Hum Genet (1997) 101 : 299–305 

ORIGINAL INVESTIGATION

**Lucia Migliore · Anna Testa · Roberto Scarpato**
**Nicola Pavese · Lucia Petrozzi · Ubaldo Bonuccelli**

# Spontaneous and induced aneuploidy in peripheral blood lymphocytes of patients with Alzheimer's disease

Received: 13 January 1997 / Accepted: 30 July 1997

**Abstract** This study was aimed at assessing whether peripheral blood lymphocytes of patients with Alzheimer's disease (AD) show significant levels of aneuploidy and high percentages of cytogenetic events in vitro, indicating a predisposition to aneuploidy spontaneously, or after chemical treatment in vitro. A group of affected individuals and a group of unaffected, age-, sex- and smoking-habit-matched controls were identified. Lymphocytes were cultured for analysis of the following cytogenetic parameters: premature centromere division (PCD), satellite associations of acrocentric chromosomes (SA) and micronuclei (MN). In a subset of subjects, the fluorescence in situ hybridization (FISH) technique was combined with the MN assay, by means of a pancentromeric DNA probe for the detection of the presence of centric material. To evaluate the sensitivity to aneuploidogenic agents, in vitro treatment of lymphocytes of affected individuals was performed by adding griseofulvin, a chemical whose supposed target is microtubule-associated protein(s). Both the spontaneous frequency of MN and the frequency of PCD was significantly higher in patient cells than in controls. Furthermore, after application of the FISH technique, we found that the majority of MN were composed of whole chromosomes (because of the phenomenon of chromosome loss). Metaphase analysis for the detection of associative events between satellite regions of acrocentric chromosomes showed no differences between the two groups under study. Analysis of sensitivity to the aneuploidogen griseofulvin showed that the patient group was characterized by lower levels of MN induction compared with controls. Our data confirm that peripheral blood lymphocytes of AD patients are prone to undergo aneuploidy spontaneously in vitro and support the hypothesis that microtubule impairment might be associated with the disease.

L. Migliore (✉) · A. Testa · R. Scarpato
Dipartimento di Scienze dell'Uomo e dell'Ambiente,
University of Pisa, Via S. Giuseppe 22, I-56100 Pisa, Italy
Tel.: +39-50-551217; Fax: +39-50-551290

N. Pavese · L. Petrozzi · U. Bonuccelli
Dipartimento di Neuroscienze, University of Pisa, Pisa, Italy

## Introduction

Alzheimer's disease (AD) has been included among common complex diseases, with environmental and genetic factors possibly playing a role (Ottman 1995; Pericak-Vance and Haines 1995). Recently, knowledge of the molecular genetics of AD has rapidly increased and several major factors determining susceptibility have been elucidated: the amyloid precursor protein (APP) gene has been mapped to chromosome 21, preseniline genes have been mapped to chromosomes 1 and 14 and the apolipoprotein E (APO E) locus is found on chromosome 19 (St. George Hyslop et al. 1987; Schellenberg 1992; Cruts et al. 1996; Jarvik et al. 1996).

Cytogenetic studies have been carried out to assess structural and numerical chromosomal aberrations in cultured cells of AD patients. An increase in aneuploid metaphases has been reported (Ward et al. 1979; Nordenson et al. 1980), but not always confirmed (White et al. 1981; Kormann-Bortolotto et al. 1993).

Aged individuals with Down's syndrome (DS) prematurely and consistently develop AD-like neuropathological lesions such as neurofibrillary tangles and senile plaques and many of them develop dementia (Cork 1990; Franceschi et al. 1990). An increased risk of AD in young mothers of DS patients has been documented (Richards et al. 1994; Schupf et al. 1994). On the other hand, in families in which AD is inherited as an autosomal dominant trait, a high frequency of DS children and haematological malignancies has been registered (Heston and Mastri 1977). The observation that all these pathologies are characterized by the presence of aneuploid cells, taken together with the findings concerning the location of the amyloid β-protein gene on chromosome 21, has led some authors to speculate that a disorder of microtubules (MTs) could explain these associations (Heston and Mastri 1977; Matsuyama and Jarvik 1989; Wright and Whalley 1984; Potter 1991; Schupf et al. 1994; Strittmatter et al. 1994).

