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GENETIC ANALYSIS OF MICROSOMAL EPOXIDE HYDROLASE IN PATIENTS WITH CARBAMAZEPINE HYPERSENSITIVITY

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Abstract—Carbamazepine therapy is occasionally complicated by hypersensitivity reactions, the mechanism of which is poorly understood. It has been suggested that affected individuals may have a genetically-determined defect of microsomal epoxide hydrolase. The aim of this study was to determine whether a single genetic mutation or pattern of mutations could be used to predict individual susceptibility to carbamazepine-hypersensitivity. DNA was isolated from 10 carbamazepine-hypersensitive patients and 10 healthy volunteers. The patients had developed various forms of toxicity with carbamazepine, including toxic epidermal necrolysis, Stevens-Johnson syndrome, hepatitis and pneumonitis. The technique of polymerase chain reaction single-strand conformation polymorphism analysis (PCR-SSCP) was used to screen for mutations in all nine exons of the microsomal epoxide hydrolase gene. Any new mutations detected by this method were characterised by direct sequencing of the DNA. In addition, in the most severely affected patient, we sequenced all nine exons of the gene. There was a higher frequency of mutations in the hypersensitive group when compared with the controls, but there was no consistent mutation (or pattern of mutations) in the microsomal epoxide hydrolase gene which was common to the hypersensitive group. DNA sequencing of all nine exons of the microsomal epoxide hydrolase gene from the most severely affected patient showed the sequence to be "wild-type," when compared to the previously published sequences. The results of this study suggest that a single mutation within the coding region of the microsomal epoxide hydrolase gene cannot be the sole determinant of the predisposition to carbamazepine hypersensitivity.

Key words: microsomal epoxide hydrolase; carbamazepine; hypersensitivity

CBZ‖ is a widely used anticonvulsant drug associated with rare (estimated at 1 in 1–10,000 exposures), but potentially fatal idiosyncratic toxicity [1]. The severity of toxicity encompasses a wide spectrum, ranging from mild erythematous rashes to the more serious hypersensitivity reactions characterised by fever, skin rash, and lymphadenopathy, which may also be accompanied by multi-organ involvement [2–5]. The mechanisms of these reactions, both in terms of factors determining the severity and individual susceptibility, are not fully understood. Several lines of evidence, outlined below, have suggested that carbamazepine forms reactive arene oxides, and a deficiency in their detoxication by microsomal epoxide hydrolase (termed HYL1 according to the nomenclature proposed by Beetham *et al.* [6]) may be important.

First, peripheral lymphocytes isolated from hypersensitive patients and controls have been used as target cells for reactive metabolites of CBZ generated *in vitro* [7]. The cells from the hypersensitive patients exhibited greater cytotoxicity than cells from the controls, suggestive of a detoxication defect [7–9]. Additionally, the sensitivity of control cells to CBZ metabolites was increased to a level seen in hypersensitive patients by using the HYL1 inhibitor, 1,1,1-trichloro-2-propene oxide (TCPO) [7], and cytotoxicity and covalent binding were reduced by the addition of purified hepatic HYL1 [10].

Second, the *in vivo* biotransformation of CBZ is extremely complex, resulting in the formation of more than 30 metabolites including the stable 10,11-epoxide and its corresponding diol [11]. Furthermore, the identification of hydroxylated metabolites and the 2,3- and 1,4-dihydrodiols is suggestive of the formation of chemically reactive arene oxides. Under normal circumstances, these epoxides are detoxified by enzymes such as glutathione transferase or, as in the case of the stable-10,11-epoxide, HYL1 [12].

Third, the teratogenicity of CBZ has been postulated to be mediated by arene oxides. This is largely based on epidemiological studies showing a marked increase in frequency of foetal abnormalities in patients concomitantly administered sodium valproate, an inhibitor of HYL1.

Finally, the other aromatic anticonvulsants, phenytoin and phenobarbitone, also cause toxicity similar to that caused by CBZ [13, 14], and some patients exhibit cross-sensitivity with the three drugs [8], suggesting a common mechanism of toxicity. Furthermore, animal studies have shown an inverse relationship between the levels of HYL1 and incidence of foetal toxicity [15]. Buehler *et al.* have also shown that infants with the foetal hydantoin syndrome have a quantitative deficiency of HYL1 [16].

