

Hypersensitivity Reactions to Carbamazepine: Characterization of the Specificity, Phenotype, and Cytokine Profile of Drug-Specific T Cell Clones

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ABSTRACT

Administration of carbamazepine (CBZ) causes hypersensitivity reactions clinically characterized by skin involvement, eosinophilia, and systemic symptoms. These reactions have an immune etiology; however, the role of T cells is not well defined. The aim of this study was to characterize the specificity, phenotype, and cytokine profile of CBZ-specific T cells derived from hypersensitive individuals. Proliferation of blood lymphocytes was measured using the lymphocyte transformation test. CBZ-specific T cell clones were generated by serial dilution and characterized in terms of their cluster of differentiation and T cell receptor V β phenotype. Proliferation, cytotoxicity, and cytokine secretion were measured by [3 H]thymidine incorporation, 51 Cr release, and enzyme-linked immunosorbent assay, respectively. HLA blocking antibodies were used to study the involvement of antigen-presenting cells. The specificity of the drug T cell receptor interaction was studied using CBZ metab-

olites and other structurally related compounds. Lymphocytes from hypersensitive patients (stimulation index: 32.1 ± 24.2 [$10 \mu\text{g ml}^{-1}$]) but not control patients (stimulation index: 1.2 ± 0.4 [$10 \mu\text{g ml}^{-1}$]) proliferated upon stimulation with CBZ. Of 44 CBZ-specific T cell clones generated, 10 were selected for further analysis. All 10 clones were either CD4+ or CD4+/CD8+, expressed the $\alpha\beta$ T cell receptor, secreted IFN- γ , and were cytotoxic. T-cell recognition of CBZ was dependent on the presence of HLA class II (DR/DQ)-matched antigen-presenting cells. The T cell receptor of certain clones could accommodate some CBZ metabolites, but no cross-reactivity was seen with other anticonvulsants or structural analogs. These studies characterize drug-specific T cells in CBZ-hypersensitive patients that are phenotypically different from T cells involved in other serious cutaneous adverse drug reactions.

Administration of carbamazepine (CBZ), which is commonly administered for the treatment of epilepsy, causes hypersensitivity reactions characterized by skin involvement and systemic manifestations such as hepatitis and eosinophilia (Leeder, 1998). Reactions vary in severity and may cause death. The frequency of CBZ hypersensitivity is between 1 in 1000 and 1 in 10,000 new exposures to the drug (Vitorrio and Mulgia, 1995; Tennis and Stern, 1997). Taken from clinical manifestations, CBZ hypersensitivity reactions are believed to have an immune etiology. In accordance with

this, a sensitization period of 3 to 4 weeks is required for the initial development of clinical symptoms, whereas rechallenge results in the recurrence of symptoms much sooner than with primary exposure (Leeder, 1998). The mechanism by which CBZ induces a hypersensitivity reaction is unclear. Although CBZ undergoes bioactivation to toxic arene oxide and quinone metabolites (Madden et al., 1996; Ju and Uetrecht, 1999), definite evidence linking this to immunogenicity is lacking (Park et al., 1998).

The generation and characterization of drug-specific T cell lines and clones from drug-allergic patients with severe skin reactions has recently led to the classification of cutaneous eruptions in terms of their cellular and molecular pathophysiology (Pichler et al., 2002) (Fig. 1). Skin-infiltrating CD4+ T cells, which secrete IL-5, perforin, and granzyme, are characteristic of maculopapular exanthemas (Pichler et al., 1997; Schnyder et al., 1998; Yawalker et al., 2000a,b), whereas

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ABBREVIATIONS: CBZ, carbamazepine; B-LCL, B-lymphoblastoid cell line; PBMC, peripheral blood mononuclear cells; SI, stimulation index; TT, tetanus toxoid; CD, circular dichroism; HLA, human leukocyte antigen; IFN- γ , interferon- γ ; IL, interleukin; MHC, major histocompatibility complex; ELISA, enzyme-linked immunosorbent assay.

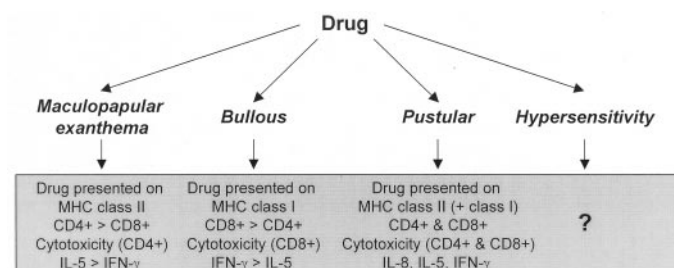


Fig. 1. Scheme depicting our current understanding of cellular and molecular pathophysiology of severe skin reactions (adapted from Pichler et al., 2002).

bullous reactions such as Stevens-Johnson syndrome and toxic epidermal necrolysis are caused by cytotoxic CD8+ T cells (Hertl et al., 1995; Hari et al., 2001; Nassif et al., 2002). Recently, Britschgi et al. (2001) have shown that CD4+ T cells from patients with pustular reactions such as generalized exanthematous pustulosis secrete high levels of the neutrophil attracting chemokine IL-8.

Laboratory investigations have shown that blood mononuclear cells (PBMC) from hypersensitive patients but not from drug-naïve patients or patients administered CBZ without detectable adverse effects proliferate on exposure to CBZ (Zakrzewska and Ivanyi, 1988; Mauri-Hellweg et al., 1995; Brown et al., 1999; Hari et al., 2001). Cells from CBZ-hypersensitive patients have been shown to produce variable but occasionally high levels of IFN- γ and IL-5 (Koga et al., 2000; Leyva et al., 2000; Villada et al., 1992; Mauri-Hellweg et al., 1995; Schnyder et al., 1998), which is consistent with the pathophysiology of the adverse reaction. However, the precise role of T cells in CBZ hypersensitivity is not known.

To provide a definition of the role of T cells in CBZ hypersensitivity and further laboratory evidence of the immune pathogenesis, we cloned drug-specific T cells from the circulation of CBZ-hypersensitive patients and analyzed their phenotype, specificity, and cytokine profile in vitro. To investigate the structural requirements of T cell receptor activation, CBZ-specific T cell clones were stimulated with nine structurally related compounds.

Materials and Methods

Donor Characteristics. PBMCs were obtained from five CBZ-hypersensitive patients, four patients who had been administered

TABLE 1
Details of the carbamazepine-hypersensitive patients

Patient	Age	Gender	Time to Reaction	Details of Reaction	Time Since Reaction	Rechallenge
	years		days		months	
1	69	Male	21	Widespread maculopapular eruption with tissue eosinophilia	16	No
2	65	Female	42	Maculopapular eruption, fever, eosinophilia, abnormal liver function tests	72	No
3	65	Female	1 (rechallenge)	Desquamating rash with fever on inadvertent rechallenge; history of rash on initial exposure	<1 ^a	Positive
4	27	Female	1	Widespread maculopapular rash with mild fever and eosinophilia after one dose; history of allergy to multiple antibiotics and possible exposure to carbamazepine in the past with development of rash	2	Previous exposure likely
5	26	Male	21	Widespread rash with fever; history of allergy to multiple drugs including lamotrigine and phenytoin	24	No

^a The time blood was taken after rechallenge with CBZ.

