Evidence linking familial thrombosis with a defective antithrombin III gene in two British kindreds

S H SACKS*, J M OLD*, S T REEDERS*, D J WEATHERALL*, A S DOUGLAS†, J H WINTER‡, AND C R RIZZA§

From *the Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU; †University Department of Medicine and Therapeutics, Aberdeen Royal Infirmary, Foresterhill, Aberdeen AB9 2ZB; ‡Royal Infirmary, Glasgow G31 2ES; and §Haemophilia Centre, Churchill Hospital, Oxford.

SUMMARY Using DNA probes in a structural study of the antithrombin III gene locus we found no evidence of gene deletion in two British kindreds with inherited antithrombin III deficiency. However, linkage analysis between a common DNA polymorphism and the antithrombin III deficiency trait showed that the defect lies at or close to the antithrombin III structural gene. The lod score for linkage within the larger Scottish kindred was 3.1 ($\theta=0$). These results are consistent with previously published data^{1,2} suggesting that mutation of the antithrombin III structural gene is the cause of inherited antithrombin III deficiency in some families.

Antithrombin III (AT III) is the main physiological inhibitor of thrombin, factor Xa, and other serine proteases generated during coagulation.³ Familial deficiency of AT III results in marked predisposition to venous thromboembolic disease in adult life. The deficiency is inherited as an autosomal dominant trait and occurs with an estimated incidence of 1 in 2000 of the general population.⁴ Of those affected, 40 to 70% become symptomatic with recurrent and sometimes fatal peripheral venous thrombosis and pulmonary embolism at some time during their lives.⁴ Since the disorder was first described in 1965,⁵ over 100 families have been described.⁶ In most patients with this disorder about half the normal levels of AT III protein are present, although on rare occasions deficiency of AT III anticoagulant activity is associated with normal levels of a structurally abnormal molecule.⁷⁻⁹ AT III deficiency trait has now been mapped to the long arm of chromosome 1.^{10,11}

In recent years, DNA probes for the AT III structural gene have been isolated.¹²⁻¹⁴ With these it has been possible to show genetic heterogeneity of inherited AT III deficiency. In one family, deficiency of AT III was attributed to deletion of the AT III structural gene,¹ whereas in two other families the disorder was not associated with deletion of the AT III gene.^{1,2} We are investigating the genetic defect in two British kindreds with AT III deficiency. Here

we report the results of structural and segregation analyses at the AT III gene locus to suggest that AT III deficiency in these kindreds is the result of a mutant AT III gene.

Materials and methods

We studied 29 members of a large Scottish kindred^{15,16} (kindred a), and five members of an Oxford kindred (kindred b) which has not previously been reported. Functional and antigenic levels of plasma AT III in members of the Scottish family were measured as previously described.^{15,16} In the Oxford family, AT III functional activity was determined by a modified progressive antithrombin assay,¹⁷ and AT III antigen levels were measured by electroimmunoassay.¹⁸ Two dimensional immunoelectrophoresis of plasma AT III was carried out by the method of Sas *et al.*¹⁹

DNA was extracted from peripheral blood cells by standard procedures²⁰ and 10 µg aliquots of DNA were digested completely with restriction enzymes. The resulting DNA fragments were separated by electrophoresis in 0.8% agarose gels, transferred to nitrocellulose by the blotting procedure of Southern,²¹ and then hybridised to nick translated ³²P labelled AT III DNA probes, as previously described.²⁰

Segregation analysis used two types of polymorphic variation found at the AT III gene locus (1) a *Pst*I restriction site polymorphism within the gene¹³ revealed by hybridisation of a partial length

TABLE 1 Functional and antigenic levels of plasma antithrombin III in members of kindred b (Oxford family). The normal range measured in a group of healthy controls was 80 to 140% of the average value in functional assays, and 80 to 120% of the average value in immunological assays.

	AT III functional assay	AT III immunological assay
	% average normal	
I.1	50	58
I.2	90	100
I.3	36	46
II.1	94	74
II.2	46	36
II.3	64	28

cDNA probe, pAT III-3 (a gift from S Orkin, Boston); and (2) a variable length polymorphism with two alleles located 345 bp upstream from the 5' end of the gene,²² revealed by *Ava*II and the plasmid pAT 1-2 containing the 5' flanking 1.2 kb *Pst*I genomic fragment (a gift from S Orkin). Maximum likelihood analysis of linkage between polymorphisms and the AT III deficiency trait was carried out using the computer programme MLINK.²³

Results

The criteria we used to assign AT III deficiency to members of kindreds a and b were: (1) symptomatic thrombosis with reduced AT III activity and antigen; (2) reduced AT III activity and antigen in relatives without symptoms of thrombosis; and (3) a history of thrombosis in dead relatives. The cut off taken to define reduced levels of AT III was <70% of the mean values obtained for a group of healthy subjects in both functional and immunological assays. Previously published data for kindred a^{15 16} show that both AT III functional activity and AT III antigen levels were reduced by about 50% in affected members (range 25 to 54% of normal in functional AT III assays, and 43 to 56% of normal in immunological assays). The results given in table 1 for kindred b show a similar reduction in both functional and antigen levels to about 50% of normal. The AT III antigen in affected members of both kindreds showed normal mobility on two dimensional gel electrophoresis, suggesting that the AT III protein was qualitatively normal. That the deficiency was inherited as an autosomal dominant trait is evident from the family pedigrees (see below).