In view of this evidence, it has been hypothesised that susceptibility to CBZ hypersensitivity may be due to a functional deficiency of HYL1. This would create an

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‖ Abbreviations: HYL1, microsomal epoxide hyrolase; CBZ, carbamazepine; PCR-SSCP, polymerase chain reaction single-strand conformation polymorphism.

imbalance between bioactivation and detoxication, allowing the reactive metabolites to bind to cellular macromolecules, and by acting as immunogens, initiate immune-mediated toxicity in the form of the hypersensitivity reactions seen in patients.

Unlike many of the drug metabolising enzymes that are coded for by superfamilies of genes, HYL1 appears to exist as one isozyme encoded by a single gene [17, 18]. Recently, Gaedigk *et al.* [19] have analysed the coding region of the *HYL1* gene in a heterogeneous group of patients who had suffered adverse reactions to one or more of the three aromatic anticonvulsant drugs. This study did find nucleotide variants, but none was common to all of the hypersensitive patients. In the present study, we have examined a homogeneous group of patients who had all experienced hypersensitivity reactions to the drug CBZ. We have used single-strand conformation polymorphism (SSCP) analysis to look for mutations in the 9 exons of the *HYL1* gene in a well-characterised group of CBZ-hypersensitive patients and controls. To characterise new mutations predicted from SSCP, direct DNA sequencing was performed. In addition, we have also sequenced the entire coding region of the *HYL1* gene in one patient (patient 1, Table 1) who had the most severe reaction to CBZ of all our patients. The principal aim of the study was to determine whether a single genetic mutation (or pattern of mutations) could be used to predict individual susceptibility to CBZ hypersensitivity.

METHODS

Patients and controls

Ten patients with a clinical history suggestive of CBZ-hypersensitivity took part in the study. Their clinical features are summarised in Table 1. In all the patients, the adverse reaction occurred shortly (between 1–5 weeks) after the start of CBZ therapy, and resolved on discontinuation of the drug. All patients had a fever associated with the reaction, and some were also recorded to have eosinophilia. Patients 1, 2, 3, 6, 7, 8, and 9 have been described before [5, 7]; their lymphocytes showed greater *in vitro* sensitivity to CBZ metabolites than cells from controls. Patients 4, 5, and 10 have never been tested in the *in vitro* cytotoxicity assay.

Patient 1 [5] is a 22-year female who developed the most severe reaction to CBZ of all our patients. Two weeks after the start of CBZ (600 mg/day), she developed a blistering rash, which rapidly progressed to toxic epidermal necrolysis. The rash was accompanied by fever, leucopenia, and abnormal liver function. The patient was hospitalised for six weeks, requiring intensive support and treatment, including high-dose steroids, plasmapheresis, and immunoglobulin infusions. Immunohistochemical examination of the patient's skin showed infiltration by $CD8^+$ cells, suggesting an immune cytotoxic reaction [5].

Ten healthy individuals who had never been exposed to anticonvulsant drugs were used as controls. Genomic DNA was isolated from whole blood (10 mL) taken from the patients and controls by a simple salting out procedure [20].

Materials

Oligonucleotide primers were synthesized by International Laboratory Services (London, U.K.) and Pharmacia Biotech (St. Albans, U.K.). Acrylamide was purchased from Appligene (Birtley, U.K.), urea from Stratagene Ltd. (Cambridge, U.K.), and Konica X-ray film from X-Ograph (Malmesbury, U.K.). *Taq* DNA polymerase was purchased from Perkin Elmer Cetus (Beaconsfield, U.K.). A USB Sequenase Kit and [α-^{35}S]dATP were from Amersham International plc (Bucks, U.K.). All other reagents were of molecular biology grade, and were obtained from Sigma Chemical Co. (Poole, U.K.).

Amplification of the exons of the microsomal epoxide hydrolase gene

All nine exons of the *HYL1* gene were amplified according to the method of Gaedigk *et al.* [19]. The reactions were performed in an Omnigene programmable thermal cycler (Hybaid, Teddington, U.K.). A negative control was included with each batch of samples in which DNA template was substituted with sterile distilled water. PCR products were resolved by electrophoresis in 1.5% (w/v) agarose, stained with ethidium bromide (0.5 μg/mL), and visualised under UV light.

