiation which makes a direct comparison between our present results and those of Lavin and Kidson very difficult.

Increased SSB disappearance in PHA-stimulated PBL has also been found after treatment with methyl methanesulfonate [Boerrigter et al., 1991a], but not after exposure to ethylnitrosourea [Boerrigter and Vijg, 1991b]. It therefore appears that mitogen-stimulation of PBL does not result in an increased removal of all types of DNA lesions.

CHAPTER 4

INDUCTION AND DISAPPEARANCE OF DNA STRAND BREAKS IN HUMAN PERIPHERAL BLOOD LYMPHOCYTES AND FIBROBLASTS TREATED WITH METHYL METHANESULFONATE[1]

4.1 Introduction

Methyl methanesulfonate (MMS) has been used extensively as a 'model' DNA-alkylating agent for studies on alkylation damage in DNA of both prokaryotic and eukaryotic organisms [see Roberts, 1978 for a review]. MMS is a directly acting methylating agent yielding the major adduct N7-methylguanine (N7-meG), which accounts for 86% of the total DNA alkylation, and several minor adducts [Hemminki, 1983]; direct alkylation of the phosphodiester chain is very infrequent [Lawley and Brookes, 1963].

Exposure of cells to alkylating agents results in the formation of DNA modifications that can be detected as single-strand breaks (SSB), after denaturation of the DNA in alkaline medium, with the highly sensitive alkaline filter elution technique [Kohn et al., 1976]. These SSB are the result of the spontaneous or enzymatic breaking of the N-glycosidic bond of alkylated purines and pyrimidines, leaving an apurinic/apyrimidinic (AP) site which is readily hydrolyzed by AP endonucleases or by alkali to yield a strand break [Kohn and Spears, 1976; Singer and Brent, 1981]. Also, the SSB detected can be a consequence of direct alkaline hydrolysis at the site of the alkylated base damages.

Human PBL offer the most readily available source of DNA for the measurement of persistent alkyl-DNA adducts in humans exposed to alkylating agents. However, only limited studies have been performed on the capability of freshly isolated quiescent cells to repair DNA-alkylation damage [Scudiero et al., 1976; Schutte et al., 1988]. Most studies have employed either fibroblast or lymphoid cell lines, which allow radioactive labelling of the cultured cells in order to detect the DNA [Scudiero et al., 1976; Bohr and Kober, 1985; Cleaver, 1985; Munzer et al., 1988]. However, cell lines may be less representative for the <u>in vivo</u> situation than freshly isolated cells. Furthermore, the pattern of DNA-damage

[1]Parts of this chapter were published previously by Boerrigter et al., 1991a.

induction and removal may differ from cell type to cell type but may also depend on the proliferative status of the cell population under study. Therefore, we investigated SSB induction and disappearance after MMS treatment in both freshly isolated and PHA-stimulated PBL in comparison to confluent and actively dividing fibroblasts, using the alkaline filter elution technique [Kohn et al., 1976]. To discriminate between certain types of repair, the effect of 1-ß-arabinofuranosylcytosine (araC) was investigated; this compound inhibits gap-filling during excision repair [Cozzarelli, 1977]. The results indicate that MMS-induced SSB disappearance (1) is generally higher in fibroblasts than in PBL; (2) is slightly influenced by the proliferative status in both cell types; (3) correlates with cell survival; and (4) probably involves different forms of repair in the 2 cell types.

4.2 Materials and Methods

4.2.1 Cell isolation and culture

Human peripheral blood was collected from healthy volunteers not taking medication. Lymphocytes were isolated using Ficoll-Paque (Pharmacia) gradients [Boyum, 1968], and washed twice in RPMI 1640 medium (Flow Laboratories) plus 2% fetal calf serum (FCS). All steps were performed at 4°C. Lymphocytes that were stimulated with PHA (Wellcome Ltd) were resuspended at a final concentration of $2x10^6$ cells/ml in RPMI 1640 medium plus 10% FCS supplemented with 2 mM glutamine and antibiotics. Stimulation with PHA (10 μg/ml) was for 3 days at 37°C.

Primary human fibroblasts were derived from skin biopsies of healthy young donors as described earlier [Vijg et al., 1986]. Cells were passaged with a 1:2 split ratio in Dulbecco's modification of Eagle's medium (DMEM, Flow Laboratories, UK) supplemented with 10% FCS, 2 mM glutamine and antibiotics. Fibroblasts were studied between 15 and 25 cumulative population doublings. For experiments with actively dividing cells, confluent fibroblasts were trypsinized, subdivided 1:2 in fresh medium and cultured for an additional 48 hr before use in the alkaline filter elution. Confluent monolayers were obtained by high-density inoculation and keeping fibroblasts for 10 days without passaging as described. Autoradiographic studies indicated less than 5% S-phase cells at that time [Vijg et al.,

1986].

4.2.2 Cell Treatments

MMS (Aldrich Chemie) was dissolved in phosphate-buffered saline (PBS) immediately before use. Both PBL suspensions and fibroblast monolayers were exposed to MMS for 45 min with or without 100 μM of the excision-repair inhibitor araC (Sigma) in RPMI 1640 medium plus 20 mM Hepes, 2 mM glutamine and 5% FCS at 37°C. Because in early experiments no additional increase in SSB was observed when araC was used in combination with 10 mM hydroxyurea, the latter was excluded from subsequent experiments. At the end of exposure, the cells were washed and resuspended in RPMI 1640 supplemented with 10% FCS and glutamine (PBL) or DMEM plus 10% FCS and glutamine (fibroblasts). Then they were either used in the alkaline filter elution or incubated at 37°C for various time periods for repair studies. For treatment with ^{60}Co-γ-rays, PBL suspensions or fibroblast monolayers were irradiated on ice in a Gamma-cell 100 (Atomic Energy of Canada Ltd.) at a dose rate of 6 Gy/min. After irradiation the cells were centrifuged and resuspended in RPMI 1640, 10% FCS, 2 mM glutamine (PBL) or incubated in DMEM, 10% FCS, 2 mM glutamine (fibroblasts).

4.2.3 Alkaline filter elution assay

The technique of alkaline filter elution was used to measure SSB as described in Chapter 2. All steps were performed under subdued lighting in order to minimize artificial induction of SSB. The results were plotted as the log percent of DNA remaining on the filter as a function of elution volume. Mean slopes of the linear initial part of elution curves were used to quantitate the SSB. Absolute numbers of SSB were calculated by comparison of the elution curves of the treated cells with calibration curves obtained with cells exposed to 4 Gy ^{60}Co-γ-radiation, which were assayed in the same experiment. At 4 Gy γ-radiation approximately 4000 SSB per diploid genome are introduced [Van der Schans et al., 1982]. Consequently, 4000 times the ratio of the experimental slope to the calibration slope yielded the number of SSB. In all experiments, mean slopes were based on at least triplicate

determinations and standardized with reference to mean slopes of untreated control cells assayed simultaneously.

4.2.4. Incorporation of ^{3}H-thymidine

The incorporation of tritiated thymidine (^{3}H-TdR) into DNA of both PHA-stimulated PBL and actively dividing fibroblasts after exposure to MMS was quantitated as a measure of cell viability. After treatment with MMS, PBL suspensions were cultured in flat-bottomed wells of a sterile 24-well culture plate (Costar) at a concentration of $1x10^6$ PBL/well in 1 ml of RPMI 1640 medium with 10% FCS, 2 mM glutamine, antibiotics and 10 μg/ml PHA at 37°C in a humidified incubator with 5% CO_2. After 60 h, 0.5 μCi of ^{3}H-TdR (25 mCi/mmol; Amersham) was added to each well. After 16 h, cells were harvested and washed twice with ice-cold PBS. Finally, after the addition of 5 ml picofluor the incorporation of ^{3}H-TdR was evaluated in a Mark III scintillation counter (Tracor Analytic). After exposure of the fibroblasts to MMS, the monolayer was washed 3 times with ice-cold PBS and incubated for 60 h at 37°C in a humidified incubator with 7% CO_2. Then, 0.5 μCi of ^{3}H-TdR was added, after which the cells were incubated for another 16 h time period. Finally, cells were washed 3 times with ice-cold PBS and scraped from the petri dish with a rubber policeman. Incorporation of ^{3}H-TdR was determined as described for PBL.

4.3 Results

By means of the alkaline elution method we determined the number of SSB induced in human fibroblasts and PBL exposed to 0.2 mM MMS for 45 min. The determinations were made immediately after treatment and after subsequent repair periods of different duration. In these studies we compared quiescent and PHA-stimulated PBL with confluent and actively dividing fibroblasts. Figure 4.1 shows typical alkaline elution curves of a non-stimulated PBL and an actively dividing fibroblast population, immediately after treatment and after 1 h repair incubation. From Table 4.1, which summarizes the results, it can be derived that comparable amounts of SSB are induced in the 2 cell types. The rate of disappearance seemed to depend on both the proliferative status and the cell type. In fibroblasts the rate of

disappearance appeared to be higher than that in PBL (Table 4.1). Confluent fibroblasts were found to have only 25 ± 2% (mean ± SD) of SSB disappearance over a 1 h repair period, whereas their actively dividing counterparts removed 50 ± 12% (Table 4.1). With PBL a small, borderline, significant difference was evident when PHA-stimulated cells from two different donors were compared with quiescent PBL from the same donors; the percentage SSB disappearance at 1 h after MMS exposure in quiescent and PHA-stimulated PBL was 9 ± 4% and 23 ± 12%, respectively (Table 4.1).

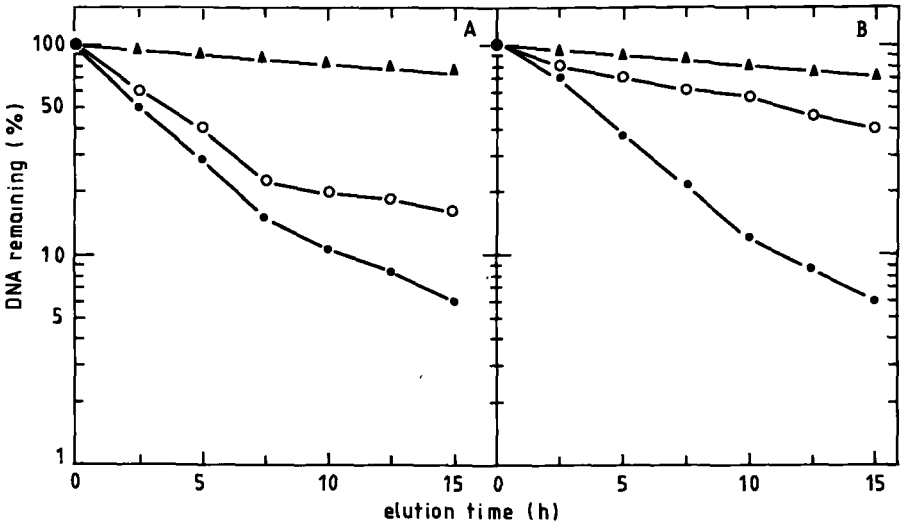

Fig. 4.1: Alkaline elution of DNA through membrane filters. Typical elution curves for human PBL (A) and human fibroblasts (B). Cells were either untreated or exposed to 0.2 mM MMS for 45 min at 37°C. Following exposure to MMS the cells were either directly used in the filter elution assay or incubated for 1 h at 37°C in fresh medium for repair incubation. (▲) untreated cells; (o) cells treated with 200 μM MMS, no repair incubation; (●) cells treated with 200 μM MMS, 1 h repair incubation.

Table 4.1 REPAIR OF MMS-INDUCED SSB IN NONDIVIDING VERSUS ACTIVELY DIVIDING PBL AND FIBROBLASTS.

	PBL			fibroblasts		
	0 h[a]	1 h[b]	repair (%)[c]	0 h[a]	1 h[b]	repair (%)[c]
nondividing	3337 ± 951	3009 ± 942	9 ± 4 (4)	3816 ± 874	2871 ± 516	25 ± 2 (4)
dividing	5084 ± 1704	3922 ± 1501	23 ± 12 (4)*	3405 ± 747	1691 ± 858	50 ± 12 (4)**

Cells were exposed to 0.2 mM MMS (45 min, 37°C). SSB were determined directly after treatment or after a subsequent 1 h repair incubation in fresh medium.
Numbers between parentheses indicate the number of independent measurements on two donors; cells from each donor were analysed twice.
*, ** = significantly different from non-dividing cells of the same type at $P < 0.05$ and $P < 0.02$, respectively (paired t-test).
[a]Number of SSB (mean ± SD) directly after treatment.
[b]Number of SSB (mean ± SD) after 1 h repair incubation.
[c]Percentage repair (mean ± SD) after 1 h repair incubation. Percentages are the mean of 4 repair values calculated from the 4 independent experiments for each cell type.

The slow rate of SSB disappearance in freshly isolated PBL was confirmed by measurements on 16 different donors (Table 4.2). Although some interindividual variation was observed, the highest percentage of SSB disappearance measured was 35%. This poor level of SSB disappearance in PBL was not due to an overall lack of repair capability of these cells. Indeed, the PBL from the same 16 donors were able to repair 89 ± 4% of the SSB within 1 h after 4 Gy γ-radiation (Table 4.2). Fibroblasts treated with the same dose of γ-radiation also removed about 90% of the induced SSB (not shown). Viability, as determined by trypan blue dye exclusion, exceeded 95% in PBL directly after treatment with 0.2 mM MMS or 4 Gy γ-radiation or after subsequent repair incubations of 1 h (not shown), indicating no hypersensitivity over these time periods and at these particular dose levels.

When repair-incubation with quiescent PBL exposed to MMS was continued for 23 h, almost no removal was observed over the first 2½ h, but eventually the induced SSB were found to disappear by approximately 50% (Figure 4.2). In actively dividing fibroblasts there was no difference in the number of SSB detected at 1, 2½ or 24 h after MMS treatment

Table 4.2 MMS-INDUCED AND γ-RAY-INDUCED SSB DISAPPEARANCE IN HUMAN PBL.

agent	0 h[a]	1 h[b]	repair (%)[c]
MMS	4185 ± 895	3680 ± 970	12 ± 12 (16)
γ	4000[d]	480 ± 210	89 ± 4 (16)

Cells were treated with 0.2 mM MMS (45 min, 37°C) or exposed to 4 Gy γ-radiation on ice. SSB were determined directly after treatment or after a subsequent 1 h repair period in fresh medium.
Numbers between parentheses indicate the number of independent measurements.
[a]Number of SSB (mean ± SD) directly after treatment.
[b]Number of SSB (mean ± SD) after 1 h repair incubation.
[c]Percentage repair after 1 h repair incubation. Percentages are the mean of 16 repair values calculated from the 16 independent experiments for each cell type.
[d]The number of SSB induced by 4 Gy γ-radiation, which was set at 4000, was used as the calibration value in the determination of the MMS-induced SSB, as described in Materials and Methods.

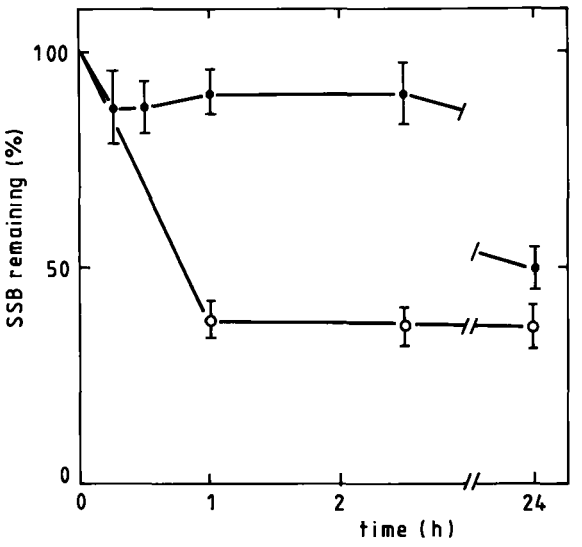

Fig. 4.2: Induction and repair of SSB in quiescent PBL and actively dividing fibroblasts. Cells were treated with 0.2 mM MMS for 45 min and incubated in fresh medium for the periods indicated on the abscissa. Points represent averages with SD of at least 2 independent experiments in triplicate. The amount of DNA damage directly after treatment was set at 100%. (●) PBL; (○) fibroblasts.

(about 60% repair; Figure 4.2). To investigate the possibility of whether saturation of repair systems in PBL might be responsible for the poor removal of MMS-induced SSB in these cells, the dose of the agent was varied. The number of SSB removed in PBL exposed to various concentrations of MMS did not change as a function of dose (Figure 4.3).