Potter (1991) has further suggested that non-disjunction may underlie both AD and DS, disorders in which an impaired, or rather an accelerated, process of ageing can be postulated.

Higher levels of premature centromere division (PCD) of a given chromosome in cells can be seen as a potential cause of improper chromosome segregation and consequently as a predisposition to aneuploidy (Fitzgerald et al. 1986; Mehes and Bühler 1995). PCD was found in some instances to be increased in AD patients, together with an increase in aneuploid metaphases (Ward et al. 1979; Nordenson et al. 1980; White et al. 1981; Moorhead and Heyman 1983).

The aim of this work has been to assess whether somatic cells of AD patients show significant levels of aneuploidy and a high percentage of cytogenetic events that characterize predisposition to aneuploidy (i.e. PCD). To verify aneuploidy frequency, the micronucleus (MN) assay was applied to peripheral blood lymphocytes of a group of patients and a group of related controls. This assay has been widely applied for the detection of aneuploidogenic compounds in vitro; by coupling the fluorescence in situ hybridization (FISH) technique with an alphoid DNA probe, specific for the centromere of all human chromosomes, it is possible to discriminate MN because of fragments (clastogenic damage) from MN containing whole chromosomes (mal-segregation events, i.e. chromosome loss; Becker et al. 1990; Migliore et al. 1993; Eastmond et al. 1995). Since we were also interested in verifying the sensitivity of cells of AD patients to aneuploidogenic agents, we specifically treated some cultures in vitro with griseofulvin (GF), a chemical whose targets are probably microtubule-associated proteins (Roobol et al. 1977).

## Materials and methods

For each affected person, an age-, sex- and smoking-habit-matched normal control subject was tested. A diagnosis of probable AD was made by using the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (McKhann et al. 1994). Because of the onset age and familial history, all patients were classified as sporadic cases. Patients were otherwise healthy and did not have a previous history of exposure to toxic metals or recent X-ray examination. All patients were drug-free for at least two months before the examination was performed. The severity of cognitive impairment was assessed by the Global Deterioration Scale (Reisberg et al. 1982). Written informed consent was obtained from patients or their legal representatives. Table 1 summarizes some characteristics of the study population. The AD patient group comprised 24 persons (21 females and 3 males) and the control group 22 persons (19 females and 3 males). Individuals of the two groups were matched with respect to age (± 2 years), sex and smoking habit (in the present trial, all individuals were non-smokers). Both the patient and control group ranged in age from 55 to 80 years, with an average age of 69.8 and 69.0 years, respectively.

Cytogenetic analyses were carried out on whole blood stimulated with phytohaemagglutinin and cultured according to standard techniques for 48 and 72 h for the metaphase analysis and MN as-

**Table 1** Demographic and clinical characteristics of the population studied (GDS, Grade deterioration scale: score (from 1 to 7) based on a panel of standardized neuropsychological tests; Reisberg et al. 1982)

| AD patients | | | | | Controls | | |
|---|---|---|---|---|---|---|---|
| Individual code | Age | Sex | GDS | Years of illness | Individual code | Age | Sex |
| 1 | 55 | F | 4 | 4 | 5 | 71 | M |
| 2 | 78 | F | 4 | 4 | 9 | 80 | M |
| 3 | 80 | F | 3 | 3 | 10 | 60 | F |
| 4 | 71 | M | 4 | 3 | 15 | 70 | F |
| 6 | 80 | F | 4 | 7 | 16 | 75 | F |
| 8 | 77 | F | 4 | 3 | 24 | 55 | F |
| 11 | 74 | F | 3 | 2 | 32 | 74 | F |
| 12 | 76 | F | 4 | 4 | 33 | 68 | F |
| 13 | 63 | F | 4 | 10 | 34 | 71 | F |
| 14 | 73 | F | 3 | 11 | 35 | 78 | F |
| 17 | 61 | M | 5 | 2 | 36 | 61 | F |
| 18 | 71 | F | 3 | 3 | 37 | 72 | F |
| 19 | 73 | F | 5 | 3 | 38 | 70 | F |
| 20 | 61 | M | 3 | 5 | 39 | 68 | F |
| 21 | 68 | F | 4 | 4 | 40 | 65 | F |
| 22 | 62 | F | 4 | 6 | 41 | 61 | F |
| 23 | 61 | F | 5 | 4 | 42 | 65 | F |
| 25 | 61 | F | 3 | 4 | 43 | 80 | F |
| 26 | 80 | F | 4 | 4 | 44 | 72 | F |
| 27 | 73 | F | 4 | 4 | 45 | 74 | F |
| 28 | 62 | F | 5 | 3 | 47 | 66 | M |
| 29 | 60 | F | 5 | 6 | 48 | 63 | F |
| 30 | 76 | F | 5 | 4 | | | |
| 31 | 80 | F | 3 | 3 | | | |
| Mean ± SD: | 69.8 ± 8.0 | | | | | 69.0 ± 6.6 | |

say, respectively. Each culture was performed in duplicate. The detailed protocols are reported elsewhere (Migliore et al. 1993). GF (15 μg/ml) treatment was performed at 24 h and lasted until harvesting (at 72 h). The FISH technique was applied only to a subset of the original population (15 patients and 14 matched controls). The pancentromeric probe, purchased from Oncor, USA, was used according to Migliore et al. (1996).

Coded slides were scored for metaphase or MN analysis. For each metaphase, chromosome aberrations, PCD and satellite associations of acrocentric chromosomes (SA) were systematically recorded. In particular, the number of metaphases with at least one separated chromosome, the number of metaphases with at least one acrocentric chromosome with PCD, the total number of PCD chromosomes and the total number of PCD acrocentric chromosomes were noted. For MN analysis, bi-nucleated cells were scored and the criteria for MN acceptance listed by Fenech (1993) were followed. Results from FISH were evaluated according to Migliore et al. (1996).

Decoded data were statistically elaborated by using multifactor analysis of variance to assess differences in the frequency of cytogenetic parameters between AD patients and controls after checking for confounding factors. Age was used as a co-variate.

## Results

The average frequencies of MN scored in human lymphocytes of both the patient and the control groups are reported in Fig. 1. There was a significant difference in the spontaneous frequency of MN in bi-nucleated cells, that of patients being higher than that in controls (average: 18.01 ± 6.62 vs 8.82 ± 2.80). Application of the FISH technique with a pancentromeric probe to a subset of individuals yielded the results shown in Fig. 2. In the lymphocytes of patients, a higher percentage (average: 76.27 ± 3.21) of centromere-positive MN was found compared with those of controls (49.79 ± 7.86). The difference was statistically significant, indicating an increase of spontaneous aneuploidy in cells of affected individuals.

Data on MN induction in lymphocytes of patients and respective controls treated in vitro with the aneuploidogen GF are shown in Fig. 3. Since the basal frequency of MN was significantly higher in AD patients, the difference between the spontaneous and induced frequency (Δf) was calculated for both groups and evaluated statistically. Figure 4 shows the mean increase ± standard deviations for both groups. The difference was statistically significant ($P < 0.001$).