PCR-SSCP analysis of the microsomal epoxide hydrolase gene

DNA from 10 hypersensitive patients (including patient 1, Table 1) and from 10 controls was successfully amplified for all nine exons of the *HYL1* gene. SSCP analysis of radiolabelled PCR products was performed as previously described by Gaedigk *et al.* [19], except that analysis was performed on 0.5%, 1%, 5%, 10%, and 15% glycerol gels. Where SSCP analysis suggested a

Table 1. Clinical characteristics of the carbamazepine-hypersensitive patients

Patient	Sex	Age	Time to adverse reaction	Manifestations
1	Female	22	2 weeks	Fever, toxic epidermal necrolysis, leucopenia, abnormal liver function
2	Male	18	5 weeks	Fever, desquamating rash, hepatitis, jaundice, eosinophilia
3	Female	49	1 week	Fever, Stevens-Johnson syndrome, abnormal liver function tests
4	Male	28	3 weeks	Fever, extensive erythematous rash, eosinophilia
5	Female	75	4 weeks	Fever, toxic erythema, eosinophilia
6	Male	20	5 weeks	Rash, pneumonitis, splenomegaly, fever, eosinophilia
7	Male	22	3 weeks	Exfoliative dermatitis, fever, eosinophilia
8	Female	82	4 weeks	Stevens-Johnson syndrome, fever, abnormal liver function tests
9	Male	20	2 weeks	Erythematous rash, fever
10	Female	65	3 weeks	Generalised desquamating rash, fever

mutation, the DNA was amplified again and analysed by direct dideoxy sequencing. SSCP analysis was performed in the 20 individuals using a blinded protocol.

Direct sequencing of DNA

DNA from previously unidentified SSCP mobility shifts, occurring for either controls or hypersensitive patients, was sequenced to identify the mutation present. In addition, all nine exons for patient 1 were amplified by PCR, and the DNA used as a template for sequencing reactions. PCR-amplified DNA (5 × 50 μL reactions) was precipitated with sodium acetate (3M, pH 5.2) and ethanol at −80°C, and the pellet resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The desired band was then purified by elution from an agarose gel (1.5% w/v) after electrophoresis. The DNA was precipitated once again and resuspended in a final volume of 25 μL TE buffer. Sequencing of PCR products was carried out according to the USB Sequenase Version 2.0 protocol with minor modifications. DNA (0.3–1.0 pmoles) was denatured at 95–100°C for 6 min in the presence of 0.5 pmoles primer, followed by cooling to room temperature for 5 min. Extension and termination steps were performed for 5 min at room temperature and 37°C, respectively, in the presence of [α-^{35}S]dATP. Sequencing reactions (3 μL) were resolved by denaturing electrophoresis in polyacrylamide gels (0.4 mm, 6% acrylamide, 7.7 M urea) at 50°C. Gels were fixed (10% methanol/10% acetic acid) for 15 min, rinsed, and dried prior to exposure to X-ray film. Both strands of the DNA were sequenced for each sample.

RESULTS

PCR amplification was successful in all of the DNA samples from the 10 control and 10 hypersensitive patients. The sizes of the PCR products obtained were of the expected size [19], suggesting no gross deletion of any coding region of the gene. PCR-SSCP analysis (Table 2) showed mobility shifts representing a total of 10 nucleotide variants. The nucleotide change responsible for the mobility shift was identified by (a) direct sequencing of both strands of DNA in the case of a previously unidentified mutation, or (b) where the mutation has been previously identified [19, 21], by the use of DNA from an individual known to possess that mutation, and subsequent comparison of their respective mobility shifts on SSCP analysis. SSCP analysis suggested the presence of mutations in 3 individuals in exon 5, which on sequencing were found to be wild-type. Of the 10

Table 2. Mutations within the *HYL1* gene as determined by single-strand conformation polymorphism analysis (SSCP) and DNA sequencing