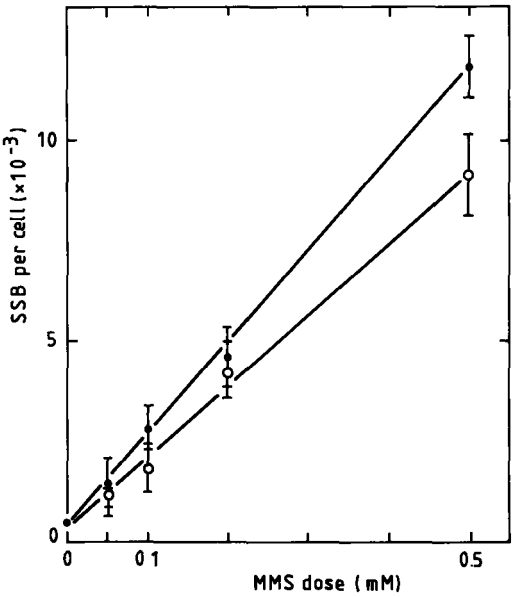

Fig. 4.3: Induction and repair of SSB in human PBL. Cells were treated with the MMS concentrations as indicated on the abscissa. Repair incubation was for 1 h at 37 °C in fresh medium. Absolute amounts of SSB were calculated as described in Materials and Methods. (●) SSB directly after treatment; (○) SSB after treatment and 1 h repair incubation. Points represent averages of 3 experiments; bars indicate standard error. Slope of the line representing SSB remaining directly after treatment is 80.3% of the slope of the line representing SSB after 1 h repair incubation.

The results presented above indicate differences between PBL and fibroblasts in the way these cells process MMS-induced DNA damage. To investigate this further we made use of araC, a deoxycytidine analog known to inhibit the polymerization step in excision repair, thereby causing an accumulation of repair-related DNA breaks [Cozzarelli, 1977]. The presence of araC during the exposure of either quiescent or PHA-stimulated PBL to MMS resulted in the accumulation of SSB (Figure 4.4A&B), indicating the occurrence of

DNA excision repair events already during treatment. No significant accumulation was observed in either confluent or actively proliferating fibroblasts, treated with MMS in the presence of araC (Figure 4.4C&D). When araC was present only during the 1 h repair period, an accumulation of breaks in quiescent or

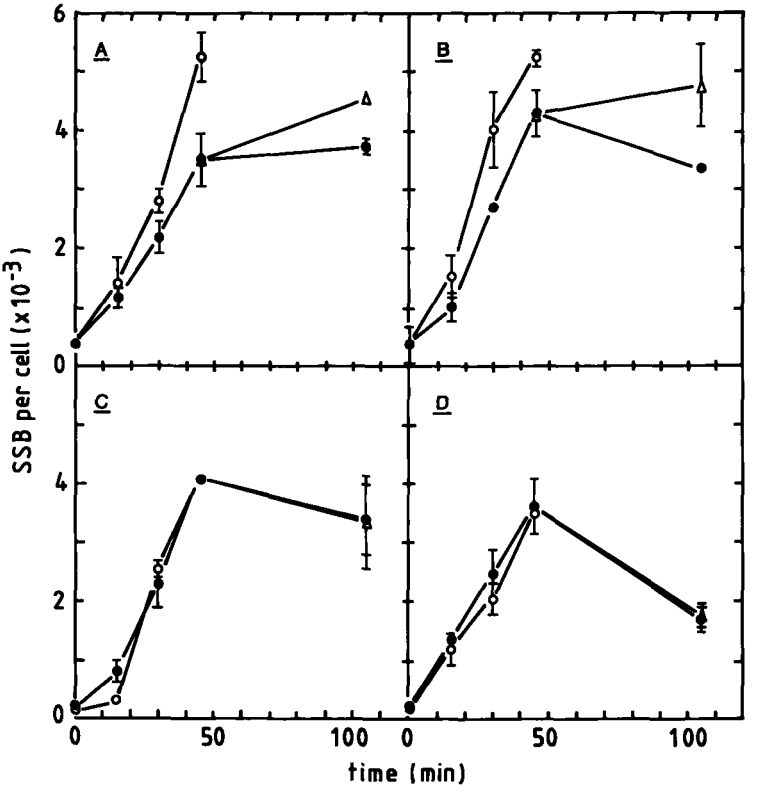

Fig. 4.4: Induction and repair of SSB in PBL and fibroblasts exposed to MMS. The 45 min exposure of quiescent (A) or PHA-stimulated (B) lymphocytes or confluent (C) or actively dividing (D) fibroblasts to MMS was either in the absence or presence of araC. SSB were determined after 15, 30 and 45 min of MMS exposure. After 45 min, cells treated with MMS, in the absence or presence of araC, were incubated in fresh medium for 1 h, either in the presence or absence of araC. (o): number of SSB directly after MMS treatment or repair incubation without araC; (•): number of SSB directly after MMS treatment in the presence of 100 μM araC; (Δ): number of SSB after 1 h repair incubation in the presence of 100 μM araC. Points represent averages of at least 3 experiments; bars indicate standard error.

mitogen-stimulated PBL, but not in confluent or proliferating fibroblasts, was observed relative to cells incubated without araC (Figure 4.4). The absence of any effect of araC on

SSB accumulation in fibroblasts was confirmed with a 10- and 100-fold higher dose (results not shown).

In an attempt to correlate the different rates of SSB disappearance in MMS-exposed PBL and fibroblasts to their survival, we studied their capacity to incorporate ^{3}H-thymidine over a 16-h period starting at 60 h after MMS treatment. The curves for PHA-stimulated PBL and actively dividing fibroblasts are shown in Figure 4.5. After a small increase in the amount of incorporated radioactive label in both cell types after a dose of 0.2 mM MMS, a sharp decline in ^{3}H-thymidine incorporation with increasing dose was observed in PBL, whereas in fibroblasts only a shallow decline was observed over the same dose-range (Figure 4.5).

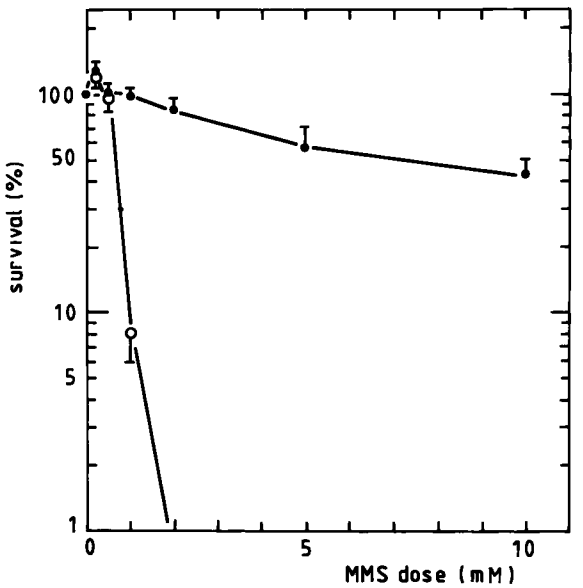

Fig. 4.5: Incorporation of ^{3}H-thymidine into DNA (survival) of PHA-stimulated PBL and actively dividing fibroblasts after treatment with various concentrations of MMS for 45 min, and plotted as the percentage of incorporated label in control cells. (o) PHA-stimulated PBL; (•) actively dividing fibroblasts. Unstimulated, untreated PBL incorporated 1216 ± 756 (mean ± SD) cpm whereas stimulated, untreated PBL incorporated 40,054 ± 5047 cpm. Points represent averages of 3 experiments; bars indicate standard error.

4.4 Discussion

Alkylating agent-induced SSB as detected by alkaline elution essentially result from the spontaneous or enzymatic hydrolysis of DNA-alkylation damage [James and Lehmann, 1982] yielding alkali-labile AP sites, or reflect the incision step of excision repair events. In addition, the SSB can represent the lesions themselves when these are alkali-labile [Shooter and Merrifield, 1978]. In this study we investigated the induction and disappearance of SSB in MMS-exposed PBL and fibroblasts by using alkaline elution. Although the amount of detectable SSB induced by MMS did not differ between PBL and fibroblasts, the rate of their disappearance was found to be much lower in the former cell type.

With respect to the type of lesions detected as SSB in this study, O^6-methylguanine and phosphotriesters, which constitute only a minor fraction of MMS-induced adducts, can be ignored since they are not alkali-labile under the conditions applied. By contrast, the major alkylation products, N7-meG and N3-meA, which account for 86% and 10% of the total DNA alkylation, respectively [Hemminki 1983], are alkali-labile; as much as 5% of these lesions could be detectable as SSB in our system [Snyder and Regan, 1981]. The percentage of DNA alkylations detectable as strand breaks by alkaline filter elution is dependent on the rate of depurination in alkali.

The SSB accumulation in PBL exposed to MMS in the presence of araC indicates the involvement of some form of excision repair which is not operative in fibroblasts, as evidenced by the virtual absence of SSB accumulation in this latter cell type during MMS treatment or during the subsequent repair period in the presence of araC (Figure 4.4). Fibroblasts might employ a form of direct repair, similar to O^6-alkylguanine DNA alkyltransferase. However, as yet there is no experimental evidence for such a mechanism.

Although hardly any SSB disappearance is observed over the first few hours after MMS exposure, araC-related breaks are readily detectable, indicating the occurrence of excision repair activities. It should be realized that, whereas only a small part of MMS-induced lesions are detected as SSB in our system (1-5%), all lesions are potentially amenable to excision repair. Hypothetically, MMS-induced damage in PBL is removed very slowly by an excision repair mechanism, while fibroblasts appear to utilize exclusively a more efficient direct repair system.

Our observation that fibroblasts do not display an accumulation of MMS-induced araC-dependent sites appears to contradict with results of Snyder and Regan [1982], who found, with alkaline sucrose gradient sedimentation, that fibroblasts exposed to 4 mM MMS accumulated 0.5 araC-associated sites/10^8 Daltons over a 1-h repair period. Assuming a molecular weight of 10^{12} Da for the total DNA of a cell and a linear accumulation of araC-sites with increasing dose [Snyder and Regan, 1982], this should have resulted in the accumulation of about 250 araC-sites per cell following treatment with 0.2 mM MMS. Such a small accumulation is not detectable under our experimental conditions. Alternatively, it is not inconceivable that araC-dependent excision repair in fibroblasts only occurs at higher doses of MMS, when any putative araC-independent repair pathway, such as direct repair, has become saturated.

Whatever the precise mechanisms underlying SSB disappearance in PBL and fibroblasts, there is some dependency on the cellular proliferative status, although in PBL this dependency is only marginally significant. One can argue that in actively proliferating cells the new high molecular weight DNA (resulting from continued replication) may negatively influence the elution rate of DNA, thereby leading to higher repair values. This, however, is unlikely to be the case over short time intervals; the amount of high molecular weight DNA appearing over a repair period of 1 h is negligible.

Interestingly, the particular excision repair pathway, which can be inhibited by araC and is observed only in PBL, is <u>not</u> dependent on the proliferative status of the cell as can be seen from the not significantly different amounts of araC-associated SSB induced over the 45-min MMS treatment or during the subsequent 1-h repair period. Recent studies have demonstrated that some DNA repair pathways are actively regulated as a function of cell proliferation. The specific activity of several DNA repair enzymes, including uracil DNA glycosylase [Vollberg et al., 1984; Duker and Grant, 1980], hypoxanthine DNA glycosylase [Dehayza and Sirover 1986a, 1986b], 3-methyladenine DNA glycosylase [Gombar et al., 1981], and O^6-alkylguanine DNA alkyltransferase [Pegg and Weist, 1983; Trey and Gerson, 1989], have been shown to be positively correlated with the proliferative state of the cell. Additionally, increased DNA repair activity in stimulated PBL has been described for cells irradiated with ultraviolet light [Lewensohn et al., 1979; Freeman and Ryan, 1988] or ionizing radiation [Lavin and Kidson, 1977; McWilliams et al., 1983] and cells exposed to

@N-acetoxy-2-acetylaminofluorene or MMS [Scudiero et al., 1976].

The lower incorporation of ^{3}H-TdR, used in our study as a measure of cell viability, into DNA of MMS-treated PBL might be the consequence of the slow removal of the most abundant candidate lesion, i.e., N7-meG. Although O^{6}-alkylation of guanine is the major premutagenic lesion of monofunctional alkylating agents [see Saffhill et al., 1985], the alkylation of the N7 of guanine may significantly contribute to mutagenesis [Lee et al., 1990], especially under conditions where excision repair is deficient [Van Zeeland et al., 1990], e.g. in PBL. Moreover, N7-meG can undergo a rearrangement to yield a ring-opened imidazole form. The ring-opened N7-meG, or formamidopyrimidine, is a block to DNA synthesis in vitro [Boiteux and Laval, 1983]; therefore, if not repaired, it probably will constitute a lethal lesion for the cell. It should be noted that the difference in the rate of disappearance of MMS-induced SSB was observed at a non-toxic dose of MMS for both cell types (0.2 mM) which precludes the possibility that the lower rate of disappearance in PBL is the effect of a lower survival. Apparently, at this particular (or any lower) dose, the number of MMS-induced SSB present is too small to exert a pernicious effects on cellular survival. Interestingly, at the lowest dose of MMS tested, both PBL and fibroblasts were slightly stimulated, i.e., they incorporated a higher amount of tritiated thymidine as compared to untreated control cells (Figure 4.5). Possibly, low doses of MMS stimulate, rather than inhibit, growth of PBL and fibroblasts as has been found for mouse epidermal cells exposed to active oxygen [Muehlmatter et al., 1988].

CHAPTER 5

INDUCTION AND DISAPPEARANCE OF DNA STRAND BREAKS AND/OR ALKALI-LABILE SITES IN HUMAN LYMPHOCYTES EXPOSED TO N-ETHYL-N-NITROSOUREA[1]

5.1 Introduction

Many alkylating agents are potent carcinogens when administered to animals. In particular N-nitroso compounds, such as N-ethyl-N-nitrosourea (ENU), produce tumors in rats, mice and hamsters [for a review, see Montesano and Bartsch, 1976]. ENU causes damage to DNA by formation of DNA-base adducts. Approximately 80% of the reaction of ENU with DNA in vivo [Den Engelse et al., 1986] or in vitro [Singer et al., 1978] is at oxygen atoms, with the ethyl phosphotriester representing 50-60% of all ethylation products, O^6-ethylguanine (O^6-etG) and O^2-ethylthymidine (O^2-etT) about 8% each and O^4-ethylthymidine (O^4-etT) and O^2-ethylcytosine (O^2-etC) 2-4% each. Minor ethylation products on the nitrogen atoms in DNA are N7-ethylguanine (N7-etG) and N3-ethyladenine (N3-etA) [Singer, 1979].

DNA ethyl-adducts may be removed by two basic mechanisms in mammalian cells, namely excision repair and direct repair. In excision repair, alkylated bases can be removed by specific DNA-glycosylases [Singer and Brent, 1981]. The resulting apurinic (AP) site in the DNA is subsequently cleaved by the action of a 5' AP endonuclease. The resulting 5'-deoxyribose-phosphate moieties can be excised by the action of a 5'-->3' exonuclease followed by the insertion of intact nucleotides into the DNA strand by DNA polymerase and ligation to the original single-strand by DNA ligase [for reviews, see Friedberg, 1985b; Strauss, 1985]. In direct repair, the damaged base is repaired by the direct transfer of the alkyl group to an alkyl-accepting protein, leaving the intact base [for a review, see Lindahl, 1982].

Interindividual variation in the ability of human cells to remove alkylating agent-induced DNA base damages may be an important factor in determining cellular susceptibility to the mutagenic and carcinogenic action of these agents. To our knowledge, interindividual variation in the repair of DNA alkyl-adducts has only been described for the direct repair of O^6-alkylguanine by O^6-alkylguanine-DNA alkyltransferase (O^6-AT) [Waldstein et al., 1982; Gerson

[1]Parts of this chapter were published previously by Boerrigter et al., 1991b.

et al., 1985; Sagher et al., 1988, 1989]. It would be of interest to determine if also the activity of excision repair pathways acting on other alkyl-adducts might differ among human individuals.

Exposure of cells to alkylating agents results in the formation of lesions in DNA that can be detected as single-strand breaks (SSB), after denaturation of the DNA in alkaline medium, with the highly sensitive alkaline filter elution technique [Kohn et al., 1976]. These SSB are either repair-associated breaks present as such, or breaks formed as a consequence of the alkali-lability of adducts, by hydrolysis at alkylated base damages or at AP sites, which are alkali-labile [Margison and O'Connor, 1978].

In this study we have determined the kinetics of induction and disappearance of ENU-induced SSB in PBL, freshly isolated from a number of apparently normal human donors, by using the alkaline filter elution technique. The results showed partial repair of the ENU-induced lesions, at a rate that varied among normal individuals from about 0% to about 55% in 1 h. To study whether the level of repair in PBL correlated with survival after ENU treatment, ^{3}H-thymidine incorporation in PHA-stimulated cells was determined as a measure of cell viability.

5.2 Materials and Methods

5.2.1 Lymphocyte preparation

Human peripheral blood was collected from healthy volunteers not taking medication. The mean age was 34 $\pm$ 7 years (range 24-47 years). Lymphocytes were isolated by using Ficoll-Paque (Pharmacia) gradients [Boyum, 1968], and washed twice in RPMI 1640 medium (Flow Laboratories) plus 2% fetal calf serum (FCS). All steps were performed at 4°C.

5.2.2 Cell Treatments

ENU (Sigma) was dissolved in dimethylsulfoxide (DMSO) immediately before use. PBL suspensions were exposed to ENU for 20 min with or without 100 μM 1-ß-D-arabinofuranosyl-cytosine (araC; Sigma) in RPMI 1640 medium plus 20 mM Hepes, 2 mM glutamine and 5% FCS at 37°C. The DMSO concentration was never higher than 1%. AraC is believed to interfere with gap filling by chain-termination [Robichaud and Fram, 1987]. At the end of the exposure period, cells were centrifuged and resuspended in RPMI 1640 supplemented with 10% FCS and glutamine and incubated for repair at 37°C. During γ-irradiation (see below), cell suspensions were kept on ice. Afterwards the cells were centrifuged and resuspended in RPMI 1640 plus 10% FCS.

5.2.3 Filter elution assay

Alkaline filter elution was used to determine existing SSB together with alkali-labile sites as described in Chapter 2. The elution results were plotted as the log percent of DNA remaining on the filter as a function of elution time. Mean slopes of the linear initial part of elution curves were used to calculate the number of SSB. Absolute amounts of SSB were calculated by calibration of mean elution curves of ENU-treated cells with mean elution curves of control cells exposed to 4 Gy ^{60}Co-γ-radiation (Gamma-cell 100, Atomic Energy of Canada Ltd.; dose rate 6 Gy/min), which were assayed in the same experiment. At 4 Gy γ-radiation approximately 4000 SSB per diploid genome are introduced [Van der Schans et al., 1982]. The absolute amount of SSB per cell was calculated by using the formula

$$\frac{\text{slope of the elution curve of treated cells}}{\text{slope of the elution curve of irradiated cells}} \times 4000$$

In all experiments, mean slopes were based on at least triplicate determinations on each sample. Mean slopes of treated cells were standardized by subtracting mean slopes of untreated control cells assayed simultaneously.

5.2.4 Incorporation of ^{3}H-thymidine

After treatment with ENU, PBL suspensions were cultured in flat-bottomed wells of a 24-well culture plate (Costar) at 10^6 PBL/well in 1 ml of RPMI 1640 medium with 10% FCS, 2 mM glutamine and antibiotics. The cells were stimulated with phytohemagglutinin (PHA; 10 μg/ml; Wellcome Foundation) at 37°C in a humidified incubator with 5% CO_2. After 60 h of culture, 0.5 μCi of ^{3}H-thymidine (25 Ci/mmole, Amersham) was added to each well. After 16 h cells were harvested and washed twice with ice-cold PBS. Finally, after the addition of 5 ml picofluor the incorporation of ^{3}H-thymidine was evaluated in a Mark III scintillation counter (Tracor Analytic).

5.3 Results

5.3.1 Induction and disappearance of SSB

We investigated the induction and disappearance of SSB in human PBL after treatment with ENU. Figure 5.1 shows the amount of SSB induced in freshly isolated PBL by various doses of ENU. The absolute number of SSB detected increased linearly with increasing dose in the dose range applied. Under these conditions low numbers of SSB and small changes therein can readily be quantitated [Boerrigter et al., 1989].

Figure 5.2A shows the time course of SSB disappearance for PBL from a given individual, representative for most of the 15 donors studied, after exposure to various doses of ENU. For the 4 doses tested 35-45% of the induced DNA damage disappeared within 1 h. For the lowest dose (0.25 mM) an additional 35% had disappeared at 24 h after treatment; at higher doses of ENU, i.e., 0.5, 1 or 2 mM, no additional disappearance of SSB was observed for up to 24 h after the exposure (Figure 5.2A). As is illustrated in Figure 5.2B, the disappearance of SSB induced to comparable levels by γ-radiation was rapid and 70-80% complete for all doses at 2½ h posttreatment.

Our observation that after exposure of PBL to ENU 35-45% of the induced SSB disappeared within 1 h could indicate that the remaining SSB represent persistent lesions. Alternatively, the remaining SSB could be a consequence of a rapidly exhausted excision

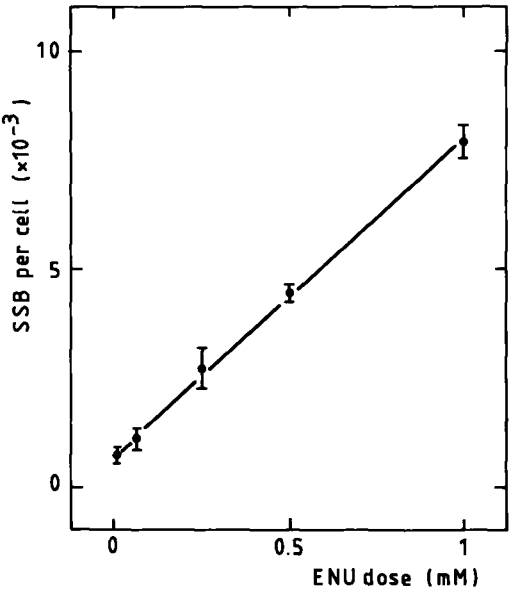

Fig. 5.1: Induction of SSB in PBL exposed to various concentrations of ENU. Exposure was for 20 min at 37°C. Results of a typical experiment are shown.

repair system. To investigate this further, we performed a split-dose experiment by repeating the treatment with equal doses of ENU at 2½-h time intervals (Figure 5.3). In these and the further experiments dosages of 0.5 mM ENU or 4 Gy γ-radiation were used, unless specified otherwise. After treatment with the first dose of ENU, 46% of the 3900 SSB induced per cell disappeared within the first 2½ hr of repair incubation. Subsequent exposure of the same cells to a second dose of ENU induced approximately the same number of SSB as the first dose, but because 47% of the SSB from the first dose were still present, the total number of SSB detected was higher, i.e. about 5600. After 1 h only 1600 of these SSB had disappeared. (Figure 5.3). After a subsequent third dose about 4500 SSB again were added to the remaining ones; only about 2600 of these SSB disappeared within 1 h (Figure 5.3). The ever increasing level of remaining SSB indicate that these are persistent lesions and do not represent an equilibrium level of excision repair events. Furthermore, the repetitious removal of 30 to 50% of the number of

additional SSB demonstrates that the repair system retains its capacity over the period studied.

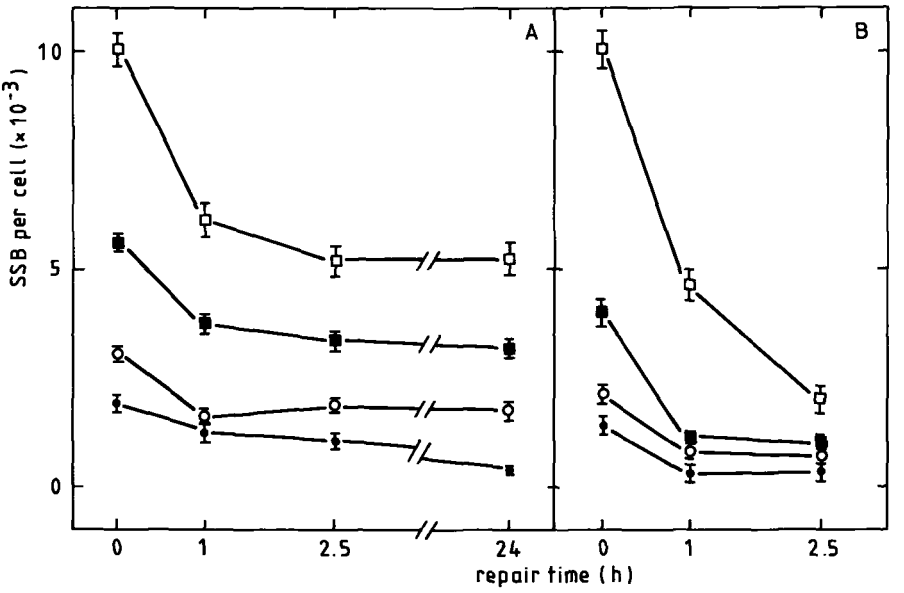

Fig. 5.2: Repair of SSB in PBL induced by ENU or γ-radiation. Cells were treated with 0.25 (•), 0.5 (○), 1 (▪) or 2 (□) *mM* ENU for 20 min at 37 °C (panel A) or with 1 (•), 2 (○), 4 (▪) or 8 Gy (□) γ-radiation (panel B) and incubated for the periods indicated on the abscissa. The number of SSB in treated cells was corrected for the number of SSB in untreated control cells. Results of a typical experiment are shown.

5.3.2 Interindividual variation in the rate of disappearance

ENU-treatment of PBL from 15 different normal human donors resulted in identical amounts of SSB induced (results not shown), but a greatly differing extent of disappearance after 1 h of posttreatment was observed. In PBL of the 15 individuals studied, 35 ± 16% (mean ± SD) of the SSB induced had disappeared at 1 h after exposure to ENU (Figure 5.4). For comparison we also determined the extent of SSB disappearance in PBL at 1 h after γ-

irradiation; for the 15 individuals studied 85 ± 3% (mean ± SD) of the SSB induced disappeared within 1 h. Apparently there are no significant interindividual differences in the disappearance of γ-ray-induced SSB (Figure 5.4). Comparison of the time course of ENU-induced SSB removal of one high (N18) and one low (N01) responder showed that it is predominantly the rate of disappearance that differs between these 2 individuals; after 2½ h, in PBL of the low responder 42% of the SSB had disappeared (Figure 5.5), not much less than the level reached in the other PBL, which remained constant after 1 h.

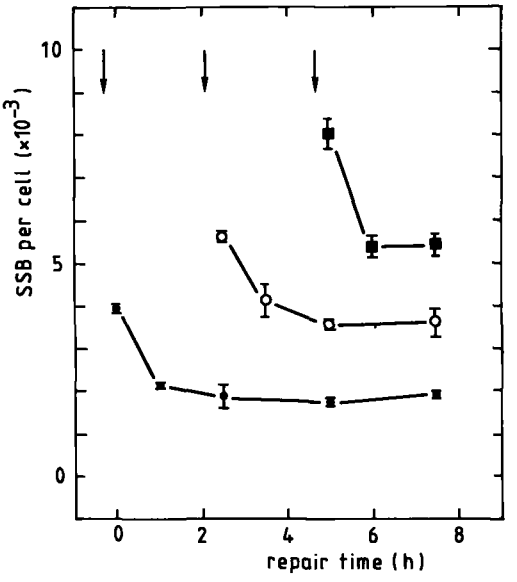

Fig. 5.3: Presence of SSB after exposure to multiple doses of ENU. PBL from donor N18 were treated 3 times with 0.5 mM ENU for 20 min at 37 °C (arrows) followed by repair incubation for the periods indicated on the abscissa. Following each ENU treatment cells were washed and incubated in fresh medium. The number of SSB in treated cells was corrected for the number of SSB in untreated control cells. Values, mean ± SD of 3 independent experiments.

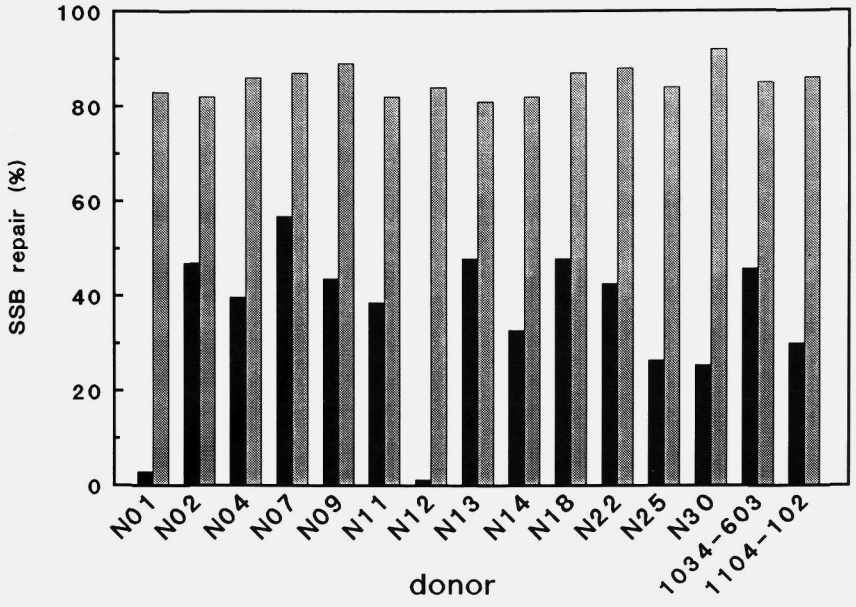

Fig. 5.4: Interindividual variation in the repair of ENU-induced (closed bars) or γ-radiation-induced (shaded bars) SSB in PBL. Cells were treated with 0.5 mM ENU for 20 min at 37°C or irradiated with 4 Gy γ-radiation at 0°C, followed by a 1-h repair incubation in fresh medium. Percentages repair were calculated from the percentual change in the number of SSB present directly after treatment and following an 1-h repair incubation. The number of SSB in treated cells was corrected for the number of SSB in untreated control cells.

To determine the reproducibility of the observed interindividual variation in the rate of SSB disappearance, we followed a high and a low responder in time. PBL from donors N01 and N18 were sampled and tested with intervals of 1 month. The results presented in Figure 5.6 show that the interindividual difference was constantly present although some intra-individual variation was observed.

5.3.3 Survival

In an attempt to correlate the difference in repair activity in PBL with their sensitivity towards ENU, we adopted the incorporation of tritiated thymidine after PHA-stimulation as a measure for survival. When PBL of donor N01 and donor N18 were treated with various doses of ENU and subsequently mitogenically stimulated with PHA, the lower level of SSB disappearance in cells of the former donor correlated with a lower level of ^{3}H-thymidine incorporation, suggesting a lower level of survival (Figure 5.7).

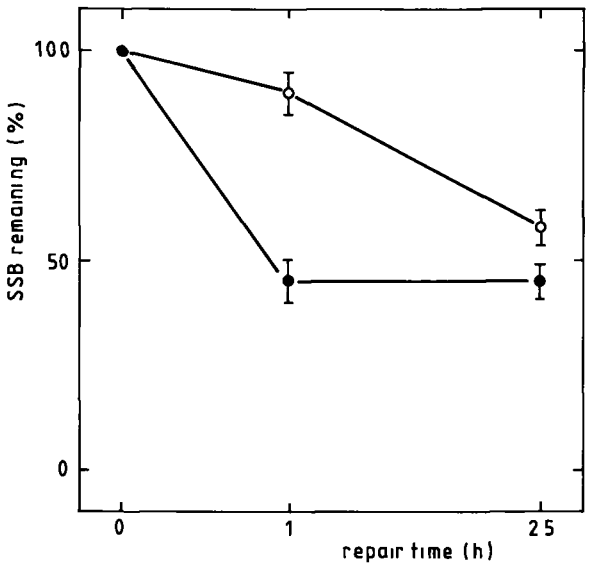

Fig. 5.5: Repair of ENU-induced SSB in PBL of individual N01 (o) or N18 (•). Cells were treated with 0.5 mM ENU for 20 min at 37 °C and incubated for the periods indicated on the abscissa. The number of SSB in treated cells was corrected for the number of SSB in untreated control cells. Bars represent SD of 3 experiments.

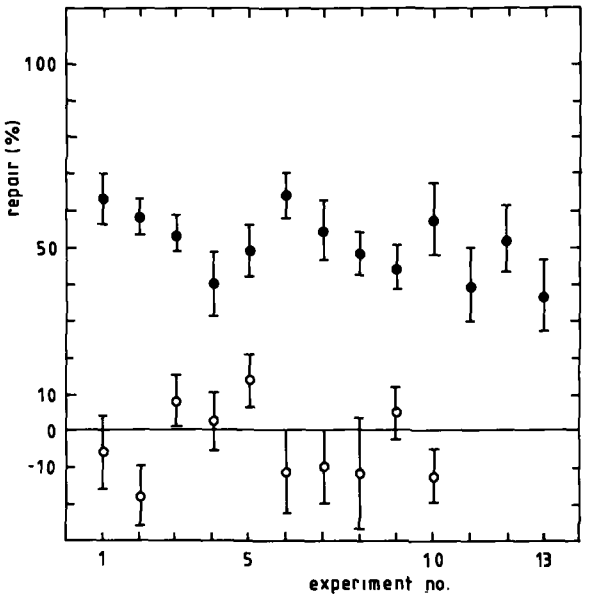

Fig. 5.6: Repair of ENU-induced SSB in PBL from individuals N01 (o) and N18 (•) sampled at intervals of 1 month. Each point represents a separate sampling. Treatment was with 0.5 mM ENU for 20 min at 37°C, followed by a 1-h repair incubation in fresh medium. Percentages repair were calculated from the percentual change in the number of SSB present directly after treatment and following an 1-h repair incubation. The number of SSB in treated cells was corrected for the number of SSB in untreated control cells. Bars represent SD within one experiment.

