Absence of Duplication of Chromosome 21 Genes in Familial and Sporadic Alzheimer's Disease

PETER H. ST GEORGE-HYSLOP, RUDOLPH E. TANZI, RONALD J. POLINSKY, RACHAEL L. NEVE, DANIEL POLLEN, DAVID DRACHMAN, JOHN GROWDON, L. ADRIENNE CUPPLES, LINDA NEE, RICHARD H. MYERS, DIANNE O'SULLIVAN, PAUL C. WATKINS, JEAN A. AMOS, CURTIS K. DEUTSCH, JAMES W. BODFISH, MARCEL KINSBOURNE, ROBERT G. FELDMAN, AMALIA BRUNI, LUIGI AMADUCCI, JEAN-FRANCOIS FONCIN, JAMES F. GUSELLA

The possibility that Alzheimer's disease (AD) is caused by overexpression or duplication of one or more genes on chromosome 21 has been raised by the observation of AD-like neuropathologic changes in individuals with Down syndrome and by the mapping of both the defect for familial AD and the amyloid β protein gene to this autosome. Possible duplication on chromosome 21 was investigated in both familial and sporadic AD by means of restriction fragment length polymorphisms for the amyloid and *SOD*I loci, as well as for DNA markers in the vicinity of the familial AD defect and in the critical Down syndrome region of chromosome 21. No evidence of increased DNA dosage was observed in either brain or leukocytes of patients with inherited or sporadic forms of AD. Duplication of these regions is therefore not a frequent event in either form of AD. Furthermore, no significant allelic association was detected between AD and any of the loci, including the amyloid and *SOD*I genes, providing no support for the hypothesis that defects in these specific genes are the primary cause of AD.

ALZHEIMER'S DISEASE (AD) IS A DEgenerative disorder of the human central nervous system involving progressive impairment of cognitive functions, leading to eventual death in mid- to late adult life (*1*). Although AD can result from an autosomal dominant inherited defect [familial AD (FAD)], it occurs most frequently as an apparently sporadic disorder that may represent an incompletely penetrant gene defect (*2*). In four pedigrees with FAD, the genetic defects resides on chromosome 21 near the DNA markers D21S1/D21S11 and D21S16 (*3*). The observation of an Alzheimer-like illness in patients with Down syndrome (DS) (trisomy 21) has led to the suggestion that duplication of individual genetic loci, or of larger segments of chromosome 21, may form the molecular basis of both types of AD (*3–5*). Reports showing duplication of the amyloid β peptide (AP) gene, the *ets2* proto-oncogene, and the superoxide dismutase gene (*SOD*I) in sporadic AD (*4, 5*) suggest that sporadic AD may result from an incompletely penetrant genetic defect (*2*) involving increased gene dosage.

To clarify these issues, we have examined gene dosage and allelic association in AD at a number of chromosome 21 loci including the AP gene. Gene dosage was examined in leukocytes of affected individuals from four pedigrees with pathologically proven FAD (*3*), as well as in peripheral leukocytes or brain tissue from 63 cases of sporadic AD. The diagnosis of "sporadic" AD was achieved by either postmortem pathological confirmation of an antemortem diagnosis (42 cases) or by antemortem diagnosis of AD using NIA and NINCDS criteria (*6*) in patients attending clinics at the Massachusetts Alzheimer's Disease Research Center (21 cases) (*7*). Gene dosage measurements were also conducted on 39 individuals without AD and on 29 individuals with karyotypically proven DS. Because of the high incidence of AD in the elderly, we accepted a diagnosis of "without AD" only if rigorous criteria were met. In the case of postmortem brain specimens, a diagnosis of "without AD" was accepted if the neuropathological examination and the available clinical records showed no evidence of AD and if the age at death was more than 60 years (69 ± 7.8 years, mean ± SD). In subjects who contributed blood specimens, a diagnosis of "without AD" was accepted if the age of the patient was more than 65 years (77 ± 8.8 years) and if he or she had been examined by a neurologist and found to be normal. The greatest source of such individuals was spouses of affected members of FAD pedigrees and members of families with other neurogenetic disorders being studied by this laboratory (*8*).