CBZ for at least 12 months without adverse effects, and four healthy volunteers with no history of previous exposure to CBZ. Approval for the study was obtained from the local ethics committee, and informed consent was obtained from each participant. The clinical details of the CBZ-hypersensitive patients are shown in Table 1.

Culture Medium and Chemicals. Culture medium consisted of RPMI 1640 supplemented with 10% pooled heat-inactivated human blood type AB serum, HEPES buffer (25 mM), L-glutamine (2 mM), transferrin (25 $\mu\text{g}\cdot\text{ml}^{-1}$), streptomycin (100 $\mu\text{g}\cdot\text{ml}^{-1}$) and penicillin (100 U $\cdot\text{ml}^{-1}$). For culture of the T cell lines and clones, the medium was enriched with human recombinant IL-2 (60 U $\cdot\text{ml}^{-1}$). The above reagents were obtained from Sigma Chemical (Poole, Dorset, UK).

Epstein-Barr virus-transformed B-lymphoblastoid cell lines (B-LCL) were generated from the drug-allergic donors by transformation of PBMC with supernatant from the Epstein-Barr virus-producing cell line B9-58 (obtained from Dr. D. Neumann-Haefelin, University of Freiburg, Freiburg, Germany) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, HEPES buffer (25 mM), and L-glutamine (2 mM).

Phenylurea, ethylurea, dicyclohexylurea, diphenylurea, iminostilbene, dihydro-CBZ, CBZ-10,11-epoxide, phenytoin (all from Sigma Chemical) and lamotrigine (GlaxoSmithKline, Uxbridge, Middlesex, UK) were used at concentrations that did not inhibit the proliferative response to the mitogen phytohemagglutinin (1 $\mu\text{g}\cdot\text{ml}^{-1}$) for T cell culture. Stock solutions (10 mg $\cdot\text{ml}^{-1}$) were prepared in a mixture of culture media and dimethyl sulfoxide (4:1 v/v) and diluted before use. Tetanus toxoid (TT; Serum and Vaccine Institute, Bern, Switzerland) was used as a control protein antigen. All general reagents were purchased from Sigma Chemical (Poole, Dorset, UK) and were of the best available grade.

Determination of Carbamazepine-Specific Proliferation In Vitro. Proliferation of PBMC from patients and control volunteers was measured using the lymphocyte transformation test as described previously (Nyfeler and Pichler, 1997). Briefly, freshly isolated PBMC (1.5×10^5 ; total volume, 0.2 ml) was incubated with CBZ (1–100 $\mu\text{g}\cdot\text{ml}^{-1}$), CBZ-10,11-epoxide (1–100 $\mu\text{g}\cdot\text{ml}^{-1}$; hypersensitive patients 1 and 2 only), or TT (0.1 and 1 $\mu\text{g}\cdot\text{ml}^{-1}$) in 96-well U-bottomed tissue culture plates for 6 days (37°C; 5% CO₂). Proliferation was determined by the addition of [³H]thymidine (0.5 μCi) for the final 16 h of the incubation period. Cells were harvested, and [³H]thymidine incorporation was measured as cpm on a β -counter (PerkinElmer Life Sciences, Boston, MA). Proliferative responses were calculated as stimulation indices (SI; cpm in drug-treated cultures/cpm in cultures with dimethyl sulfoxide alone).

After a 6-day incubation with CBZ (10 $\mu\text{g}\cdot\text{ml}^{-1}$), PBMC from hypersensitive patients 1 and 2 were stained with phosphatidylethanolamine-labeled anti-CD4+/CD8+ and fluorescein isothiocyanate-labeled anti-HLA-DR antibodies (Serotec, Oxford, UK). Cells were

stained in phosphate-buffered saline (50 μ l) containing fetal calf serum (1%) and NaN_3 (0.02%) at 4°C. After 30 min, the cells were washed, and fluorescence was measured on a Coulter EPICS XL flow cytometer (Beckman Coulter Inc., Fullerton, CA).

Determination of Carbamazepine-Specific Cytotoxic Activity In Vitro. To enrich the quantity of CBZ-specific T cells, PBMC (5×10^6 ; total volume, 1 ml) from hypersensitive patients 1 and 5 were incubated with CBZ ($25 \mu\text{g}\cdot\text{ml}^{-1}$) in 24-well tissue culture plates (37°C; 5% CO_2); on day 4, IL-2 (60 $\text{U}\cdot\text{ml}^{-1}$) was added to preserve the CBZ-specific response. After 7 days, growing cells were restimulated with irradiated (45 Gy) autologous PBMC (1.5×10^5 /well), CBZ ($25 \mu\text{g}\cdot\text{ml}^{-1}$), and IL-2 (60 $\text{U}\cdot\text{ml}^{-1}$). This procedure was repeated for a further 3 weeks, and cytotoxic activity was measured on weeks 3 and 4 using a standard ^{51}Cr release assay (Brunner et al., 1968). Autologous B-LCLs (1×10^6) were labeled with ^{51}Cr (50 μ l; room temperature). After 1 h, the B-LCLs were washed (3×50 ml) to remove free ^{51}Cr and then incubated (2.5×10^3 ; total volume, 0.2 ml) with T cell-enriched PBMC (0.6×10^5) in the presence and absence of CBZ (10 and 50 $\mu\text{g}\cdot\text{ml}^{-1}$). Specific lysis was calculated as $100 \times (\text{experimental release} - \text{spontaneous release})/\text{maximal release} - \text{spontaneous release}$. Direct CBZ-specific toxicity was investigated by incubating CBZ (50 $\mu\text{g}\cdot\text{ml}^{-1}$) with ^{51}Cr -loaded B-LCL (2.5×10^3) for 4 h, in the absence of T cells.

The ^{51}Cr release assay also was used to assess the cytotoxic activity of three CBZ-specific T cell clones. In these experiments, the concentration of CBZ used was 50 $\mu\text{g}\cdot\text{ml}^{-1}$ and the effector/target ratios were 3:1, 10:1, and 30:1.

Generation of Carbamazepine-Specific T Cell Lines and Clones. For the generation of T cell lines, PBMC (2×10^6 ; total volume, 1 ml) from hypersensitive patients 1 and 2 were incubated with CBZ ($25 \mu\text{g}\cdot\text{ml}^{-1}$) in 24-well tissue culture plates. On days 6 and 9, IL-2 (60 $\text{U}\cdot\text{ml}^{-1}$) was added to maintain drug-specific proliferation. After 14 days, T cell lines were cloned by serial dilution, as described previously (Schnyder et al., 1997). In brief, cells from the above cultures were diluted to 3 to 30 $\text{cells}\cdot\text{ml}^{-1}$, dispensed (100 μ l/well) into 96-well U-bottomed tissue culture plates, and stimulated with irradiated (45 Gy) allogenic PBMC (0.5×10^5 /well) and the mitogen phytohemagglutinin (5 $\mu\text{g}\cdot\text{ml}^{-1}$). Growing cells were expanded in culture media containing IL-2 (60 $\text{U}\cdot\text{ml}^{-1}$) and restimulated on day 14.

To test the specificity of the clones (28 days after serial dilution), T cell clones (0.5×10^5 ; total volume, 0.2 ml) were incubated with autologous-irradiated (60 Gy) B-LCL (0.1×10^5) and CBZ (25 $\mu\text{g}\cdot\text{ml}^{-1}$) in U-bottomed 96-well tissue culture plates. After 48 h, [^3H]thymidine was added, and proliferation was measured 16 h later by scintillation counting as described above. Cell cultures with an SI of greater than or equal to 2.5 were expanded further.