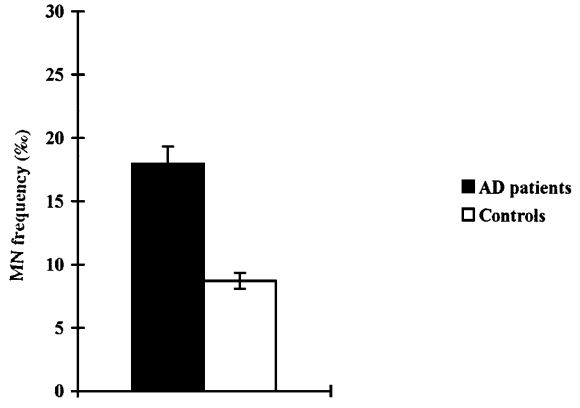


**Fig. 1** Mean MN frequency in AD patients ($n$ = 24) and in the control group ($n$ = 22)

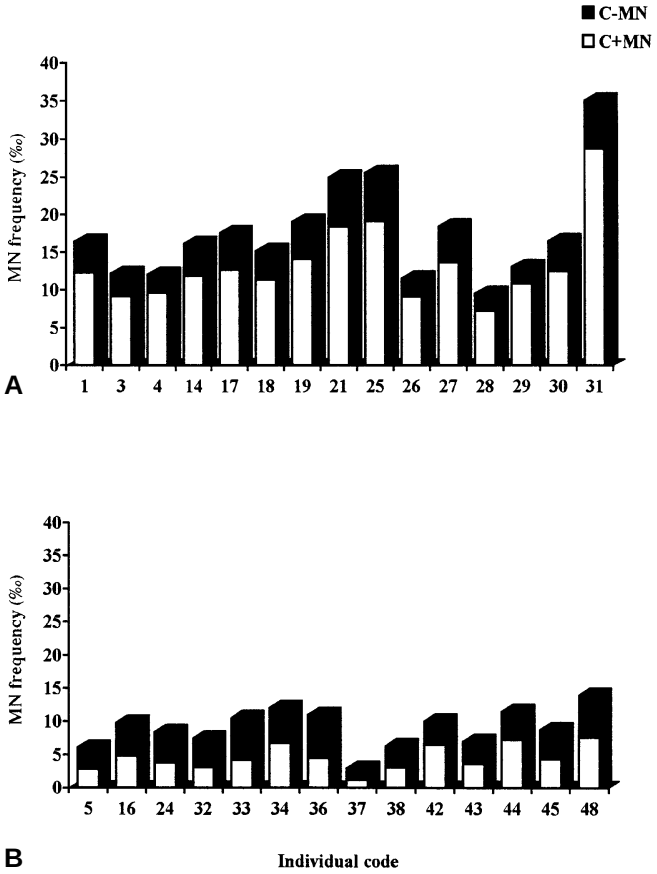


**Fig. 2A, B** Percentages of centromere-positive (C+MN) and centromere negative MN (C-MN) in **A** AD patients ($n$ = 15) and **B** controls ($n$ = 14)

The cytogenetic results shown in Table 2 refer to a subset of 12 patients and 11 controls. In AD patients, a higher frequency of metaphases with PCD was found compared with the control group. Acrocentrics (chromosomes belonging to both the D and G groups) were found to be preferentially involved. In contrast, the frequency of SA showed no statistically significant increase in the AD group (data not shown). None of the cytogenetic parameters considered were found to be correlated with age. When metaphases were analysed, chromosome aberrations were also recorded, although no significant difference in frequency between affected and unaffected individuals was observed (data not shown).

## Discussion

A higher frequency of MN was found in peripheral lymphocytes of patients affected by AD, compared with healthy matched controls. Since MN can contain fragments or whole chromosomes, the application of FISH with the alphoid satellite probe can help to distinguish