The approach used previously to detect increased dosage of the AP gene in AD relied on measurement of the density of single autoradiographic bands from the locus in AD and normal individuals and the use of a second non–chromosome 21 band as a standard for intersample comparison (*4*). We reasoned that the question of duplication on chromosome 21 could be more easily addressed by examining gene dosage in subjects who are heterozygous for a restriction fragment length polymorphism (RFLP) at the locus in question. In the presence of an extra copy of chromosome 21, the signal intensities will bear an abnormal ratio of approximately 2:1 compared to the 1:1 ratio present in normal DNA samples. For both DS and AD, this should confer greater sensitivity and reliability in the detection of extra chromosome 21 material than the 50% difference (3:2) previously employed (*4*).

Incomplete digestion of genomic DNA and variations in DNA transfer during Southern blotting can potentially lead to artifactual differences in signal intensities in gene dosage experiments (*9*). To ensure complete enzymatic digestion of the samples, we exhaustively purified the high molecular weight genomic DNA from both brain and leukocyte samples (Fig. 1) and carried out restriction enzyme reactions in large volumes with excess enzyme (10- to 30-fold). Unequal efficiency for transfer of DNA fragments with different molecular weights during blotting of agarose gels can also be a source of variation in signal, and considerable differences in degree of transfer are often evident from gel to gel. To control for this, each gel was loaded with samples from all test groups, providing internal comparisons in all cases.

Gene dosage measurements were performed with DNA from frontoparietal cerebral cortex (42 sporadic AD, 2 FAD, 20 without AD) or from buffy coat leukocytes or skin fibroblasts (21 sporadic AD, 13

P. H. St George-Hyslop, R. E. Tanzi, J. F. Gusella, Neurogenetics Laboratory, Massachusetts General Hospital, and Department of Genetics and Neuroscience Program, Harvard Medical School, 10 Fruit Street, Boston, MA 02114.
R. J. Polinsky and L. Nee, Clinical Neuropharmacology Section, Medical Neurology Branch, National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, MD 20892.
R. L. Neve, Division of Genetics and Mental Retardation Center, The Children's Hospital, and Department of Pediatrics, Harvard Medical School, Boston, MA 02115.
D. Pollen and D. Drachman, Department of Neurology, University of Massachusetts Medical School, and University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01605.
J. Growdon and D. O'Sullivan, Department of Neurology, Massachusetts General Hospital, and Harvard Medical School, 32 Fruit Street, Boston, MA 02114.
L. A. Cupples, School of Public Health, Boston University Medical School, 80 East Concord Street, Boston, MA 02118.
R. H. Myers and R. G. Feldman, Department of Neurology, Boston University Medical School, 80 East Concord Street, Boston, MA 02118.
P. C. Watkins, Integrated Genetics Inc., 31 New York Avenue, Framingham, MA 01701.
J. A. Amos, C. K. Deutsch, J. W. Bodfish, M. Kinsbourne, Departments of Behavioral Neurology, Genetics, and Molecular Neurogenetics, Eunice Kennedy Shriver Center, Waltham, MA 02154.
A. Bruni and L. Amaducci, Instituto di Mallatie Nervose e Mentali, University of Florence, Florence, Italy.
J.-F. Foncin, Laboratoire de Neurohistologie, Ecole Pratique des Hautes Etudes, Hopital de la Salpetriere, 47 Boulevard de l'Hopital, Paris, France.