5.3.4 Influence of araC

To determine whether the disappearance of ENU-induced SSB over the first 1-h period was due to DNA excision repair, the induction and disappearance of SSB in PBL treated with ENU were investigated both in the presence and absence of araC. AraC, a cytosine analog, is known to inhibit the polymerization step of base/nucleotide excision repair by chain termination, which results in the accumulation of repair-related SSB [Robichaud and Fram, 1987]. The presence of araC during the treatment of PBL from donors N18 and N25, which have a

relatively high rate of disappearance of ENU-induced SSB, resulted in the accumulation of SSB, indicating that excision repair was already active during the 20 min exposure of PBL to ENU (Table 5.1). This accumulation was much less when PBL from N01, characterized by a low rate of disappearance of ENU-induced SSB, were treated in the presence of araC (Table 5.1). When araC was present only during the 1 h repair period after ENU-treatment of PBL from donors N01, N18 and N25 no decrease, or even a small increase, in the number of SSB was observed during that period (Table 5.1). This indicates that the SSB observed to disappear are breaks associated with lesions that are removed by excision repair. The addition of 100 μM araC during the repair of SSB induced by γ-radiation resulted in a 29% inhibition of repair (Table 5.1), indicating that also a part of γ-ray-induced lesions are repaired via excision repair pathways.

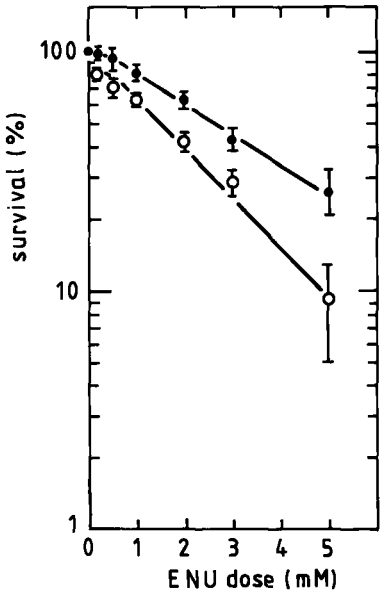

Fig. 5.7: Incorporation of ^{3}H-thymidine by PBL (survival) from donor N01 (○) and N18 (●) after exposure to various concentrations of ENU (20 min, 37°C). Following treatment, PBL were stimulated with PHA for 3 days at 37 °C and the amount of ^{3}H-thymidine incorporation was determined over the last 16 h of culture. Data are the mean ± SD of three experiments plotted against ENU dose.

5.4 Discussion

Any SSB detected in ENU-treated cells with alkaline elution either can result from the continuous breaking and rejoining of DNA by excision repair mechanisms known to be acting on alkylation damage in DNA [Morgan and Cleaver, 1983], or can represent certain lesions themselves, provided these are unstable in alkali [Margison and O'Connor, 1978]. Several studies have used high performance liquid chromatography (HPLC) in order to elucidate which of the base damages induced by ENU could be related to the breaks as detected by alkaline filter elution. However, comparison of the induction and removal of ethyl-adducts as measured by HPLC with the induction and repair of SSB as measured with alkaline filter elution did not indicate any particular adduct that could be responsible for the observed disappearance of SSB measured with alkaline filter elution [Dogliotti et al., 1987; Schutte et al., 1988].

The results obtained in our study indicate the presence of at least two categories of ENU-induced lesions with regard to their susceptibility to repair processes. Both categories can be detected as SSB, but only one is easily removed from the DNA, at a rate which can vary greatly among different individuals. This component represents about half of the lesions that yield SSB. Removal of the other half has not been observed to occur within 24 h after treatment except for a 35% disappearance that was only apparent after the lowest dose applied (Figure 5.2A). The inhibition of disappearance of ENU-induced SSB by araC suggests that the first, rapid process involves steps of the excision repair pathway. The SSB disappearance observed in PBL from high responders, such as N18, may in part be masked by the accumulation of araC-associated SSB as a consequence of the inhibition of excision repair acting on lesions which are not alkali-labile. However, the absence of araC-associated SSB in PBL from donor N01 after an 1-h repair period in the presence of araC indicates that a defective excision repair is responsible for the persistence of the SSB detected at the end of the ENU treatment (in the absence of araC), and

Table 5.1 EFFECT OF ARAC ON THE INDUCTION AND DISAPPEARANCE OF SSB IN PBL AFTER EXPOSURE TO ENU OR GAMMA-RADIATION.

donor	treatment	araC[a] induction	araC[a] repair	repair time (h)	SSB/cell
N25	none[b]	-		0	265 ± 119
N25	none[b]	+		0	419 ± 99
N01	ENU	-		0	3944 ± 206
N01	ENU	+		0	4293 ± 97[ns]
N01	ENU	-	-	1	4121 ± 555
N01	ENU	-	+	1	4387 ± 811[ns]
N18	ENU	-		0	4076 ± 531
N18	ENU	+		0	5606 ± 533*
N18	ENU	-	-	1	2081 ± 791
N18	ENU	-	+	1	4010 ± 349*
N25	ENU	-		0	2846 ± 160
N25	ENU	+		0	3836 ± 40*
N25	ENU	-	-	1	2292 ± 144
N25	ENU	-	+	1	2807 ± 185*
N25	4 Gy[b]	-		0	4000
N25	4 Gy[b]	-	-	1	655 ± 39
N25	4 Gy[b]	-	+	1	1887 ± 401*

Cells were treated with 0.5 mM ENU for 20 min at 37°C or irradiated with 4 Gy ^{60}Co-γ-radiation, and incubated for 1 h at 37°C. The data are the mean ± SD of 3 determinations on each donor. Experiments with lymphocytes from N01 and N18 were always performed at the same time in parallel.

[a]Presence (+) or absence (-) of 100 μM araC during the 20 min treatment or the subsequent 1 h repair.

[b]Mean ± SD of 3 independent experiments representative for all three donors.

*Significantly different at $P < 0.05$ from PBL of the same donor treated identically but in the absence of araC (one-sided t test).

[ns]Not significantly different from PBL of the same donor treated identically but in the absence of araC.

possibly also of other adducts which are not detected in our system.

The larger number of SSB induced during ENU exposure of PBL from donors N18 and N25 in the presence of araC indicates the occurrence of excision repair events already during ENU treatment. This confirms comparable findings by Dogliotti et al. [Dogliotti et al., 1987]. However, it should be realized that the araC-related breaks over this 20-min treatment will for the large majority reflect excision repair events involving other lesions than the ones detected here as SSB. Indeed, the ENU-induced SSB detected in this study may reflect only 1-3% of the total amount of lesions induced by this agent [Schutte et al., 1988].

The process of slow removal of the more persistent SSB might reflect the loss of alkali-labile ethyl-adducts, such as N3-etA, through spontaneous hydrolysis in the cell. These SSB do not represent phosphotriesters, which are the major lesions following exposure of cells to ENU, because under the conditions of the alkaline elution these lesions are not detected [Shooter and Merrifield, 1978]. Similarly, the observed SSB disappearance is probably not a result from the repair of O^6-etG by the action of O^6-alkylguanine-DNA alkyltransferases (O^6-AT); this form of direct repair does not generate SSB as repair intermediates. In addition, O^6-etG does not destabilize the N-glycosidic bonds in the DNA and is, as such, not detectable as an alkali-labile lesion in the alkaline elution assay. However, indirect evidence has implicated excision repair in the removal of O^6-alkylguanine (O^6-alkylG). Putative evidence for excision repair of O^6-alkylG has been reported in *Escherichia coli* [Samson et al., 1988] and human fibroblasts which lacked O^6-AGT activity [Boyle et al., 1987; Kalamegham and Ebisuzaki, 1990]. The formation of AP sites, following ENU treatment of CHO cells, as observed by Fontini et al. [Fontini et al., 1990] might potentially result from the deamination of cytosines located opposite to the O^6-alkylG, leading to the formation of uracil and subsequent excision of the uracil by uracil-DNA glycosylase [Fix et al., 1990].

It should be realized that no direct correlation between the disappearance of O^6-alkylG and excision repair has been reported. For example, Fix et al. inferred the existence of "cross-strand deamination", i.e. deamination of cytosines opposite O^6-alkylated guanines, from the increased mutagenesis in uracil-DNA glycosylase deficient Escherichia coli [Fix et al., 1990]. In contrast, Vogel et al. reported that excision-defective mutants of Drosophila melanogaster were not hypersensitive to the mutagenic effects of high doses of O^6-alkylating agents (ENU, DEN, ENNG) supporting their conclusion that excision repair does not affect the O^6-alkylG in

DNA [Vogel et al., 1985]. In addition, mutability was greatly increased in repair-deficient Drosophila following treatment with N-alkylating agents (MMS, EMS). The putative involvement of excision repair in the removal of O^6-alkylG, as proposed by Fontini et al., was based on their observation that the fast-resealed ENU-induced SSB were probably due to the formation of AP sites [Fontini et al., 1990], and that SSB disappearance as detected by alkaline elution seems to some degree to be dependent on the ability of the alkylating agents to modify DNA oxygen atoms. This ability is often indicated by the Swain-Scott constant s [see Beranek, 1990], which more or less distinguishes alkylating agents reacting via an S_N1 or S_N2-mechanism. Low values of s are generally associated with high S_N1 reactivity and a relative higher percentage of O-alkylation. The rate of SSB disappearance in quiescent PBL exposed to MNNG [Hartshorn and Robison, 1990] or ENU [Dogliotti et al., 1987; Schutte et al., 1988; Boerrigter et al., 1991b], both agents with a relatively low s value of 0.42 and 0.26, respectively, is quite rapid, whereas exposure to MMS [Boerrigter et al., 1991a] or DES [Schutte et al., 1988], which have a s value of 0.83 and 0.64, respectively, leads to SSB removal that is rather slow. Although we cannot exclude the possibility that some excision repair of O^6-alkylG may occur following exposure of PBL to ENU, we propose that the great majority of these SSB represent alkylation damages other than O^6-etG.

An important conclusion that can be derived from our present results is that PBL from different individuals can vary greatly in their repair activity. This was most convincingly demonstrated by the observation that among the 15 apparently normal young individuals participating in this study, two low responders were found. That the observed differences in repair capacity were not caused by strong intra-individual variation was shown by monitoring one low and one high responder over many months (Figure 5.6). Our finding of interindividual variation in DNA repair is not new. Interindividual variation has been noted in the activity of O^6-AT [Waldstein et al., 1982; Gerson et al., 1985; Sagher et al., 1988] and also the ability to repair ultraviolet light-induced [Lambert et al., 1976; Freeman, 1988] or benzo(a)pyrene-induced [Oesch et al., 1987] DNA damage has been shown to vary considerably among individuals [for reviews, see Setlow, 1983; Harris 1989 and references cited therein]. However, our present data indicate such interindividual variation in the disappearance of ENU-induced SSB which thus far has not been reported.

In order to obtain more insight in the nature of this variation we have used the chain-

terminator araC at a concentration which inhibits > 90% of replicative and > 75% of repair DNA synthesis [Collins and Johnson, 1984]. From the results we can conclude that the defect in repair as observed, for example, in PBL of donor N01 cannot be attributed to disturbances in post-incision steps of the excision repair process. In that case we would expect to find a larger number of SSB in PBL from donor N01 directly after the 20-min ENU treatment (in the absence of araC). Instead, N01 seems to have a defect in the capacity for incision. This can be derived from the observation that in the presence of araC, the number of SSB is not increased during the 20-min period of treatment with ENU, a phenomenon which was clearly present in N18 (Table 5.1). Our observation that in PBL of those individuals characterized by a low repair of ENU-induced SSB, the repair of SSB induced by γ-rays is proficient, also suggests that some initiating, damage-specific step in the repair pathway is defective. Indeed, although both γ-radiation and ENU induce lesions that can be detected as SSB, the type of damages involved are entirely different [Den Engelse et al., 1986; Téoule, 1987]. Therefore, the most likely type of defect responsible for our present findings is a lesion-specific step in excision repair, e.g. the activity of a specific DNA glycosylase. At present we have no clue as to the type of glycosylase that might be involved. However, its activity is not dependent on the proliferative status of the cells. ENU-induced SSB repair does not differ between PHA-stimulated and quiescent PBL. Therefore, interindividual differences are not caused by variability between individuals in the degree to which PBL are activated [Boerrigter and Vijg, 1991b]. Nevertheless, the low rate of ENU-induced SSB disappearance in PBL of individuals like N01, possibly has an influence on the incorporation of ^{3}H-thymidine upon mitogenic stimulation. This follows from our observation that the adverse effects of ENU on the incorporation of ^{3}H-thymidine after PHA-stimulation, used as a measure of cell survival, are more severe with PBL of N01 than with PBL of N18. This indicates that the observed DNA repair defect has some functional relevance for cellular maintenance.

CHAPTER 6

INDUCTION AND DISAPPEARANCE OF DNA SINGLE-STRAND BREAKS IN HUMAN B AND T LYMPHOCYTES AFTER EXPOSURE TO ETHYLNITROSOUREA[1]

6.1 Introduction

DNA repair activities represent an important category of host factors relevant for cancer and other diseases [Friedberg, 1985a; Bohr et al., 1989]. In order to assess variations in DNA repair among human individuals, peripheral blood lymphocytes (PBL) are the most readily obtainable cells. However, it should be realized that PBL constitute a heterogeneous cell population which might be subject to variations in the response to DNA damage. More specifically, DNA excision repair synthesis in PBL has been found to increase after phytohemagglutinin (PHA)-stimulation [Scudiero et al., 1976; Lavin and Kidson, 1977; Lewensohn et al., 1979; Freeman and Ryan, 1988], while differences in DNA repair between B and T cells have also been reported [Yew and Johnstone, 1978; Van Rensburg et al., 1985, 1989]. Thus, any observed variability in DNA repair activities among individuals might reflect different degrees of in vivo lymphocyte activation or differences in sizes of lymphocyte subsets. The contribution of these factors to interindividual variation in DNA repair should be thoroughly evaluated before one may assign prognostic and/or pathogenetic implications to differences in DNA repair.

Using the alkaline filter elution method we have recently described the kinetics of SSB induction and disappearance in human PBL exposed to a single, low dose of ethylnitrosourea (ENU) [Boerrigter et al., 1991b]. Results obtained in this previous study indicated interindividual variation in the rate of SSB disappearance. Moreover, a low rate of ENU-induced SSB disappearance appeared to correlate with a low cellular survival after ENU exposure, as measured by the incorporation of ^{3}H-thymidine (^{3}H-TdR) into the DNA of PHA-stimulated PBL.

In the present study we applied the alkaline filter elution method to determine the

[1]Parts of this chapter were published previously by Boerrigter and Vijg, 1991b.

levels of SSB disappearance in freshly isolated quiescent PBL, in B and T lymphocyte-enriched lymphocyte populations, and in cultured T cells, after exposure to ENU. The results indicate that the level of ENU-induced SSB disappearance in total PBL closely correlated with that found in quiescent T lymphocytes; in B lymphocytes no significant SSB disappearance was observed at 1 h after exposure to ENU. Removal of ENU-induced SSB in T lymphocytes was not dependent on the proliferative status of the cells; the repair of SSB induced by this agent was the same in both quiescent PBL and actively proliferating T cells.

6.2 Materials and Methods

6.2.1 Lymphocyte preparation.

Human peripheral blood was collected from healthy volunteers not taking medication. Lymphocytes were isolated by using Ficoll-Paque (Pharmacia) gradients [Boyum, 1968], and washed twice in RPMI 1640 medium (Flow Laboratories) plus 2% fetal calf serum (FCS). All steps were performed at 4°C.

6.2.2 Isolation of B and T lymphocytes.

For obtaining B and T lymphocyte-enriched lymphocyte populations the procedure as described by Pellegrino et al. [1976] was used with small modifications. In brief, sheep red blood cells (SRBC) were washed twice with phosphate buffered saline (PBS) and adjusted to a 1 per cent concentration in PBS in a total volume of 10 ml. Ten ml of a suspension containing about 10^8 washed human PBL was added to the SRBC. This mixture was centrifuged for 10 min at 1000 g at 4°C and incubated on ice for 1 h. Then, the pellet was gently resuspended and T and B lymphocytes were separated using Ficoll-Paque (Pharmacia) gradients. B lymphocytes were removed from the interphase of the gradient, washed three times with RPMI 1640 medium plus 5% FCS and suspended in RPMI 1640 medium containing 2 mM glutamine and 10% FCS. The pellet containing the rosettes of SRBC with the T lymphocyte fraction was suspended in 10 ml RPMI 1640 medium plus 10% FCS. After lysis of the SRBC by the addition of water, the T lymphocytes were centrifuged and

resuspended in RPMI 1640 medium plus 10% FCS and 2 mM glutamine.

