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Family Study of Platelet Membrane Fluidity in Alzheimer's Disease

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The fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene in labeled platelet membranes, an index of membrane fluidity, identifies a prominent subgroup of patients with Alzheimer's disease who manifest distinct clinical features. In a family study, the prevalence of this platelet membrane abnormality was 3.2 to 11.5 times higher in asymptomatic, first-degree relatives of probands with Alzheimer's disease than in neurologically healthy control subjects chosen without regard to family history of dementia. The pattern of the platelet membrane abnormality within families was consistent with that of a fully penetrant autosomal dominant trait. Thus, this abnormality of platelet membranes may be an inherited factor that is related to the development of Alzheimer's disease.

MOUNTING EVIDENCE SUGGESTS that Alzheimer's disease is associated with pathologic changes in cells outside the central nervous system (*1, 2*). Several of the abnormalities described in nonneural cells reflect an alteration in cell membrane structure or function. Among these is an increase in platelet membrane fluidity, as revealed by a reduction in the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) in labeled membranes. This method provides a measurement of the degree to which the rotation of DPH molecules in labeled membranes is hindered and, therefore, provides an index that is inversely related to membrane fluidity (*3*). An increase in platelet membrane fluidity associated with Alzheimer's disease has been observed by us in blind studies of patients from Boston (*4, 5*) and Pittsburgh (*6, 7*), and has been replicated by others in London (*8*). Abnormalities of cell membrane composition and structure have also been found in brain tissue obtained at autopsy from patients who died with confirmed Alzheimer's disease. These abnormalities include changes in brain phospholipid metabolism as revealed by nuclear magnetic resonance spectroscopy (*9*), disordering of cortical myelin as indicated by x-ray diffraction (*10*), and an alteration in the molecular dynamics of hippocampal membranes as reflected by fluorescence spectroscopy (*11*).

Initial assessments of the specificity of this platelet membrane alteration in other neuropsychiatric disorders have been promising. The increase in platelet membrane fluidity associated with Alzheimer's disease was not found in platelets from patients with depression, a common cause of reversible dementia in the elderly (*7*), in patients with mania, which may also be accompanied by a secondary dementia (*12*), or in patients with multi-infarct dementia (*8*).

A cutoff point for DPH anisotropy of 0.1920 at 37°C (90th percentile for healthy elderly controls) (Fig. 1) segregates patients with Alzheimer's disease into two clinical subtypes. As a group, patients with increased platelet membrane fluidity (DPH anisotropy less than 0.1920) suffer from an earlier symptomatic onset and have a more rapidly progressive course. A family history of dementia also appears to be a more common feature of patients in this group. (*7*).

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At the cellular level, several lines of evidence suggest that the increase in platelet membrane fluidity associated with Alzheimer's disease results from an accumulation of internal membranes rather than a generalized abnormality of cell membranes (*13*). Ultrastructural studies have revealed an excess of atypical cells containing an overabundant system of trabeculated cisternae bounded by smooth membrane in platelet preparations from patients with Alzheimer's disease. Menashi *et al.* have reported that internal membranes exhibit higher membrane fluidity than external platelet membranes, as reflected by DPH anisotropy (*14*). Therefore, a relative increase in internal membranes may account for the increase in platelet membrane fluidity associated with Alzheimer's disease. In support of this hypothesis, Cohen *et al.* found that platelets that exhibit increased membrane fluidity also manifest a reduction in the cholesterol: phospholipid ratio that could be accounted for by an approximate doubling of the usual mass of internal membranes per cell (*15*). In addition, when intact platelets from patients with Alzheimer's disease are labeled with DPH, a process that preferentially labels external membrane, they fail to exhibit an alteration in fluorescence anisotropy (*13*). Moreover, erythrocyte ghosts, which lack internal membranes, also fail to exhibit an alteration in membrane fluidity as reflected by fluorescence (*13*) or electron spin resonance spectroscopy (*16*). This evidence suggests that the increase in platelet membrane fluidity associated with Alzheimer's disease may result from a dysregulation of platelet membrane biogenesis or turnover.