Characterization of Carbamazepine-Specific T Cell Clones. CD phenotype of the T cell clones was measured by flow cytometry using fluorescent-labeled anti-CD3, anti-CD4, and anti CD8 antibodies. Monoclonality was demonstrated by T cell receptor $\text{V}\beta$ -chain staining using an anti-CD3 antibody and a panel of 25 antibodies that detect more than 75% of all known $\text{V}\beta$ families (Immunotech,

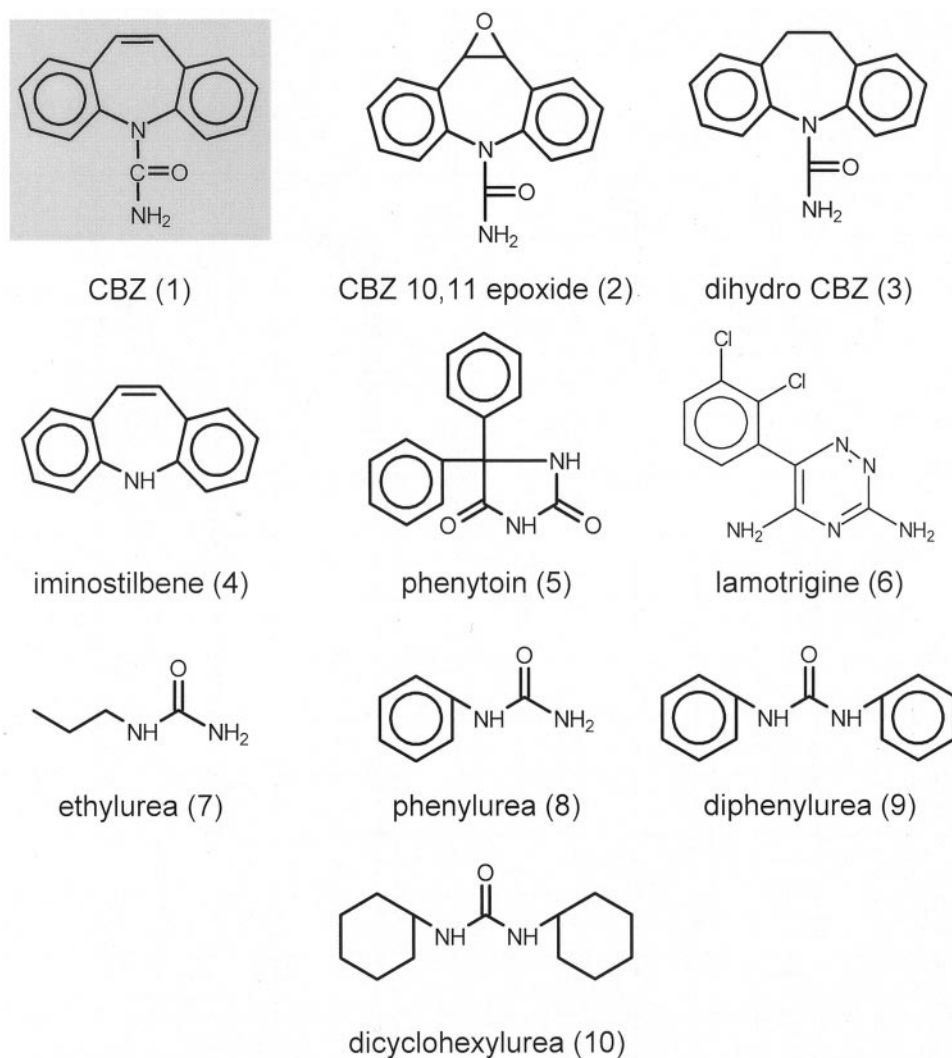


Fig. 2. Chemical structures of CBZ, CBZ metabolites, CBZ analogs, and structurally unrelated anticonvulsants. Each compound has been given a number, which is used in Fig. 7.

Marseille, France) (Zanni et al., 1997). Cells were stained as described above, and fluorescence was measured by flow cytometry.

To investigate the specificity of the interaction between CBZ and the T cell receptor, T cell clones were incubated with CBZ ($1\text{--}100\ \mu\text{g}\cdot\text{ml}^{-1}$) and nine structurally related compounds (10 and $50\ \mu\text{g}\cdot\text{ml}^{-1}$) (Fig. 2). These compounds include the widely used anticonvulsants phenytoin and lamotrigine and CBZ-10,11-epoxide, a major metabolite formed in humans after administration of CBZ (Maggs et al., 1997). Proliferation was determined after 48 h, as described above.

Restriction of the CBZ-specific T cell clones was determined: first, by the addition of specific anti-HLA blocking antibodies (anti-class I, anti-DR, anti-DP, and anti-DQ [all obtained from Dr. E. Padovan, University of Basel, Basel, Switzerland]) to the proliferation assay at concentrations known to inhibit MHC-restricted stimulations of CD8+ and CD4+ T cell clones (Schnyder et al., 1997); and second, by measuring proliferation with partly HLA-matched and unmatched B-LCLs as antigen-presenting cells. To exclude self-presentation by

HLA-DR+ T cells, certain incubations contained T cells and CBZ in the absence of antigen-presenting cells. For matching HLA alleles, HLA-typing was performed by PCR in our tissue-typing laboratories.

Measurement of Cytokine Levels in Cell Culture Supernatant. Cell culture supernatant from the proliferation assay was taken after 48 h to measure cytokine levels by ELISA. Supernatant of incubations containing T cells and B-LCLs in the absence of CBZ was taken as a control. The following ELISA kits were used: IL-4, IL-10, IFN- γ (Diaclone, Besancon, France), and IL-5 (BD PharMingen, San Diego, CA). Samples were diluted in duplicate (1:10, 1:100, and 1:1000; v/v), and cytokine levels were measured according to standard protocols of the corresponding ELISA kit. The detection limits were $1\ \text{pg}\cdot\text{ml}^{-1}$ for IL-4, $8\ \text{pg}\cdot\text{ml}^{-1}$ for IL-5, and $13\ \text{pg}\cdot\text{ml}^{-1}$ for IL-10 and IFN- γ .

Statistical Analysis. All data are expressed as mean \pm S.D. The Mann-Whitney test was used for comparison of control and test values, accepting $P < 0.05$ as significant.

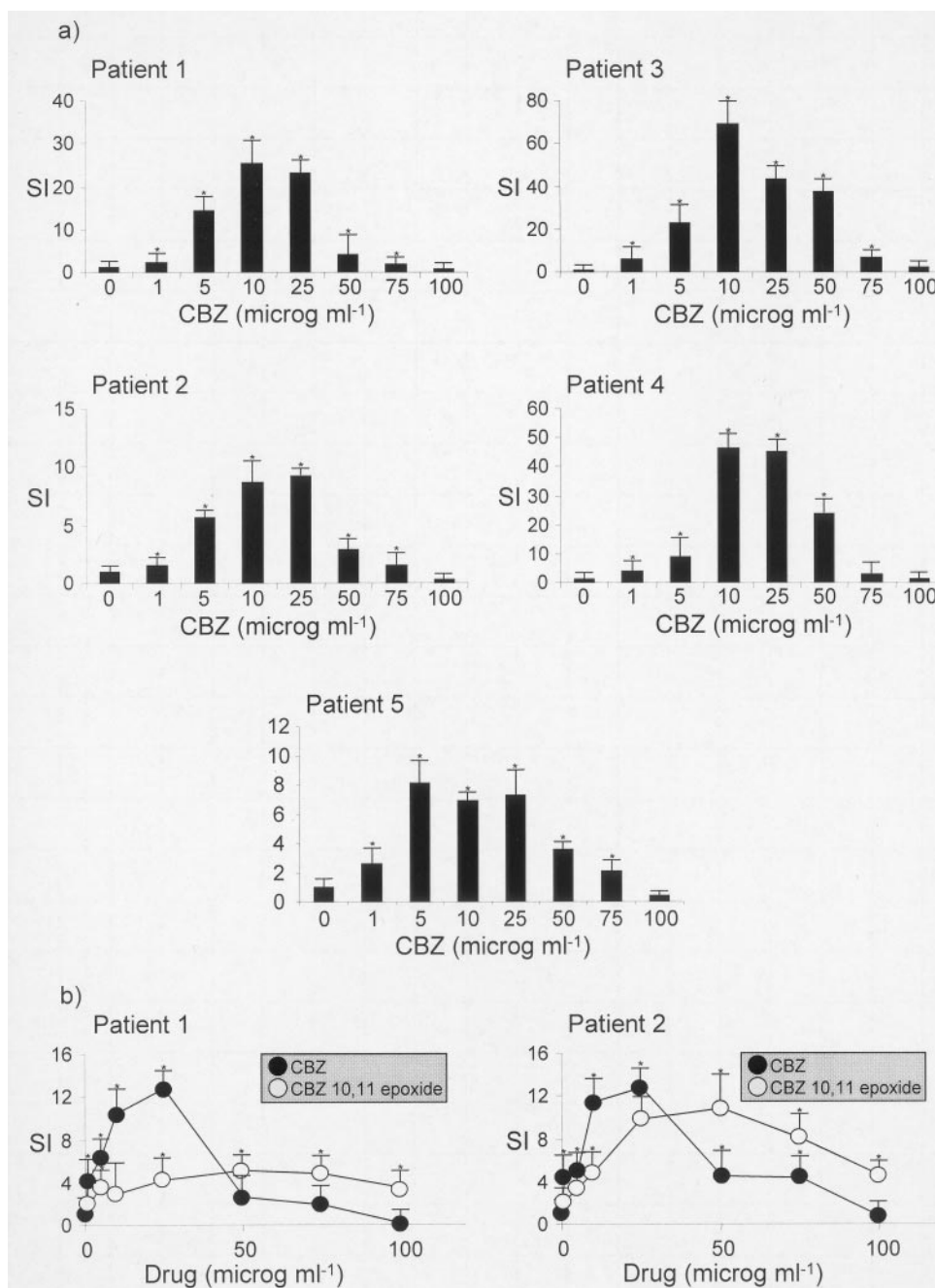


Fig. 3. Demonstration of dose-dependent (a) CBZ-specific and (b) CBZ-10,11-epoxide-specific PBMC proliferation in vitro. PBMC ($1.5 \times 10^5/\text{well}$) from hypersensitive patients were incubated with CBZ ($1\text{--}100\ \mu\text{g}\cdot\text{ml}^{-1}$) or CBZ-10,11-epoxide ($1\text{--}100\ \mu\text{g}\cdot\text{ml}^{-1}$) for 6 days, and proliferation was measured by [^3H]thymidine incorporation. Results are expressed as mean SI \pm S.D. of triplicate cultures. The cpm in control incubations was consistently less than 1500. Statistical analysis was performed by comparing incubations in the presence of drug with those in the absence of drug (*, $P < 0.05$).

Results

In Vitro Stimulation of PBMC by Carbamazepine.

Incubation of CBZ with PBMC from hypersensitive patients, but not from the two control groups (SI: 1.3 ± 0.4 for CBZ-exposed nonhypersensitive; SI: 1.1 ± 0.3 for nonexposed; $10 \mu\text{g}\cdot\text{ml}^{-1}$), caused a strong proliferative response, as measured by the incorporation of [^3H]thymidine. Proliferation was seen with 1 to $50 \mu\text{g}\cdot\text{ml}^{-1}$ CBZ (Fig. 3a), whereas concentrations of $75 \mu\text{g}\cdot\text{ml}^{-1}$ and greater inhibited proliferation without causing direct toxicity (determined by trypan blue dye exclusion; results not shown). Flow cytometric analysis of CBZ-stimulated PBMC on day 6 revealed an up-regulation in the expression of HLA-DR molecules on CD4+ and CD8+ T cell subsets from hypersensitive patients 1 ($3.8 \pm 0.5\%$ CD4+ and $7.7 \pm 1.2\%$ CD8+) and 2 ($6.2 \pm 0.4\%$ CD4+ and $0.8 \pm 0.2\%$ CD8+). PBMC also proliferated in the presence of the major metabolite of CBZ, CBZ-10,11-epoxide (Fig. 3b). No significant difference in TT ($1 \mu\text{g}\cdot\text{ml}^{-1}$)-specific proliferation was seen when patient and control PBMCs were compared ($P > 0.05$).

The proliferative response of PBMC to CBZ from hypersensitive patients was reproduced in further experiments using fresh PBMC approximately 6 months after the first blood donation (results not shown).

Carbamazepine-Specific T Cells Display Cytotoxic Activity Against Autologous B-LCL. T-cell-enriched PBMC from CBZ hypersensitive patients showed a dose-dependent cytotoxic response against autologous B-LCLs (Fig. 4a). In addition, two of three CBZ-specific T cell clones incubated with CBZ were able to kill ^{51}Cr -loaded B-LCL with an effector/target ratio of 10:1 and greater (Fig. 4b). Incuba-

tion of CBZ with ^{51}Cr -loaded B-LCL alone did not cause significant ^{51}Cr release ($P > 0.05$).

Phenotype and Specificity of Carbamazepine-Specific T Cell Clones. More than 500 T cell clones were generated from PBMCs of hypersensitive patients 1 and 2. Thirty-nine CBZ-specific T cell clones were obtained from patient 1 (SI: 11.2 ± 15.7 ; range, 2.3–80.3), and 5 were obtained from patient 2 (SI: 20.1 ± 11.2 ; range, 3.0–31.4); 10 wells growing clones were chosen for further analysis. Seven clones were of the CD4+ phenotype, whereas three clones stained double-positive (i.e., the same cells expressed both CD4+ and CD8+); all clones expressed the $\alpha\beta$ T cell receptor. Of the 10 clones, 7 expressed a single V β chain (Fig. 5), whereas the V β chain of the other clones was not detected with our panel of 25 antibodies. Four clones expressed the T cell receptor V β 5.1, which suggests a preferential usage in CBZ hypersensitivity reactions.

All of the clones responded to CBZ in a dose-dependent manner (Fig. 5). The concentration required for maximal proliferation ranged between 5 and $50 \mu\text{g}\cdot\text{ml}^{-1}$; proliferation was also seen with $100 \mu\text{g}\cdot\text{ml}^{-1}$, a concentration that inhibited proliferation of PBMC (Fig. 3). Concentrations of CBZ greater than $100 \mu\text{g}\cdot\text{ml}^{-1}$ inhibited proliferation (results not shown).

T Cell Receptor Recognition of Carbamazepine by Antigen-Presenting Cells is HLA-DR- and HLA-DQ-Restricted. There was no proliferation when the T cell clones were stimulated with CBZ in the absence of B-LCL. These data indicate that T cells cannot present CBZ to each other (data not shown). In the presence of autologous B-LCL, the response to CBZ was HLA class II-restricted. Antibodies

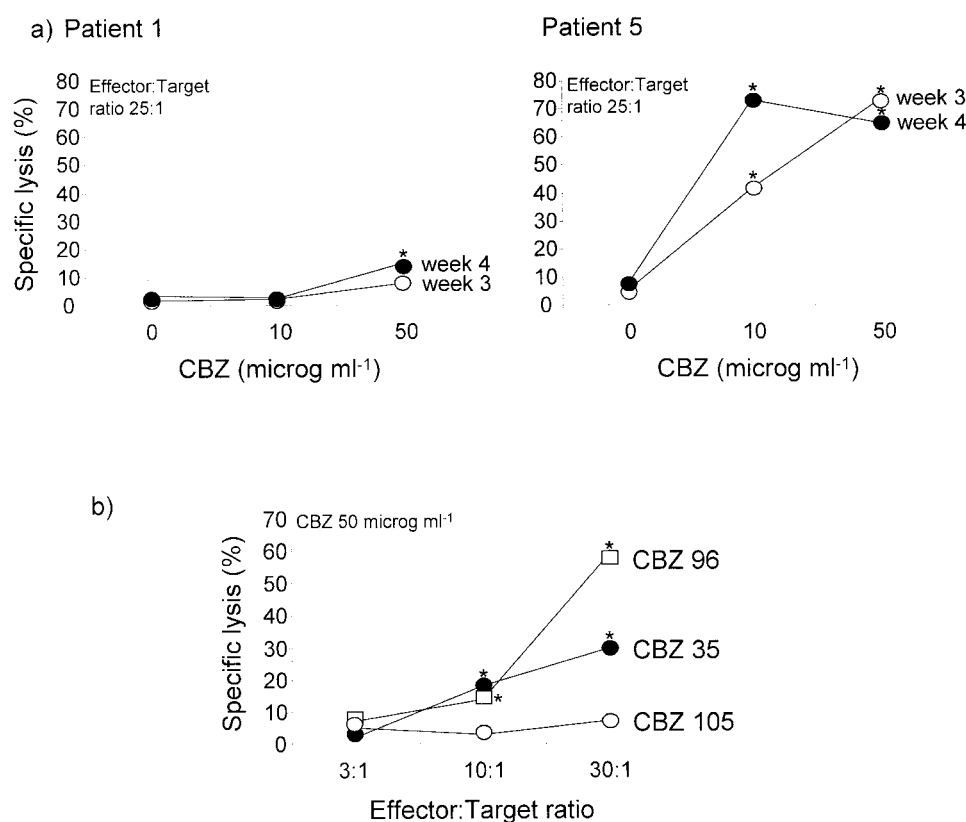


Fig. 4. Cytotoxic activity of (a) T cell-enriched PBMC (PBMC stimulated for 3–4 weeks with irradiated autologous PBMC and CBZ) and (b) T cell clones stimulated with CBZ. Cell death was measured in a 4-h ^{51}Cr -release assay. T cells were incubated with CBZ (10 – $50 \mu\text{g}\cdot\text{ml}^{-1}$) and ^{51}Cr -loaded B-LCL (2.5×10^3) at the indicated dilutions. Specific lysis was calculated as $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$. The value of spontaneous release from ^{51}Cr -loaded B-LCL was between 124 and 860 cpm, and the ratio of maximal release to spontaneous release ranged between 3.1 and 5.8. Statistical analysis was performed by comparing incubations in the presence of drug to those in the absence of drug (*, $P < 0.05$). The coefficient of variation was consistently less than 20%.

against HLA-DR and HLA-DQ, but not HLA-DP, inhibited proliferation. CBZ was presented to eight clones on HLA-DR and two clones on HLA-DQ. Figure 6 shows a panel of representative T cell clones.

Previous studies have demonstrated that approximately 75% of HLA-DR-restricted drug-specific T cell clones (von Greyerz et al., 2001) recognize drug antigens in the presence of HLA-matched antigen-presenting cells, and the other clones proliferated irrespective of the HLA phenotype of the antigen presenting cell used. In these studies, hypersensitive

patient 1 was positive for HLA-DRB1*15. All of the HLA-DR-restricted T cell clones from this patient showed a CBZ-specific proliferative response in the presence of allogenic HLA-matched B-LCL, whereas no proliferation was seen with HLA-unmatched B-LCL. Hypersensitive patient 2 was positive for HLA-DRB1*04 and *09. CBZ was not presented by unmatched B-LCL or B-LCL partly matched with either allele.

Cytokine Production by Carbamazepine-Specific T Cell Clones. Analysis of CBZ-specific T cell clones revealed