6.2.3 Cell culture.

The initiation and expansion of T cell lines was performed as described [Van der Griend and Bolhuis, 1984]. Briefly, freshly isolated PBL were cultured at 3 x 10^3 cells/well with 2 x 10^4 PBL feeder cells and a mixture of two types of lymphoblastoid B cell lines (5 x 10^3 cells/well) in 96-well, round-bottomed microtiter plates (Greiner Labor Technik). Both PBL feeder cells and lymphoblastoid B cells had been γ-irradiated with 30 Gy in a Gamma-cell 200 (Atomic Energy of Canada Ltd.) at a dose rate of 30 Gy/min. Culture medium, RPMI 1640 buffered with 20 mM Hepes was supplemented with 10% FCS, 4 mM glutamine, indomethacin (1 μg/ml; Sigma), penicillin-streptomycin (100 U/ml), interleukin 2 (25 U/ml; Boehringer) and leucoagglutinin (1 μg/ml; Pharmacia). After 7 days of culture at 37°C, cells were harvested and washed twice with RPMI 1640 medium with 2% FCS and 2 mM glutamine. After washing, the amplified T lymphocytes were incubated overnight in fresh medium immediately before experiments. Typical yields were 1.5 x 10^5 cells/well. At the end of the culture period the contamination of the dividing T lymphocytes with the feeder cells APD and BSM was always less than 2%.

6.2.4 Cell treatments.

ENU (Sigma, St. Louis, MO) was dissolved in dimethylsulfoxide (DMSO) immediately before use. Cell suspensions were exposed to ENU for 20 min in RPMI 1640 medium plus 20 mM Hepes, 2 mM glutamine and 5% FCS at 37°C. The DMSO concentration was never higher than 1%. At the end of the exposure period, cells were centrifuged and resuspended in RPMI 1640 supplemented with 10% FCS and glutamine and incubated for repair at 37°C. During γ-irradiation (Gamma-cell 100, Atomic Energy of Canada Ltd.; dose rate 6 Gy/min), cell suspensions were kept on ice. Afterwards the cells were centrifuged and resuspended in RPMI 1640 plus 10% FCS.

6.2.5 Alkaline filter elution.

Alkaline filter elution [Kohn et al., 1976] with spectrofluorometric detection of the DNA [Stout and Becker, 1982] was used to determine existing SSB together with alkali-labile sites as described previously [Boerrigter et al., 1989; see Chapter 2]. The elution results were plotted as the log percent of DNA remaining on the filter as a function of elution time. Mean slopes of the linear initial part of elution curves were used to calculate the number of SSB. Absolute amounts of SSB were calculated by calibration of mean elution curves of ENU-treated cells with mean elution curves of control cells exposed to 4 Gy ^{60}Co-γ-radiation in the Gamma-cell 100 source (see above), which were assayed in the same experiment. At 4 Gy γ-radiation approximately 4000 SSB per diploid genome are introduced [Van der Schans et al., 1982]. The absolute amount of SSB per cell was calculated by using the formula

$$\frac{\text{slope of the elution curve of treated cells}}{\text{slope of the elution curve of irradiated cells}} \times 4000$$

In all experiments, mean slopes were based on at least triplicate determinations and standardized with reference to mean slopes of untreated control cells assayed simultaneously.

6.3 Results

In this study PBL of 4 unrelated, apparently healthy human individuals were used for repair studies. The number of SSB induced during a 20-min treatment with 0.5 mM ENU was not significantly different between quiescent PBL, cultured T cells, and B and T lymphocyte-enriched lymphocyte populations (Tables 6.1 and 6.2). In quiescent PBL

Table 6.1 PERCENTAGE SSB DISAPPEARANCE IN DIFFERENT LYMPHOCYTE POPULATIONS AFTER EXPOSURE TO 0.5 mM ENU (20 MIN, 37°C) OR 4 GY GAMMA-RADIATION

		ENU-induced SSB[a,b]		repair (%)[b]	γ-induced SSB		repair (%)
donor	cell type	t = 0	t = 1		t = 0[c]	t = 1	
N18	PBL	3579 ± 211	1474 ± 421	58.0 ± 14.3	4000	488 ± 238	88.0 ± 5.6
	B cell	3790 ± 422	3590 ± 432	5.4 ± 0.9	4000	582 ± 524	85.5 ± 13.4
	T cell	3274 ± 116	1579 ± 105	51.8 ± 1.5	4000[d]	308	92
N32	PBL	3541 ± 224	3131 ± 399	12.0 ± 5.7	4000[d]	769	81
	B cell	3579 ± 520	3266 ± 442	8.6 ± 0.9	4000[d]	612	85
	T cell	3932 ± 167	2871 ± 447	27.4 ± 8.3	4000[d]	333	92

[a]Number of SSB (mean ± SD) per cell present directly after the ENU treatment (t=0) or following a 1-h repair incubation in fresh medium (t=1).
[b]Percentages are the mean (± SD) of at least 3 independent experiments.
[c]The number of SSB induced by 4 Gy γ-radiation was set at 4000 SSB per cell as described in Materials and Methods.
[d]Numbers represent values of one experiment.

of 2 of the donors, N13 and N18, approximately 50% of the SSB induced had disappeared after 1 h, whereas in PBL of the other 2 donors, N01 and N32, only about 10% SSB disappearance was observed (Tables 6.1 and 6.2). This low level of SSB disappearance in PBL of some individuals after ENU treatment has been described previously [Boerrigter et al., 1991b].

Determination of ENU-induced SSB disappearance in B and T lymphocyte-enriched lymphocyte populations from a low and a high responder, N32 and N18, respectively, indicated that only T lymphocytes of the high responder were capable of repairing ENU-induced SSB; the percentage SSB disappearance in B lymphocytes after exposure to ENU was low (5-9%) for both donors, while for donor N32 also the T-cell fraction appeared to be deficient in SSB removal (Table 6.1).

To exclude the possibility that a low SSB disappearance was the result of a differential

sensitivity to the cell killing effects of ENU we assessed cell viability by means of trypan blue dye exclusion. Cell viability directly after ENU exposure and after the subsequent 1 h repair period was always more than 95% in both the B and T lymphocyte-enriched lymphocyte populations and in freshly isolated PBL (results not shown).

In order to assess whether the inability of B lymphocytes to remove a significant fraction of SSB induced by the radiomimetic agent ENU was caused by an overall defect in the repair of X-ray-like DNA damage, quiescent PBL and B and T lymphocytes were exposed to 4 Gy of γ-radiation and allowed to repair the induced SSB for 1 h. Repair of γ-radiation-induced SSB was not noticeably different between quiescent PBL and the B and T lymphocyte-enriched lymphocyte populations; in all three lymphocyte populations, 85-90% of the induced SSB were repaired at 1 h after irradiation (Table 6.1).

Table 6.2 PERCENTAGE SSB DISAPPEARANCE IN QUIESCENT PBL AND T CELL LINES AFTER EXPOSURE TO 0.5 mM ENU (20 MIN, 37°C)

	quiescent			proliferating		
	SSB[a,b]		repair (%)[b]	SSB		repair (%)
donor	t = 0	t = 1		t = 0	t = 1	
N01	3846 ± 154	3407 ± 22	11.3 ± 3.0	3430 ± 295	3258 ± 53	4.5 ± 6.7
N13	3654 ± 125	1948 ± 170	46.5 ± 6.5	3873 ± 6	1896 ± 105	51.1 ± 2.8
N18	3739 ± 462	1735 ± 255	53.5 ± 4.6	4261 ± 271	2310 ± 556	46.4 ± 10.2

[a]Number of SSB (mean ± SD) present directly after the ENU treatment (t=0) or following a 1-h repair incubation in fresh medium (t=1).
[b]Percentages are the mean (± SD) of at least 2 independent experiments.

The possible influence of the proliferative status on the level of SSB disappearance was studied by comparing freshly isolated PBL with actively proliferating T cell populations, i.e. cultured T-cell lines. The results in Table 6.2 indicate that ENU-induced SSB disappearance does not vary with cellular proliferative capacity; no differences in the amount of SSB disappearance were found between quiescent PBL and cultured T cells, irrespective of the donor (Table 6.2). In addition, the results presented in Figure 6.1 indicate that also the rate of SSB disappearance after ENU treatment was not different between quiescent PBL and proliferating T-cells.

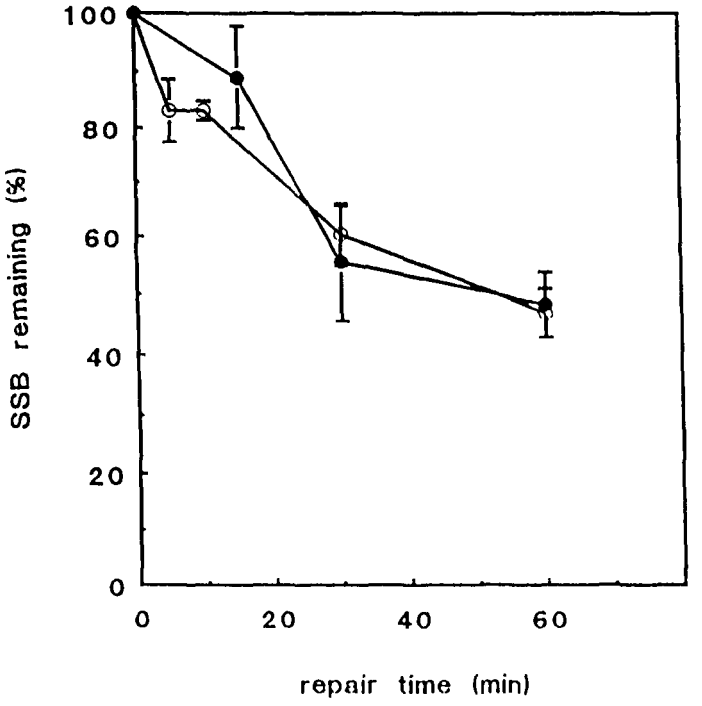

Fig. 6.1: SSB disappearance of ENU-induced SSB. Lymphocytes were exposed to 0.5 mM ENU (20 min, 37°C) and incubated for the periods indicated on the abscissa to allow repair of the induced SSB. The number of SSB remaining in quiescent PBL (o) or cultured T cells (•) is expressed as the percentage of SSB initially induced by the ENU treatment. Bars indicate SD.

6.4 Discussion

In the present study we used alkaline filter elution to determine whether interindividual variation in ENU-induced SSB disappearance in PBL might be a consequence of a differential repair in lymphocyte subpopulations or caused by variability in the degree of in vivo lymphocyte activation. Previously we have described the induction and disappearance of ENU-induced SSB in quiescent PBL from 15 normal, young individuals, two of which had a low ENU-induced SSB disappearance [Boerrigter et al. 1990b]. Here we show that the capacity to remove ENU-induced SSB resides in the T and not in the B lymphocytes. Since T lymphocytes may represent 80-90% of all lymphocytes, the percentage ENU-induced SSB disappearance in freshly isolated PBL will in most cases reflect the amount of SSB disappearance in T lymphocytes. However, our observations demonstrate the necessity to measure the T/B cell ratio before drawing definite conclusions on individual differences in DNA repair in PBL.

Differences in SSB disappearance upon ENU treatment of PBL (and T lymphocytes) can be explained by differences in DNA repair activities, as was indicated by the use of the excision repair inhibitor araC in a previous study [Boerrigter et al., 1991b]. Our previous observation that the presence of araC during the 20-min ENU exposure resulted in the accumulation of araC-associated SSB only in PBL from the high responder indicated the lack of incision in PBL from the low responder, i.e. PBL deficient in SSB removal after ENU treatment. Moreover, the occurrence of a proficient repair of γ-radiation-induced SSB in PBL of those individuals characterized by a low repair of ENU-induced SSB also suggested that some initiating, damage-specific step in the repair of ENU-induced SSB is defective [Boerrigter et al., 1991b]. In contrast, a defect in post-incision events of excision repair in PBL from N01 would have resulted in a higher number of SSB, in the absence of araC, after exposure to ENU, which was not observed. At present we have no indication which adduct(s) and what type of DNA repair enzymes might be involved in the removal of ENU-induced SSB. Nevertheless, a similar defect in incision activity might also be present in B lymphocytes.

In order to investigate whether ENU-induced SSB disappearance in PBL was dependent on the proliferative status of the cells, we cultured T-cells from one low and two high

responders and compared their capacity to remove ENU-induced SSB to that observed in their quiescent counterparts. It appeared that in actively proliferating T lymphocytes from all 3 donors, SSB disappearance after ENU treatment did not differ from that in their quiescent counterparts over the 1 h repair period studied.

Increased excision repair synthesis has been observed in PHA-stimulated PBL irradiated with X-rays [Lavin and Kidson, 1977] or UV light [Lewensohn et al., 1979; Freeman and Ryan, 1988], or treated with N-acetoxy-2-acetylaminofluorene or methyl methanesulfonate (MMS) [Scudiero et al., 1976]. Also, our own data on MMS-induced SSB disappearance in PBL indicate an increase in SSB disappearance in PHA-stimulated PBL exposed to MMS as compared to unstimulated cells [Boerrigter et al., 1990a]. In relative terms this increase was comparable to the reported 3-fold increase in repair replication in mitogenically stimulated PBL [Scudiero et al., 1976]. Also, our own data on the removal of MMS-induced SSB in PHA-stimulated PBL indicate an increased SSB disappearance in these cells as compared to unstimulated PBL exposed to MMS [Boerrigter et al., 1990a]. Moreover, PHA-stimulation has been found to increase the activity of uracil DNA glycosylase [Sirover, 1979] and O^6-AGT [Waldstein et al., 1982; Gerson et al., 1988]. We recently noticed that the rate of SSB disappearance following exposure of mitogenically stimulated PBL to γ-radiation was 2-3 fold increased when assessed after repair periods of 5 and 15 min [Boerrigter and Vijg, 1991a]. Thus, although most studies indicate that DNA repair is positively regulated with increased proliferative capacity [for a recent review, see Sirover, 1990], this does not seem to apply to the SSB disappearance observed in PBL exposed to ENU.

In conclusion, SSB disappearance after ENU treatment is seemingly absent from B lymphocytes whereas T lymphocytes exposed to ENU display a percentage SSB disappearance comparable to that found in PBL of the same donor. ENU-induced SSB disappearance was not increased in proliferating T cells, indicating that this particular repair pathway is not regulated as a function of cellular proliferative capacity.

CHAPTER 7
DECREASED DNA REPAIR CAPACITY IN FAMILIAL, BUT NOT IN SPORADIC ALZHEIMER'S DISEASE[1]

7.1 Introduction

Alzheimer's disease (AD) is a common neurodegenerative disorder characterized by the premature death of neurons [Katzman, 1986]. It has been found that AD patient-derived fibroblast and lymphoblastoid cell lines have an increased sensitivity to the cell killing effects of alkylating agents and X-rays, as compared to cell lines from normal age-matched controls [Robbins et al., 1983b, 1985a; Scudiero et al., 1986]. This phenomenon has been attributed to some defect in the capacity to remove brain-specific DNA damages [Robbins et al., 1983b, 1985a; Robison et al., 1987]. It appears possible that in vitro such a defect may become apparent also in cells other than brain cells after exposure to certain DNA-damaging agents, but that in vivo the defect will be prominent only in the neuronal cells of AD patients [Robbins et al., 1983b]. Direct comparison of DNA-damage levels in neuronal tissue of AD patients and controls indicated an at least twofold higher level of DNA breaks in cortex of AD patients as compared to controls [Mullaart et al., 1990a].

In keeping with the observations mentioned above, decreased levels of DNA repair have been found in fibroblast and lymphoid cell lines of AD patients after treatment with methyl methanesulfonate (MMS) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) [Li and Kaminskas, 1985; Robison et al., 1987; Bradley et al., 1989; Jones et al., 1989], although some of these results have been challenged [Kinsella et al., 1987a, 1987b]. Thus far, DNA repair determinations have not been made on freshly isolated cells from AD patients. Such direct testing for general DNA repair defects would circumvent artifacts that may be associated with the use of cell lines and may offer new diagnostic criteria.

In the present study a highly sensitive alkaline filter elution assay was used to determine the induction and disappearance of SSB in peripheral blood lymphocytes (PBL) of AD patients and normal, age-matched controls exposed to N-ethyl-N-nitrosourea (ENU), methyl methanesulfonate (MMS), or gamma (γ)-radiation. Initially, the amount of SSB

[1]Parts of this chapter were published previously by Boerigter et al. 1991c.

disappearance in PBL from normal young or old controls or from AD patients was analyzed at 1 and 2½ hr after treatment of PBL with either ENU or MMS, and at 15 min and 1 h after γ-irradiation. The results of these experiments indicated that PBL of AD patients displayed a significantly lower percentage SSB disappearance, as compared to PBL from young and old controls, only after exposure to ENU followed by a 1-h repair incubation. Consequently, PBL from additional old controls and AD patients were assessed for their ability to remove ENU-induced SSB over a 1-h repair period. We found that in PBL from sporadic AD patients with no or only one first-degree relative with dementia (n=16 and n=12, respectively) and in PBL of 48 age-matched controls the mean percentage SSB disappearance was about 40%, whereas in PBL from 15 AD patients with at least 2 first-degree relatives the mean percentage SSB disappearance of about 23% was significantly lower as compared to the former two groups.

