

Characterization of drug-specific T cells in lamotrigine hypersensitivity

Dean J. Naisbitt, PhD,^a John Farrell, BSc,^a Gavin Wong, MBChB,^b
Jan P. H. Depta, BSc,^c Charlotte C. Dodd, PhD,^a Josephine E. Hopkins, BSc,^a
Claire A. Gibney, BSc,^a David W. Chadwick, FRCP,^d Werner J. Pichler, MD,^c
Munir Pirmohamed, FRCP,^a and B. Kevin Park, PhD^a *Liverpool, United Kingdom, and
Bern, Switzerland*

Background: Lamotrigine is associated with hypersensitivity reactions, which are most commonly characterized by skin rash. An immune etiology has been postulated, though the nature of this is unclear.

Objectives: The aim of this study was to characterize the role of T cells in lamotrigine hypersensitivity.

Methods: A lymphocyte transformation test was performed on 4 hypersensitive patients. Lymphocytes from 3 of 4 lamotrigine-hypersensitive patients proliferated when stimulated with lamotrigine. T-cell clones were generated from one patient to further characterize the nature of the T-cell involvement. Cells were characterized in terms of their phenotype, functionality, and mechanisms of antigen presentation and cytotoxicity.

Results: Of the 44 drug-specific T-cell clones generated, most were CD4⁺ with occasional CD8⁺ cells. All clones expressed the $\alpha\beta$ T-cell receptor; several V β 5.1⁺ or 9⁺ T-cell clones were generated. All clones also expressed the skin-homing receptor cutaneous lymphocyte antigen. Lamotrigine-stimulated T cells were cytotoxic and secreted perforin, IFN- γ , IL-5, and macrophage inflammatory protein 1 α , macrophage inflammatory protein 1 β , RANTES, and I-309. Lamotrigine was present on HLA-DR and HLA-DQ by antigen-presenting cells in the absence of drug metabolism and processing. The T-cell receptor of certain clones could accommodate analogs of lamotrigine, but no cross-reactivity was seen with other anticonvulsants.

Conclusions: Our data provide evidence that T cells are involved in the pathogenesis of some lamotrigine-hypersensitivity reactions. The identification of drug-specific cells that express cutaneous lymphocyte antigen and type 1 cytokines after T-cell receptor activation is consistent with the clinical symptoms. Furthermore, identification of large numbers of V β 5.1⁺ T cells suggests that polymorphisms within T-cell receptor genes might act as determinants of susceptibility. (*J Allergy Clin Immunol* 2003;111:1393-1403.)

Key words: Lamotrigine, anticonvulsant hypersensitivity, T cells, antigen processing, cytokines, chemokines, T-cell receptors

Lamotrigine (LTG) is a widely used, broad-spectrum anticonvulsant with efficacy comparable to that of other major antiepileptic drugs. LTG is associated with a number of adverse effects, most of which are dose-dependent. LTG has also been reported to cause skin rashes in up to 10% of patients, the frequency of rashes can be reduced by a low starting dose and slow dose escalation. The cutaneous eruptions often occur as part of a generalized syndrome that is referred to as *hypersensitivity*¹ or with the acronym *DRESS* (drug rash with eosinophilia and systemic symptoms).² These reactions are similar to those reported with the aromatic anticonvulsants phenytoin, phenobarbital, and carbamazepine.

In general, drug-induced severe skin reactions can be divided into maculopapular exanthemas, bullous eruptions, and pustular eruptions on the basis of their clinical, cellular, and molecular pathophysiology.³ Demonstration of drug-specific proliferation of PBMCs from allergic individuals, but not individuals administered the drug in the absence of an adverse event, is consistent with immune system involvement.^{4,5} Classical immunochemical theory states that drugs are not antigenic per se but must covalently modify protein to stimulate an immune response.^{6,7} For most drugs, metabolic activation, a process often referred to as *bioactivation*,⁸ is a prerequisite to protein binding. Once formed, the drug-protein conjugate is taken up by antigen-presenting cells, and through a complex series of enzymatic reactions, peptide fragments are transported to the cell surface and presented to T cells in the context of MHC.^{9,10} However, recent in vitro evidence suggests that drugs can also be presented directly to T cells in an MHC-restricted manner.^{11,12} This pathway apparently avoids the requirement for drug metabolism and antigen processing.¹³

In relation to anticonvulsant hypersensitivity, PBMCs from patients hypersensitive to carbamazepine, phenobarbital, and phenytoin have been shown to proliferate in the presence of the suspect drugs,¹⁴⁻¹⁶ whereas immunohistochemical studies have shown the presence of activated CD4⁺ and CD8⁺ T cells.^{17,18} An increase in the number of circulating PBMCs that express the skin-homing molecule cutaneous lymphocyte antigen (CLA) has also been shown in the acute phase of carbamazepine

From the Departments of ^aPharmacology and Therapeutics and ^bDermatology, University of Liverpool, Liverpool, England; University of Liverpool; ^cthe Clinic for Rheumatology and Clinical Immunology/Allergology, Inselspital, University of Bern; and ^dthe Department of Neurological Sciences, University of Liverpool.

Supported by the Wellcome Trust. D.J.N. is a Wellcome Trust Research Career Development Fellow.

Received for publication November 11, 2002; revised February 26, 2003; accepted for publication March 4, 2003.

Reprint requests: Dean J. Naisbitt, PhD, Department of Pharmacology and Therapeutics, Sherrington Building, Ashton Street, The University of Liverpool, PO Box 147, Liverpool, L69 3GE, England.

© 2003 Mosby, Inc. All rights reserved.

0091-6749/2003 \$30.00 + 0

doi:10.1067/mai.2003.1507

TABLE I. Details of the lamotrigine hypersensitive patients

Patient no.	Age (y)	Sex	Time between start of drug and development of reaction (d)	Time between reaction and blood donation (y)	Details of reaction
1	32	Female	10	10	Generalized erythematous maculopapular eruption, fever, lymphadenopathy, eosinophilia
2	59	Female	30	9	Stevens-Johnson syndrome, fever
3	46	Female	25	9	Maculopapular eruption, fever
4	36	Male	21	5	Toxic epidermal necrolysis, renal failure, adult respiratory distress syndrome

Abbreviations used

B-LCL: EBV-transformed B-lymphoblastoid cell line
 CLA: Cutaneous lymphocyte antigen
 LTG: Lamotrigine
 MIP: Macrophage inflammatory protein
 SI: Stimulation index
 TT: Tetanus toxoid

hypersensitivity.¹⁹ We have recently shown that carbamazepine-specific T-cell clones from hypersensitive patients were CD4⁺ and secreted IFN- γ .²⁰ Preliminary data indicate that LTG hypersensitivity might also be immune-mediated,²¹ though the nature of the immune response and whether it is similar to or distinct from that observed with the other anticonvulsants is not known.

The aim of this study was therefore to explain the nature of the T-cell response in LTG hypersensitivity by characterizing drug-specific T cells in terms of their phenotype, functionality, and mechanisms of antigen presentation and cytotoxicity.

METHODS

Donor characteristics

PBMCs were obtained from the peripheral blood of 4 LTG-hypersensitive patients (Table I). All patients were free of immunosuppressive therapy for at least 24 months before blood donation. Controls were patients administered LTG for a long term without adverse effects ($n = 4$) and unexposed individuals ($n = 4$). Two LTG-exposed controls were on LTG therapy; 2 had not been exposed to LTG for greater than 12 months. Approval for the study was obtained from the local ethics committee, and informed consent was obtained from each participant.

Culture medium and chemicals

Culture medium consisted of RPMI-1640 supplemented with 10% pooled heat-inactivated human AB serum, HEPES buffer (25 mmol/L), L-glutamine (2 mmol/L), transferrin (25 μ g mL⁻¹), streptomycin (100 μ g mL⁻¹), and penicillin (100 U mL⁻¹). For culture of the T-cell lines and clones, the media were enriched with human recombinant IL-2 (60 U mL⁻¹).

Autologous EBV-transformed B-lymphoblastoid cell lines (B-LCLs) were generated from drug-allergic donors by transformation of PBMCs with supernatant from the EBV-producing cell line B9-58 (obtained from Dr D. Neumann-Haefelin, University of Freiburg, Freiburg, Germany). B-LCLs were cultured in RPMI-1640 supplemented with 10% FCS, HEPES buffer (25 mmol/L), and L-glutamine (2 mmol/L).

LTG and LTG analogs (2-methyl LTG, 3-methyl LTG, 4-methyl LTG, and LTG *N*-oxide) were donated by Glaxo Wellcome plc (Stevenage, United Kingdom). Carbamazepine and phenytoin were obtained from Sigma Chemical Co (Poole, United Kingdom). Tetanus toxoid (TT; Serum and Vaccine Institute, Bern, Switzerland) was used as a control protein antigen.

Determination of LTG-specific blood mononuclear cell proliferation in vitro

Freshly isolated PBMCs (1.5×10^5 ; total volume 0.2 mL) were incubated with LTG (1 to 100 μ g mL⁻¹) or TT (1 μ g mL⁻¹) in 96-well U-bottomed tissue culture plates for 6 days (37°C; 5% CO₂). Proliferation was measured by the addition of [³H] thymidine (0.5 μ Ci) for the last 16 hours of the incubation. Cells were harvested, and [³H] thymidine incorporation was measured as counts per minute on a β -counter (Perkin Elmer Life Sciences, Cambridge, United Kingdom). Proliferative responses were calculated as stimulation index (SI; counts per minute in drug-treated cultures/counts per minute in cultures with dimethyl sulfoxide alone).

In a separate experiment, PBMCs from hypersensitive patient 1 were incubated with LTG (5–100 μ g mL⁻¹), structural analogs of LTG (2-, 3-, and 4-methyl LTG and LTG *N*-oxide [5–100 μ g mL⁻¹]), and structurally unrelated anticonvulsants (carbamazepine and phenytoin [5–100 μ g mL⁻¹]). Proliferation was measured through use of the method described previously.

Generation and characterization of LTG-specific T-cell lines and clones

For generation of T-cell lines, PBMCs (2×10^6 ; total volume 1 mL) from hypersensitive patient 1 were incubated with LTG (10 μ g mL⁻¹). On days 6 and 9, IL-2 was added to maintain antigen-specific proliferation. After 14 days, T-cell lines were cloned by serial dilution, as described previously.¹¹ In addition, PBMCs were incubated with TT (1 μ g mL⁻¹), and IL-2 and TT-specific clones were generated. Hypersensitive patient 1 had a generalized erythematous maculopapular eruption develop 10 days after starting LTG therapy (Table I).

To test the specificity of the clones, T cells (0.5×10^5 ; total volume, 0.2 mL) were incubated with autologous irradiated (60 Gy) B-LCLs (0.1×10^5) and LTG (10 and 25 μ g mL⁻¹) or TT (1 and 10 μ g mL⁻¹). After 48 hours, [³H] thymidine was added and proliferation was measured by scintillation counting, as described previously. T-cell clones with an SI greater than or equal to 2.5 were restimulated and expanded in IL-2-containing medium.

LTG-specific T-cell clones were characterized in terms of CD phenotype and T-cell receptor V β expression by flow cytometry through use of a previously described protocol.²² Measurement of skin-homing receptor CLA expression was also measured by flow cytometry through use of a rat IgM anti-CLA antibody conjugated to FITC (Pharmingen, San Diego, Calif). Expression of CLA on the surface of LTG-specific clones was compared with CLA expressed on the surface of T-cell clones from the same patient that proliferate

in the presence of the recall antigen TT. TT-specific T-cell clones expressed a single V β chain and, as described previously,¹¹ proliferated in the presence of TT and irradiated, but not fixed, B-LCLs.

Determination of the functionality of LTG-specific T-cell clones

The functionality of the T-cell clones was investigated by measurement of LTG-specific (1–100 $\mu\text{g mL}^{-1}$) proliferation by thymidine incorporation (see earlier) and cytotoxicity through use of a 4-hour [⁵¹Cr] release assay (LTG, 10–50 $\mu\text{g mL}^{-1}$).²³ To measure immune-mediated killing, autologous B-LCLs (1×10^6) were labeled with [⁵¹Cr] (50 μL ; rt) for 1 hour. B-LCLs were washed ($3 \times 50 \text{ mL}$) to remove free [⁵¹Cr] and incubated (2.5×10^3 ; total volume 0.2 mL) with T cells at effector/target ratios of 3:1, 10:1, and 30:1 in the presence and absence of LTG (10 and 50 $\mu\text{g mL}^{-1}$). Specific lysis was calculated as $100 \times (\text{experimental release} - \text{spontaneous release})/(\text{maximal release} - \text{spontaneous release})$. Direct toxicity of LTG was excluded by incubating LTG (50 $\mu\text{g mL}^{-1}$) with [⁵¹Cr]-loaded B-LCLs (2.5×10^3) for 4 hours in the absence of T cells. To investigate the mechanism of immune-mediated killing, brefeldin A (10 $\mu\text{g mL}^{-1}$; inhibitor of FAS-mediated cytotoxicity) and concanamycin A (100 nmol/L; inhibitor of perforin-mediated cytotoxicity) were added to certain incubations. Perforin release by LTG-stimulated T-cell clones was confirmed by immunostaining with a mouse anti-perforin antibody (Pharmingen, San Diego, Calif) through use of a previously described protocol.²⁴

ELISA was used to measure cytokine levels in cell culture supernatants of LTG-stimulated T-cell clones. Cell culture supernatant from incubations containing T cells and B-LCLs in the absence of LTG was taken as a control. The following ELISA kits were used: IL-4, IL-10, IFN- γ (Diacclone, Besancon, France), and IL-5 (PharMingen, San Diego, Calif). The detection limits were 1 pg mL⁻¹ for IL-4, 8 pg mL⁻¹ for IL-5, and 13 pg mL⁻¹ for IL-10 and IFN- γ .

Chemokine mRNA expression in resting and LTG-stimulated T-cell clones was measured by RNase protection. T cells (1×10^6 ; total volume, 4 mL) were stimulated with LTG (25 $\mu\text{g mL}^{-1}$) or solvent alone and autologous irradiated (60 Gy) B-LCLs (2×10^5). Control incubations contained irradiated B-LCLs and LTG without T cells. After 4 and 16 hours, cells were harvested and dissolved in Trizol reagent (Gibco, Paisley, United Kingdom). RNA was prepared according to the manufacturer's instructions and hybridized overnight with the RiboQuant human chemokine hCK5 multiprobe template set, which detects Ltn, RANTES, IP-10, macrophage inflammatory protein (MIP) 1 α , MIP-1 β , monocyte chemoattractant protein 1 α , IL-8, and I-309. A custom-made template set that detects eotaxin in addition to the chemokines in the standard hCK5 kit was used in certain experiments. Densitometric analysis was carried out through use of Image 1.6.1 software (National Institutes of Health, Bethesda, Md). Quantitative data were expressed as a percentage of the RNA species derived from glyceraldehyde-3-phosphate dehydrogenase.

Determination of the mechanism of LTG presentation to T cells

LTG presentation to T cells was characterized in terms of (1) the nature of the drug antigen (ie, metabolism-dependent or -independent), (2) the role of antigen-presenting cells (ie, processing-dependent or -independent and HLA restriction), and (3) the interaction between LTG and the T-cell receptor. The role of metabolism in LTG presentation to T cells was studied by prepulsing B-LCLs (1×10^6 ; total volume 1 mL) with LTG (25 $\mu\text{g mL}^{-1}$) for 2 to 16 hours. Pulsed B-LCLs, which present covalently bound antigen but not parent drug,²⁵ were washed ($3 \times 4 \text{ mL}$), suspended in drug-free media, and added to the proliferation assay. In some experiments,

reduced glutathione (1 mmol/L), which binds irreversibly to protein-reactive LTG metabolites,²⁶ *N*-acetyl cysteine (1 mmol/L), or both were added.

The involvement of MHC in LTG presentation was assessed 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, Switzerland]) to the proliferation assay at concentrations known to inhibit MHC-restricted stimulations of CD8⁺ and CD4⁺ T-cell clones.⁹ To exclude self-presentation by HLA⁺ T cells, certain incubations contained T cells and LTG in the absence of antigen-presenting cells.