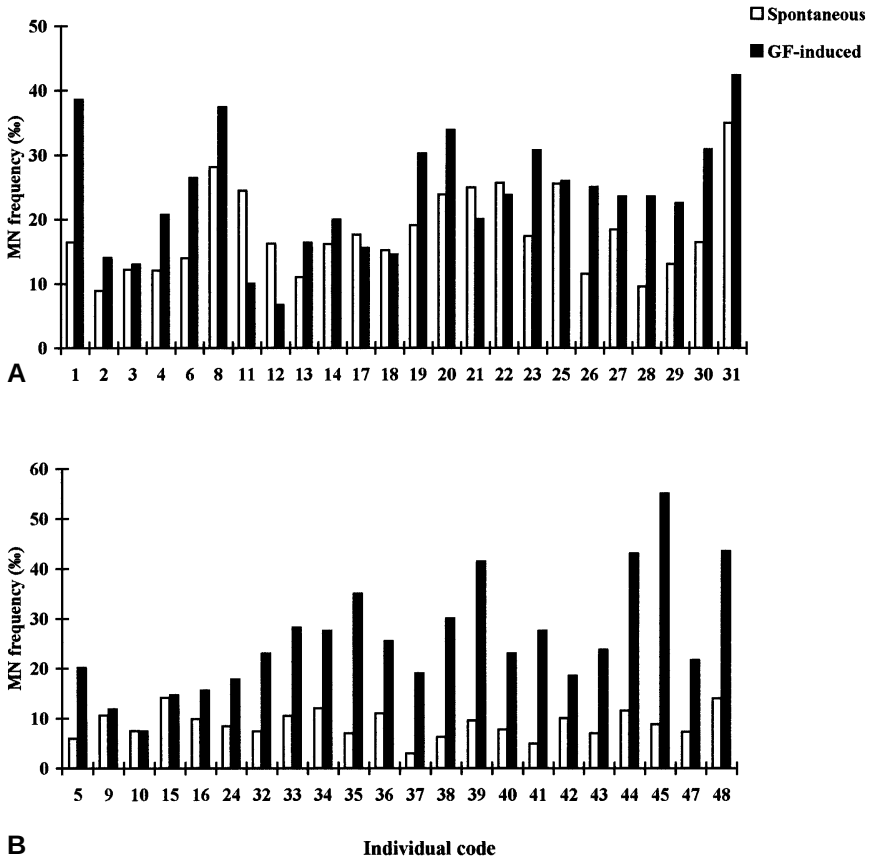


**Fig. 3A, B** MN frequencies induced by griseofulvin (GF, 15 μg/ml) in peripheral lymphocytes from **A** AD patients ($n$ = 24) and **B** normal individuals ($n$ = 22)

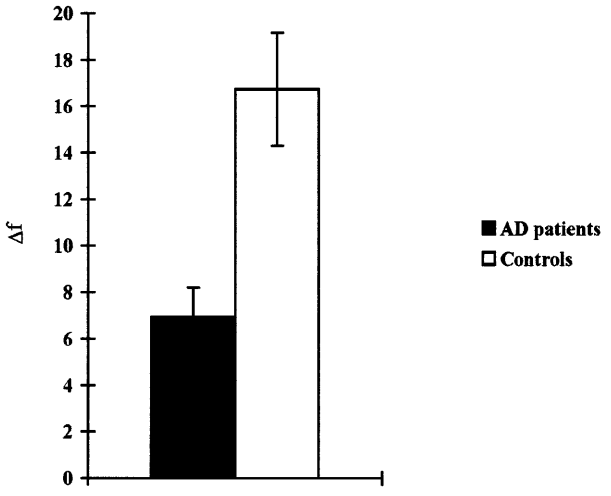


**Fig. 4** Mean increase of MN frequency in human lymphocytes of AD patients ($n$ = 24) and related controls ($n$ = 22) after GF treatment. $\Delta f$ represents the difference between the induced and spontaneous MN levels

between MN originating from chromosome (chromatid) breakage and MN of another origin. Our results show a higher percentage of centromere-positive MN, indicating more frequent involvement of aneuploidy (specifically chromosome loss) phenomena in the origin of spontaneous MN.

Previous findings from cytogenetic studies performed to assess chromosomal aberrations in cells of AD patients have been controversial (Ward et al. 1979; Nordenson et al. 1980; White et al. 1981; Kormann-Bortolotto et al. 1993). A clear trend of a positive correlation between aneuploidy and both forms of the disease (sporadic and familial) in female AD patients compared with unaffected subjects has been found by Moorhead and Heyman (1983). However, the differences were not statistically significant, probably because of the small number of cells analysed. In our experiments employing the human lymphocyte MN assay, which is easier to perform and less time-consuming than the conventional analysis in metaphase, we analysed a larger number of cells, totalling 2000 interphase cells for each individual. This increase in number of observations is likely to have influenced the power of the statistical analysis.