Subject	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8	Exon 9
				Controls					
A			G → A			C → T			G → C
B			T → C						
C			T → C	A → G				C → T	
D	**G → A**	g → a	T → C						
E	**G → A**	g → a	G → A						
F									
G			T → C/G → A	A → G		C → T		C → T	G → C
H									
I			T → C/G → A						
J									
				Carbamazepine-hypersensitive patients					
1									
2			G → A	g → a					
3	**G → A**		T → C/T → C			C → T			G → C
4			G → A	g → a					
5			T → C/T → C	A → G				**t → c**	
6			T → C	A → G/A → G				C → T	
7			T → C	A → G					
8			G → A	A → G/g → a					
9				g → a					
10	**G → A**	g → a	G → A			C → T			G → C

Positions of nucleotide variants in the microsomal epoxide hydrolase gene as detected by SSCP analysis and direct sequencing. All mutations are wt/mutation unless otherwise indicated. Novel mutations are shown in bold type. For exonic mutations (upper case) the position corresponds to the cDNA sequence, where start = 1 and stop = 1368. For intronic mutations (lower case) the positions are in bold type and are denoted by −(upstream) or +(downstream) from the start or finish respectively, of the exonic sequence. For exon 1 the position of the mutation (italics) is given from the start of exon 1. The only mutations to result in amino acid substitutions are the T → C in exon 3, which results in a Tyr → His at codon 113, and the A → G mutation in exon 4, which results in a His → Arg at codon 139.

EXON	1	2	3	4	5	6	7	8	9
Nucleotide change	$G_{66} \rightarrow A$	$g_{+20} \rightarrow a$	$T_{337} \rightarrow C$ $G_{357} \rightarrow A$	$A_{416} \rightarrow G$ $g_{+34} \rightarrow a$		$C_{994} \rightarrow T$		$C_{1071} \rightarrow T$ $t_{+31} \rightarrow c$	$G_{1350} \rightarrow C$

variants identified, 2 resulted in amino acid substitutions, 4 were silent mutations, 3 were located in the flanking introns, and 1 was located in exon 1 which is a non-coding exon (Table 2). Although there was a higher incidence of nucleotide mutations within the group of hypersensitive patients (total 28 variants) compared with controls (21 variants), there was no single mutation or pattern of mutations present in the patients that could be related to the occurrence of CBZ hypersensitivity.

All 9 exons for patient 1, who had the most severe reaction to CBZ of all our patients (Table 1), were amplified by PCR and sequenced. SSCP analysis had suggested the presence of a previously unidentified mutation in exon 9. However, amplification of this exon and sequencing revealed that there was no mutation. In addition, none of the other exons were found to possess any mutations when sequenced. Thus, the whole of the coding region of the *HYL1* gene in patient 1 had the wild-type sequence when compared to the 6 previously published sequences [17, 21, 22].

DISCUSSION

A recent study by Hassett *et al.* [21] compared three *HYL1* cDNA sequences [17, 22], a genomic *HYL1* sequence [18], and the sequences of two clones from a λgt11 library. From the comparison of these 6 sequences, a cDNA with the most frequently occurring nucleotide at each position was denoted the "wild-type" *HYL1* sequence. We have used this wild-type sequence as a standard against which to compare *HYL1* sequences from our CBZ-hypersensitive patients.

Analysis of the entire coding region of the *HYL1* gene in 10 hypersensitive patients revealed no single mutation that could be responsible for altered enzyme activity, and furthermore, there was no consistent difference in the *HYL1* gene between the patients and controls. This suggests that a functional alteration in *HYL1* activity due to a mutation in the coding region of the gene cannot solely be responsible for CBZ hypersensitivity. This conclusion is supported by a parallel study (Davis *et al.*, this issue), which showed no difference in lymphocyte *HYL1* activity (using *cis*-stilbene oxide as a substrate) between hypersensitive patients and controls. No significant difference in either K_m or V_{max} for lymphocyte *HYL1* was observed between the controls and hypersensitive patients. Several of the patients (patients 1–5; Table 1) were included in both studies. Our results are also in agreement with those of Gaedigk *et al.* [19], in which it was not possible to relate hypersensitivity reactions to anticonvulsants with an alteration in the *HYL1* gene. However, these conclusions are not in accordance with earlier studies, which had implied that susceptibility to these idiosyncratic reactions may be due to the lack of functional *HYL1* activity [7–9].

