

Detection of an autoantibody directed against human liver microsomal protein in a patient with carbamazepine hypersensitivity

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A 16-year old patient with carbamazepine-induced hepatotoxicity, associated with the hypersensitivity manifestations of fever, rash and eosinophilia, is described. Mononuclear leucocytes from the patient were more sensitive to oxidative metabolites of carbamazepine generated by induced murine and human hepatic microsomes, than cells from controls. On immunoblot analysis, serum from the patient recognised a single protein band (94 kDa) on human liver microsomes, but none out of 25 control sera recognised this band. No bands were recognised by the patient serum on human kidney microsomes or on microsomes from mouse and rat liver.

Keywords carbamazepine toxicity hypersensitivity autoantibody

Introduction

The liver is the main site of drug metabolism in the body. Since it is thought that many idiosyncratic drug reactions result from the production of toxic, reactive metabolites by the cytochrome P450 enzyme system (Park, 1986), it is not surprising that hepatotoxicity has been reported with many chemically related and unrelated drugs (Kaplowitz *et al.*, 1986). The toxic metabolite may lead to hepatic injury either by direct toxicity (Park, 1986) or by an immunological mechanism (Park *et al.*, 1987).

Carbamazepine, a widely used anti-epileptic agent, is associated with a wide range of adverse reactions (Pellock, 1987), including hepatotoxicity. Since all individuals may be capable of metabolising CBZ to a toxic metabolite (Pirmohamed *et al.*, 1991), the critical factor in predisposing individuals to such idiosyncratic toxicity may be a deficiency in cellular detoxification, postulated to be a deficiency of epoxide hydrolase (Shear *et al.*, 1988). The liver injury associated with CBZ is thought to have an immunoallergic basis (Dreifuss & Langer, 1987), although the direct evidence for an immunological mechanism such as the presence of specifically committed immune cells and/or antibodies directed against the drug-protein conjugate, is limited (Zakrzewska & Ivanyi, 1988). In certain circumstances, autoantibody formation, particularly when associated with idiosyncratic drug toxicity, may also be used as evidence for the immunological nature of an adverse reaction. However, to the best of our knowledge, no specific autoantibodies have been demonstrated in

patients who have developed idiosyncratic toxicity with CBZ.

In this paper, we report a patient who had severe hepatotoxicity with carbamazepine, and in whom we have been able to demonstrate *in vitro* susceptibility to CBZ metabolites generated by murine and human liver microsomes and the presence of a specific autoantibody directed against a human liver microsomal protein.

Case report

A 16-year old boy (termed patient AB) was started on carbamazepine (400 mg day⁻¹) for generalised epilepsy. Four weeks after the start of therapy, he developed a fever, followed by a generalised rash (after 5 weeks) and jaundice (6 weeks).

On admission to hospital, the patient had a temperature of 39° C, and had a generalised erythematous, desquamating rash. He was icteric and had palpable cervical lymph nodes and liver. Laboratory investigations showed a mild leucocytosis ($11.3 \times 10^9 \text{ l}^{-1}$) with an eosinophilia (39%) and atypical lymphocytes on a peripheral blood film. The liver function tests were as follows: total serum bilirubin 45 $\mu\text{mol l}^{-1}$ (normal range (NR) 2–17), alkaline phosphatase 617 iu l^{-1} (NR 35–130), alanine aminotransferase 328 iu l^{-1} (NR 7–45), and gamma-glutamyl transferase 310 iu l^{-1} (NR 0–65). Immunological screening, including anti-nuclear antibody, anti-

mitochondrial antibody, anti-smooth muscle antibody and anti-reticulin antibody were all negative. Tests for EB virus (including glandular fever slide test) and hepatitis viruses were negative, while cytomegalovirus antibodies were weakly positive.

Carbamazepine-induced hepatotoxicity was diagnosed and the carbamazepine withdrawn. The patient's clinical condition and abnormal laboratory investigations improved over the subsequent 6 weeks, the abnormal physical signs disappearing in the same order in which they appeared.

Methods

Antisera

Serum from the patient AB was obtained on three occasions: at the time of the adverse reaction, 2 months and 6 months after the reaction.

Sera for comparison with the sera from patient AB (hereafter called 'control sera') were obtained from 25 individuals, as follows: 12 normal, healthy volunteers who had never been exposed to CBZ, three patients on chronic CBZ therapy without adverse effects, six patients with hepatitis induced by drugs other than CBZ (three due to lofepramine, one due to sulphasalazine, one due to phenytoin, and one due to norfloxacin). In addition, sera from four other patients with CBZ-induced toxicity were also used for comparison with patient AB; in three of these patients, the sera were collected during the acute phase, although they did not have hepatic manifestations of toxicity (two with Stevens-Johnson syndrome and one with aplastic anaemia), while in the other patient who had CBZ-induced hepatitis (full clinical details of severity of the reaction were unavailable), the serum was collected one year after the occurrence of his reaction.

Preparation of microsomes and liver cytosol

Microsomes were prepared from human liver ($n = 9$), human kidney ($n = 1$), rat liver ($n = 2$ rats), untreated mouse liver ($n = 2$ mice) and phenobarbitone-treated (PB) mouse liver ($n = 6$ mice pre-treated with phenobarbitone 60 mg kg^{-1} body weight day^{-1} i.p. in 0.9% w/v saline for 3 days) as reported previously (Purba *et al.*, 1987). The supernatant after the first 100,000 g centrifugation step was used as cytosol. Ethical approval for use of human tissue was granted and informed consent obtained from donors' relatives. The protein content was determined by the method of Lowry *et al.* (1951). The microsomes and cytosol were stored at -80°C until required for use.

Determination of metabolism-dependent cytotoxicity of carbamazepine

Mononuclear leucocytes (MNL; viability $> 95\%$) were isolated from patient AB (6 months after the adverse reaction) and two male controls (Riley *et al.*, 1988).

The MNL ($1 \times 10^6 \text{ ml}^{-1}$) were incubated with CBZ ($50 \mu\text{M}$) and PB mouse liver microsomes (0.5 mg) or

human liver microsomes (2 mg) in the presence or absence of NADPH (1 mM), the cytotoxicity being determined by trypan blue dye exclusion. Incubations were performed in quadruplicate and the results are presented as the increase in cell death above the baseline.

SDS-PAGE electrophoresis and immunoblotting of microsomal and cytosolic proteins

This was performed according to the method of Laemmli (1970). Briefly, either $10 \mu\text{g}$ or $25 \mu\text{g}$ of protein was loaded onto a 7% polyacrylamide gel and electrophoresis was performed for 1 h at 30 mA/gel at 10°C .

The protein was transferred to nitrocellulose electrophoretically at 100 mA overnight (Towbin *et al.*, 1979). The nitrocellulose sheets were either stained for protein with amido black or taken for antibody overlay.

Prior to blotting with antibody, non-specific binding sites were blocked with casein buffer (2.5% (w/v) casein, 154 mM NaCl , 10 mM tris , $0.5 \text{ mM thimerosal}$; pH 7.6) for 1 h at room temperature. The blots were then incubated with sera from the hypersensitive patient or controls diluted 1:20, 1:50 or 1:100 in casein washing buffer (casein 0.5% (w/v), 0.02% (w/v) thimerosal, 154 mM NaCl , 5 mM tris ; pH 7.6), followed by successive washes with casein washing buffer for 10 min, casein buffer containing 0.1% (w/v) SDS and 0.5% (w/v) triton X-100 for 5 min (to remove non-specifically bound plasma proteins and antibodies) and then twice for 10 min each in casein washing buffer again. The second antibody, horseradish peroxidase conjugated goat anti-human IgG (diluted 1:250 in casein washing buffer; Tissue Culture Services, Bucks., England) was then incubated with blots overnight at room temperature. This was followed by successive washes with casein washing buffer ($4 \times 10 \text{ min}$) and tris-saline buffer (50 mM tris , 0.2 mM NaCl ; pH 7.4; $4 \times 10 \text{ min}$). Sites of antibody binding were visualised by incubation of the blots with 4-chloro-1-naphthol, the apparent molecular mass of any bands being determined by comparison of their electrophoretic mobilities with mobilities of marker proteins of known molecular mass.