Chromosome loss (also evaluated as MN-centromere-positive) is known to increase in healthy people with increasing age; it is also known to be higher in the female sex (Nowinski et al. 1990; Migliore et al. 1991; Guttenbach et al. 1994; Stone and Sandberg 1995; Scarpato et al. 1996). In our sample, a positive correlation with age has not been found, possibly because the age range may have been too narrow to detect an influence of age. However, it should be noted that multivariate analysis has allowed us to consider our data apart from the age effect. Furthermore, the sex parameter shows no effect attributable to the patients being almost exclusively female. On the other hand, prevalence epidemiological studies have found higher rates of AD in women than in men; indeed, sex seems to

**Table 2** Analysis of premature centromere division (PCD)

| | Code | No. of PCD metaphases[a] | | No. of PCD chromosomes | | |
|---|---|---|---|---|---|---|
| | | All chromosomes | Acrocentrics[b] | All chromosomes | Acrocentrics[c] | |
| AD patients | | | | | | |
| | 1 | 10 | 1 | 23 | 1 | (D) |
| | 2 | 25 | 11 | 63 | 14 | (7G, 7D) |
| | 3 | 23 | 1 | 37 | 1 | (D) |
| | 4 | 26 | 1 | 51 | 1 | (D) |
| | 6 | 27 | 3 | 46 | 4 | (G) |
| | 11 | 25 | 5 | 37 | 5 | (4D, 1G) |
| | 12 | 14 | 0 | 22 | 0 | |
| | 13 | 29 | 5 | 47 | 5 | (4D, 1G) |
| | 14 | 29 | 2 | 50 | 2 | (1D, 1G) |
| | 17 | 27 | 4 | 60 | 5 | (4D, 1G) |
| | 18 | 22 | 0 | 24 | 0 | |
| | 19 | 20 | 0 | 29 | 0 | |
| Mean ± SD: | | 23.1 ± 5.9* | 2.7 ± 3.2** | 40.7 ± 14.3* | 3.2 ± 4.0** | |
| Controls | | | | | | |
| | 5 | 3 | 0 | 3 | 0 | |
| | 9 | 7 | 0 | 12 | 0 | |
| | 10 | 13 | 0 | 14 | 0 | |
| | 15 | 13 | 1 | 18 | 1 | (D) |
| | 16 | 18 | 0 | 22 | 0 | |
| | 36 | 9 | 0 | 12 | 0 | |
| | 42 | 19 | 0 | 22 | 0 | |
| | 43 | 12 | 1 | 20 | 1 | (G) |
| | 44 | 6 | 0 | 7 | 0 | |
| | 45 | 3 | 0 | 3 | 0 | |
| | 48 | 16 | 0 | 24 | 0 | |
| Mean ± SD: | | 10.8 ± 5.7 | 0.2 ± 0.4 | 14.3 ± 7.6 | 0.2 ± 0.4 | |

[a] 100 metaphases scored for each individual
[b] No. of metaphases with at least one PCD acrocentric
[c] The number of acrocentrics belonging to the two groups G and D is reported in brackets
*$P < 0.001$ vs controls
**$P < 0.05$ vs controls

play a role in the pathogenesis of AD and to influence the risk of developing the disease (Blass 1993; Payami et al. 1996).

The second cytogenetic endpoint assessed, viz. PCD, gave positive results. AD patients showed a higher percentage of PCD in their metaphases. Among prematurely separated chromosomes, acrocentric chromosomes, which were easily distinguishable by the conventional staining technique, were found to be preferentially involved in PCD phenomenon.