The data presented here suggest that no structural change in the *HYL1* gene can be the cause of CBZ hypersensitivity. This conclusion, as in the study by Gaedigk *et al.*, is mainly based on SSCP analysis of the exons of the *HYL1* gene. It has been reported that SSCP analysis will detect 80–90% of single-base mutations in DNA fragments of up to 400 bp in length [23, 24]. However, a more recent systematic analysis of the technique of SSCP has shown that sensitivity varies dramatically with the size of the DNA fragment being analysed, the optimal size being 150 bp. The sensitivity of detecting a single base substitution was less than 60% when the DNA fragment was about 400 bp [25]. The PCR fragment sizes in our study ranged from 208 bp for exon 5 to 438 bp for exon 1 [19]. Furthermore, SSCP analysis in the present study falsely indicated the presence of mutations in exons 5 and 9, which in fact were wild-type when investigated by direct sequencing. Thus, it is possible that an undetected mutation could be present in the *HYL1* gene in one or more of our patients. However, this seems unlikely, since we also sequenced the entire coding region of the *HYL1* gene in one patient (patient 1, Table 1) who developed a well-characterised, severe, immune-mediated reaction to CBZ [5], and found it to be identical to the consensus wild-type.

It is still possible that the regulation of expression of the *HYL1* protein may differ among individuals. Induction of *HYL1* in humans has been demonstrated by using CBZ-10,11-epoxide as an *in vivo* functional probe [26], and this may be a necessary response to protect against toxicity. Differences in induction may therefore be involved in predisposition to such toxicity. Gene expression is a complicated process, and induction of the gene by drugs may involve a different sequence of events to those seen during constitutive expression. For example, positive regulation by sequences such as xenobiotic response elements [27, 28] or electrophile response elements [29] may be involved. Furthermore, in the present study, only the coding regions have been analysed; apart from the flanking intronic sequences, we also cannot exclude the possibility that an intronic mutation is responsible for individual predisposition to hypersensitivity. The importance of intronic mutation has been observed with other genes, such as the angiotensin converting enzyme gene [30]. Further studies are therefore essential before the role of *HYL1* in this toxicity can be completely eliminated.

In some cases of hypersensitivity to aromatic anticonvulsants, cross-reactivity to drugs occurs. This results in some patients experiencing adverse reactions to CBZ, phenytoin, and phenobarbitone, whereas others may be sensitive to only one of the drugs [1, 7, 8]. Although this indicates a similar mechanism of toxicity, the factor(s) responsible for determining the phenotype may vary among individuals. A deficiency of *HYL1* may adversely alter the critical balance between the bioactivation of CBZ to its chemically reactive metabolite and its detoxication, resulting in toxicity. However, other factors could also affect this balance in different ways to produce the same overall effect, and thus identification of these alternative factors is as important. For example, a relative increase in the bioactivation of CBZ by the P450 enzymes to electrophilic intermediates could lead to the same end-point as observed with a decrease in detoxication. In this respect, it is important to note that CBZ causes both auto and hetero-induction [31], which occurs mainly because of induction of CYP3A *in vivo* [32, 33] and *in vitro* in human hepatocytes [34]. CBZ is known to be metabolised by both CYP3A and CYP2C8 [35, 36]. An alternative possibility is that deficiency resides in a metabolic pathway other than *HYL1*. As our hypothesis assumes electrophilic CBZ metabolites are involved in the pathogenesis of the toxicity, it is also important to consider other detoxication enzymes such as glutathione *S*-transferase (GST). Of particular importance may be GSTM1 (GST μ), which metabolises epoxides [37], and is polymorphically expressed with 50% of the popula-