7.2 Materials and Methods

7.2.1 Selection of patients and control subjects

Patients and age-matched controls were participants in an ongoing epidemiological study of risk factors of clinically diagnosed AD at the Department of Epidemiology and Biostatistics, Erasmus University Medical School [Hofman et al., 1989]. All patients with AD were selected according to the criteria established by the NINCDS-ADRDA [McKhann et al., 1984]. Systemic disorders or brain diseases other than AD, such as multi-infarct dementia, and dementia secondary to alcoholism, depression, metabolic disorders, epilepsy, Parkinson's disease and other conditions were excluded on the basis of a clinical history, neurological examination and neuropsychological and laboratory tests [Hughes et al., 1982]. All patients fulfilled the following criteria: (1) slow progressive decline of intellectual function; (2) a score on the Clinical Dementia Rating scale of more than 0.5 [Hughes et al., 1982]; (3) a score on the Short Portable Mental Status Questionnaire (SPMSQ) of less than 20 [Pfeiffer, 1975]; (4) a score of 7 or less on the Hachinski-scale [Hachinski et al., 1974]; (5) no evidence for abnormalities on CT-scan other than cerebral atrophy; and (6) no evidence for focal dysfunction in the EEG. Full pedigree information was obtained by a

structured interview of the next of kin of the patient or control [Hofman et al., 1989]. Patients and controls were classified according to the number of affected relatives. Patients were considered familial if there were at least 2 or more individuals known in their family with clinically diagnosed AD. Within the family of these patients, the disease was apparently inherited as an autosomal dominant disorder. According to a breakpoint of 58 years in order to distinguish between the early and late onset type of familial AD [Farrer et al., 1990], all familial AD patients in the present study were classified as belonging to late-onset families. AD patients and control subjects were similar with respect to age range (51-81 and 50-79 years, respectively).

7.2.2 Lymphocyte isolation and treatment

Informed consent was obtained from the responsible family members of the subjects with AD and/or directly from the control subjects. Lymphocytes were isolated from 10 ml of coded blood samples, using Ficoll-Paque (Pharmacia) gradients [Boyum, 1968], and washed twice in RPMI 1640 medium (Flow Laboratories) plus 2% fetal calf serum (FCS). All steps were performed at 4°C. Viability of recovered PBL was always higher than 95% as measured by trypan blue dye exclusion. ENU (Sigma) was dissolved in dimethylsulfoxide (DMSO) immediately before use. MMS was dissolved in phosphate buffered saline. A constant number of cells ($3x10^6$/ml) was used for each treatment in order to exclude variations in the amount of damage initially induced. Lymphocyte suspensions were exposed to either 0.5 mM ENU (20 min) or 0.2 mM MMS (45 min) in RPMI 1640 medium plus 20 mM Hepes, 2 mM glutamine and 5% FCS at 37°C. The DMSO concentration during ENU exposure was never higher than 1%. For treatment with ^{60}Co-γ-rays, PBL suspensions were irradiated on ice in a Gamma-cell 100 (Atomic Energy of Canada Ltd.) at a dose rate of 6 Gy/min. With respect to ENU, control cells were treated with RPMI containing the same DMSO concentration. At the end of the exposure period, cells were centrifuged and resuspended in RPMI 1640 supplemented with 10% FCS and glutamine and incubated for repair at 37°C.

7.2.3 Alkaline filter elution assay

The technique of alkaline filter elution was used to measure ENU-induced DNA lesions, detected as single-strand breaks (SSB) as described in Chapter 2. The elution results were plotted as the log percent of DNA remaining on the filter as a function of elution time. Mean slopes of elution curves were used to calculate the percentage SSB disappearance. In all experiments, mean slopes were based on at least triplicate determinations and standardized with reference to mean slopes of untreated control cells assayed in the same experiment. Variation between the triplicate determinations of a single sample was typically less than 2%.

7.3 Results

The alkylating agents MMS and ENU were used at doses of 0.2 mM and 0.5 mM, respectively, which induce approximately 4000 SSB per cell (i.e. the equivalent of the DNA damage detected as SSB by alkaline filter elution after exposure of PBL to 4 Gy of γ-rays). The initial amount of SSB, as represented by the mean slope of the elution curves of PBL exposed to MMS, ENU or γ-radiation, was not different between young

Table 7.1 INDUCTION OF SSB IN PBL FROM NORMAL YOUNG AND OLD CONTROLS AND AD PATIENTS EXPOSED TO 0.2 mM MMS (45 MIN, 37°C), 0.5 mM ENU (20 MIN, 37°C) OR 4 GY γ-RADIATION.

agent	young controls	old controls	AD patients
MMS	0.217 ± 0.047 (12)	0.211 ± 0.059 (8)	0.248 ± 0.056 (8)
ENU	0.202 ± 0.031 (15)	0.203 ± 0.059 (8)	0.168 ± 0.043 (8)
γ	0.186 ± 0.024 (10)	0.210 ± 0.037 (9)	0.209 ± 0.038 (8)

[a]Mean (± SEM) slope of the elution curves of PBL treated with MMS, ENU or γ-radiation. Numbers between parentheses indicate the different number of donors analyzed. The slope is calculated from the linear part of the elution curve and is proportional to the number of SSB present.

Table 7.2 PERCENTAGE SSB DISAPPEARANCE IN PBL FROM YOUNG AND OLD CONTROLS AND AD PATIENTS EXPOSED TO 0.2 mM MMS (45 MIN, 37°C), 0.5 mM ENU (20 MIN, 37°C) OR 4 GY γ-RADIATION.

agent	repair time (h)	repair (%)[a]		
		young	old	AD
MMS	1	13.9 ± 5.0 (12)	7.7 ± 5.4 (8)	8.9 ± 4.3 (8)
	2½	12.7 ± 5.4 (9)	5.1 ± 5.6 (7)	22.0 ± 14.0 (2)
ENU	1	35.1 ± 4.1 (15)[b]	35.8 ± 5.3 (8)	18.0 ± 4.7 (8)[**]
	2½	35.1 ± 5.4 (9)	39.6 ± 10.8 (8)	34.9 ± 6.7 (6)
gamma	¼	74.8 ± 5.8 (8)	67.2 ± 3.9 (8)	67.3 ± 6.4 (8)
	1	86.5 ± 2.2 (10)	90.0 ± 1.8 (9)	87.3 ± 2.4 (7)

Freshly isolated PBL were treated with either 0.5 mM ENU (20 min, 37°C), 0.2 mM MMS (45 min, 37°C), or 4 Gy gamma-radiation and subsequently incubated in fresh medium for the indicated repair times.
[a]Mean ± SEM. Numbers between parentheses indicate the number of different donors analysed for each respective agent and repair time.
[b]Data from Chapter 5.
[**]Significantly different from young and old controls at $P < 0.01$ (two-sided t test). None of other comparisons between young and old, young and AD, and old and AD, respectively were significantly different.

and old controls or between either young or old controls and AD patients (Table 7.1). Table 7.2 presents the exploratory assessment of the amount of SSB disappearance in PBL from 15 young and 9 old controls and 8 AD patients after exposure of the cells to ENU, MMS or γ-radiation and subsequent incubation in fresh medium for the indicated repair times. No significant age-related differences in the percentage SSB disappearance were noted following the different repair incubations after exposure of PBL to the three different DNA-damaging agents. MMS-induced SSB disappearance was low at both the 1 and 2½-h repair incubation for all three groups studied (see also Chapter 4), whereas SSB disappearance after exposure of PBL to γ-radiation was invariably high; at 15 min and 1 h after irradiation, PBL of both young and old controls and of AD patients had removed about 70% and 85% of the induced SSB, respectively (Table 7.2). The percentage SSB disappearance in PBL treated with ENU and incubated for a 2½-h repair period was approximately 40% in PBL of all three groups. However, a significant ($P < 0.01$) lower extent of SSB disappearance was observed in PBL of AD patients, as compared to normal young and old controls, at 1 h after treatment with ENU (Table 7.2).

Table 7.3 PERCENTAGE SSB DISAPPEARANCE IN PBL FROM AD PATIENTS AND MATCHED CONTROL SUBJECTS AFTER IN VITRO EXPOSURE TO 0.5 MM ENU (20 MIN, 37°C) FOLLOWED BY AN 1-H REPAIR INCUBATION IN FRESH MEDIUM.

subjects	number of first-degree relatives with dementia	number	age (years)[a]	female (%)	SSB induction[b] t=0	SSB induction[b] t=1	ENU repair (%)[c]
controls	0	35	68.2 ± 1.15	72	0.181 ± 0.011	0.096 ± 0.007	40.8 ± 3.4
	1	13	70.0 ± 1.55	70	0.173 ± 0.013	0.100 ± 0.012	44.3 ± 5.5
		48	68.9 ± 0.93	71	0.179 ± 0.009	0.097 ± 0.006	41.4 ± 2.9
AD patients	0	16	67.1 ± 1.47	88	0.164 ± 0.011	0.084 ± 0.009	42.5 ± 8.2
	1	12	69.3 ± 0.82	83	0.180 ± 0.011	0.101 ± 0.009	43.0 ± 4.4
	2	15	68.8 ± 1.74	75	0.181 ± 0.017	0.131 ± 0.011[=]	23.6 ± 5.8[*,d]
		43	68.3 ± 0.84	81	0.176 ± 0.008	0.105 ± 0.006	36.1 ± 4.0

[a]Mean ± SEM. [b]Number of SSB represented by the slope of the elution curve (mean ± SEM) at the indicated times (in hr) after the 20 min ENU treatment. [c]Percentages are the mean (± SEM) of the repair values as calculated for each individual subject. [=]Not significantly different from the 48 control subjects. [*]Significantly different from the 48 control subjects ($P < 0.01$, two-sided *t*-test) [*,d]Significantly different from the 48 control subjects ($P < 0.01$) and the AD patients with 0 or 1 first-degree relative with dementia ($P < 0.02$).

Consequently, in the subsequent studies SSB disappearance in PBL from additional old controls and AD patients was assessed after exposure to ENU followed by an 1-h repair incubation, in order to determine whether a decreased ENU-induced SSB disappearance was consistently detectable in a larger number of AD patients. An extension of the number of subjects was important also since in an earlier study we observed that PBL of about 10% of normal young subjects suffer from a low capacity to remove ENU-induced SSB (see also Chapter 5).

Table 7.3 shows the general characteristics of both the AD and control group. Care was taken that there were no significant differences in the mean age between AD patients and control subjects. Table 7.3 shows that 12 out of 43 AD patients (28%) had one first-degree relative with dementia, as compared to 13 out of 48 controls (27%). Fifteen of the 43 AD patients (35%) and none of the controls had at least 2 first-degree relatives with dementia. The 15 familial AD patients were not related to each other in first, second or third degree.

The number of SSB induced by ENU over the 20-min treatment period was not significantly different between PBL of AD patients and control subjects (Table 7.3). For the

significantly different between PBL of AD patients and control subjects (Table 7.3). For the 48 normal controls the percentage SSB disappearance was 41.4 ± 2.9% (mean ± SEM) which was not significantly different from the 36.1 ± 4.0% found in 43 AD patients ($P > 0.75$; Table 7.3). AD patients who did have no or only one first-degree relative with dementia (sporadic AD), had a mean percentage of SSB disappearance of 42.5 ± 8.2% and 43.0 ± 4.4%, respectively, which was not significantly different from that found in the control subjects (Table 7.3). However, AD patients with at least two first-degree relatives with dementia (considered as familial AD), had a significantly lower percentage SSB disappearance (23.6% ± 5.8%) than controls ($P < 0.01$) or AD patients with no or only one first-degree relative with dementia ($P < 0.02$; Table 7.3). There was no statistically significant correlation between age and the percentage SSB disappearance at 1-h after exposure to ENU for either the AD groups, the control groups, or all groups combined. The amount of SSB disappearance was not associated with the age of onset, the degree or the duration of the disease. Also, no differences were observed between males and females of either group (results not shown).

7.4 Discussion

A deficiency in the ability of cells to repair alkylating agent-induced SSB could reflect a hypersensitivity to one or more specific DNA lesions induced by those agents. The initial observations by Robbins et al [1983b]. of a significantly lower survival of fibroblast and lymphoid cell lines from AD patients appeared to be confirmed by recent reports of DNA repair deficiencies in AD cell lines with regard to exposure to MMS or MNNG [Li and Kaminskas 1985; Robison et al. 1987; Bradley et al., 1989; Jones et al., 1989]. However, the limited number of patient-derived cell lines used in those studies (n=4-7), the application of long-term cell culture instead of using freshly isolated cells and some conflicting evidence reported later [Kinsella et al., 1987a, 1987b] made it necessary to verify and extend these early observations.

Our present set of data on large groups of AD patients and matched control subjects considerably extends earlier findings and definitely establishes the existence of a DNA repair defect in familial, but not in sporadic AD. The earlier determinations have not been made on

freshly isolated cells from AD patients, but on a small number of cell lines derived from AD patients. It should be noted that Jones et al. reported a decreased MMS-induced SSB disappearance in lymphoblastoid cell lines from AD patients as compared to cell lines from normal control subjects which removed 70-80% of the SSB initially induced by MMS [Jones et al., 1989]. This seems to contradict not only the absence of a similar reduction in MMS-induced SSB disappearance in freshly isolated PBL from AD patients (Table 7.2), but also the low percentage SSB disappearance in PBL following exposure to MMS (Table 7.2, see also Chapter 4). However, the disappearance of SSB was previously found to be dependent upon the proliferative state of the cell population under study (Chapter 4) and the high degree of MMS-induced SSB disappearance in lymphoblastoid cell lines may have resulted from the increased proliferative capacity of these cells. It is not inconceivable that changes in growth characteristics, such as doubling time, length of S phase etc., of the lymphoblastoid cells from AD patients may have influenced MMS-induced SSB disappearance. The use of freshly isolated cells in the present study refutes the possibility that observed differences among individuals within a group or between different groups are an artefact of culture. However, it should be realized that in our study no histopathological examination has as yet confirmed the clinical diagnosis of AD. This could partly explain the large interindividual variation observed in the groups.

In a previous study, we have used the excision repair inhibitor 1-ß-arabinofuranosyl-cytosine (araC) to provide evidence that the most likely type of defect responsible for the low ENU-induced SSB repair observed in some individuals is a lesion-specific step in excision repair, e.g. glycosylation [Boerrigter et al., 1991b].

Whether defective DNA repair is related to or closely linked to the etiology and/or pathophysiology of familial AD is not clear at this time. A diminished DNA repair in familial but not in sporadic AD could either be a consequence of a different pathogenesis of familial AD or could represent a genetic defect only present in familial AD. Medical treatment was similar for the familial and sporadic patients involved in our investigations. Therefore, the decrease in DNA repair cannot be ascribed to AD medication. Although the repair of the ENU-induced DNA lesions studied in sporadic AD patients is quantitatively similar to that observed in normal control subjects, this does not necessarily exclude differences in other pathways, or qualitative differences in for example the fidelity of DNA

repair processes. Both a decreased rate of repair and/or a lower fidelity of DNA repair in non-dividing cells, such as neurons, could have adverse effects on the expression of genes important for neuronal function and cell survival.

CHAPTER 8

SUMMARY AND GENERAL DISCUSSION

The ability of mammalian cells to remove DNA damage, induced as a consequence of the exposure of cells to various endogenous and exogenous DNA-damaging agents, is of the utmost importance in counteracting the detrimental effects of persistent DNA damage on cellular functioning and survival. Insufficient or erroneous DNA repair, resulting in the gradual accumulation of non-repaired or misrepaired DNA bases, has been postulated to be a major cause of cellular aging [Gensler and Bernstein, 1981; Vijg, 1987; Mullaart, 1989, 1990b]. Moreover, a decreased capacity to correctly repair DNA damage could be an important risk factor in the etiology of various age-related diseases, including cancer and neurodegenerative diseases such as Alzheimer's disease [Robbins et al., 1985b; Kidson and Chen, 1986; Vijg et al., 1990].

Several human diseases are associated with specific alterations in DNA repair mechanisms which predispose cells of affected individuals to cellular dysfunction. Xeroderma pigmentosum (XP) and Cockayne's syndrome (CS) are two diseases which are both characterized by a hypersensitivity to UV light resulting from defective excision repair of UV-induced cyclobutane dimers [Friedberg, 1985a, Weeda et al., 1990]. XP patients are classified as belonging to one of eight complementation groups, depending on the ability to complement one another, i.e. to restore DNA repair to normal levels, when genetically combined in heterokaryons [De Weerd-Kastelein et al., 1972; Friedberg, 1985a]. Recently, the genes thought to be the primary defect in group A and group B XP patients and CS patients were cloned and identified as DNA-binding proteins, presumably as a part of an enzyme complex that makes an incision near damaged sites in the DNA [Tanaka et al., 1990, Weeda et al., 1990]. Individuals suffering from XP or CS often display neurological abnormalities and mental impairment. Therefore, it is not inconceivable that other diseases with clinical features of neurodegenerative changes are similarly associated with defective DNA repair, albeit of a different type than that found in XP and CS.