Our family study was conducted to determine whether increased platelet membrane fluidity aggregates in the families of patients with Alzheimer's disease and, if so, whether this platelet abnormality selectively runs in the families of probands who themselves exhibit the abnormality. A series of 23 patients with probable Alzheimer's disease who had at least one available first-degree relative living in the Pittsburgh area were chosen as probands from the large cohort of patients described in Fig. 1. The diagnosis of probable Alzheimer's disease was made according to currently accepted clinical criteria as applied conjointly by Board-certified neurologists and psychiatrists (*17*). An additional 15 patients with Alzheimer's disease that had been confirmed by autopsy, from whom platelet membranes could not be obtained but who had at least one available first-degree relative, also served as probands. Neuropathologic diagnoses were made by Board-certified neuropathologists according to current consensus criteria (*18*). The participation of all neurologically healthy first-

degree relatives of the 38 probands was solicited, and an age- and sex-matched cohort of neurologically healthy controls, chosen without regard to family history of dementia, was recruited through local blood drives, media promotions, and presentations at community meetings. All asymptomatic relatives and controls had Mini-Mental State (MMS) scores (on scale of 0, worst, to 30, best) that were greater than or equal to 26 (*19*) and Dementia Rating Scale (DRS) scores (on scale of 0, best, to 28, worst) less than or equal to 4 (*20*). Disorders that affect blood cell membrane lipid composition or serum lipid profiles, restrictive diets, and exposure to medications that affect platelet membrane fluidity in vitro or in vivo served as additional exclusion criteria (*7, 12, 21*).