Results

Metabolism-dependent cytotoxicity of carbamazepine with phenobarbitone-induced mouse and human liver microsomes

In the presence of PB mouse microsomes, the increase in cell death above the baseline for MNL from the CBZ-hypersensitive patient AB (15.3%) was greater than for the two controls (3.0%; 5.6%). Similarly, with human liver microsomes, cytotoxicity for the cells from the patient (16.1%) was again higher than for the two controls (5.4%; 9.1%). Taken with the results from a previous study (Pirmohamed *et al.*, 1991), these data indicate that patient AB may be categorized within a group of CBZ-hypersensitive patients whose lymphocytes are more susceptible to CBZ metabolites *in vitro* than lymphocytes from appropriate controls.

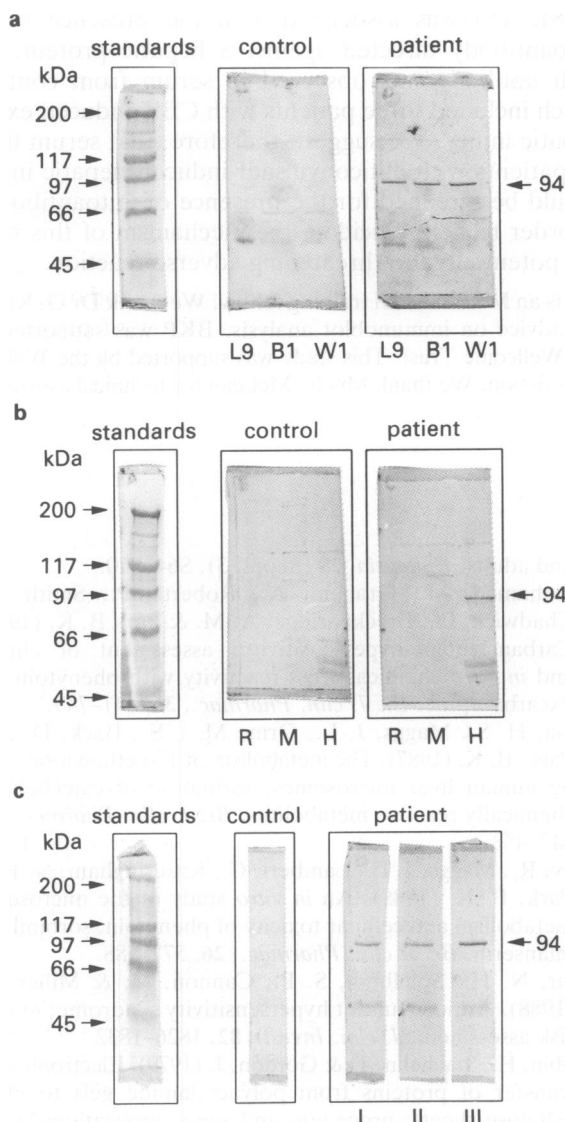


Figure 1 Immunoblots of liver microsomal proteins exposed to either control or patient serum. Microsomal proteins were separated on 7% polyacrylamide gels and transferred to the nitrocellulose support electrophoretically. In each case 'patient' refers to serum from patient AB, while the 'control' sera were obtained from a normal volunteer, a patient treated with carbamazepine without adverse effects, and a patient with lofepramine-induced hepatotoxicity in Figure 1a, b and c respectively. Figure 1a shows recognition of a band with an apparent molecular mass of 94 kDa by the serum (dilution 1:20) from patient AB in three different human liver microsomal samples (L9, B1 and W1). Figure 1b indicates the presence of the 94 kDa band in human (H), but not rat (R) or mouse (M) microsomal protein. Figure 1c shows the same band in human liver microsomes exposed to patient AB's sera obtained at the height of the reaction (I), 2 months after the reaction (II) and 6 months after the reaction (III).