Metaphase chromosomes characterized by PCD have been observed in short-term cultures of elderly women and AD patients, among parents of trisomic children and in subfertiles families (Fitzgerald et al. 1975; Fitzgerald and McEwan 1977; Moorhead and Heyman 1983; Gabarron et al. 1986). Fitzgerald and McEwan (1977) have assumed that PCD is associated with a mechanism leading to X-chromosome aneuploidy in elderly women. Moreover, PCD has been considered to be age- and sex-related, analogously to aneuploidy. Some of the earlier studies on cultured leucocytes from AD patients have documented the presence of acentric extra chromosomes but other studies have not confirmed this finding (Ward et al. 1979; Nordenson et al. 1980). These long acentric fragments or chromosomes without centromeres have subsequently been considered to be a manifestation of PCD. Moorhead and Heyman (1983), in contrast to the lack of aneuploidy, have found an increase of PCD in AD female patients compared with controls, but not in cultures of affected males. PCD in AD female patients has therefore been interpreted as an epiphenomenon of ageing. The existence of a constitutional predisposition to abnormal centromere separation has been suggested by Fitzgerald and coworkers (1986), because PCD affecting chromosomes X and 21 was found in a young woman who had three conceptuses with trisomy 21.

Finally, we have found a statistically non-significant decrease of SA in metaphases of AD patients compared with unaffected controls (data not shown). A linkage is assumed between rDNA copy number, transcription, nucleolar formation and preferential association between specific chromosomes (McDowell et al. 1994). In contrast, a reduction in the amount and activity of ribosomal genes in chromosome 21 of AD patients has been suggested by Payão et al. (1994) who have found a significantly lower frequency of silver staining and SA for chromosome 21 in a group of AD patients than in controls.

We have also used AD lymphocytes as an experimental model to study the influence and behaviour of aneuploidy-inducing compounds. GF was chosen as a standard

compound, since it has previously been found to induce a high level of centromere-positive MN in human lymphocyte cultures (Migliore et al. 1996). In AD patient cultures, GF, whose dose was selected in accordance with previous results, induced significantly smaller increases in MN frequency than in controls. The probable target of GF would appear to be proteins linked to MTs or MTs themselves (Roobol et al. 1977; Wehland et al. 1977; Sloboda et al. 1982). A systemic defect in MT assembly in AD patients because of abnormal phosphorylation of the MT-associated protein tau has therefore been invoked (Grundke-Iqbal et al. 1986). Accordingly, our data can be explained in terms of the already abnormal target being only partially damaged by GF.

Evidence supporting the hypothesis of a defect in MT assembly comes from experiments utilizing agents known to disrupt them. Such experiments have reproduced, in rodent cells, certain abnormalities observed in cells from AD patients. When infused into the rat hippocampus, colchicine induces the same abnormalities as seen in the brain of AD patients (Matsuyama and Jarvik 1989). Treatment of experimental animals and cultured neurons with MT inhibitors (colchicine, vinblastine, maytanprine) results in the depolymerization of cytoplasmic MTs and the formation of neurofibrillary tangles. Following colchicine treatment, skin fibroblasts from AD patients show a delayed reappearance of the cellular MT network compared with controls. AD lymphoblasts also exibit a slower rate of MT re-polymerization following exposure to colcemid (Matsuyama and Jarvik 1989). Our findings concerning a predisposition to spontaneous aneuploidy in the lymphocytes of AD patients indicate MT impairment in this cell type. This abnormality could thus be expressed not only in neuronal cells, but also in other cells. In this regard, it is known that amyloid β-protein deposition is also present in non-neuronal tissues of AD patients (Joachim et al. 1989).

Since the majority of MN found in our study contain whole chromosomes, a high frequency of chromosome loss can be assumed. Thus, our data deal with an increase of spontaneous aneuploidy in lymphocytes of AD patients and support the hypothesis of microtubule instability underlying the disease. This feature could be more than an epiphenomenon, since a constitutive alteration in some factor also involved in chromosome segregation can be suggested (i.e. AD patients carrying the APOε4 allele do not bind hyperphosphorylated tau, thereby destabilizing MTs; Strittmatter et al. 1994). A model resembling that of Potter (1991), in which AD and most DS cases are held to result from unequal chromosome 21 segregation in somatic and germ cells, respectively, may now appear too simplistic. Recently, many genes have been found to be involved and numerous mutant alleles could thus contribute to the phenotype. However, one of these constitutional or induced genetic defects may be expressed through a defect in the regulation of MT organization, leading to altered sister chromatid segregation and consequently to aneuploidy.

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