Fig 1 shows the *Pst*I restriction site polymorphism and the *Ava*II variable length polymorphism used as

FIG 1 AT III DNA polymorphisms revealed by Southern blotting experiments. Blots of *Pst*I digested DNA from eight subjects from kindreds a and b were hybridised with ³²P labelled probe pAT III-3, and similarly *Ava*II digested DNA was hybridised with the probe pAT 1-2. The genotype with respect to each polymorphism is shown above each track and the numbers to the right indicate the approximate band sizes in kilobases (kb). The upper polymorphic bands of 10.5 and 3.0 kb are designated -, the lower bands of 5.5+5.0 and 2.8 kb are designated +.

FIG 2 Pedigree of kindred a showing segregation of the two AT III DNA polymorphisms. Genotypes for the *Pst*I and *Ava*II polymorphisms are indicated below each subject studied by DNA analysis.

the two genetic markers in this analysis. From this and other autoradiographs we were unable to detect any gross rearrangement of AT III gene structure that could not be accounted for by normal variation.

The relationship between these markers and the AT III deficiency trait in kindred a is shown in fig 2. Major deletion of the AT III gene of one chromosome could be discounted in seven affected members of kindred a because they were heterozygous ($-/+$) for at least one of the two markers. Of the remaining five apparently homozygous affected members ($-/-$ or $+/+$), who could conceivably be hemizygous at this locus, two (III.16 and IV.6) had affected parents who were heterozygous for these markers. One of these markers must have been passed on to the affected child, and therefore subjects III.16 and IV.6 must be truly homozygous. Similarly, any major deletion in kindred b could be discounted in two affected members who were heterozygous for the *Pst*I marker and in one apparently homozygous patient (II.2) who had an affected parent with heterozygosity for the *Pst*I marker (fig 3).

The information from the two two-allele polymorphisms can be combined to generate four possible haplotypes ($--$, $-+$, $+-$, $++$). It is

apparent from the family pedigrees shown in figs 2 and 3 that all affected members possess the *Pst*I ($-$), *Ava*II ($+$) haplotype, suggesting linkage of the AT III deficiency trait with these gene markers. To test the strength of association between the AT III deficiency trait and the AT III structural gene we

FIG 3 Pedigree of kindred b showing segregation of the two AT III DNA polymorphisms. Affected subjects are indicated by solid symbols and genotypes for the *Pst*I and *Ava*II polymorphisms are shown for each subject studied.

TABLE 2 Lod scores for linkage of AT III deficiency and *AvaII* (+) length polymorphism in kindred a.

θ	Lod
0	3.13
0.1	2.65
0.2	2.11
0.3	1.50
0.4	0.80

carried out linkage analysis in the larger Scottish kindred (kindred a) between the AT III deficiency trait and the *AvaII* + polymorphism. The results given in table 2 show that the maximum lod score for kindred a was 3.13 at a recombination fraction $\theta=0$ (95% confidence limits: 0 to 20%). This is consistent with close linkage between the genetic defect which is responsible for AT III deficiency and the AT III structural gene.

Discussion

Deletion of an entire AT III structural gene has been implicated as the cause of AT III deficiency in three kindreds.¹⁻¹⁰ In contrast, we were unable to show any structural abnormality of this gene in two British kindreds with AT III deficiency, even though phenotypically (proportionate decrease in activity and antigen) they were the same as the cases just cited. In both our kindreds two copies of the AT III structural gene were present and both alleles were normal. Extensive deletion was excluded by identification of the $-/+$ *PstI* genotype in seven affected members of kindred a and two affected members of kindred b. These results add to existing reports of two other kindreds in whom no evidence of AT III gene deletion was detectable by gene probing.^{1,2}

The precise molecular defect in our families was not evident from the Southern blot data presented here. Small DNA deletions and single nucleotide changes can only be detected by Southern blotting if they occur at a restriction enzyme site. The majority of known molecular defects of single gene disorders do not occur at such sites, as in, for example, the thalassaemia disorders.²⁴ These may only be characterised by gene cloning and DNA sequencing. Alternatively, the genetic defect in these families may be unlinked to the AT III structural gene. A precedent exists in the case of type I familial isolated growth hormone deficiency where disease trait and growth hormone structural gene markers segregated independently in linkage studies.²⁵

Linkage analysis in the present study which compared segregation of the AT III deficiency trait and the *AvaII* DNA polymorphism clearly

established linkage between AT III deficiency and the AT III structural gene, at a significance level of $p<0.001$. This strongly suggests that AT III deficiency in our families was caused by a subtle defect at or close to the AT III gene locus. If this situation exists the $-$, + haplotype that we identified in all the affected family members we studied must encode the defect on the basis of the linkage study of the affected parent II.9 and her affected offspring III.16 (fig 2). Cloning and sequencing of genomic DNA from an affected heterozygote with this haplotype might help to determine the precise molecular defect in this kindred.

During the course of this study, Bock *et al*² published data on a Utah kindred, showing tight linkage between the same two AT III gene markers and AT III deficiency. Our data confirm these results, but interestingly AT III deficiency in our kindreds is linked to a different haplotype from the one reported by Bock *et al* (*PstI*+, *AvaII*-). This implies that more than one mutation has occurred leading to the non-deletion type of AT III deficiency. Furthermore, these differences at the genomic level may reflect heterogeneity that has been observed at the protein level. Most families with AT III deficiency display reduced levels of an apparently normal AT III protein,⁴ but on rare occasions the AT III molecule has been found to be structurally abnormal.⁷⁻⁹ Our families belong to the first category, since we found no evidence of protein abnormality on two dimensional gels. In contrast, Bock *et al*² were able to show abnormal electrophoretic behaviour of the AT III protein in affected members of the Utah kindred, suggesting that this family belongs to a different category from the families we have reported.

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Correspondence and requests for reprints to Dr S H Sacks, Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU.