# Generation and characterization of antigen-specific $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T-cell clones from patients with carbamazepine hypersensitivity

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**Background: Hypersensitivity is a serious manifestation of anticonvulsant therapy characterized by infiltration of the epidermis and dermis by activated $CD8^+$ and $CD4^+$ T-cells, respectively. Attempts to characterize drug-specific $CD8^+$ T cells have been largely unsuccessful.**

**Objectives: The aim of these studies was to generate and characterize $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells in patients with carbamazepine hypersensitivity.**

**Methods: Carbamazepine-specific T-cell clones were generated from 5 patients by using modified cloning methodologies. Cell surface receptor phenotype, functionality, and mechanisms of antigen presentation were then compared.**

**Results: Ninety $CD4^+$, 23 $CD8^+$, and 14 $CD4^+CD8^+$ carbamazepine-specific T-cell clones were generated. $CD4^+$ T-cell clones proliferated vigorously with carbamazepine associated with MHC class II but exhibited little cytotoxic activity. In contrast, most $CD8^+$ T cells proliferated weakly but effectively killed target cells via an MHC class I or MHC class II restricted, perforin-dependent pathway. $CD4^+CD8^+$ T cells displayed characteristics similar to those of $CD4^+$ T cells; however, drug stimulation was demonstrable in the absence of antigen-presenting cells. Carbamazepine was presented to $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells in the absence of antigen processing. Drug stimulation resulted in the secretion of IFN-γ and IL-5. A panel of $CD11a^+CD27^-$ clones differentially expressed the receptors CXCR4, CCR4, CCR5, CCR8, CCR9, and CCR10.**

**Conclusion: Carbamazepine-specific $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells exist in the peripheral circulation of hypersensitive patients, often many years after the resolution of clinical manifestations.**

**Clinical implications: Carbamazepine-specific $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells displaying different effector functions and homing characteristics persist in hypersensitive patients' blood for many years after resolution of clinical symptoms.**

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***Key words:*** *Drug allergy, T cells, anticonvulsant hypersensitivity syndrome*

T-cell–mediated cutaneous hypersensitivity reactions to drugs are a significant cause of patient morbidity and mortality. They are associated with a spectrum of clinical manifestations, brought about by different immunologic mechanisms.[1,2] Blistering reactions such as toxic epidermal necrolysis are thought to be caused by the cutaneous infiltration of cytotoxic $CD8^+$ T cells,[3-5] whereas exanthemas seem to be mediated largely by drug-specific $CD4^+$ T cells.[4-8] However, this apparent association between $CD4^+$ and $CD8^+$ cells and the type of cutaneous manifestation is not clear-cut. For instance, Kuechler et al[9] identified an additional rare outgrowth of $CD8^+$ T cells from patients with several different forms of cutaneous hypersensitivity to anti-infective drugs.

To investigate further the role of different T-cell populations in drug hypersensitivity, we have focused on carbamazepine for several reasons. First, carbamazepine administration is associated with a high incidence of clinically well defined immune reactions.[10] The features of these reactions include skin rash, fever, eosinophilia, and internal organ involvement in some cases. Second, histologic analyses of skin from hypersensitive patients reveal an infiltration of recently activated $CD4^+$ and $CD8^+$ into the dermis and epidermis, respectively.[11,12] Third, detection of drug-specific proliferation of lymphocytes from sensitive patients, but not from drug-exposed controls, has verified the specificity of the T-cell response.[13-16] Finally, we were recently successful in cloning carbamazepine-specific $CD4^+$ T cells from a panel of hypersensitive patients.[14,15] T cells proliferated on drug exposure by direct interaction of the compound with the MHC and the T-cell receptor in the absence of antigen processing, but displayed limited cytotoxicity. Drug stimulation also resulted in the secretion of high levels of the proinflammatory cytokine IFN-γ, and low to moderate levels of IL-4 and IL-5. Importantly, drug-specific stimulation of $CD8^+$ T-cell clones was seen rarely and could not be fully characterized because the individual clones could not be expanded *in vitro*.

It is important to consider why previous studies of carbamazepine hypersensitivity have been largely unsuccessful in generating drug-specific $CD8^+$ T-cell clones.[14,15] First, it is possible that $CD8^+$ T cells found in skin might not be

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**TABLE I.** Clinical details of hypersensitive patients

| Patient* | Age (y) | Sex | Days to reaction | Details of reaction | Months since reaction | Lymphocyte transformation test (SI) |
|---|---|---|---|---|---|---|
| 1 | 34 | M | 21 | Exfoliative dermatitis, facial swelling, fever, and eosinophilia | 192 | 29.8 |
| 2 | 68 | F | 6 | Widespread erythematous rash, Lymphocytosis, and eosinophilia | 192 | 13.2 |
| 3 | 32 | M | 28 | Eythematous rash, jaundice, and hepatomegaly | 172 | 8.4 |
| 4 | 34 | M | 21 | Widespread severe skin rash | 33 | 4.1 |
| 5 | 71 | M | 21 | Widespread maculopapular eruption with tissue eosinophilia | 40 | 9.6 |

*F*, Female; *M*, male.
*Number assigned to patient throughout manuscript.

*Abbreviations used*
APC: Antigen-presenting cell
B-LCL: B-lymphoblastoid cell line
MFI: Median fluorescent intensity
SI: Stimulation index
UK: United Kingdom

antigen-specific, but are stimulated via some form of protein antigen, as has been reported recently with $CD8^+$ cytomegalovirus-specific T-cell responses in a patient with tribenoside hypersensitivity.[17] Second, antigen-specific $CD8^+$ T cells might become depleted after resolution of the disease, and most patients have been studied retrospectively. Third, it may reflect a technical deficiency in standard *in vitro* cell culture cloning methodologies. This is a likely explanation. $CD8^+$ clones are incredibly difficult to culture because they do not proliferate readily, and therefore, expansion and determination of antigen specificity are not possible. Furthermore, antigen-specific $CD8^+$ T-cell clones have a propensity to lose specificity after repeated mitogen stimulation, a procedure that is critical for cloning T cells.[9]

Thus, the aim of the current study was to modify the cloning procedure in 2 ways to generate $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells from carbamazepine-hypersensitive patients to assess their phenotype and function *in vitro*. First, $CD4^+$ or $CD8^+$ T cells were purified from T-cell lines before serial dilution. Second, in certain experiments, antigen specificity after serial dilution was determined via analysis of cytotoxicity rather than proliferation.

## METHODS

### Materials and cell culture

Basal cell culture medium has been described elsewhere.[14,15] For culture of the T-cell lines and clones, the media was enriched with human recombinant IL-2 (120 U/mL; Peprotech, London, United Kingdom [UK]). B-lymphoblastoid cell lines (B-LCLs) were generated by transformation of lymphocytes with supernatant from the EBV-producing cell line B9-58. Fresh stock solutions of carbamazepine (10 mg/mL) were prepared in a mixture of culture media and dimethyl sulfoxide (4:1 vol/vol) and diluted before use. Unless otherwise stated, reagents were obtained from Sigma Chemical Co (Poole, Dorset, UK).

### Patients' characteristics and lymphocyte transformation test

Blood lymphocytes were obtained from 5 carbamazepine-hypersensitive patients and 5 carbamazepine-exposed nonhypersensitive controls (3 men, 2 women; age, 42.0 ± 14.7 years; minimum length of carbamazepine exposure, 6 months). Clinical characteristics for each hypersensitivity reaction are described in Table I. Approval for the study was obtained from Liverpool Local Research Ethics Committee, and informed consent was obtained from each participant. Proliferation of patients' lymphocytes on exposure to carbamazepine (10-100 μg/mL) was measured by using the lymphocyte transformation test, as described previously.[18] Proliferative responses were calculated as stimulation indices (SIs; cpm in drug-treated cultures/cpm in cultures with dimethyl sulfoxide alone).

### Generation of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T-cell clones

In initial experiments, T-cell lines were generated by incubating lymphocytes ($2 \times 10^6$; total volume, 1 mL) from 5 carbamazepine-hypersensitive patients (patients 1-5) with carbamazepine (10 μg/mL). On days 6 and 9, IL-2 was added to maintain proliferation. On day 14, T cells were cloned by serial dilution as described previously.[14] To test the specificity of the clones, T cells ($5 \times 10^4$; total volume, 0.2 mL) were incubated with autologous irradiated (60 Gy) B-LCLs ($1 \times 10^4$) and drug (10 and 25 μg/mL). After 48 hours, [$^3$H]-thymidine was added and proliferation measured by scintillation counting. Clones with an SI greater than or equal to 2 were restimulated, expanded in IL-2–containing medium, and assayed for phenotype and functionality.

In subsequent experiments, $CD8^+$ and $CD4^+$ T cells were positively selected from T-cell lines derived from patients 4 and 5 by incubating cells with immunomagnetic microbeads coated with anti-$CD4^+$ or anti-$CD8^+$ antibody and magnetic cell sorting (Miltenyi Biotec, Bisley, UK) before serial dilution. The purity of the sorted T-cell lines was determined by flow cytometry (Coulter EPICS XL-MCL flow cytometer; Beckman Coulter Inc, Fullerton, Calif) with conjugated $CD3^+$, $CD4^+$, and $CD8^+$ antibodies (BD Biosciences, Oxford, UK).