Cell lines from patients with Alzheimer's disease (AD) are hypersensitive to the cell-killing effects of ionizing radiation and alkylating agents [Scudiero et al., 1982a; Robbins et

al., 1983b; Robbins et al., 1985; Scudiero et al., 1986], but not to the cell-killing effects of UV light. This suggests that AD might be associated with defects in DNA-repair pathways involved in the removal of small base damages that have been found to be induced by ionizing radiation and alkylating agents. Indeed, evidence has been obtained for a defective repair of single-strand breaks (SSB), induced by the alkylating agents N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or methyl methanesulfonate (MMS), in both fibroblast [Li and Kaminskas, 1985; Robison et al., 1987] and lymphoblastoid [Jones et al., 1989] cell lines from AD patients.

The objective of this study was to evaluate whether AD is associated with a defective repair of DNA damage in freshly isolated quiescent peripheral blood lymphocytes (PBL). The sensitive alkaline filter elution technique has been found to be especially suitable for analyzing the induction and disappearance of SSB in cells exposed to ionizing radiation or alkylating agents. On the basis of the reported hypersensitivity of cell lines from AD patients to ionizing radiation [Robbins et al., 1983b] and the observed decreased MMS-induced SSB-disappearance in such cell lines from AD patients [Li and Kaminskas, 1985; Robison et al., 1987; Jones et al., 1989], both γ-radiation and MMS were chosen to investigate DNA repair in freshly isolated PBL. In addition, SSB induction and disappearance was studied after exposure to another alkylating agent, i.e. ethylnitrosourea (ENU).

In order to do so, it is necessary to ascertain which factors may potentially influence the removal of DNA damage in human PBL and how a possible DNA repair defect in peripheral cells might relate to the etiology of AD. A limited characterization of the three DNA-damaging agents employed in this study involved the analysis of (1) SSB induction and disappearance in both quiescent and mitogen-stimulated PBL, (2) the presence of interindividual variation in SSB disappearance and (3) the functional relevance of decreased SSB disappearance. Finally, SSB disappearance was determined in freshly isolated PBL of a large number of sporadic and familial AD patients after in vitro treatment with MMS, ENU or γ-radiation.

8.1 SSB disappearance in lymphocytes from Alzheimer patients and normal control subjects

For a number of cell types, including PBL, the ability to remove DNA damage is positively associated with proliferative capacity [for a review, see Sirover, 1990]. In this study it was found that MMS- and γ-ray-induced SSB, but not ethylnitrosourea (ENU)-induced SSB disappear at a faster rate in mitogen-stimulated PBL, as compared to their quiescent counterparts [Chapters 3 and 4]. In several studies age-related changes have been observed in the proliferative kinetics of mitogen-stimulated PBL [McCarron et al., 1987; Lucivero et al., 1988; Schindler et al., 1988] which may be even more pronounced in cells from aged AD patients [Carmeliet et al., 1990]. Differences in the growth characteristics of cells from AD patients, as compared to normal control subjects may thus give rise to differences in DNA repair [Li and Kaminskas, 1985; Robison et al., 1987; Jones et al., 1989] and/or cellular survival [Scudiero et al., 1982a; Robbins et al., 1983b; Robbins et al., 1985; Scudiero et al., 1986]. Hence, the particular cell type chosen for comparing repair capacities between individuals or between groups of individuals should preferably be limited to those cell types which do not need in vitro culturing before induction and repair of DNA damage can be monitored. The use of freshly isolated cells in the present study refutes the possibility that observed differences among individuals within a group or between different groups are an artefact of culture.

The presumed presence of large numbers of preexistent SSB in quiescent PBL [Johnstone and Williams, 1982], which might potentially interfere with the accurate assessment of DNA repair capacity, was refuted in our present study [Chapter 2]. The low number of "preexistent" SSB in quiescent PBL (100-500 SSB per cell) is representative for the number of background SSB detected in quiescent PBL from normal young and old control subjects and AD patients (results not published).

The proposal that AD may represent the most severe form of brain aging in a continuum of "normal" aging, benign senescent forgetfulness, and dementia, and in this respect should be considered as accelerated aging [Mann et al., 1985; Brayne and Calloway, 1988], more or less obliges one to monitor AD-associated changes in relation to changes which also occur during "normal" aging. A causal relation between altered DNA-repair

responses and the aging process has often been postulated [for reviews, see Gensler and Bernstein, 1981; Vijg, 1987; Mullaart, 1989, 1990b]. Although experimental data seem to indicate that there is no evidence for a substantial age-related decline in DNA-repair activities [Tice and Setlow 1985; Hanawalt 1987; Vijg, 1987; Mullaart, 1989; 1990b], an accumulation of DNA damage, as a consequence of *inherently* inefficient DNA-repair processes, might lead to compromised cellular functioning and ultimately cell death. The present study failed to observe an age-associated decline in DNA repair capacity in human quiescent PBL measured as SSB disappearance after exposure of cells to MMS, ENU, or γ-rays and subsequent incubation for various repair periods [Chapter 7]. As a consequence of the relatively short lifespan of human PBL, as compared to liver parenchymal cells or neuronal cells, an age-associated accumulation of DNA damage was not expected to occur, and was indeed not observed (results not published). However, DNA from cerebral cortex of AD patients does appear to contain a significantly higher amount of alkali-labile sites in comparison to the DNA from healthy controls, possibly as the result of inefficient DNA-repair mechanisms [Mullaart et al., 1990a]. This increased DNA breakage in cerebral cortex of AD patients might herald the advancement of neuronal degeneration, the most prominent hallmark of AD.

In the present study the ability of freshly isolated PBL from AD patients to remove DNA damage was investigated after exposure to ionizing radiation or the alkylating agents MMS and ENU [Chapter 7]. The ethylating agent ENU and the methylating agent MMS are representative of S_N1- and S_N2-alkylating agents, respectively. In general, S_N1-alkylating compounds display a greater tendency to alkylate oxygen atoms, whereas agents which react according to an S_N2-type mechanism predominantly alkylate nitrogen sites in the DNA. Selection of the S_N2-type agent MMS was based on a study which indicated that MMS-induced SSB disappearance was defective in fibroblast cell lines from AD patients [Robison et al., 1987]. With respect to the selection of an S_N1-alkylating agent, ENU was preferred over the methylating agent MNNG because the former displays a stronger S_N1-character; i.e. ENU alkylates oxygen more effectively than MNNG [Hemminki, 1983].

In contrast to the literature data on lymphoblastoid cell lines, freshly isolated PBL from AD patients did not display a decreased disappearance of MMS-induced SSB. Quiescent PBL showed only a low extent of SSB disappearance after exposure to MMS [Chapters 4

and 7], whereas lymphoblastoid cell lines from normal controls removed about 70-80% of the SSB initially induced by MMS [Jones et al., 1989]. In view of our observation that MMS-induced SSB disappearance is dependent on the proliferative state of the lymphocyte population [Chapter 4], it is not inconceivable that the previously reported decreased SSB disappearance in MMS-exposed, AD-derived lymphoblastoid cell lines is actually caused by subtle changes in the growth characteristics of these cells. The similar percentage SSB disappearance in MMS-treated quiescent PBL from AD patients and matched controls [Chapter 7] suggests that previous observations of a decreased MMS-induced SSB disappearance in AD-derived lymphoblastoid cell lines may indeed be an artefact of culturing conditions. It should be noted that the high percentage SSB disappearance in MMS-exposed, actively dividing fibroblasts of normal donors is in close agreement with values reported for this cell type in another study [Robison et al., 1987; Chapter 4]. This refutes the possibility that the observed low MMS-induced SSB disappearance in quiescent PBL [Chapter 4], as compared to the high percentage SSB disappearance in MMS-treated lymphoblastoid cell lines [Jones et al., 1989], is an artefact of the present experimental conditions.

Interestingly, disappearance of ENU-induced SSB was found to be decreased in PBL from familial AD patients, whereas no such defect was observed in PBL from sporadic AD cases [Chapter 7]. Although at present there is no indication as to which enzyme(s), involved in the removal of ENU-induced DNA lesions, are partially defective in familial AD, it is tempting to speculate that the defect might be of the same nature as that observed in PBL from some normal individuals [Chapter 5]. The decreased removal of ENU-induced SSB is hypothesized to be the consequence of defective DNA glycosylation activity [Chapter 5]. In this regard it should be noted that different alkylating agents induce a different spectrum of DNA lesions, the removal of which may require different repair pathways and possibly different glycosylases. Therefore, the previously reported DNA-repair defect in cells from sporadic AD patients, detected after treatment with MMS (Robison et al., 1987) is not necessarily in conflict with our present results but could be the consequence of the different alkylating agents used.

8.2 DNA repair in relation to the pathogenesis of Alzheimer's disease

A recent study has indicated that during neuronal development of the rat a rapid decrease occurs in uracil DNA-glycosylase activity which could impair the removal of uracil present in the DNA of adult neurons as a consequence of the spontaneous deamination of cytosine [Focher et al., 1990]. Assuming a similar DNA repair defect in neuronal cells as has been observed for ENU-induced SSB in PBL in the present study [Chapters 5-7], this might lead to an accumulation of DNA damage which might predispose neuronal cells to cellular dysfunction and cell death, ultimately resulting in the AD phenotype. It might seem surprising that a systemic defect in DNA repair in familial AD patients in vivo would manifest itself only in neuronal cells. However, the expression of such a defect will greatly depend on the specific environment of the affected cell types. Possibly, specific metabolic processes occurring only, or to a greater extent, in neuronal cells may contribute to the formation of the DNA damage that cannot be efficiently removed by these cells.

Assuming that a decreased disappearance of ENU-induced SSB in PBL from certain normal, young controls [Chapter 5] and familial AD (FAD) patients [Chapter 7] is based on the same underlying genetic defect, the observed DNA-repair defect in ENU-treated PBL from FAD patients might be rather specific, i.e. a defective DNA glycosylase [Chapters 5-7]. Thus, it is necessary to discuss how the observed defect in the removal of ENU-induced single-strand breaks might relate to the pathogenesis of AD.

The only alkylation damages induced by ENU which are alkali-labile and/or formed in sufficient quantities in order to contribute to the formation of SSB as detected by alkaline elution, are N7-ethylguanine and N3-ethyladenine. The presence of DNA glycosylase activities for N7-alkylguanine and N3-alkyladenine in extracts from human lymphoblasts indicates that alkali-labile apurinic sites may be formed via excision repair processes [Singer and Brent, 1981]. A decreased activity of one or both of these DNA glycosylases in quiescent PBL might thus explain the slower removal of ENU-induced SSB as observed in quiescent PBL from some normal individuals [Chapters 5 and 6] and familial AD patients [Chapter 7].

A defective removal of N7-alkylguanine and/or N3-alkyladenine takes on added relevance when one realizes that DNA in vivo can be alkylated by S-adenosyl-L-methionine

(SAM), the normal intracellular methylgroup donor [Barrows and Magee, 1982]. In particular, SAM alkylates DNA at the N7 position of guanine and N3 position of adenine [Rydberg and Lindahl, 1982]. The spontaneous formation of N7-methylguanine and N3-methyladenine by SAM might be higher in neurons as compared to peripheral cells. The in vitro activity of SAM has been shown to exhibit statistically significant differences among brain regions of normal subjects [Erdely et al., 1978]. It is therefore not inconceivable that similar differences exist for different cell types. The absence of sufficiently efficient DNA glycosylase activities for these particular alkylation damages might result in the accumulation of N3-methyladenine and/or N7-methylguanine in DNA of neuronal cells and might ultimately cause the neuronal degeneration as observed in brains of AD patients.

A complication arises when one considers the more or less delineated neuronal degeneration in AD. Possibly, the most severely affected regions in the AD brain, i.e. the parieotemporal cortex and hippocampus, are exposed to unusually high amounts of DNA-damaging agents as compared to those brain regions that appear to be spared from neuronal degeneration. Alternatively, differences in the spatiotemporal regulation of the activity of DNA repair enzymes might account for the degeneration of specific regions of the brain. For this purpose, highly specific monoclonal antibodies directed against DNA alkylation products and cell-type specific markers may be used on sections of freeze-dried blocks of brain tissue to quantitate the amount of alkylation damage present in the DNA of specific cell populations.

Whether defective DNA repair is related to or closely linked to the etiology and/or pathophysiology of FAD is not clear at this time. A diminished DNA repair in familial, but not in sporadic AD could either be a consequence of a different pathogenesis of FAD or could represent a genetic defect only present in FAD. The expression of a genetic defect in DNA repair might be modulated by environmental factors, e.g. the availability of intracellular metabolites to contribute to the induction of DNA damage. In this regard, it can be hypothesized that PBL from sporadic AD patients possess a DNA repair defect similar to that observed in FAD patients which, however, only becomes manifest upon the induction of higher levels of the relevant DNA lesions. This hypothesis seems to be supported by the previous observation of DNA-repair defect in cells from sporadic [Robison et al., 1987] and familial [Jones et al., 1989] AD patients exposed to MMS. This particular alkylating agent

induces N7-methylguanine and N3-methyladenine at about 86% and 10% of total alkylation, respectively. In contrast, exposure of cells to ENU results in the formation of the ethylated analogues of these 2 lesions at only 13% and 4% of total alkylation, respectively [Hemminki, 1983]. The expression of a DNA repair defect in cells from sporadic AD patients following exposure to MMS [Robison et al., 1987], but not after treatment with ENU [Chapter 7] might thus be the consequence of the relatively higher level of N7-alkylguanine and N3-alkyladenine induced by MMS, at an equimolar concentration, as compared to ENU. Hence, the observed later onset of the disease process in most, but not all, sporadic AD patients as compared to familial cases might be due to the longer time period needed for the accumulation of a sufficient amount of N7-methylguanine and N3-methyladenine in neuronal cells from sporadic AD patients before neuronal degeneration ensues (assuming similar rates of DNA damage accumulation in neurons from familial and sporadic AD patients). However, as yet there is no experimental evidence for this hypothesis. Although the repair of ENU-induced DNA lesions in sporadic AD patients is quantitatively similar to that observed in normal control subjects [Chapter 7], this does not necessarily exclude differences in other pathways, or qualitative differences in, for example, the fidelity of DNA repair processes. Both a decreased rate of repair and/or a lower fidelity of DNA repair in non-dividing cells, such as neurons, could have adverse effects on the expression of genes important for neuronal function and survival.

8.3 Conclusions

The present study was concerned with the characterization of DNA damage induction and disappearance in human PBL, exposed to the alkylating agents MMS or ENU, or γ-radiation, by using the sensitive alkaline filter elution technique. This was done in order to evaluate the possibility that freshly isolated PBL from AD patients might display an impaired ability in the removal of such damage, as compared to PBL from matched controls. Several factors that can influence DNA repair in PBL, i.e. proliferative status, pool sizes of lymphocyte subpopulations, and in vivo aging, were considered before interpreting the results in relation to AD. The experimental data presented in this study can be summarized as follows:

1. Quiescent human PBL do not contain a sizable amount of preexistent DNA strand breaks.
2. Human fibroblasts employ an efficient form of direct repair for the removal of MMS-induced, alkali-labile DNA lesions; this form of direct repair is correlated with cellular survival and seems to be absent in PBL.
3. There is a marked interindividual variation in the removal of ENU-induced DNA lesions in quiescent human PBL which is associated with cellular survival after ENU exposure.
4. Mitogen-stimulation of PBL does not result in an increased removal of all types of DNA lesions.
5. Quiescent human PBL do not suffer from a general age-related decline in their ability to remove small base damages such as induced by MMS, ENU, or γ-radiation.
6. Quiescent PBL from familial, but not from sporadic, AD patients appear to be defective in the removal of ENU-induced DNA damage possibly as the consequence of defective DNA glycosylase activity.

SAMENVATTING

Zoogdiercellen bezitten de mogelijkheid om een verscheidenheid aan DNA-schades te verwijderen na blootstelling aan een endogeen en/of exogeen DNA-beschadigend agens. Deze DNA-herstelprocessen zijn van groot belang voor het correct functioneren en de overleving van de cel. Indien deze DNA-herstelsystemen te weinig DNA schades verwijderen of te veel fouten maken bij de herstelprocedure zou dit tot cellulaire veroudering kunnen leiden [Gensler en Bernstein, 1981; Vijg, 1987; Mullaart, 1989, 1990b]. Een inefficiënte verwijdering van DNA-schades kan bovendien een belangrijke risicofactor in de ontwikkeling van verschillende verouderingsgerelateerde ziekten, bijvoorbeeld kanker en de ziekte van Alzheimer, vormen [Robbins e.a., 1985b; Kidson en Chen, 1986; Vijg e.a., 1990].

Een aantal verschillende ziekten kunnen worden geassocieerd met specifieke afwijkingen in een DNA-herstelsysteem. Het cellulaire metabolisme van lichaamscellen van patiënten die een defect DNA-herstel hebben wordt door dit defecte DNA-herstelsysteem dusdanig ontregeld dat een afwijkend metabolisme het uiteindelijke gevolg is. De ziekten Xeroderma pigmentosum (XP) en Cockayne's syndroom (CS) worden allebei gekarakteriseerd door een overgevoeligheid voor ultraviolette (UV) straling. Dit is het gevolg van een defect DNA herstel van UV-geïnduceerde cyclobutaandimeren [Friedberg, 1985a; Weeda e.a., 1990]. XP patiënten worden onderverdeeld in 8 complementatiegroepen welke is gebaseerd op de mogelijkheid van de lichaamscellen van een bepaalde patient om een normaal DNA-herstel uit te voeren na fusie met lichaamscellen van een andere patient tot een zogenaamd heterokaryon [De-Weerd Kastelein e.a., 1972; Friedberg, 1985a]. De genen die betrokken zijn bij het DNA-herstel defect in XP, groep A en B, en CS zijn recentelijk gecloneerd. Analyse van de genetische code gaf aan dat beide genen een DNA-bindend eiwit coderen welke waarschijnlijk het DNA knippen in de buurt van een beschadigde DNA-base [Tanaka e.a., 1990; Weeda e.a., 1990]. Patiënten die aan XP of CS lijden vertonen vaak neurologische afwijkingen. Het klinische patroon van deze afwijkingen vertoont sterke overeenkomsten met die van patiënten met een neurodegeneratieve ziekte. Het zou dus niet verbazend zijn wanneer zou blijken dat lichaamscellen van patiënten met bepaalde neurodegeneratieve ziekten ook een afwijkend DNA herstelsysteem hebben,

alhoewel wellicht anders dan het defect in XP of CS.

Gekweekte cellen van patiënten met de ziekte van Alzheimer zijn overgevoelig voor het celdodende effect van ioniserende straling of alkylerende agentia [Scudiero e.a., 1982a; Robbins e.a., 1983b; Robbins e.a., 1985; Scudiero e.a., 1986]. UV-straling heeft echter geen buitensporig effect op zulke cellen. Een defect DNA-herstel van DNA-schades, geïnduceerd door ioniserende straling of alkylerende agentia, zou dus een rol kunnen spelen in de etiologie van de ziekte van Alzheimer. Inderdaad is er een verlaagd herstel gevonden van DNA-breuken in fibroblasten [Li en Kaminskas, 1985; Robison e.a., 1987] en getransformeerde B lymfocyten [Jones e.a., 1989] van Alzheimerpatiënten na in vitro behandeling met de alkylerende agentia N-metyl-N'-nitro-N-nitrosoguanidine (MNNG) of methylmethaansulfonaat (MMS).

De studie beschreven in dit proefschrift richtte zich in eerste instantie op de mogelijkheid dat ongetransformeerde, perifere bloedlymfocyten (PBL) van Alzheimerpatiënten een defect DNA herstelsysteem bezitten. De uiterst gevoelige alkalische elutie methode is uitermate geschikt voor de meting van DNA-enkelstrengsbreuken in cellen na behandeling met ioniserende straling of alkylerende agentia. In het licht van de al eerder gerapporteerde overgevoeligheid van cellen van Alzheimerpatiënten voor ioniserende straling en alkylerende agentia [Robbins e.a, 1983b] en het verlaagde herstel van MMS-geïnduceerde DNA-enkelstrengsbreuken in zulke cellen [Li en Kaminskas, 1985; Robison e.a., 1987; Jones e.a., 1989], werden zowel gammastraling en MMS geselecteerd om het DNA-herstel in PBL te karakteriseren. Het herstel van DNA-enkelstrengsbreuken na behandeling met een ander alkylerend agens, N-ethyl-N-nitrosoureum (ENU), werd eveneens bestudeerd.

Voordat men de invloed van een mogelijk DNA-hersteldefect op de ontwikkeling van de ziekte van Alzheimer kan beoordelen, is het van belang om die factoren, die de verwijdering van DNA-schades kunnen beïnvloeden, te karakteriseren. De karakterisatie van het DNA herstel in PBL na behandeling met gammastralen, MMS of ENU beperkte zich in deze studie tot (1) de vorming en verwijdering van DNA-enkelstrengsbreuken in zowel delende als niet-delende PBL, (2) mogelijke interindividuele variatie in de verwijdering van DNA-enkelstrengsbreuken en (3) de mogelijke invloed van een defect DNA-herstel op cellulaire overleving. Uiteindelijk werd de verwijdering van DNA-enkelstrengsbreuken bepaald in PBL van een groot aantal sporadische en familiaire Alzheimerpatiënten na de in

vitro behandeling met MMS, ENU, of gammastralen.

De verwijdering van DNA-schades is in een aantal celtypen, waaronder PBL, positief gecorreleerd met de delingscapaciteit [Sirover, 1990]. Uit het huidige onderzoek bleek dat delende PBL DNA-enkelstrengsbreuken geïnduceerd door gammastralen en MMS vlugger verwijderen dan niet-delende PBL, terwijl de verwijdering van ENU-geïnduceerde DNA-breuken nagenoeg gelijk bleek in delende en niet-delende PBL [Hoofdstukken 3 t/m 6]. Het gebruik van ongetransformeerde, niet-delende PBL in de huidige studie sluit uit dat verschillen in DNA-herstel tussen individuen van een bepaalde groep, of tussen verschillende groepen, het gevolg is van verschillen in de celcyclus van de bestudeerde cellen.

De aanwezigheid van een groot aantal "achtergrondbreuken" in het DNA van niet-delende PBL, zoals gepostuleerd door Johnstone en Williams [1982] kan in principe de DNA-herstelcapaciteit beinvloeden. Echter, met behulp van de alkalische filter elutie methode kon in de huidige studie slechts een relatief laag aantal (100-500) DNA-enkelstrengsbreuken in PBL van gezonde jonge en oude donoren en Alzheimerpatiënten worden aangetoond [zie Hoofdstuk 2].

De opvatting dat de ziekte van Alzheimer een "versnelde" vorm van hersenveroudering is [Mann e.a., 1985; Bayne en Calloway, 1988] maakt het interessant om verschillen in de cellen van Alzheimerpatiënten te vergelijken met veranderingen die optreden tijdens normale veroudering. De hypothese dat veroudering het gevolg zou kunnen zijn van een afwijkend DNA-herstel [Gensler en Bernstein, 1981] wordt niet ondersteund door de meerderheid van experimentele data [Tice en Setlow, 1985; Hanawalt, 1987; Vijg, 1987; Mullaart, 1989, 1990b]. Ook in de huidige studie zijn geen aanwijzingen gevonden die zo'n opvatting staven. De verwijdering van DNA-enkelstrengsbreuken in niet-delende PBL na behandeling met gamma-stralen, MMS, of ENU nam niet af tijdens in vivo veroudering [zie Hoofdstuk 7]. Niettemin, de opeenhoping van DNA schades als gevolg van een onvolledig DNA-herstel, kan in principe tot celdegeneratie en celdood leiden. In verband met de relatief korte levensduur van PBL in vivo is het niet verbazingwekkend dat dit celtype niet onderhevig is aan een verouderingsgerelateerde accumulatie van DNA-schades. Deze werden in de huidige studie dan ook niet gevonden. Neuronale cellen uit de cerebrale cortex van Alzheimerpatiënten echter, bezitten een hoger aantal alkalisch labiele DNA-schades in vergelijkijng tot het DNA van gezonde controles [Mullaart e.a., 1990a]. Dit verhoogde

niveau van DNA-schades in de cerebrale cortex van Alzheimerpatiënten is misschien een van de onderliggende oorzaken van de neuronale degeneratie bij de ziekte van Alzheimer.

De verwijdering van DNA-enkelstrengsbreuken in niet-delende PBL van Alzheimerpatiënten na behandeling met MMS was, in tegenstelling tot wat eerder werd gevonden met getransformeerde B-lymfocyten, niet lager dan de verwijdering in cellen van gezonde controlepersonen. Het totale percentage verwijdering in niet-delende PBL na blootstelling aan MMS bleek tamelijk laag te zijn [Hoofdstukken 4 en 7], terwijl getransformeerde PBL van controlepersonen zo'n 70-80% van de DNA- enkelstrengsbreuken konden verwijderen [Jones e.a., 1989]. De verwijdering van MMS-geïnduceerde DNA-enkelstrengsbreuken in PBL wordt echter geregeld door de delingssnelheid van de celpopulatie [Hoofdstuk 4]. De lagere verwijdering van DNA-enkelstrengsbreuken in getransformeerde, delende PBL van Alzheimerpatiënten na behandeling met MMS, zou dus kunnen worden veroorzaakt door kleine verschillen in de proliferatieve capaciteit van de cellen.

De verwijdering van ENU-geïnduceerde DNA-enkelstrengsbreuken in niet-delende PBL van familiaire Alzheimerpatiënten bleek wel beduidend lager, terwijl niet-delende PBL van sporadische Alzheimerpatiënten deze DNA-breuken net zo snel en efficiënt verwijderden als PBL van normale, gezonde controlepersonen [Hoofdstuk 7]. Een defect herstel van ENU-geïnduceerde DNA breuken werd ook gevonden in niet-delende PBL van enkele normale personen [Hoofdstuk 5]. Het is echter nog niet mogelijk om vast te stellen of beide defecten aan éénzelfde genetische afwijking ten grondslag liggen. Een mogelijkheid is dat een defect in de verwijdering van ENU-geïnduceerde DNA-enkelstrengsbreuken het gevolg is van een defect in een DNA-glycosylase. Omdat verschillende alkylerende agentia een verschillend spectrum van DNA-schades induceren, is het defecte DNA-herstel van MMS-breuken in delende fibroblasten van sporadische Alzheimerpatiënten [Robison e.a., 1987], niet noodzakelijkerwijs in tegenspraak met de huidige resultaten, maar kan het gevolg zijn van een ander DNA-schadepatroon.

Tijdens de ontwikkeling van het centrale zenuwstelsel van de rat neemt de activiteit van uracil DNA-glycosylase dramatisch af. Een inefficiënte verwijdering van uracil uit het DNA, gevormd door de spontane deaminatie van cytosine, kan kwade gevolgen hebben voor de neuronale cellen tijdens in vivo veroudering van de rattehersenen [Focher e.a., 1990]. Indien het defecte herstel van ENU-geïnduceerde DNA-enkelstrengsbreuken zowel in niet-delende

PBL [zie Hoodstukken 5 t/m 7] als in niet-delende neuronen voorkomt, kan dit tot een opeenhoping van DNA-breuken in neuronen leiden. Op een gegeven moment tijdens de levensduur van het individu zal de accumulatie van deze DNA-schades in de neuronen een kritische drempel bereiken en, uiteindelijk, misschien aanleiding geven tot celdood en de ontwikkeling van de ziekte van Alzheimer. Het moet worden opgemerkt dat de openbaring van een DNA-hersteldefect, aanwezig in alle lichaamscellen, niet noodzakerlijkerwijs de functie van alle lichaamscellen beïnvloedt. De specifieke condities waaronder de DNA-schades worden gevormd, zoals die misschien alleen in de hersenen voorkomen, beïnvloeden mogelijk de expressie van het DNA-hersteldefect. Als we aannemen dat het defecte herstel van ENU-geïnduceerde DNA enkelstrengsbreuken in niet-delende PBL van sommige normale controlepersonen [Hoofdstuk 5] en familiaire Alzheimerpatïenten [Hoofdstuk 7], het gevolg is van één en hetzelfde defect, namelijk een defect DNA-glycosylase [Hoofdstukken 5-7], moeten we de relevantie van zo'n defect m.b.t de pathogenese van de ziekte van Alzheimer proberen te verklaren.

De enige typen alkylschades die door de behandeling met ENU worden gevormd en als alkalische-labiele plaatsen in het DNA kunnen worden gedecteerd door de alkalische filter elutie methode én in een voldoende aantal worden gevormd, zijn N7-ethylguanine en N3-ethyladenine. Deze DNA-adducten vormen een alkalische labiele plaats in het DNA nadat de base door een DNA-glycosylase, welke voorkomt in menselijke bloedcellen, is verwijderd [Singer en Brent, 1981]. De lagere verwijdering van ENU-geïnduceerde DNA-breuken in niet-delende PBL van sommige controlepersonen [Hoofdstukken 5 en 6] en familiaire Alzheimerpatiënten [Hoofdstuk 7], zou dus kunnen worden verklaard door een defect in een DNA-glycosylase dat deze alkylschades verwijdert.

De vorming van N7-alkylguanine en N3-alkyladenine in vivo kan o.a. plaatsvinden door S-adenosyl-L-methionine (SAM) [Rydberg en Lindahl, 1982]. Verschillende gebieden in de hersenen vertonen in vitro een verschillende activiteit van SAM [Erdely e.a., 1978]. Naar alle waarschijnlijkheid zullen verschillende celtypen verschillen in de activiteit van intracellulair SAM. Indien de DNA-alkylschades zich ophopen, als het gevolg van een defect DNA-glycosylase, zou dit misschien een oorzaak voor de neuronale degeneratie in de ziekte van Alzheimer kunnen zijn. De hersengebieden in Alzheimerpatiënten die de meeste celdood bevatten (pariotemporale cortex en hippocampus) worden misschien blootgesteld aan extreem

grote hoeveelheden van het DNA-beschadigend agens. Anderzijds is het mogelijk dat het DNA-herstel in deze gebieden van nature al laag is zodat voornamelijk deze cellen sterven.

De conclusie dat een defect DNA-herstelsysteem één (of de) oorzaak is van de ziekte van Alzheimer is, kan op dit moment nog niet worden getrokken. Een defect DNA-herstel in PBL van familiaire, maar niet in PBL van sporadische, Alzheimerpatiënten, kan worden veroorzaakt door een ander ziektepatroon of door een genetische afwijking die alleen in de familiaire vorm voorkomt. Indien eenzelfde defect in sporadische en familiaire Alzheimer-patiënten aanwezig is, zal dit zich in de sporadische patiënten pas dan openbaren als er een grotere hoeveelheid van de specifieke DNA-schades is gevormd. Dit lijkt te worden bevestigd door de bevinding dat cellen van sporadische en familiaire Alzheimerpatiënten een lager herstel van MMS-geïnduceerde DNA-breuken hebben [Robison e.a, 1987; Jones e.a., 1989]. De vorming van N7-alkylguanine en N3-alkylguanine door MMS is respectievelijk 6 en 2 keer hoger in vergelijking tot ENU [Hemminki, 1983]. Bij dezelfde molaire concentratie van de twee alkylerende agentia zal MMS dus beduidend meer DNA schades vormen waarna een eventueel DNA-hersteldefect in de cellen van sporadische Alzheimerpatiënten waarneembaar is. Als we aannemen dat de opeenhoping van de DNA-schades in vivo hetzelfde patroon volgt in sporadische en familiaire Alzheimerpatiënten, zullen de eerste symptomen van de ziekte van Alzheimer zich later openbaren in de sporadische patiënten omdat de accumulatie van een zodanige hoeveelheid DNA-schades, die de overleving van de neuronen kunnen beïnvloeden, een langere tijd nodig heeft in de sporadische gevallen. Dit zou betekenen dat de eerste symptomen van sporadische Alzheimer's zich pas op een latere leeftijd openbaren, wat inderdaad voor de meeste, doch niet alle, sporadische patiënten, lijkt op te gaan.

Samengevat kunnen uit de in dit proefschrift beschreven resultaten de volgende conclusies getrokken worden.

1) Het DNA van niet-delende humane PBL bevat geen hoog aantal "achtergrondbreuken".
2) De verwijdering van MMS-geïnduceerde DNA-enkelstrengsbreuken vindt op een zeer efficiënte wijze plaats in menselijke huidcellen en is ogenschijnlijk gecorreleerd met de overleving van de huidcellen; in niet-delende PBL is dit DNA herstelproces klaarblijkelijk niet of nauwelijks aanwezig.

3) De interindividuele variatie in de verwijdering van DNA-enkelstrengsbreuken uit het DNA van niet-delende PBL na een in vitro behandeling met N-ethyl-N-nitrosoureum is geassocieerd met de overleving van deze cellen.

4) Het DNA-herstel in delende PBL is niet voor alle typen DNA-schades hoger dan in niet-delende cellen.

5) De verwijdering van kleine DNA-schades, zoals geïnduceerd door gammastraling, MMS, of ENU, vertoont geen verouderingsgerelateerde afname in niet-delende PBL.

6) De verwijdering van ENU-geïnduceerde DNA-enkelstrengsbreuken is lager in niet-delende PBL van familiaire, maar niet in PBL van sporadische, Alzheimerpatiënten en is mogelijk het gevolg van een defect DNA-glycosylase.

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