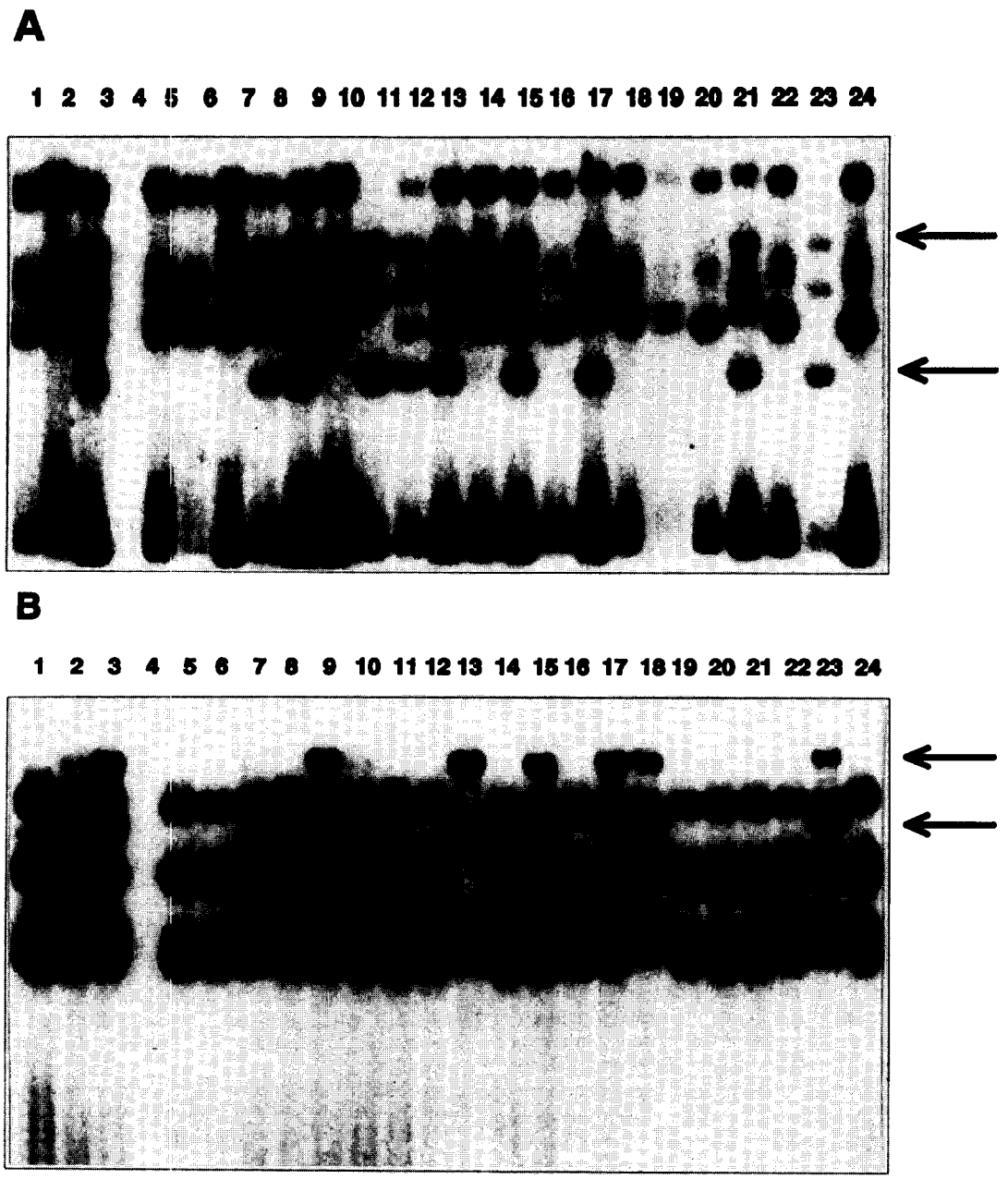

Fig. 1. Detection of mutations by single-strand conformation polymorphism (SSCP) analysis in exons 3 (photograph A) and 4 (photograph B) of the microsomal epoxide hydrolase gene. The numbers at the top of the figure indicate the different subjects as follows: The carbamazepine-hypersensitive patients (Table 1) 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 are in lanes 6, 10, 11, 7, 23, 13, 15, 18, 22, and 19, respectively. The controls subjects A, B, C, D, E, F, G, H, I, and J are in lanes 5, 8, 9, 12, 14, 16, 17, 20, 21, and 24, respectively. Lanes 1, 2, and 3 were control DNA samples known to possess specific mutations. The arrows indicate shifted bands. (A) Exon 3 was amplified by oligonucleotide-driven PCR, and the resulting products separated in a 10% glycerol/6% acrylamide gel. Lanes 1, 2, and 3 represent wt/wt, wt/G → A, and wt/T → C, respectively. Lanes 6, 16, 20, 22, and 24 are wt/wt; lanes 5, 7, 10, 14, 18, and 19 are wt/G → A; lanes 8, 9, 12, 12, 13, and 15 are wt/T → C; lanes 17 and 21 are T → C/G → A; and, lanes 11 and 23 are T → C/T → C. (B) Exon 4 was amplified by oligonucleotide-driven PCR, and the resulting products separated in a 1% glycerol/6% acrylamide gel. Lanes 1, 2, and 3 represent wt/wt, wt/g → a and wt/A → G, respectively. Lanes 5, 6, 8, 11, 12, 14, 16, 19, 20, 21, and 24 are wt/wt; lanes 7, 10, and 22 are wt/g → a; lanes 9, 15, 17, and 23 are wt/A →G; lane 18 is g → a/A → G; and, lane 13 is A → G/A → G.

tion lacking the enzyme [38, 39]. However, to date we have found no association between CBZ hypersensitivity and GSTM1 status [40]. Covalent binding of the reactive metabolite of CBZ may be followed by involvement of the immune system resulting in the typical hypersensitivity reactions observed in our patients (Table 1), and thus, it is important to consider whether the differences in susceptibility may lie with immune responsiveness

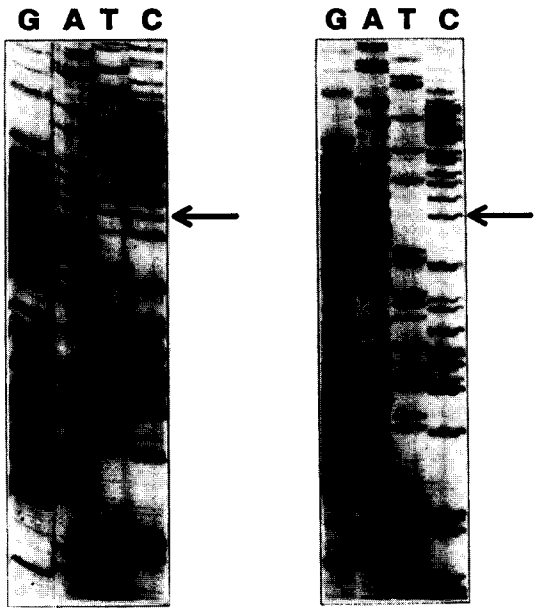

Fig. 2. Sequencing autoradiograph for the antisense strand of exon 1. DNA fragments were separated by electrophoresis on a 0.4 mm/6% acrylamide/7.7 M urea gel. (1) Sequence from the carbamazepine-hypersensitive patient 3 (Table 1). The arrow indicates a wt/C → T mutation which corresponds to the G → A mutation in exon 1, as shown in Table 2. (2) Sequence from patient 6, which shows wt/wt at this position.

rather than with drug metabolism. It is possible that many patients may form drug-protein adducts during anticonvulsant therapy, but that only in a small percentage do these act as immunogens and initiate an immune response. Such a hypothesis has been put forward to explain individual predisposition to halothane hepatitis [41]. Finally, it is also conceivable that rather than any one single factor predisposing an individual to anticonvulsant hypersensitivity, several factors acting in unison may be responsible. Thus, it may be the overall balance between bioactivation, detoxication, and immune responsiveness that determines whether a hypersensitivity reaction will occur. If this is the case, it may not be possible to define the genetic basis of carbamazepine hypersensitivity, because the low frequency of the reaction would necessarily preclude the recruitment of sufficient patients for a study having acceptable statistical power.

In conclusion, the present study has identified several mutations in the coding region of the *HYL1* gene, although there was no single mutation or pattern of mutations that correlated with the occurrence of hypersensitivity. Thus, it is unlikely that hypersensitivity reactions to CBZ are due to a structural change in the *HYL1* protein. Predisposition to the toxicity may be a multifactorial process, involving several enzyme systems as well as individual differences in immune responsiveness.

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