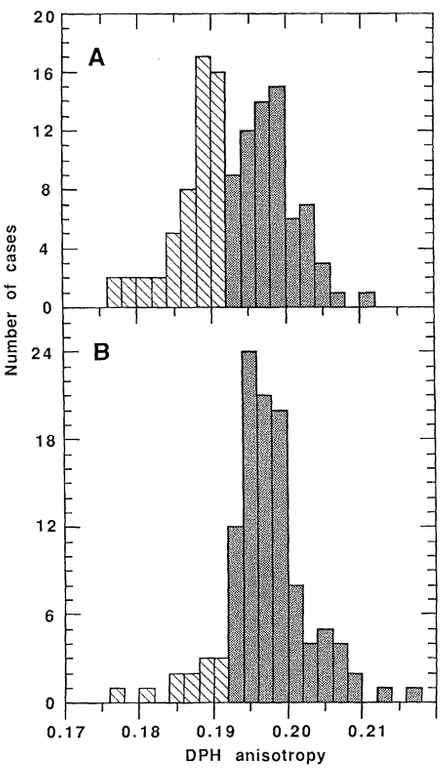

Fig. 1. Distribution of fluorescence anisotropy for DPH-labeled platelet membranes from 123 (41 males, 82 females) demented patients with probable Alzheimer's disease (**A**) and 114 (41 males, 73 females) neurologically healthy controls (**B**). Subjects with normal membrane fluidity (DPH anisotropy ≥0.1920) are shown by shaded bars and subjects with increased platelet membrane fluidity (DPH anisotropy <0.1920) are shown by diagonal line bars. One patient with a DPH anisotropy value of 0.1646 is not shown. The mean age (±SD) of the patient group was 69.8 ± 8.3 years, and the patients were moderately demented as reflected by mean MMS and DRS scores (±SD) of 16.9 ± 6.2 and 7.6 ± 5.3, respectively. The mean age (±SD) of symptomatic onset was 67.1 ± 8.2 years, and mean duration of illness (±SD) was 3.9 ± 2.3 years. The mean age of the control group (±SD) was 64.3 ± 11.0 years, and the mean MMS and DRS scores (±SD) for this group were 29.0 ± 2.3 and 1.3 ± 2.5, respectively. The mean DPH value and associated variance (SD) for the group of subjects with Alzheimer's disease, 0.1933 ± 0.0070, were significantly different than the respective values for the control group, 0.1972 ± 0.0057 (comparison of means, $P = 1.3 \times 10^{-5}$, two-tailed Mann-Whitney-Wilcoxon test; comparison of variances, $P < 5 \times 10^{-2}$, two-tailed F test). Moreover, the shapes of the distributions differed significantly ($P = 2.0 \times 10^{-6}$, Kolmogorov-Smirnov statistic). The cutoff point for normal fluidity, 0.1920, was based on the lowest 90th percentile of anisotropy values in the control group. By using this cutoff, 45% (55 of 123) of the patients with Alzheimer's disease exhibited increased platelet membrane fluidity (compared to controls, $P < 1 \times 10^{-4}$, χ^2 or Fisher's exact test). One demented patient with normal platelet membrane fluidity and one with increased platelet membrane fluidity have since died, and the clinical diagnoses of Alzheimer's disease have been confirmed at autopsy in both cases. The size of the alteration in platelet membrane fluidity associated with Alzheimer's disease is at least 1 SD from the mean for controls and is considerable from a physiologic perspective (*5, 11, 12, 21*).

Fasting blood samples were collected and platelet isolation was performed at room temperature according to a minor modification of the method of Corash and co-workers (*22*), as described (*13*). Laboratory analyses (*6, 7, 13*) were carried out by staff who were blind to demographic and clinical histories of the subjects. Platelet yields were greater than 90% in all cases, and platelet preparations contained less than 0.5% red cells or leukocytes. After hypotonic lysis and homogenization, platelet membranes were isolated by centrifugation at 4°C. Labeling of membranes was carried out in the dark in the presence of 1 μ*M* DPH for 60 minutes at 37°C. Labeled membranes contained approximately 1 probe molecule to 100 phospholipid molecules. Fluorescence measurements were made at 37.0°C (range, ±0.1°C) on an SLM 4800 spectrofluorometer equipped as described (*7, 13*). Autofluorescence and excitation light scattering were negligible. Steady-state anisotropy measurements of DPH-labeled membranes provided a reliable and valid index of membrane fluidity over the range of values reported (*3*).

Data for the high-risk cohort of asymptomatic, first-degree relatives of demented probands along with the neurologically healthy controls are presented in Table 1. Because most of the relatives were offspring of the probands rather than sibs, the mean ages of the relatives were younger than those of the probands. However, since platelet membrane fluidity was not significantly correlated with subject age between 35 and 90 years in previous studies (*6, 7*) or in any of the subject groups described in this study, the same cutoff for increased platelet membrane fluidity was used. Both groups of

Table 1. Platelet membrane fluidity in asymptomatic first-degree relatives of probands with autopsy-proved or probable Alzheimer's disease. The probands with probable Alzheimer's disease consisted of 1 male and 22 females with a mean age (±SD) of 73.3 ± 6.2 years. Their mean age (±SD) of symptomatic onset was 69.1 ± 7.6 years with a mean duration of illness (±SD) of 4.4 ± 2.3 years. The group was moderately to severely demented as reflected by a mean MMS score (±SD) of 12.7 ± 4.6 and a DRS score (±SD) of 10.9 ± 5.8. Because gender does not affect platelet membrane fluidity for subjects over the age of 35 years, the excess of females in the proband group was not expected to affect the results. The mean DPH anisotropy value and SD for this group, 0.1925 ± 0.0081, were similar to the respective values for the larger demented cohort from which they were recruited, 0.1933 ± 0.0070. Forty-eight percent of the proband group exhibited increased platelet membrane fluidity (DPH anisotropy < 0.1920), similar to the respective proportion of the larger demented cohort, 45%. Means for subject age and DPH anisotropy for labeled platelet membranes are presented with standard deviations in parentheses. AD, Alzheimer's disease.

Group	*n*	Sibs/ off-spring	Male/ female	Age (years)	DPH anisotropy	DPH anisotropy <0.1920	
						Total	Percent
Probands							
Autopsy-proved AD	15	3/29	8/24	49.1 (9.5)	0.1952 (0.0068)	10/32	31.3
Probable AD	23	10/33	19/24	50.0 (12.0)	0.1948 (0.0075)	15/43	34.9
Total	38	13/62	27/48	49.6 (10.9)	0.1950 (0.0071)*	25/75†	33.3
Controls	34		13/21	51.0 (7.5)	0.1976 (0.0037)*	1/34†	2.9

*Total relatives compared to controls: for comparison of means, $P = 1.7 \times 10^{-2}$, one-tailed Mann-Whitney-Wilcoxon test; for comparison of variances, $P < 5.0 \times 10^{-3}$, one-tailed F test. †Total relatives compared to controls: $P = 2.0 \times 10^{-3}$, one-tailed Fisher's exact test.

Table 2. Relationship of platelet membrane fluidity in probands with probable Alzheimer's disease to platelet membrane fluidity in asymptomatic first-degree relatives. Relatives with increased platelet membrane fluidity (DPH anisotropy <0.1920) were significantly more likely to be related to probands with increased platelet membrane fluidity than probands with normal platelet membrane fluidity (DPH anisotropy ≥0.1920); $P = 0.0015$, one-tailed Fisher's exact test. M, male; F, female.

Probands	First-degree relatives	
	Increased fluidity	Normal fluidity
Increased fluidity	13 (8M/5F)	10 (4M/6F)
Normal fluidity	2 (1M/1F)	18 (6M/12F)

relatives as well as the control group were well matched for age and gender. The platelet membrane fluidity data for the two groups of relatives were similar. The combined group of first-degree relatives exhibited an increase in platelet membrane fluidity as reflected in a significant reduction in mean DPH anisotropy value as well as in the increased proportion of relatives who exhibited increased platelet membrane fluidity (DPH anisotropy less than 0.1920). The prevalence of increased platelet membrane fluidity in the combined sample of asymptomatic first-degree relatives was 11.5 times higher than the respective value for the age- and sex-matched controls chosen without regard to family history and 3.2 times as high as the respective value for the older control population (Fig. 1). This range reflected the estimates of the baseline rate of the platelet abnormality in the two control populations (2.9% or 1 of 34, 10.5% or 12 of 114), which did not significantly differ from one another. Furthermore, the variance in the anisotropy data from the combined group of relatives was significantly increased relative to the control value but was similar to the respective variances for the demented probands and the larger cohort of demented patients from which they were chosen (Fig. 1). In summary, the increase in platelet membrane fluidity associated with Alzheimer's disease is a familial trait that aggregates in neurologically healthy, first-degree relatives of probands with Alzheimer's disease.

The implications of these results are manifold. Population and family studies conducted in the United States (*23*) and abroad (*24*) have uniformly found that first-degree relatives of patients with Alzheimer's disease are at a significantly higher lifetime risk of developing dementia, especially if the affected family member is a parent and the age of onset occurs before age 70. The observed 3.2- to 11.5-fold increase in the prevalence of the platelet membrane abnormality among first-degree relatives (primarily offspring) of probands with Alzheimer's disease strongly suggests that this cellular change is related to, but antedates, the onset of symptoms of dementia. Furthermore, this finding excludes nonspecific concomitants of chronic illness or medication history as the cause of the increased platelet membrane fluidity observed in patients with Alzheimer's disease.

More than 50 pedigrees have been reported in which Alzheimer's disease appears to be segregating as an autosomal dominant disorder (*25*). Moreover, population and family studies have suggested that a significant fraction, even a majority, of the cases of Alzheimer's disease may result from a familial disorder that places first-degree relatives at a lifetime risk approaching 50% (*23, 26*). On the basis of these reports, we have analyzed our family data for evidence that the increased platelet membrane fluidity associated with Alzheimer's disease might potentially provide a useful genetic marker for familial Alzheimer's disease. The observed 3.2- to 11.5-fold increase in the frequency of cases with increased platelet membrane fluidity among the first-degree relatives of probands with Alzheimer's disease is similar to the reported 3.6- to 6.9-fold increase in the 85- to 90-year lifetime risk of developing Alzheimer's disease in this high-risk group (*23, 24, 26*). Moreover, the mean of the 2.9 to 10.5% range of cases with increased platelet membrane fluidity in the control group is approximately half of the respective value for the 8 to 18% estimates of the 85- to 90-year lifetime risk of Alzheimer's disease in the general population (*23, 26*). This finding is consistent with the approximate 50% prevalence of the platelet membrane abnormality in our patients with Alzheimer's disease.

As predicted by an autosomal dominant mode of inheritance, relatives with increased platelet membrane fluidity were almost exclusively related to probands who exhibited this phenotype (Table 2). Moreover, the proportion of the individuals exhibiting increased platelet membrane fluidity among the first-degree relatives of probands of the same phenotype, 57% (13 of 23), did not significantly differ from the 50% expected for a rare but fully penetrant autosomal dominant trait. On the assumption that Alzheimer's disease is produced by the inheritance of an autosomal dominant gene, Larrson *et al.* have estimated that such a gene would be present in the population at a frequency of approximately 0.06 (*24*). If a putative dominant gene for increased platelet membrane fluidity existed in our population at a similar frequency, 55% of first-degree relatives of probands with increased platelet membrane fluidity would be expected to manifest this phenotype (*23*), a value even closer to the 57% value observed. Males and females in this group of relatives were affected at approximately equal frequencies, consistent with this pattern of inheritance. Furthermore, in several large families in which the proband with Alzheimer's disease exhibited the abnormal platelet membrane phenotype, we have observed a similar aggregation pattern of increased platelet membrane fluidity (*27*). Finally, we have examined platelet membranes from a pair of monozygous twins with Alzheimer's disease, and the resulting DPH anisotropy values, 0.1866 and 0.1870, were identical within experimental limits (*27*). In summary, our results suggest that increased platelet membrane fluidity is a genetic marker for familial Alzheimer's disease and that the platelet membrane abnormality is inherited as an autosomal dominant trait. However, further assessments of specificity and sensitivity are necessary to adequately evaluate the role of increased platelet membrane fluidity as an aid in the diagnosis of Alzheimer's disease, and a prospective study of a high-risk cohort seems warranted to assess the risk of Alzheimer's disease associated with this biological marker.

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physiologic temperatures. For steady-state anisotropy measurements, the spectrofluorometer was configured in the T-format, labeled suspensions were excited at 360 nm, and emission light was measured through Schott long-pass filters. Observation of a narrow emission band was unnecessary since the fluorescence anisotropy of DPH is constant across the emission spectrum. Steady-state fluorescence anisotropy (r_s) was calculated from observed fluorescence intensities (I) by the following equation:

$$r_s = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \text{ where } G = \frac{I_{hv}}{I_{hh}}$$

First and second subscripts refer to the vertical (v) or horizontal (h) positions of the excitation and emission polarizers, respectively. This formula corrects for any asymmetry in the detection of vertically and horizontally polarized light. The usefulness of the steady-state anisotropy of DPH in labeled membranes as an inferential index of membrane "fluidity" or "order" has been documented widely in the literature.

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A Cytoplasmic Protein Stimulates Normal N-*ras* p21 GTPase, but Does Not Affect Oncogenic Mutants

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The role of guanine nucleotides in *ras* p21 function was determined by using the ability of p21 protein to induce maturation of *Xenopus* oocytes as a quantitative assay for biological activity. Two oncogenic mutant human N-*ras* p21 proteins, Asp12 and Val12, actively induced maturation, whereas normal Gly12 p21 was relatively inactive in this assay. Both mutant proteins were found to be associated with guanosine triphosphate (GTP) in vivo. In contrast, Gly12 p21 was predominantly guanosine diphosphate (GDP)–bound because of a dramatic stimulation of Gly12 p21-associated guanosine triphosphatase (GTPase) activity. A cytoplasmic protein was shown to be responsible for this increase in activity. This protein stimulated GTP hydrolysis by purified Gly12 p21 more than 200-fold in vitro, but had no effect on Asp12 or Val12 mutants. A similar factor could be detected in extracts from mammalian cells. It thus appears that, in *Xenopus* oocytes, this protein maintains normal p21 in a biologically inactive, GDP-bound state through its effect on GTPase activity. Furthermore, it appears that the major effect of position 12 mutations is to prevent this protein from stimulating p21 GTPase activity, thereby allowing these mutants to remain in the active GTP-bound state.

Ras P21 PROTEINS BIND GUANINE nucleotides and convert bound guanosine triphosphate (GTP) to guanosine diphosphate (GDP) by an intrinsic guanosine triphosphatase (GTPase) activity (*1, 2*). In these respects, *ras* proteins resemble signal-transducing G proteins, as well as initiation and elongation factors. All of these proteins cycle between active, GTP-bound forms, and inactive, GDP-bound forms (*3*). The observation that many oncogenic mutants of p21 are reduced in GTPase activity led to the proposal that transformation by p21 is the result of abnormal levels of p21 in an active, GTP-bound state (*2*). However, analysis of GTPase activities associated with a large number of p21 mutants has failed to reveal a quantitative relationship between GTPase activity and transforming potential (*4, 5*). It therefore appears that biochemical properties of p21 proteins measured in vitro do not adequately account for their different biological properties. This could be because in vitro measurements do not accurately reflect conditions in vivo, so that estimates of GTPase activities are misleading, or because oncogenic mutations have additional effects that have not yet been identified. To resolve these issues, and to develop a quantitative model for the effects of oncogenic mutations, we examined the roles of guanine nucleotide binding and hydrolysis on p21 function in living cells. *Xenopus* oocytes were used because of the relative ease with which these large cells can be manipulated and because it has been shown previously that human p21 proteins are able to induce oocyte maturation, thus providing a convenient quantitative assay for biological activity (*6*).

As shown in Fig. 1a, Asp12 and Val12 N-*ras* p21 proteins efficiently induced maturation of stage VI *Xenopus* oocytes, as determined by the fraction of oocytes that undergo germinal vesicle breakdown (GVBD) after injection of p21 protein. Normal Gly12 p21 was much less potent, requiring at least 150 ng of pure protein per oocyte to induce maturation. These effects are similar to those of Gly12 and Val12 H-*ras* p21 (*6*).

The effects of guanine nucleotides on the ability of p21 proteins to induce oocyte maturation were examined by binding either GDP or Gpp(NH)p (a nonhydrolyzable GTP analog) to p21 proteins prior to injection into oocytes. GDP had no effect on the ability of normal Gly12 p21 to induce maturation. However, binding of Gpp(NH)p greatly increased its biological activity (Fig. 1b). Under these conditions, Gly12 p21 was as active as either the Asp12 or the Val12 mutant (*7*). These results indicate that the function of normal Gly12 p21 is controlled by association with GDP or GTP. They also suggest that the different biological potencies of normal and mutant p21 proteins may reflect their differing association with GDP and GTP in vivo.

To determine whether GTP or GDP became associated with injected p21 proteins in vivo, [^{32}P]phosphate was co-injected so that cellular nucleotide pools would rapidly become radiolabeled (*8*), and p21 proteins were recovered from oocyte extracts by immunoprecipitation with monoclonal antibody to p21 (anti-p21) Y13-259 (*9*). Immune complexes were treated with EDTA and SDS and analyzed for radiolabeled nucleotide by thin-layer chromatography. Nor-

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