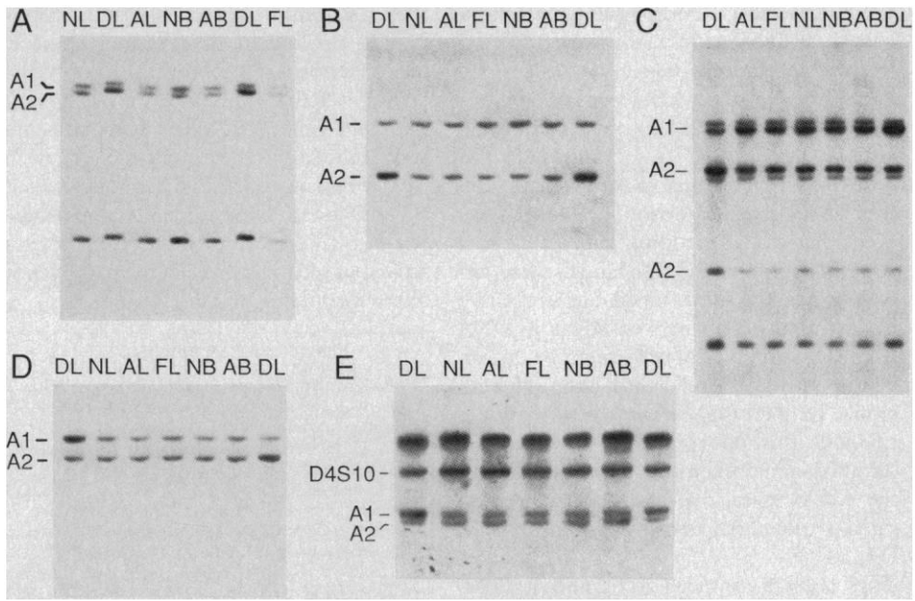

Fig. 1. Representative autoradiograms of the chromosome 21 loci in AD. Autoradiograms assessing dosage for (**A**) D21S16 (detected by probe E9) (*3, 11*); (**B**) D21S1 (probe pPW228C) (*3, 16*); (**C**) *SOD1* (probe SOD4A) (*17*); (**D**) D21S17 (probe H8) (*11*); and (**E**) the AP gene (probe FB63) (*10*). In the case of the AP gene, a fragment from the D4S10 locus on chromosome 4 (probe R7) (*12*) was hybridized to the filters simultaneously with the AP probe to allow direct comparison with a non–chromosome 21 locus. DNA samples were from leukocytes from subjects without AD (NL), brains from subjects without AD (NB), sporadic AD leukocytes (AL), sporadic AD brain (AB), FAD leukocytes (FL), and DS leukocytes (DL). Cell nuclei were obtained from frozen cerebral cortex (*18*) or from fresh buffy coat leukocytes by lysis of the cells in sucrose plus Triton X-100 solution with gentle homogenization in a Potter homogenizer, and DNA was prepared by sequential proteinase K digestion and multiple phenol-chloroform extractions (*19*). Restriction endonuclease (New England Biolabs, Beverly, Massachusetts) digestion was carried out with a 10- to 30-fold excess of restriction enzyme in the buffer recommended by the supplier. Five micrograms of DNA were digested to completion with the appropriate restriction endonuclease, and the resultant fragments were separated according to size and bound to a nylon membrane by Southern blotting (*3*). DNA probes were labeled and hybridized for 48 hours to the filters, which were then washed to high stringency and exposed to x-ray film (*3*).