The role of processing in LTG presentation was determined first by chemical fixation of antigen-presenting cells²⁷ and second by measurement of the kinetics of T-cell receptor downregulation, which is an early parameter of antigen T-cell receptor engagement.¹³ B-LCLs (2×10^6 ; 0.5 mL) were fixed by the addition of glutaraldehyde (2%; 12.5 μL) for 30 seconds at room temperature. To determine the kinetics of T-cell receptor downregulation, T-cell clones (0.5×10^5 ; total volume, 0.2 mL) and B-LCLs (0.25×10^5) were incubated with LTG (10 and 50 $\mu\text{g mL}^{-1}$) in 96-well U-bottomed tissue culture plates. After 1, 4, and 16 hours, cells were harvested, washed, and stained for 30 minutes at 4°C with an FITC-labeled anti-CD3 mAb (Serotec Ltd., Oxford, United Kingdom). CD3 fluorescence was measured by flow cytometry (Coulter Epics, XL software; Beckman Coulter, Luton, United Kingdom). One hundred percent values represent the mean CD3 fluorescence of T cells incubated with B-LCLs in the absence of LTG.

The specificity of the interaction between LTG and the T-cell receptor was studied by incubating T-cell clones (0.5×10^5 ; total volume 0.2 mL) and B-LCLs (0.1×10^5) with LTG (10–75 $\mu\text{g mL}^{-1}$), structural analogs of LTG (2-, 3-, and 4-methyl LTG and LTG *N*-oxide [10–75 $\mu\text{g mL}^{-1}$]), and structurally unrelated anticonvulsants (carbamazepine and phenytoin [10 and 50 $\mu\text{g mL}^{-1}$]). Proliferation was measured 2 days after antigen stimulation through use of the method described previously.

Statistical analysis

Values to be compared were analyzed for non-normality by means of the Shapiro-Wilks test. Because the data were found to be non-normally distributed, we used the Mann-Whitney test for comparison of the 2 groups, accepting $P < .05$ as significant.

RESULTS

In vitro stimulation of blood mononuclear cells by LTG

PBMCs from 3 of 4 LTG-hypersensitive patients proliferated after in vitro stimulation with LTG (1–100 $\mu\text{g mL}^{-1}$; Fig 1, *a*). No proliferation was seen with PBMCs from hypersensitive patient 4 or the 2 control groups (Fig 1, *b*). PBMCs from the patients and controls also proliferated in the presence of TT used as a positive control (1 $\mu\text{g mL}^{-1}$).

PBMCs from LTG-hypersensitive patient 1 also proliferated in the presence of LTG-*N*-oxide (Fig 1, *c*). No cross-reactivity was seen with 2-, 3-, and 4-methyl LTG, carbamazepine, or phenytoin.

Phenotype of LTG-specific T-cell clones

Forty-four LTG-specific T-cell clones were generated from PBMCs of hypersensitive patient 1. Most of the T-cell clones were CD4⁺ (SI, 11.8 ± 11.1 [range, 2.9–55.0]; 50 $\mu\text{g mL}^{-1}$ LTG) with occasional CD8⁺ (SI, 2.5 and 8.9;

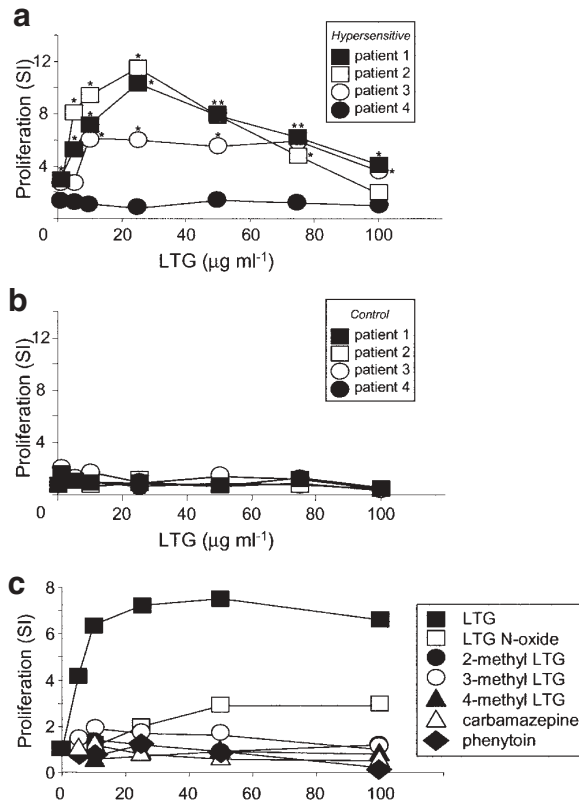


FIG 1. **a** and **b**, Proliferation of PBMCs from LTG-hypersensitive patients and controls. **c**, Proliferation of PBMCs from patient 1 with LTG and related compounds. Statistical analysis compares incubations with and without antigen ($*P < .05$).

50 $\mu\text{g mL}^{-1}$ LTG) cells. A significant increase in the expression of the skin-homing receptor CLA was seen on the surface of LTG-specific T-cell clones when resting LTG- and TT-specific T cells were compared (Fig 2).

All clones expressed the $\alpha\beta$ T-cell receptor. Flow cytometric analysis was used to assess T-cell receptor V β chain expression of 22 clones, and these clones were used in all subsequent experiments. Seventeen clones expressed a single V β chain. The V β chain of 4 clones was not detected with our panel of 25 antibodies, and 1 clone contained 50% V β 17-positive and 50% V β 19-positive cells and is hereinafter referred to as a cell line (these cells did not stain double-positive for V β 17 and 19). T-cell receptors V β 5.1 and 9 were expressed on the surface of 6 and 4 clones, respectively.

Despite repeated attempts, we could not generate T-cell clones from hypersensitive patient 2. Hypersensitive patients 3 and 4 refused to donate second blood samples.

Functionality of LTG-specific T-cell clones

Proliferation of the CD4 $^{+}$ and CD8 $^{+}$ LTG-specific T-cell clones was dose-dependent. Maximal proliferation was observed with 10 to 100 $\mu\text{g mL}^{-1}$ LTG (Fig 3, *a*); LTG concentrations greater than 100 $\mu\text{g mL}^{-1}$ inhibited prolifer-

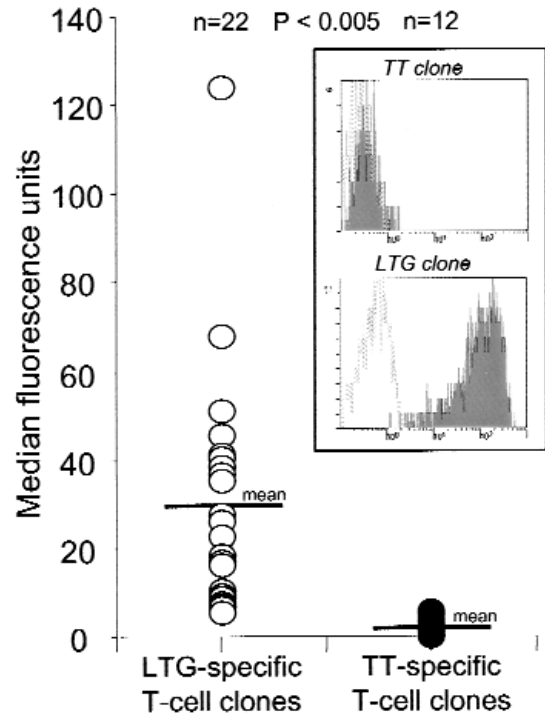


FIG 2. Cell surface expression of the skin-homing molecule CLA on LTG- and TT-specific T-cell clones.

ation (results not shown). CD8 $^{+}$ T-cell clones could not be expanded with phytohemagglutinin and, except for T-cell receptor characterization, were not analyzed further.

The cytotoxic activity of 4 CD4 $^{+}$ T-cell clones was tested through use of a 4-hour [^{51}Cr] release assay. Three LTG-stimulated (10 and 50 $\mu\text{g mL}^{-1}$) T-cell clones killed [^{51}Cr]-loaded autologous B-LCLs at an effector/target ratio of 10:1 and above (Fig 3, *a*). No [^{51}Cr] release was seen when B-LCLs were incubated with LTG (50 $\mu\text{g mL}^{-1}$) in the absence of T cells, which rules out a direct drug-mediated effect. Concanamycin A, which inhibits perforin-mediated killing, but not brefeldin A (inhibitor of Fas-mediated killing), inhibited cytotoxicity (Fig 3, *b*). In accordance with this, immunostaining revealed perforin staining in the cytoplasm of LTG-specific T-cell clones (Fig 3, *c* and *d*).

When culture supernatants from incubations containing T cells, B-LCLs, and LTG were analyzed for the presence of cytokines, high concentrations of IFN- γ (8118 ± 17105 pg mL^{-1} ; range, 0-56200; $n = 10$ T-cell clones; $P < .05$), IL-5 (674 ± 659 pg mL^{-1} ; range, 0-1760; $n = 10$ T-cell clones; $P < .05$), and IL-10 (807 ± 1213 pg mL^{-1} ; range, 0-3750; $n = 10$ T-cell clones; $P < .05$) but not IL-4 (39 ± 82 pg mL^{-1} ; range, 0-214; $n = 10$ T-cell clones), were observed (Table II).

In preliminary experiments, chemokine mRNA expression from LTG-stimulated T-cell clones was measured at 4 and 16 hours. Chemokine mRNA levels were higher after 4 hours (results not shown); therefore, in all subse-

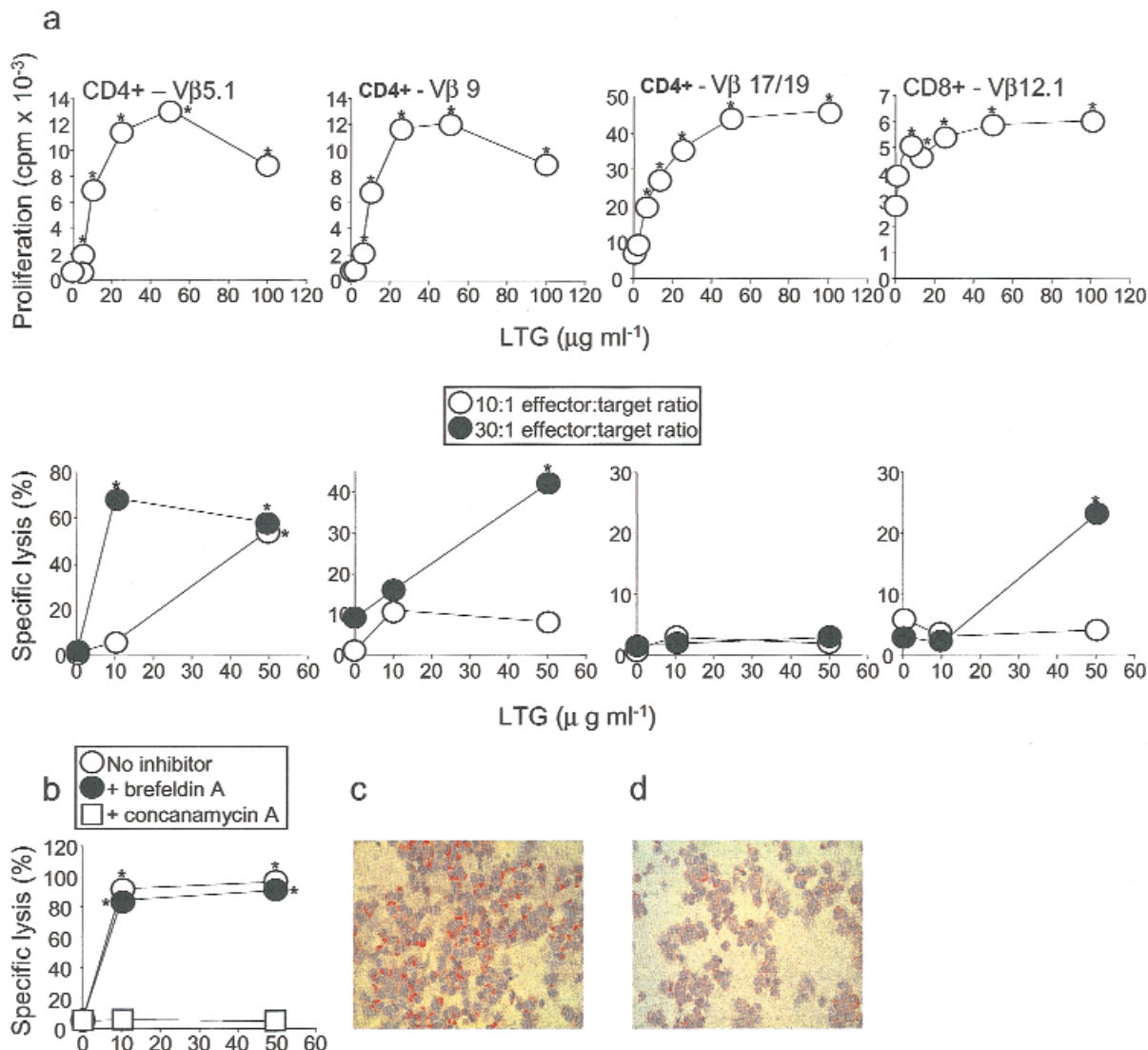


FIG 3. **a**, Phenotype, proliferation, and cytotoxicity of LTG-specific T-cell clones. **b**, Cytotoxicity was inhibited by concanamycin A, an inhibitor of perforin killing. **c**, Perforin was secreted by LTG-stimulated T-cell clones. **d**, No staining was found in incubations without T cells. Statistical analysis compares incubations with and without antigen (**P* < .05).

quent experiments chemokine expression was quantified at 4 hours. Five of 6 T-cell clones expressed high levels of MIP-1α, MIP-1β, and I-309 (Fig 4). RANTES was expressed constitutively in activated and resting T-cell clones. No chemokines were found in B-LCLs (in the presence or absence of LTG). The T-cell clone that did not produce chemokine mRNA with LTG stimulation no longer proliferated (results not shown). Eotaxin expression was not demonstrated (results not shown).

Presentation of LTG to T-cell clones is HLA-DR- and HLA-DQ-restricted and processing-independent and metabolism-independent

T-cell clones did not proliferate in the absence of B-LCLs (results not shown). To study whether the LTG T-

cell receptor interaction is MHC-restricted, proliferation assays were performed with 12 T-cell clones and B-LCLs in the presence of specific HLA-blocking antibodies. Proliferation of LTG-specific T-cell clones was blocked by the addition of antibodies against HLA-DR and HLA-DQ (Fig 5, *a* and *b*). By contrast, antibodies against HLA-DP and MHC class I had no effect. LTG was presented to 11 clones on HLA-DR and to 1 clone on HLA-DQ.

To study the role of oxidative metabolism in the presentation of LTG to T cells, 12 T-cell clones were incubated with LTG, B-LCLs, and glutathione or *N*-acetyl cysteine, or LTG-pulsed B-LCLs. Pulsing B-LCLs (ie, B-LCLs are washed free of drug before T-cell co-incubation) with protein-reactive drug (metabolites) generates a B-LCL drug conjugate, which might activate T cells.²⁵ By contrast, nonreactive drugs are easily removed from

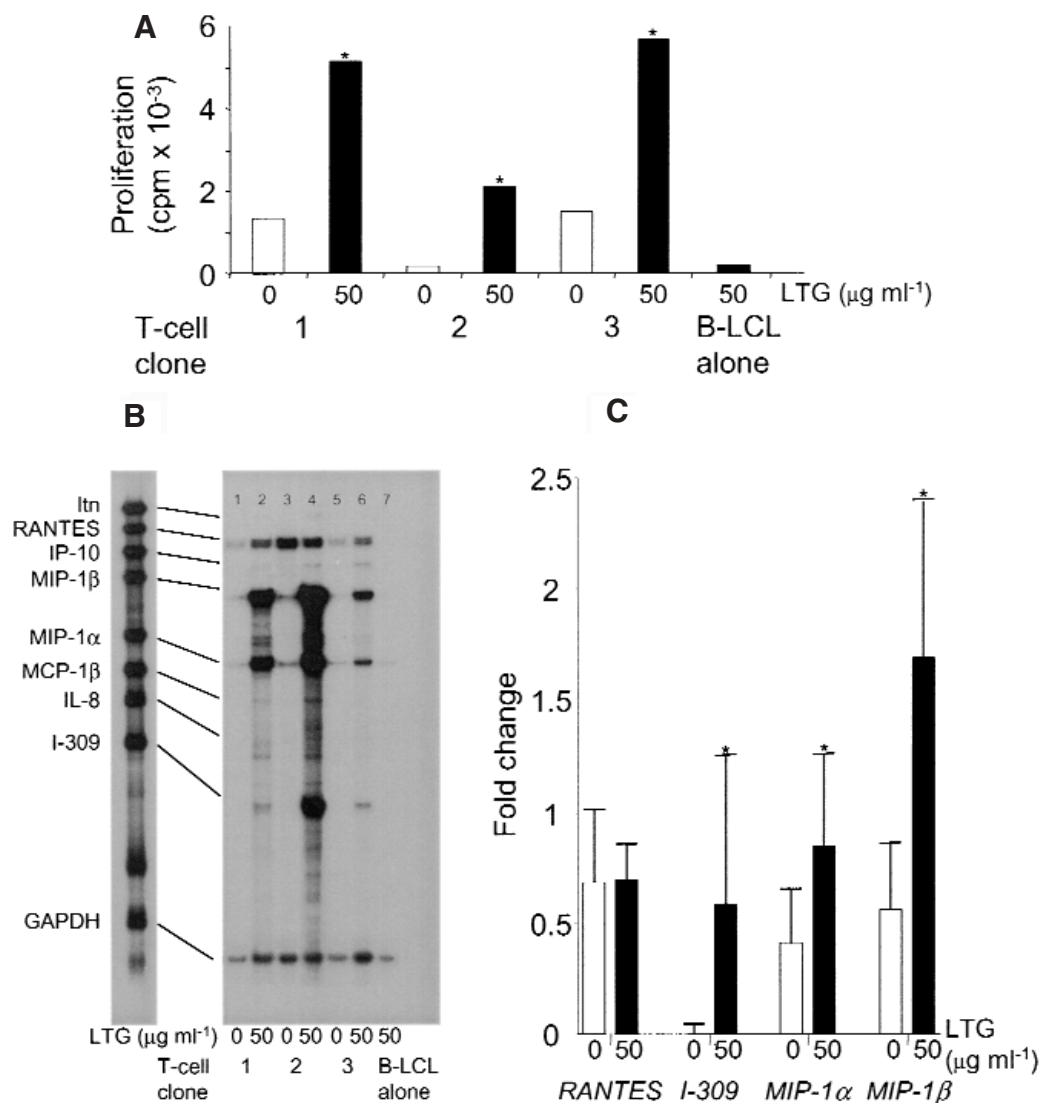


FIG 4. a, Proliferation and chemokine secretion (b and c) by LTG-specific T-cell clones. Statistical analysis was performed by comparing proliferation or chemokine release in the presence and absence of antigen (* $P < .05$).

the surface of MHC by washing.²⁵ LTG-specific T-cell clones did not proliferate in the presence of B-LCLs pulsed with LTG for 2 or 16 hours (Fig 5, c). In addition, proliferation of LTG-specific T-cell clones, which is seen when LTG is present for the duration of the experiment, was not inhibited by co-incubation with glutathione (Fig 5, d) or *N*-acetyl cysteine (results not shown). To determine whether LTG presentation to T cells requires processing, B-LCL was fixed with glutaraldehyde. After fixing, B-LCL was still able to present LTG (Fig 5, e). To confirm this finding, downregulation of T-cell receptor expression after LTG stimulation was measured at 1, 4, and 16 hours. Downregulation of T-cell receptor expression was seen within 1 hour, which is indicative of processing-independent presentation (Fig 5, f).

Cross-reactivity of LTG-specific T-cell clones

To study the structural requirements for T-cell receptor activation, we analyzed the cross-reactivity of 10 LTG-specific T-cell clones with structural analogs of LTG and the anticonvulsants carbamazepine and phenytoin. Four clones proliferated weakly in the presence of LTG-*N*-oxide, and 1 clone proliferated with 3-methyl LTG (Fig 6). No cross-reactivity was seen with 2- and 4-methyl LTG, carbamazepine, or phenytoin.

DISCUSSION

Anticonvulsant hypersensitivity syndrome, which can be severe and cause deaths, is characterized clinically by

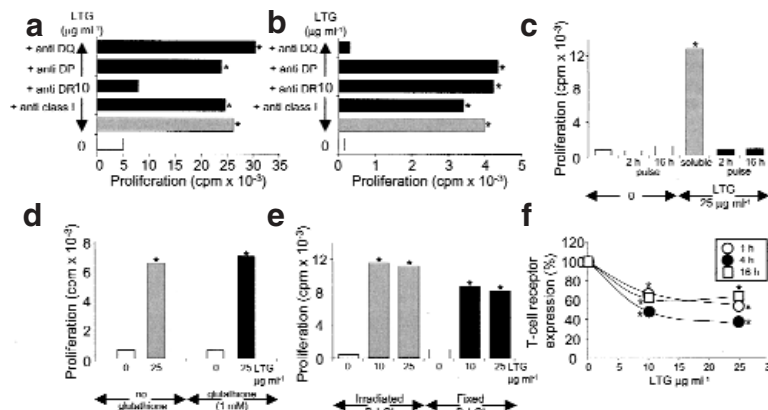


FIG 5. Mechanism of LTG presentation to T-cell clones. **a** and **b**, LTG is presented on HLA-DR and HLA-DQ by HLA-matched antigen-presenting cells. **c** and **d**, LTG is presented to T cells by B-LCLs in the absence of drug metabolism. **e** and **f**, LTG is presented by B-LCLs in the absence of antigen processing. Statistical analysis compares incubations with and without antigen (**P* < .05).

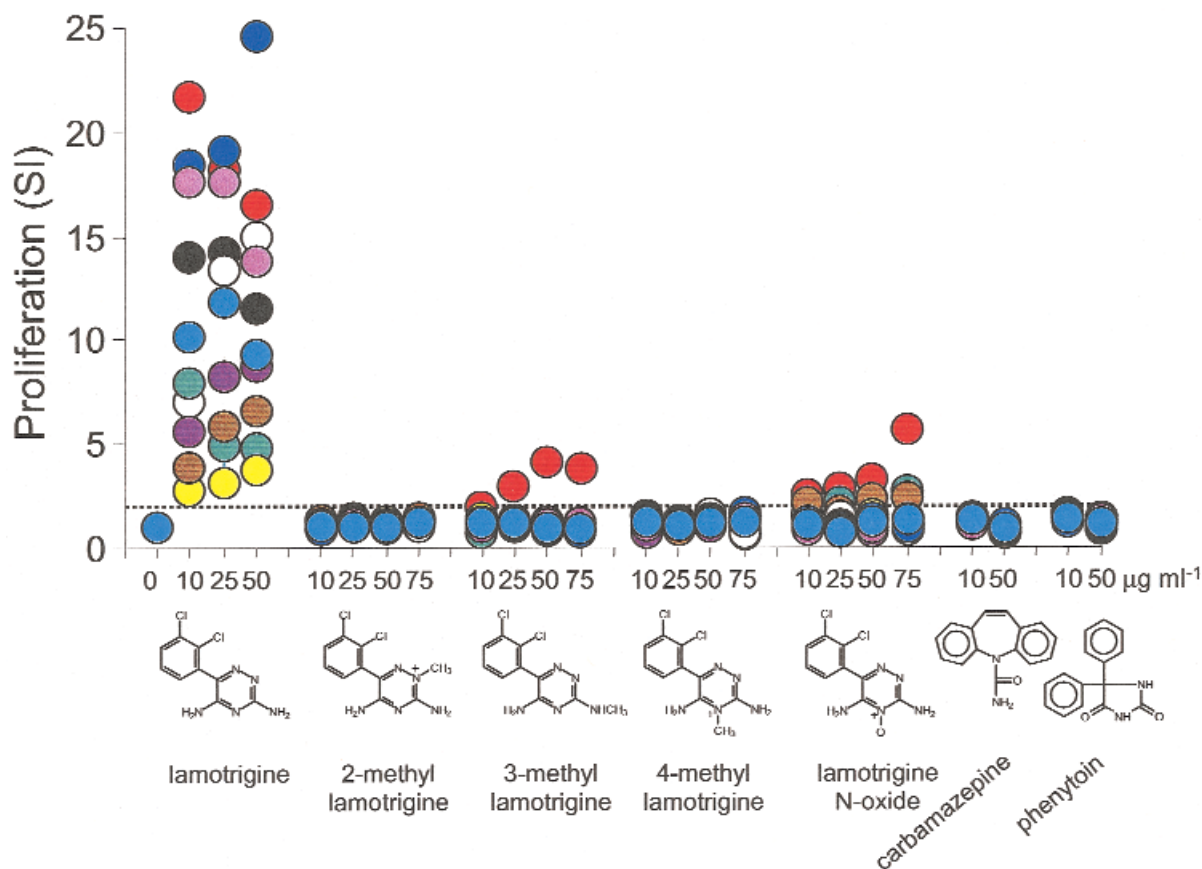


FIG 6. Proliferation of LTG-specific T-cell clones stimulated with LTG and related compounds. Colors refer to individual clones.

skin rash (of variable severity), fever, arthralgia, lymphadenopathy, and eosinophilia.¹ The demonstration of drug-specific clonal proliferation of lymphocytes from hypersensitive patients^{14-17,21,28} is indicative of the requirement of immune system activation in the develop-

ment of the clinical symptoms. In this study, we have shown the presence of LTG-specific proliferation of lymphocytes taken from 3 of 4 LTG-hypersensitive patients. Lymphocyte proliferation was dose-dependent and seen at therapeutic concentrations of LTG. Positive lympho-

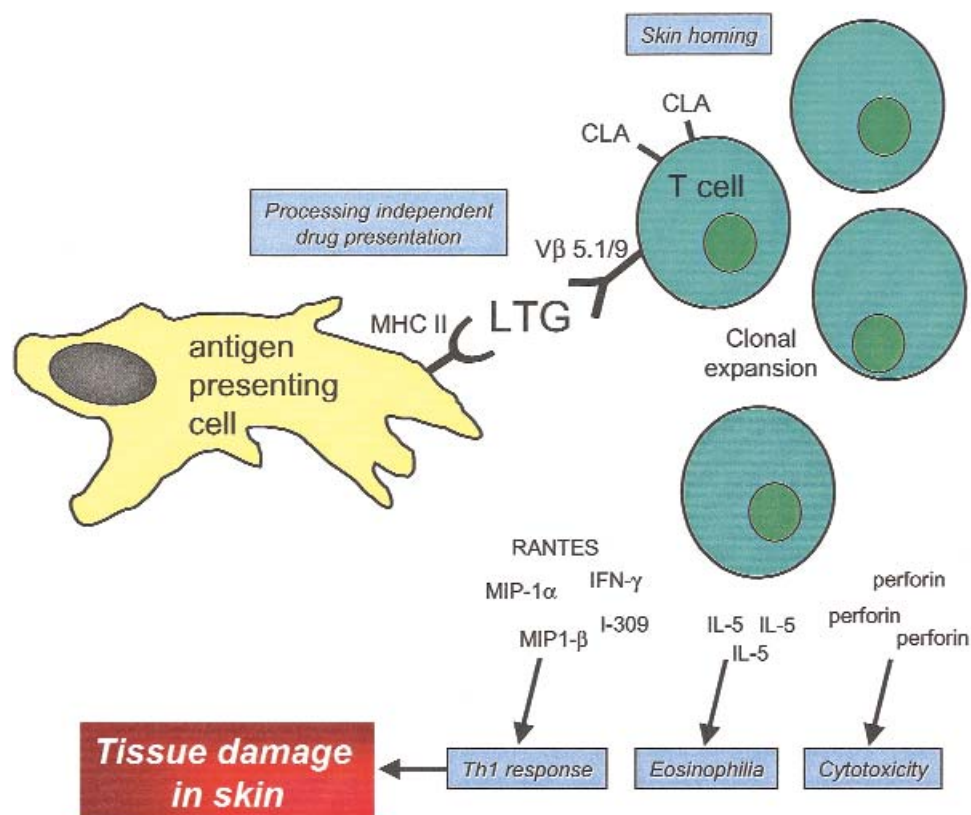


FIG 7. Scheme depicting the chemical, cellular, and molecular pathophysiology of LTG hypersensitivity.

TABLE II. Proliferation, phenotype, and cytokine profile of lamotrigine-specific T-cell clones

T-cell clone	SI (control cpm)	CD phenotype	IL-4	IL-5	IL-10	IFN- γ
1	7.3 (2159)*	4	nd	462	nd	2,426
2	13.3 (1205)	4	nd	321	nd	4,458
3	21.9 (1850)	4	nd	23	nd	4,162
4	19.4 (851)	4	nd	1,609	1,118	310
5	103.6 (152)	4	nd	1,210	nd	nd
6	19.2 (900)	4	nd	881	270	35
7	10.5 (1258)	4	nd	1,763	1,797	219
8	2.8 (1305)	4	214†	512	1,069	7,511
9	2.1 (2854)	8	176	nd	68	5,860
10	5.9 (7890)	4	nd	nd	3,750	56,200

nd, Not detectable.

*Numbers in parenthesis refer to cpm in control incubations that contained T cells and irradiated B-LCLs but no LTG.

†Cytokine levels are expressed as pg mL⁻¹ subtracted from control levels.

cyte proliferation in LTG hypersensitivity has been reported,^{21,29} though the nature of the immune response was not characterized. Therefore, to assess the cellular and molecular features of LTG hypersensitivity, we cloned T cells from one LTG-hypersensitive patient who had maculopapular exanthema develop as part of a generalized hypersensitivity reaction.

T-cell clones stimulated with LTG in vitro secreted high levels of the pro-inflammatory cytokine IFN- γ . These data agree with our previous study in carbamazepine-hypersensitive patients.²⁰ IFN- γ -producing T cells also secreted the chemokines MIP-1 α , MIP-1 β , and

RANTES. Co-secretion of IFN- γ , MIP-1 α , MIP-1 β , and RANTES alongside activation-induced, T-cell-derived, and chemokine-related cytokine is thought to constitute a group of "type 1 cytokines" that act together as a functional unit on cells of the innate and adaptive immune system to drive antigen-specific responses in vivo.³⁰ I-309, which was also secreted by LTG-specific T cells, functions as an antiapoptotic factor³¹ and attracts regulatory CD4⁺ T-cells expressing the receptors CCR4 and/or CCR8. Thus, a combination of these effects might generate a potent signal that controls the strength and duration of the IFN- γ response in LTG hypersensitivity.

Eosinophilia and cytotoxicity, which are common features of anticonvulsant hypersensitivity syndrome, are thought to be due to the secretion of IL-5 and perforin, respectively.^{18,32,33} The LTG-stimulated T cells also secreted perforin and killed autologous target cells (Fig 3); the specificity of the cytotoxic mechanism was confirmed by the use of concanamycin A, an inhibitor of perforin secretion. LTG-stimulated T-cell clones also produced moderate levels of IL-5, which might be sufficient to account for the eosinophilia that is observed in LTG hypersensitivity. In the clones that we generated, we did not demonstrate secretion of eotaxin, which is also associated with eosinophilia³³; clearly, it is possible that there might be other clones that we did not characterize that did secrete eotaxin.

Most LTG-specific T cells were CD4⁺; however, detection of 2 CD8⁺ T-cell clones suggests that CD8⁺ T cells might also be involved in the pathogenesis of the hypersensitivity reaction. Indeed, in similar studies from phenobarbital-hypersensitive patients with bullous skin eruptions (Stevens-Johnson syndrome [n = 3]/toxic epidermal necrolysis [n = 1]), Hashizume et al³⁴ have recently shown the presence of drug-specific CD4⁺ and CD8⁺ T-cells. Interestingly, and in contrast to our findings, the phenobarbital-stimulated T cells secreted IL-4 but not IFN- γ . The implication of these findings is unclear but might be related to differences in the implicated drug, ethnicity, or clinical manifestations between the 2 studies. In contrast to the findings with phenobarbital, lymphocytes from our LTG-hypersensitive patient with toxic epidermal necrolysis did not proliferate in vitro in the presence of LTG (Fig 1). This phenomenon has been observed previously in patients with drug-induced toxic epidermal necrolysis, in which the sensitivity of the test has been quoted to be approximately 10%.^{18,35}

It is becoming increasingly apparent that cellular trafficking plays a key role in tissue-specific inflammatory diseases. CLA is a skin-homing receptor expressed on T cells involved in targeting sites of chronic inflammation.³⁶ Several groups have reported increased levels of CLA on the surface of activated T cells from drug-hypersensitive patients, which parallels the severity of the cutaneous eruption.^{19,37-39} However, these studies did not identify whether the CLA⁺ T cells were drug-specific and/or caused tissue damage. Our findings confirm the expression of CLA on most CD4⁺ LTG-specific T-cell clones but, importantly, also show a significant increase in CLA expression on LTG-specific T-cell clones compared with TT-specific T-cell clones from the same patient (Fig 2). It is important to note, however, that not all LTG-specific T-cell clones expressed CLA. It is possible that these cells might be involved in other aspects of the immune response against LTG (ie, eosinophilia, hepatitis, and immune regulation), though this requires further investigation.

Further experiments in this study were aimed at explaining the mechanism of antigen presentation and processing. First, assessment of the T-cell receptor phenotype showed that all of the LTG-specific T-cell clones

had the $\alpha\beta$ T-cell receptor phenotype, and polyclonal activation was confirmed by the T-cell receptor V β use. T-cell receptor V β 5.1 and 9 were expressed on the surface of 6 and 4 clones, respectively. Use of V β 5.1 seems to be of particular importance in anticonvulsant hypersensitivity, because a number of T-cell clones from carbamazepine-hypersensitive and phenobarbital-hypersensitive patients have also been shown to be T-cell receptor V β 5.1-positive.^{20,33}

Second, antibody-blocking experiments highlighted the critical role of antigen-presenting cells and MHC in the LTG T-cell receptor interaction; antibodies against HLA-DR and HLA-DQ, but not HLA-DP or MHC class I, inhibited proliferation. HLA-DR-restricted antigen presentation is a common feature of allergic drug reactions^{9,11,24,34}; however, drug presentation in the context of HLA-DQ has so far been found only with the anticonvulsants LTG and carbamazepine.²⁰ These observations are consistent with the recently observed MHC gene polymorphisms in patients with severe anticonvulsant hypersensitivity.⁴⁰

Third, it has been proposed that metabolic activation and formation of a covalently linked LTG-protein adduct is a prerequisite for the development of clinical symptoms.¹ Furthermore, it is feasible that the small amount of metabolism seen in skin might generate sufficient quantities of a reactive species to haptenate protein and initiate an immune response. In support of this hypothesis, Wolkenstein et al⁴¹ demonstrated covalent binding of carbamazepine metabolites to CYP3A4, which is present in skin. We have previously shown that LTG undergoes metabolic activation of the dichlorophenyl ring in an in vivo rodent model.²⁶ However, though LTG did accumulate in skin,²⁶ it was not possible to measure oxidative drug metabolism. In this study, we show that T cells from LTG-hypersensitive patients recognize LTG in the absence of any apparent drug metabolism, covalent binding, or antigen processing (Fig 5). These results add LTG to the increasing number of drugs (sulfamethoxazole,^{11,42} lidocaine,⁴³ and celecoxib)²² that have been shown to directly stimulate T cells from hypersensitive patients. It is possible that further studies might reveal T cells that proliferate in the presence of LTG haptenated protein; however, these studies will require either a synthetic, protein-reactive LTG metabolite(s) and/or a modification of the present assay so that it incorporates a metabolite-generating system. We are currently pursuing both avenues.

Fourth, the specificity of T cells for LTG itself was exemplified by the fact that PBMCs and the T-cell clones did not proliferate in the presence of the anticonvulsants carbamazepine and phenytoin. Furthermore, the *N*-methyl and *N*-oxide LTG derivatives, which represent minor structural modifications to the core structure of LTG, were not associated with a strong proliferative response (Fig 6). These data illustrate that the T-cell receptor of our LTG-specific T cells was highly specific for the diaminotriazine ring structure. Interestingly, these minor structural modifications are distal to the site of metabolic activation of LTG, which involves conjugation

of proteins to the dichlorophenyl side chain.²⁶ Although the orientation of LTG within the MHC-restricted T-cell receptor and the nature of the bidirectional bridging interaction are not known, recent studies showing that docking of MHC and the T-cell receptor is largely independent of the nature of the antigen⁴⁴ provide a framework to explain how a reversible drug interaction activates T cells.

In summary, our study highlights several important features of LTG hypersensitivity. Skin-homing CD4⁺ T-cell clones (from a patient that had an LTG-mediated maculopapular skin reaction develop) that proliferated in the presence of LTG that was noncovalently bound to the MHC and secreted perforin and high levels of type 1 cytokines were identified. These cells are likely to be crucial in the development of the clinical symptoms of LTG hypersensitivity (Fig 7). Interestingly, identification of a large number of drug-specific V β 5.1+ T cells from a patient hypersensitive to LTG (see the Results section), as well as patients hypersensitive to carbamazepine²⁰ and phenobarbital,³³ suggest that T-cell receptor gene polymorphisms might act as determinants of susceptibility to anticonvulsant hypersensitivity.

We thank the Departments of Immunology and Tropical Medicine (University of Liverpool) for the use of equipment. We also thank all blood donors.