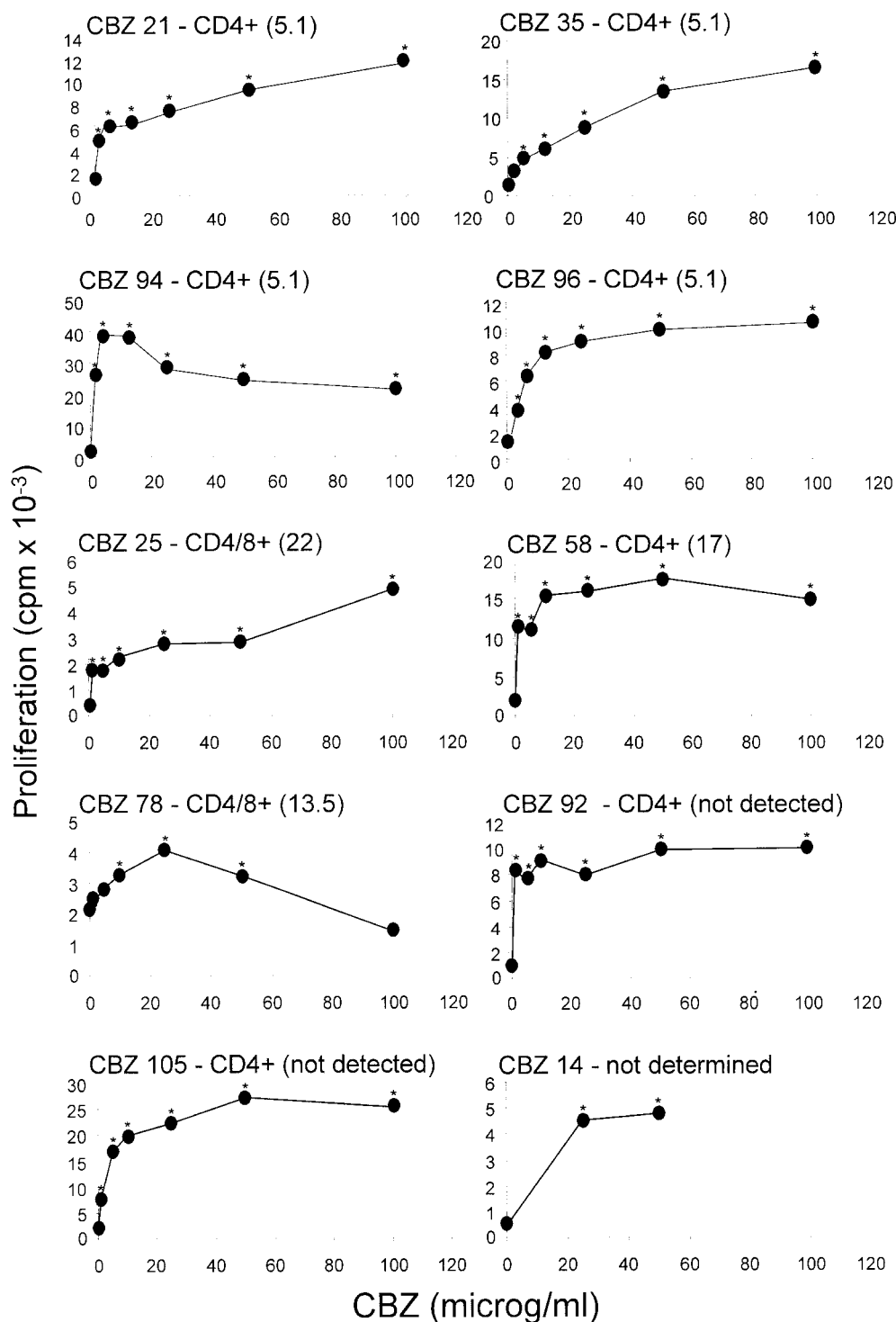
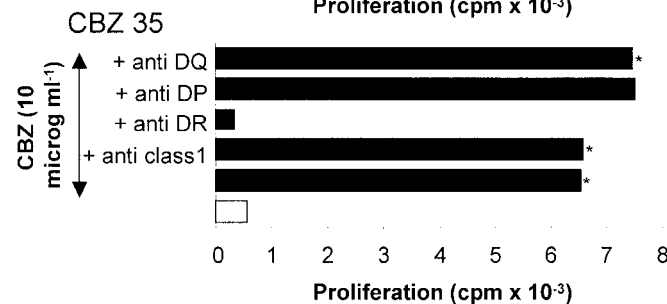
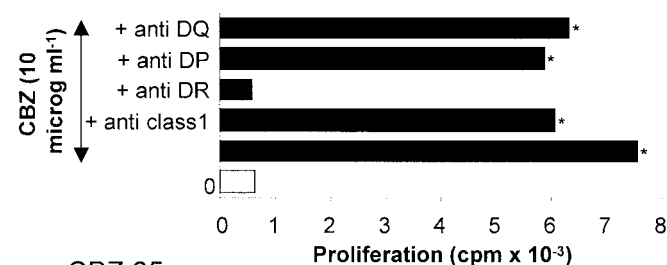


Fig. 5. Phenotype and dose-dependent proliferation of CBZ-specific T cell clones. Each T cell clone is presented by the clone name and CD phenotype followed by the V β phenotype in parentheses. CD and T cell receptor V β phenotype were measured by flow cytometry using fluorescent-labeled antibodies. To measure proliferation, T cell clones (0.5×10^5 /well) were incubated with CBZ ($1-100 \mu\text{g}\cdot\text{ml}^{-1}$) and irradiated (60 Gy) autologous B-LCL (0.1×10^5) for 48 h as described under *Materials and Methods*. Results are given as mean [³H]thymidine incorporation of duplicate cultures. Statistical analysis was performed by comparing incubations in the presence of drug to those in the absence of drug (*, $P < 0.05$). The coefficient of variation was consistently less than 20%.

three distinct cytokine patterns: four clones produced low to moderate levels of IL-4, -5, and IFN- γ ; three clones produced particularly high amounts of the proinflammatory cytokine IFN- γ ; and two clones produced moderate levels of IFN- γ , but no IL-4, -5, or -10 (Table 2). High levels of IFN- γ were seen with T cell clones from hypersensitive patients 1 and 2.

Cross-Reactivity of Carbamazepine-Specific T Cell Clones. To study the structural requirements of drug-antigen recognition by the MHC-restricted T cell receptor, we analyzed the cross-reactivity pattern of CBZ-specific T cell clones with nine structurally related compounds (Fig. 2).

a) CBZ 21



b) CBZ 58

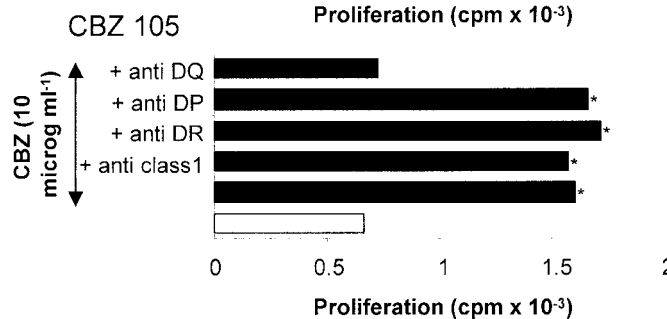
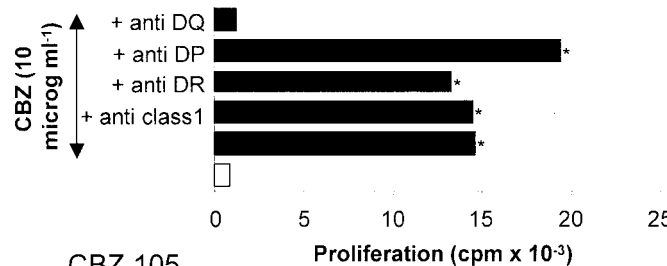


Fig. 6. HLA restriction of CBZ-specific T cell clones. T cell clones (0.5×10^6 /well) were incubated with irradiated (60 Gy) autologous B-LCL (0.1×10^6) and CBZ ($10 \mu\text{g}\cdot\text{ml}^{-1}$) in the presence of monoclonal antibodies against MHC class I or MHC class II (DR, DP, and DQ). Results are given as mean [^3H]thymidine incorporation of duplicate cultures. Statistical analysis was performed by comparing incubations in the presence of drug to those in the absence of drug (*, $P < 0.05$). The coefficient of variation was consistently less than 20%.

Cross-reactivity was seen with dihydro-CBZ (structure 3) and CBZ-10,11-epoxide (structure 2); however, any further structural modification inhibited proliferation. Four clones proliferated in the presence of dihydro-CBZ, five clones proliferated in the presence of CBZ-10,11-epoxide, and three clones were cross-reactive. Four clones did not recognize any compounds other than CBZ. Figure 7 shows the cross-reactivity profile of three representative T cell clones.

With the exception of clone CBZ 25, the capacity of dihydro CBZ and CBZ-10,11-epoxide to stimulate proliferation was significantly lower than that seen with CBZ itself. Clone CBZ 25, which responded weakly with CBZ (results represent the mean of three separate experiments), proliferated more vigorously in the presence of CBZ-10,11-epoxide (Fig. 8).

Discussion

Although relatively rare, CBZ hypersensitivity reactions are a cause of significant patient morbidity and mortality and can prevent effective drug therapy, especially if the drug has to be withdrawn (Leeder, 1998). Hypersensitivity reactions occur as a result of an individual's immune system responding to an inappropriate stimulus, which may take the form of the parent drug or a drug-modified self-protein subsequent to drug bioactivation.