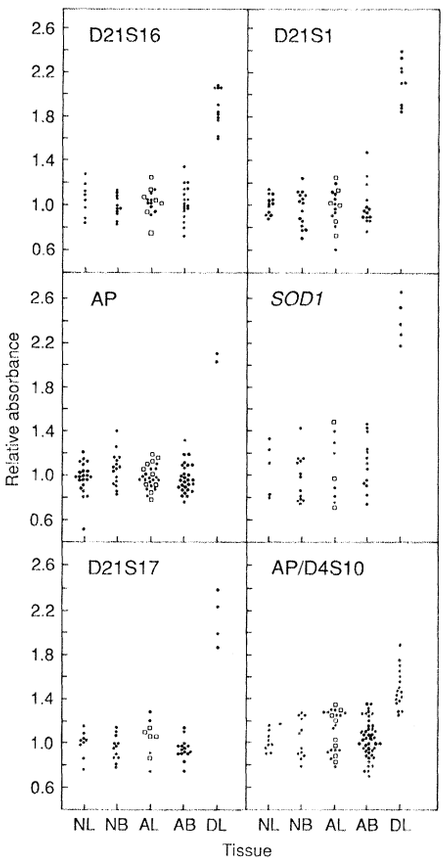

Fig. 2. Densitometric analysis of chromosome 21 loci in AD. A laser densitometer (LKB Ultroscan XL) was used to measure light absorbance of the band for each allelic fragment in sequential 50-μm by 3200-μm fields, tracking vertically through the center of each autoradiogram lane. The area under each signal peak was then calculated for each lane and used to assess the presence or absence of duplication. At each locus, dosage was measured in DNA from leukocytes from subjects without AD (NL), brains from subjects without AD (NB), AD leukocytes (AL), AD brain (AB), and DS leukocytes (DL). FAD cases are denoted by □. For each probe, the mean ratio of the absorbances measured for the two allelic fragments in the two normal groups was computed and set at 1 as a standard reference point for the purposes of this figure. Each dot represents the absorbance ratio for the two alleles in an individual sample, relative to this normal mean reference point. For the markers D21S1 and D21S17 each data point represents a single DNA sample from a different individual. For the remaining loci at which heterozygotes are infrequent, an accurate measure of variability was achieved by performing one or more independent trials with independent restriction digests and hybridizations of DNA samples from the following numbers of different individuals: D21S16—4NL, 5NB, 9AL (6AD, 3FAD), 7AB, 3DL; *SOD1*—4NL, 5NB, 9AL (6AD, 3FAD), 7AB, 3DL; AP gene—6NL, 6NB, 10AL (7AD, 3FAD), 8AB, 2DL. For comparison of the AP gene and the D4S10 locus, multiple independent trials were performed with the following samples: 9NL, 10NB, 23AL (15AD, 4FAD), 42AB, 17DS. Multiple exposures of the autoradiograms were obtained to ensure comparable absorbances for the allelic fragments relative to a standard set of control Southern blots with DNA samples loaded at varying DNA concentrations to estimate the approximate linear response range of the film. A critical internal control on each experimental filter was the observation of a 2:1 ratio for the allelic fragments in DS individuals.

FAD, 19 without AD, and 29 DS). We monitored duplication of the AP gene with an Eco RI RFLP detected by the AP complementary DNA (cDNA) clone FB63, containing the coding sequence of amyloid β peptide (*10*). Possible duplication in the vicinity of the FAD defect was examined with two DNA markers, D21S16 and D21S1, which have shown linkage to FAD (*3*). DNA dosage in the critical DS region of chromosome 21 was examined with an RFLP for *SOD1* and the anonymous DNA marker D21S17 (*11*), which is located near the *ets*-2 locus (*12*).

Contrary to previous reports (*4, 5*), we found no evidence of gene duplication in either sporadic AD or in FAD at any of the genetic loci or in any of the tissues examined (Figs. 1 and 2). All loci were heterozygous and therefore informative for a minimum of 13 independent sporadic cases (range of 13 to 28 cases) and at least three independent FAD cases. Visual inspection of the autoradiograms or densitometric analysis revealed no evidence for increased dosage in either form of AD. However, duplication was readily detected in DS at all loci, both visually (Fig. 1) and by densitometry (Fig. 2). Two-way analysis of variance with the raw ratios of signal intensities for the allelic fragments revealed no significant difference between leukocyte and brain DNA for any of the probes ($P > 0.05$). Multivariate analysis across diagnostic groups and tissue origin showed no significant difference between normal and AD ($P > 0.05$) but showed a highly significant difference between AD and DS ($P < 0.0001$) and between normal and DS ($P < 0.0001$) at all five chromosome 21 loci tested. We calculated that the statistical power of this analysis was sufficient to detect a difference between AD and normal of 20 to 40% with an 80% probability ($\alpha = 0.05$). These results suggest that duplications in the AP gene, the *SOD1* gene, the FAD region, and the DS region are not a common event in either

sporadic or FAD. Furthermore, no unique pattern of duplication was observed at these loci in four clinically demented DS patients (mean age ± SD, 61 ± 5.4 years), compared to four nondemented DS patients (59 ± 5.4 years).