The specificity of T-cell clones was determined by analysis of antigen-specific proliferation, as outlined, or by immune-mediated killing through the use of a 4-hour [$^{51}$Cr] release assay.[14] Briefly, [$^{51}$Cr] loaded B-LCL ($5 \times 10^3$; total volume, 0.2 mL) were incubated with T cells ($5 \times 10^4$) in the presence or absence of carbamazepine (10 and 25 μg/mL). Specific lysis was calculated as 100 × (experimental release − spontaneous release)/(maximal release − spontaneous release).

## Determination of the phenotype and functionality of T-cell clones

Antigen-specific T-cell clones were characterized in terms of CD phenotype and T-cell receptor Vβ expression by flow cytometry through the use of a previously described protocol.[14] Cell surface receptor (CD11a, CD18, CD27, cutaneous lymphocyte antigen) and chemokine receptor expression were also measured by flow cytometry using a panel of 13 antibodies (CXCR4 [12G5; Serotec, Oxford, UK], CXCR6 [56811; R&D Systems, Minneapolis, Minn], CCR3 [61828.111; R&D Systems], CCR4 [1G1; BD Pharmingen, Oxford, UK], CCR5 [45531.111; Serotec], CCR7 [3D12; BD Pharmingen], CCR8 [Alexis Biochemicals, Nottingham, UK], CCR9 [112509; R&D Systems], CCR10 [clone 37; kind gift from Dr E. Bowman, DNAX Research, Palo Alto, Calif], CD11a [38; Serotec], CD18 [YFC118.3; Serotec], CD27 [LT27; Serotec], cutaneous lymphocyte antigen [HECA-452; BD Pharmingen]), and relevant isotype controls.

The functionality of T-cell clones was investigated by measurement of carbamazepine-specific (1-100 μg/mL) proliferation by thymidine incorporation. To measure immune-mediated killing, [$^{51}$Cr] loaded B-LCLs ($2.5 \times 10^3$) were incubated with T cells at effector:target ratios of 5:1 to 50:1 in the presence or absence of carbamazepine. Mechanisms of killing were determined by the addition of brefeldin A (10 μg/mL; an inhibitor of FAS-mediated cytotoxicity) and concanamycin A (100 nmol/L; an inhibitor of perforin-mediated cytotoxicity) to certain incubations. ELISA was used to determine cytokine (IL-4, IL-5, IL-10, and IFN-γ) levels in cell culture supernatant 48 hours after drug stimulation of the clones.

## Determination of the mechanism of drug presentation to T cells

Specific anti-HLA blocking antibodies (BD Biosciences) were added to proliferation and cytotoxicity assays to assess the involvement of MHC in drug presentation to $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T-cell clones. Certain incubations contained T cells and drug in the absence of B-LCL.

The role of processing in drug presentation to $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T-cell clones was determined by chemical fixation of B-LCL with glutaraldehyde (0.05%; 30 seconds).[19] Fixed antigen-presenting cells (APCs) express MHC molecules and actively present antigenic peptides and drugs that interact directly with MHC; however, their ability to process and present protein antigens is completely inhibited.[19,20]

To evaluate the role of covalent binding in the presentation of carbamazepine to T cells, pulsing experiments that involved the incubation of carbamazepine with B-LCLs ($0.2 \times 10^6$) for 2 hours, followed by repeated washing steps to remove noncovalently bound drug, before the addition of the B-LCLs to T cells, were performed as described previously.[14,15]

## Statistical analysis

The Mann-Whitney test was used for comparison of control and test values, accepting $P < .05$ as significant.

# RESULTS

## *In vitro* stimulation of lymphocytes with carbamazepine

Lymphocytes from all 5 carbamazepine-hypersensitive patients proliferated *in vitro* after drug stimulation (Table I). Lymphocytes from volunteers exposed to carbamazepine without hypersensitivity did not proliferate (SI less than 1.5).

## Phenotypic characteristics and functionality of carbamazepine-specific $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T-cell clones

Cloning of T-cell lines from 5 patients produced 94 αβ clones that were stimulated with carbamazepine and irradiated B-LCL as APCs (Table II). Approximately 90% (n = 84) of these clones expressed high levels of the $CD4^+$ coreceptor (13.2 ± 4.0; median fluorescence intensity [MFI], with isotype subtracted), but low levels of $CD8^+$ (MFI < 0.3). Seven T-cell clones preferentially expressed $CD8^+$ ($CD8^+$ MFI, 19.1 ± 11.1; $CD4^+$ MFI, < 0.3), whereas 3 clones expressed high levels of both receptors ($CD4^+$ MFI, 8.8, 9.7, and 8.4; $CD8^+$ MFI, 11.2, 18.5, and 30.1).