Zakrzewska and Ivanyi (1988) and others (Mauri-Hellweg et al., 1995; Brown et al., 1999; Hari et al., 2001) have shown CBZ-specific proliferation of PBMC isolated from the peripheral blood of hypersensitive patients. Our data extend these initial findings: PBMC from five hypersensitive patients proliferated vigorously when challenged in vitro with therapeutic CBZ concentrations that are estimated to be in the range of 5 to $10 \mu\text{g}\cdot\text{ml}^{-1}$ (Tomson et al., 1980) ($\text{SI}: 32.1 \pm 24.2$; CBZ $10 \mu\text{g}\cdot\text{ml}^{-1}$) (Fig. 3). Proliferation was dose-dependent, and both CD4+ and CD8+ T cells expressed elevated levels of the activation marker HLA-DR. PBMCs were also stimulated by CBZ-10,11-epoxide ($25\text{--}75 \mu\text{g}\cdot\text{ml}^{-1}$), a major metabolite formed after administration of CBZ to patients (Madden et al., 1996). Previous work failed to detect T cells responsive to the epoxide metabolite (Zakrzewska and Ivanyi, 1988); however, Zakrzewska and Ivanyi used only three concentrations of CBZ-10,11-epoxide (1, 10, and $100 \mu\text{g}\cdot\text{ml}^{-1}$), which in our study caused nominal proliferation.

In a retrospective study derived from the clinical history of 923 drug allergic patients, the lymphocyte transformation test was shown to have a sensitivity (i.e., frequency of a positive result in drug-allergic patients) and specificity (i.e.,

TABLE 2

Cytokine secretion from CBZ-stimulated T cell clones

T-cell clone CBZ 14 was not studied. Numbers in parentheses refer to cpm in control incubations that contained T cells and irradiated B-LCL but not CBZ.

T Cell Clone	SI	IL-4	IL-5	IL-10	IFN- γ
<i>pg/ml</i>					
21	6.4(1872)	280	1,176	1,140	119
25	13.9(342)	51	390	110	498
35	11.6(1450)	280	1,176	1,140	44,900
58	9.3(1863)	27	83	N.D.	1,147
78	1.8(2241)	N.D.	N.D.	N.D.	585
92	12.5(824)	261	N.D.	N.D.	34,470
94	15.7(2371)	N.D.	N.D.	N.D.	153,000
96	4.7(1896)	N.D.	N.D.	N.D.	1,175
105	12.8(2174)	250	164	750	1,655

N.D., not detectable.

frequency of a negative result in nonallergic patients) of 78% and 85%, respectively (Nyfeler and Pichler, 1997). A recent prospective study of 22 patients by Hari et al. (2001) produced similar findings, with the lymphocyte transformation test being positive in 67% of drug-allergic patients. In our small cohort of CBZ-hypersensitive patients and controls, the

lymphocyte transformation test was positive in only the hypersensitive patients. The potential use of this test in a clinical situation requires further investigation, but it could include the identification of the culprit drug in a patient on multiple drugs and determination of cross-reactivity.

To further investigate the nature of the cellular immune response, drug-specific T cells were cloned and characterized. CD4⁺ and CD4⁺/CD8⁺ T cell clones were generated, and drug stimulation triggered proliferation and cytotoxicity (Figs. 3 and 4). All of the CBZ-specific clones expressed the $\alpha\beta$ T cell receptor, and the use of at least four different V β genes shows that the response to CBZ was polyclonal. Four clones expressed V β chain 5.1 (three from hypersensitive patient 1, one from hypersensitive patient 2), which suggests a preferential use in CBZ-hypersensitivity reactions. It is possible that clones which express the same V β chain derive from a single cell, with its outgrowth being a consequence of the cloning procedure; this, however, seems unlikely because CBZ was presented on HLA-DR and HLA-DQ, and cross-reactivity and cytokine profiles were heterogeneous (see below).

A high level of the proinflammatory cytokine IFN- γ was detected in the supernatant of several CBZ-stimulated T cell clones. These data are in line with previous investigations by Leyva et al. (2000) showing the overexpression of IFN- γ mRNA in PBMC isolated from CBZ-hypersensitive patients, and they explain the enhanced expression of HLA-DR on epidermal keratinocytes at the time of the hypersensitivity reaction (Friedmann et al., 1994). In mice, IFN- γ production by CD4⁺ T cells is involved in delayed-type cutaneous reactions observed after the topical exposure of dinitrochloroben-

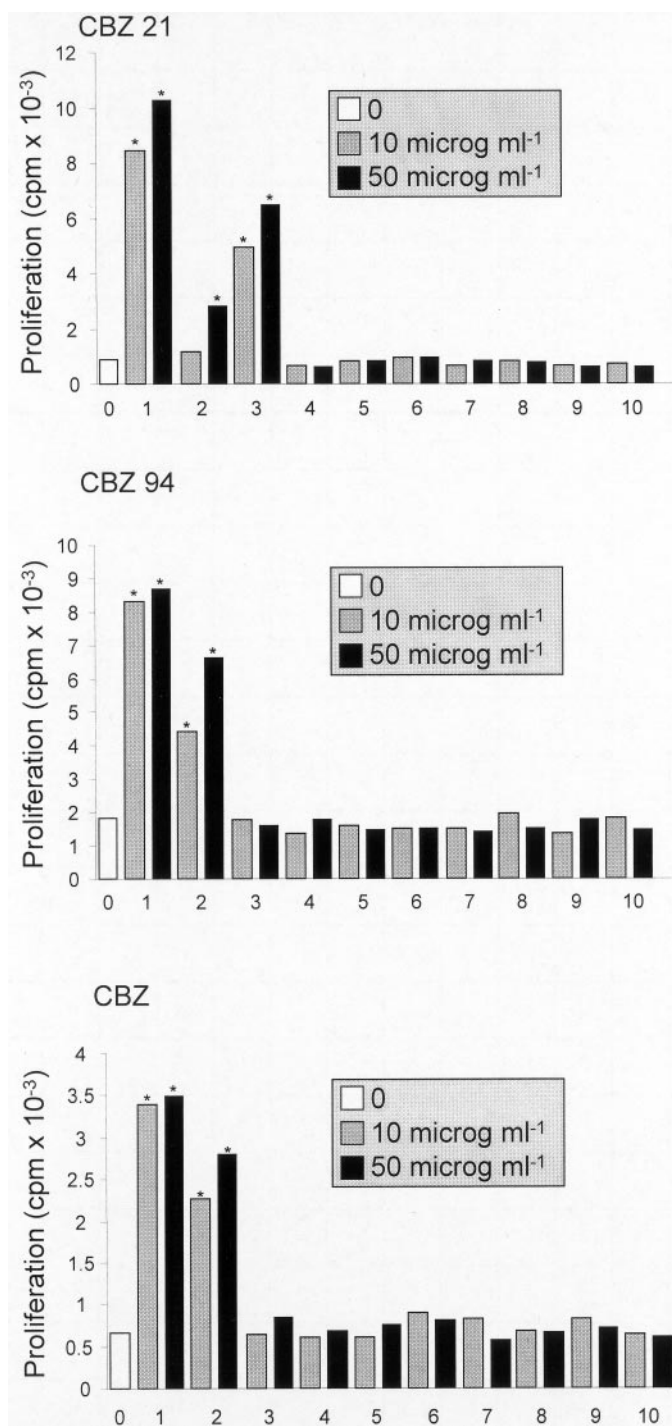


Fig. 7. Stimulation of T cell clones by CBZ, CBZ metabolites, structural analogs, and structurally unrelated anticonvulsants. Proliferation was measured after 48 h by [³H]thymidine incorporation. Results are given as mean [³H]thymidine incorporation \pm S.D. of duplicate cultures. Numbers refer to the structures displayed in Fig. 2. Statistical analysis was performed by comparing incubations in the presence of drug to those in the absence of drug (*, $P < 0.05$). The coefficient of variation was consistently less than 20%.