Immunoblot analysis of microsomal and cytosolic proteins

The serum from patient AB (up to the maximum dilution tested 1:100) recognised a protein band with an apparent molecular mass of 94 kDa on all human liver microsomes ($n = 9$) used but none of the control sera recognised this band (Figure 1). Serum from the patient was taken on three occasions: all three sera recognised the same

band with no apparent diminution in intensity (Figure 1). The band was not recognised by either the patient or control sera in human kidney microsomes, mouse and rat liver microsomes, or cytosol from human and mouse liver. Preincubation of the human liver microsomes with CBZ (50 μM ; in the presence or absence of NADPH) at 37° C for 2 h prior to immunoblotting did not affect the recognition of the 94 kDa protein band by serum from patient AB. In addition, preincubation of free CBZ (2 mM) with serum from patient AB did not inhibit antibody binding to the 94 kDa band.

Discussion

Drug hypersensitivity is essentially a clinical diagnosis based on symptomatology rather than direct laboratory evidence. Thus, our patient satisfies the clinical criteria of CBZ hypersensitivity, with fever, rash and eosinophilia occurring 4 weeks after the start of therapy. Shear *et al.* (1988) have shown that patients who have had idiosyncratic reactions to anticonvulsants can be identified by showing *in vitro* chemical cellular sensitivity to oxidative drug metabolites. Consistent with the results of the study by Shear *et al.* (1988) and our study with eight CBZ hypersensitive patients (Pirmohamed *et al.*, 1991), patient AB also showed *in vitro* chemical sensitivity to CBZ metabolites generated by a murine microsomal system. In addition, similar *in vitro* sensitivity was shown to metabolites generated by human liver microsomes, suggesting that the same toxic metabolite (possibly an epoxide) is generated by the human and mouse microsomes. Although, these results provide further supportive evidence for the clinical diagnosis of CBZ-induced hepatotoxicity, they do not provide any evidence that the reaction was immunologically-mediated.

The antibody described in patient AB, which was directed against a 94 kDa human liver microsomal protein supports the clinical evidence which suggested that the hepatic injury was immunologically-mediated. Such specific autoantibodies to hepatic proteins have been described in drug-induced hepatitis, for example with tienilic acid (Beaune *et al.*, 1987) and dihydralazine (Bourdi *et al.*, 1990). However, these antibodies were directed against the cytochrome P450 enzymes which normally hydroxylate the drug, while the antibody in our patient is directed towards a 94 kDa protein, which is above the molecular weights of known cytochrome P450 enzymes. The nature of the protein is not known; it appears to reside in the endoplasmic reticulum, although we cannot exclude the possibility of contamination with other subcellular fractions, such as cell membrane. However, the latter possibility is unlikely since this band was observed only in human liver microsomes and not in human kidney microsomes or microsomes prepared from rat or mouse liver.

The reason why an antibody should be directed against an intracellular protein is not clear. It is unlikely to be due to the protein being liberated as a result of cellular lysis, since six other patients with hepatitis induced by drugs other than CBZ who all had elevated transaminases at the time the blood was taken, did not

demonstrate the presence of this autoantibody. It is possible that a reactive metabolite of CBZ, which was inadequately detoxified by epoxide hydrolase as demonstrated by the results of the cytotoxicity assay, can bind to this protein, behave as a hapten and elicit an immune response against the carrier protein. Such a mechanism has been postulated for the occurrence of anti-cytochrome P450 antibodies due to tienilic acid (Beaune *et al.*, 1987) and dihydralazine (Bourdi *et al.*, 1990).

In summary, the cells from the hypersensitive patient showed increased *in vitro* chemical sensitivity to oxidative drug metabolites, which is thought to be due to a deficiency of epoxide hydrolase (Shear *et al.*,

1988), and was associated with the presence of an autoantibody directed against a hepatic protein. No such antibody was observed in serum from controls which included three patients with CBZ-induced extra-hepatic injury. We suggest, therefore, that serum from all patients with anticonvulsant-induced hepatic injury should be screened for the presence of autoantibodies in order to help elucidate the mechanism of this rare, but potentially life-threatening adverse reaction.

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