It is conceivable that in AD, somatic cells may suffer nondisjunction or some other event that would randomly duplicate either copy of chromosome 21. Under these circumstances, the signal intensities of the two allelic fragments for a given RFLP in DNA from large numbers of cells might be expected to be approximately equal, although both would be increased relative to normal. To rule out this possibility, we assessed the summed signal intensities of both alleles at the AP locus relative to the hybridization signal of a control locus on chromosome 4, D4S10 (Fig. 1) (*13*). Again, the analysis (Figs. 1 and 2) revealed no significant difference between DNA from AD and normal individuals ($P > 0.05$) but readily yielded evidence of increased dosage in DS relative to AD ($P < 0.0001$) and normal ($P < 0.0001$) (*14*).

Since gene duplication is only one of several mechanisms by which putative inherited defects on chromosome 21 might cause sporadic AD, we also examined the possibility of allelic association between sporadic AD and RFLPs at each of the genetic loci discussed above. Allelic association, which often results from linkage disequilibrium, provides a potential strategy to determine the proximity of polymorphic DNA segments to the sites of disease-producing mutations. In the case of sporadic AD, the demonstration of a significant allelic association at one of the chromosome 21 loci, especially the AP gene, would provide a persuasive argument for both a genetic etiology in sporadic AD and a primary role for this region of the chromosome in the pathogenesis of AD. This approach has been used to identify a candidate gene for cystic fibrosis (*15*). However, when the frequency of individual alleles in the 63 AD patients, all of whom were Caucasian, was compared to the frequency of the same alleles in the normal Caucasian population, no significant difference was observed for any of the chromosome 21 loci ($P > 0.10$ in all cases). The lack of an allelic association for RFLPs at the AP and *SOD*I loci with AD does not support the view that either of these genes plays a role in predisposition to the disorder. In the case of the anonymous markers linked to FAD, the lack of allelic association with AD is not surprising, because these markers are not located precisely at the site of the FAD mutation and are not in apparent linkage disequilibrium with FAD (*3*).

The absence of either gene duplication or allelic association, together with the deposition of amyloid in normal aged brain and the observation that FAD is not tightly linked to RFLPs in either the AP gene or the *SOD1* gene (*2, 10*), indicates that neither gene is the site of the primary defect causing AD. It remains unclear if the appearance of amyloid in AD brain is merely a by-product of neuronal death or if this protein plays an active but secondary role in the pathogenesis of the disorder.

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The Amyloid β Protein Gene Is Not Duplicated in Brains from Patients with Alzheimer's Disease

RUDOLPH E. TANZI, EDWARD D. BIRD, SAMUEL A. LATT, RACHAEL L. NEVE*

Complementary DNAs (cDNAs) encoding portions of the amyloid β protein were used to investigate possible amyloid gene duplication in sporadic Alzheimer's disease. A strategy employing two Eco RI restriction fragment length polymorphisms (RFLPs) detected by the amyloid cDNAs was used. RFLPs allow the detection of a 2:1 gene dosage in the DNA of any individual who is heterozygous for a particular RFLP. The amyloid gene regions homologous to the cDNAs used were not duplicated in the DNA from brains of individuals with sporadic Alzheimer's disease. Similar results were also obtained with a strategy employing a test for 3:2 gene dosage.

THE AMYLOID β PROTEIN (AP) IS A 4.2-kD peptide present in amyloid plaques, neurofibrillary tangles, and cerebrovascular deposits in the brains of individuals affected with Alzheimer's disease (AD) (*1, 2*) and in older patients with Down syndrome (DS) (trisomy 21) (*2*). Complementary DNAs (cDNAs) encoding AP have been isolated and mapped to the chromosomal region 21q11.2–21q21 (*3,*

R. E. Tanzi and R. L. Neve, Division of Genetics and Mental Retardation Center, The Children's Hospital, Department of Pediatrics, and Program in Neuroscience, Harvard Medical School, Boston, MA 02115.
E. D. Bird, Department of Neuropathology, Harvard Medical School, Boston, MA 02115, and Mailman Research Center, McLean Hospital, Belmont, MA 02178.
S. A. Latt, Division of Genetics and Mental Retardation Center, The Children's Hospital, Department of Pediatrics, and Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115.

*To whom correspondence should be addressed.