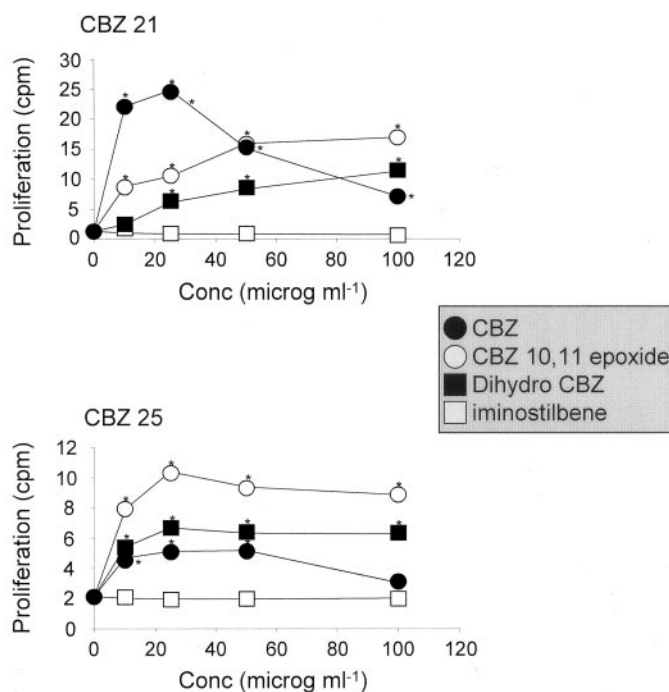


Fig. 8. Dose-dependent proliferation of two representative T cell clones stimulated with CBZ, CBZ-10,11-epoxide, dihydro-CBZ, and iminostilbene. Proliferation was measured after 48 h by [³H]thymidine incorporation. Results are given as mean [³H]thymidine incorporation of duplicate cultures. Statistical analysis was performed by comparing incubations in the presence of drug with those in the absence of drug (*, $P < 0.05$). The coefficient of variation was consistently less than 20%.

zene (Dearman et al., 1996a,b) and in hypersensitivity reactions solely affecting the liver (Ohta et al., 2000).

A prominent clinical feature of CBZ hypersensitivity is an increase in the number of circulating eosinophils (Leeder, 1998). IL-5 is essential for the growth and activation of eosinophils, and most drug-specific CD4⁺ T cells from patients with drug-induced maculopapular eruptions (the eosinophil count is also increased in these reactions) release high levels of IL-5 (Pichler et al., 1997; Yawalker et al., 2000b). Our panel of CBZ-specific T cell clones secreted low to moderate amounts of IL-5; this may have been adequate to account for the eosinophilia observed in these reactions. Clearly, it is also possible that there may be other clones, which we did not characterize, that are particularly high secretors of IL-5.

Although most T cells recognize drug antigens in the context of MHC expressed on the surface of antigen-presenting cells (von Greyl et al., 1999), chemicals such as isopentenyl pyrophosphate and fluorescein activate T cells via a direct interaction with the T cell receptor (Diamond et al., 1991; Morita et al., 1995). In our studies, there was no proliferation when the T cell clones were incubated with CBZ in the absence of antigen-presenting cells. These data indicate that T cells do not present CBZ to each other. Unlike previous studies investigating the involvement of T cells in drug-mediated allergic disease, in which certain clones responded in an MHC-restricted but MHC-allele unrestricted way (von Greyl et al., 2001), no stimulation was seen when the T cell clones were incubated with CBZ- and MHC-mismatched antigen-presenting cells. Most clones recognized CBZ in the context of HLA-DR; however, CBZ was presented to two clones on HLA-DQ. These data are in accordance with the recently observed MHC gene polymorphism associated with severe CBZ-hypersensitivity reactions (Pirmohamed et al., 2001).

In CBZ hypersensitivity, the nature of the drug antigen presented on MHC (i.e., CBZ or a CBZ metabolite peptide conjugate) to T cells is not known. It is possible that CBZ (and CBZ-10,11-epoxide) is metabolized to a reactive intermediate in situ in the proliferation assay; however, using the bioanalytical techniques that are currently available, it is not possible to detect such low levels of metabolic activation. Therefore, to attain a greater understanding of the structural requirements of T cell receptor activation, CBZ-specific T cell clones were stimulated with a panel of nine structurally related compounds, which included the commonly administered anticonvulsants lamotrigine and phenytoin. Structural modification alters the pharmacokinetic properties of CBZ, which may in turn affect its subcellular distribution within the MHC-restricted T cell receptor and/or the active site of cytochrome P450. Our data show that the drug-T cell receptor interaction is highly regulated (i.e., small structural changes inhibited proliferation) and are in accordance with previous studies with sulfamethoxazole (Schnyder et al., 1997; von Greyl et al., 1999). Modification of the nonaromatic double bond on the CBZ molecule (i.e., dihydro-CBZ [structure 3] and CBZ-10,11-epoxide [structure 2]) could be accommodated by the MHC T cell receptor binding site; however, further structural modification inhibited the interaction with either the MHC or T cell receptor. Removal of the amide side chain (iminostilbene [4], a known CBZ metabolite) (Csetenyi et al., 1973), which is not necessary for the interaction of CBZ with the active site of cytochrome P450

(Riley et al., 1989), or the aromatic ring completely abolished T cell receptor activation. The apparent direct stimulation of T cells by parent drug suggests that metabolic activation is not a prerequisite for T cell receptor activation, at least in vitro. However, further experiments using specific inhibitors of drug metabolism and/or antigen processing alongside more sophisticated and sensitive bioanalytical methodology is required to confirm this concept. Because the T cell clones were generated by culturing PBMC with soluble CBZ, these studies do not exclude the possibility that hypersensitive patients have T cells that recognize a CBZ metabolite protein conjugate; in ongoing investigations, we are trying to select clones derived from proliferation with CBZ metabolites and/or CBZ-modified protein. One clone that proliferated weakly with CBZ showed a more vigorous proliferation in the presence of CBZ-10,11-epoxide (Fig. 8). These data suggest that the patient may have T cells that respond to both CBZ and CBZ-10,11-epoxide in vivo.

In conclusion, CBZ was presented on MHC class II expressed on the surface of antigen-presenting cells to the T cell receptor of CD4⁺ T cells. T cell-receptor activation resulted in proliferation, cytotoxicity, and secretion of high levels of IFN- γ . These studies show that patients with CBZ have a higher proportion of IFN- γ -secreting cells, which might be relevant for the more general clinical symptoms seen in patients with CBZ hypersensitivity, particularly the accompanying hepatitis. Additionally, the cells had a phenotype that was qualitatively different from those seen in other serious cutaneous adverse drug reactions, such as bullous and pustular rashes.

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