REFERENCES

- Leeder JS. Mechanisms of idiosyncratic hypersensitivity reactions to antiepileptic drugs. *Epilepsia* 1998;39:S8-16.
- Bocquet H, Bagot M, Roujeau JC. Drug-induced pseudolymphoma and drug hypersensitivity syndrome (Drug Rash with Eosinophilia and Systemic Symptoms: DRESS). *Semin Cutan Med Surg* 1996;15:250-7.
- Pichler WJ, Yawalkar N, Britschgi M, Depta J, Strasser I, Schmid S, et al. Cellular and molecular pathophysiology of cutaneous drug reactions. *Am J Clin Dermatol* 2002;3:229-38.
- Nyfelor B, Pichler WJ. The lymphocyte transformation test for the diagnosis of drug allergy: sensitivity and specificity. *Clin Exp Allergy* 1997;27:175-81.
- Hari Y, Frutig-Schnyder K, Hurni M, Yawalkar N, Zanni MP, Schnyder B, et al. T cell involvement in cutaneous drug eruptions. *Clin Exp Allergy* 2001;31:1398-408.
- Landsteiner K, Jacobs J. Studies on the sensitization of animals with simple chemical compounds. *J Exp Med* 1935;61:643-56.
- Naisbitt DJ, Gordon SF, Pirmohamed M, Park BK. Immunological principles of adverse drug reactions: the initiation and propagation of immune responses elicited by drug treatment. *Drug Saf* 2000;23:483-507.
- Park BK, Naisbitt DJ, Gordon SF, Kitteringham NR, Pirmohamed M. Metabolic activation in drug allergies. *Toxicology* 2001;158:11-23.
- Kalish RS, Askenase PW. Molecular mechanisms of CD8⁺ T cell-mediated delayed hypersensitivity: implications for allergies, asthma, and autoimmunity. *J Allergy Clin Immunol* 1999;103:192-9.
- Naisbitt DJ, Farrell J, Gordon SF, Maggs JL, Burkhardt C, Pichler WJ, et al. Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation. *Mol Pharmacol* 2002;62:628-37.
- Schnyder B, Mauri-Hellweg D, Zanni M, Bettens F, Pichler WJ. Direct, MHC-dependent presentation of the drug sulfamethoxazole to human alphabeta T cell clones. *J Clin Invest* 1997;100:136-41.
- von Greysz S, Zanni MP, Frutig K, Schnyder B, Burkhardt C, Pichler WJ. Interaction of sulfonamide derivatives with the TCR of sulfamethoxazole-specific human alpha beta+ T cell clones. *J Immunol* 1999;162:595-602.
- Zanni MP, von Greysz S, Schnyder B, Brander KA, Frutig K, Hari Y, et al. HLA-restricted, processing- and metabolism-independent pathway of drug recognition by human alpha beta T lymphocytes. *J Clin Invest* 1998;102:1591-8.
- Zakrzewska JM, Ivanyi L. In vitro lymphocyte proliferation by carbamazepine, carbamazepine-10,11-epoxide, and oxcarbazepine in the diagnosis of drug-induced hypersensitivity. *J Allergy Clin Immunol* 1988;82:110-5.
- Troger U, Brandt W, Rose W. Development of a pulmonary phenytoin-associated hypersensitivity reaction despite concomitant dexamethasone and prednisolone administration. *Int J Clin Pharmacol Ther* 2000;38:452-6.
- Kahn HD, Faguet GB, Agee JF, Middleton HM, 3rd. Drug-induced liver injury. In vitro demonstration of hypersensitivity to both phenytoin and phenobarbital. *Arch Intern Med* 1984;144:1677-9.
- Mauri-Hellweg D, Bettens F, Mauri D, Brander C, Hunziker T, Pichler WJ. Activation of drug-specific CD4⁺ and CD8⁺ T cells in individuals allergic to sulfonamides, phenytoin, and carbamazepine. *J Immunol* 1995;155:462-72.
- Friedmann PS, Strickland I, Pirmohamed M, Park BK. Investigation of mechanisms in toxic epidermal necrolysis induced by carbamazepine. *Arch Dermatol* 1994;130:598-604.
- Leyva L, Torres MJ, Posadas S, Blanca M, Besso G, O'Valle F, et al. Anticonvulsant-induced toxic epidermal necrolysis: monitoring the immunologic response. *J Allergy Clin Immunol* 2000;105:157-65.
- Naisbitt DJ, Britschgi M, Wong G, Farrell J, Depta JPH, Chadwick DW, et al. Hypersensitivity reactions to carbamazepine: characterization of the specificity, phenotype and cytokine profile of drug-specific T-cell clones. *Mol Pharmacol* 2003;63:732-41.
- Sachs B, Ronnau AC, von Schmiedeberg S, Ruzicka T, Gleichmann E, Schuppe HC. Lamotrigine-induced Stevens-Johnson syndrome: demonstration of specific lymphocyte reactivity in vitro. *Dermatology* 1997;195:60-4.
- Britschgi M, Steiner UC, Schmid S, Depta JP, Senti G, Bircher A, et al. T-cell involvement in drug-induced acute generalized exanthematous pustulosis. *J Clin Invest* 2001;107:1433-41.
- Brunner KT, Mauel J, Cerottini JC, Chapuis B. Quantitative assay of the lytic action of immune lymphoid cells on ⁵¹Cr-labelled allogenic target cells in vitro: Inhibition by isoantibody and by drugs. *Immunology* 1968;14:181-6.
- Schnyder B, Frutig K, Mauri-Hellweg D, Limat A, Yawalkar N, Pichler WJ. T-cell-mediated cytotoxicity against keratinocytes in sulfamethoxazole-induced skin reaction. *Clin Exp Allergy* 1998;28:1412-7.
- Schnyder B, Burkhardt C, Schnyder-Frutig K, von Greysz S, Naisbitt DJ, Pirmohamed M, et al. Recognition of sulfamethoxazole and its reactive metabolites by drug-specific CD4⁺ T cells from allergic individuals. *J Immunol* 2000;164:6647-54.
- Maggs JL, Naisbitt DJ, Tetley JN, Pirmohamed M, Park BK. Metabolism of lamotrigine to a reactive arene oxide intermediate. *Chem Res Toxicol* 2000;13:1075-81.
- Shimonkevitz R, Kappler J, Marrack P, Grey H. Antigen recognition by H-2-restricted T cells. I. Cell-free antigen processing. *J Exp Med* 1983;158:303-16.
- Brown KL, Henderson DC, Nadel S, Tanveer A, Booy R. Carbamazepine hypersensitivity and the use of lymphocyte proliferation responses. *Dev Med Child Neurol* 1999;41:267-9.
- Schaub N, Bircher AJ. Severe hypersensitivity syndrome to Lamotrigine confirmed by lymphocyte stimulation in vitro. *Allergy* 2000;55:191-3.
- Dorner BG, Scheffold A, Rolph MS, Huser MB, Kaufmann SH, Radbruch A, et al. MIP-1alpha, MIP-1beta, RANTES, and ATAC/lymphotactin function together with IFN-gamma as type 1 cytokines. *Proc Natl Acad Sci U S A* 2002;99:6181-6.
- Van Snick J, Houssiau F, Proost P, Van Damme J, Renauld JC. I-309/T cell activation gene-3 chemokine protects murine T cell lymphomas against dexamethasone-induced apoptosis. *J Immunol* 1996;157:2570-6.
- Pichler WJ, Zanni M, von Greysz S, Schnyder B, Mauri-Hellweg D, Wendland T. High IL-5 production by human drug-specific T cell clones. *Int Arch Allergy Immunol* 1997;113:177-80.
- Yawalkar N, Shrikhande M, Hari Y, Nievergelt H, Braathen LR, Pichler WJ. Evidence for a role for IL-5 and eotaxin in activating and recruiting eosinophils in drug-induced cutaneous eruptions. *J Allergy Clin Immunol* 2000;106:1171-6.
- Hashizume H, Takigawa M, Tokura Y. Characterization of drug-specific T cells in phenobarbital-induced eruption. *J Immunol* 2002;168:5359-68.
- Roujeau JC, Albengres E, Moritz S, Piacentino A, Cuny M, Revuz J, et

- al. Lymphocyte transformation test in drug-induced toxic epidermal necrolysis. *Int Arch Allergy Appl Immunol* 1985;78:22-4.
36. Picker LJ, Michie SA, Rott LS, Butcher EC. A unique phenotype of skin-associated lymphocytes in humans. Preferential expression of the HECA-452 epitope by benign and malignant T cells at cutaneous sites. *Am J Pathol* 1990;136:1053-68.
37. Gonzalez FJ, Carvajal MJ, Leiva L, Juarez C, Blanca M, Santamaria LF. Expression of the cutaneous lymphocyte associated antigen in circulating T cells in drug-allergic reactions. *Int Arch Allergy Immunol* 1997;113:345-7.
38. Gonzalez FJ, Leyva L, Posadas S, Luque I, Blanca M, Santamaria L, et al. Participation of T lymphocytes in cutaneous allergic reactions to drugs. *Clin Exp Allergy* 1998;28:3-6.
39. Blanca M, Posadas S, Torres MJ, Leyva L, Mayorga C, Gonzalez L, et al. Expression of the skin-homing receptor in peripheral blood lymphocytes from subjects with nonimmediate cutaneous allergic drug reactions. *Allergy* 2000;55:998-1004.
40. Pirmohamed M, Lin K, Chadwick D, Park BK. TNFalpha promoter region gene polymorphisms in carbamazepine-hypersensitive patients. *Neurology* 2001;56:890-6.
41. Wolkenstein P, Tan C, Lecoeur S, Wechsler J, Garcia-Martin N, Charue D, et al. Covalent binding of carbamazepine reactive metabolites to P450 isoforms present in the skin. *Chem Biol Interact* 1998;113:39-50.
42. von Greylitz S, Bultmann G, Schnyder K, Burkhart C, Lotti B, Hari Y, et al. Degeneracy and additional alloreactivity of drug-specific human alpha beta(+) T cell clones. *Int Immunol* 2001;13:877-85.
43. Zanni MP, von Greylitz S, Hari Y, Schnyder B, Pichler WJ. Recognition of local anesthetics by alphabeta+ T cells. *J Invest Dermatol* 1999;112:197-204.
44. Wu LC, Tuot DS, Lyons DS, Garcia KC, Davis MM. Two-step binding mechanism for T-cell receptor recognition of peptide MHC. *Nature* 2002;418:552-6.