To increase the likelihood of generating $CD8^+$ clones, $CD4^+$ and $CD8^+$ T cells were purified from T-cell lines of patients 4 and 5 before serial dilution (patients were selected on the basis of their willingness to donate additional blood). From equal numbers of seeded $CD4^+$ and $CD8^+$ T cells, 6, 15, and 11 $CD4^+$ ($CD4^+$ MFI, 16.4 ± 4.6; $CD8^+$ MFI, < 0.3), $CD8^+$ ($CD4^+$ MFI, < 0.3; $CD8^+$ MFI, 17.6 ± 7.8) and $CD4^+CD8^+$ ($CD4^+$ MFI, 9.4 ± 5.1; $CD8^+$ MFI, 16.3 ± 7.4) antigen-specific T-cell clones were produced, respectively (Table II). Most $CD4^+CD8^+$ T-cell clones expressed high to moderate levels of both $CD4^+$ and $CD8^+$ receptors; however, 2 clones expressed high levels of $CD8^+$ and low but detectable levels of $CD4^+$, and 2 clones expressed high levels of $CD4^+$ and low but detectable levels of $CD8^+$ (Fig 1).

Nineteen T-cell clones with a good growth pattern were selected for more detailed phenotypic analysis and determination of functionality. Without exception, concentration-dependent stimulation of carbamazepine-specific $CD4^+$ T-cell clones was detected as a function of incorporated [$^3$H]-thymidine (SI, 2.6-15.9; Table III). No correlation was observed between the level of $CD4^+$ expression and degree of elevation of proliferation with carbamazepine. Furthermore, detection of $CD4^+$ T-cell proliferation was not associated immune-mediated killing of [$^{51}$Cr] loaded B-LCL. In fact, carbamazepine-specific cytotoxicity at a low level was detected in only 3 of the 8 $CD4^+$ T-cell clones, and only at a high effector:target cell ratio (Table IV). By contrast, carbamazepine-stimulated $CD8^+$ T-cell clones displayed strong cytotoxicity against [$^{51}$Cr] loaded B-LCL (Table IV). The strength of the proliferative response with the $CD8^+$ clones was particularly variable. A strong carbamazepine-specific proliferative response was observed with a number of $CD8^+$ clones (eg, clones 45 and 50), but not with others (eg, 49 and

**TABLE II.** Generation of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T-cell clones from patients hypersensitive to carbamazepine

| Patient | Treatment before cloning | CD | No. of clones* | Mean proliferative response (cpm) 0 | 10 μg/mL | 50 μg/mL |
|---|---|---|---|---|---|---|
| Standard cloning procedure (serial dilution of CBZ-stimulated lymphocytes) | | | | | | |
| 1 | No treatment | $CD4^+$ | 19 | 6896 ± 8323† | 23,680 ± 21,042 | 17,527 ± 14,552 |
| | | $CD8^+$ | 1 | 17,094 | 28,265 | 29,181 |
| | | $CD4^+CD8^+$ | 1 | 5426 | 9041 | 8593 |
| | | $CD4^+$ | 42 | 2566 ± 2912 | 8203 ± 6915 | 6595 ± 6071 |
| 2 | No treatment | $CD8^+$ | 4 | 7778 ± 8934 | 14,781 ± 13,900 | 9274 ± 10,314 |
| | | $CD4^+CD8^+$ | 1 | 4199 | 8872 | 6000 |
| | | $CD4^+$ | 7 | 10,792 ± 2629 | 21,971 ± 7305 | 13,455 ± 8920 |
| 3 | No treatment | $CD8^+$ | 1 | 31,849 | 54,679 | 56,268 |
| | | $CD4^+CD8^+$ | 0 | — | — | — |
| | | $CD4^+$ | 10 | 3386 ± 3405 | 7158 ± 5275 | 6656 ± 5506 |
| 4 | No treatment | $CD8^+$ | 1 | 271 | 2131 | 1078 |
| | | $CD4^+CD8^+$ | 0 | — | — | — |
| | | $CD4^+$ | 6 | 3183 ± 2029 | 32,866 ± 19,784 | 32,791 ± 21,339 |
| 5 | No treatment | $CD8^+$ | 0 | — | — | — |
| | | $CD4^+CD8^+$ | 1 | 845 | 9617 | 8960 |
| Revised cloning procedure (serial dilution of CBZ-stimulated $CD4^+$ or $CD8^+$ lymphocytes) | | | | | | |
| 4 | $CD4^+/CD8^+$ purification | $CD4^+$ | 2 | 548 ± 205 | 967 ± 289 | 1514 ± 278 |
| | | $CD8^+$ | 11 | 2235 ± 3006 | 6095 ± 4433 | 4923 ± 4256 |
| | | $CD4^+CD8^+$ | 9 | 4727 ± 6983 | 13,140 ± 16,729 | 10,498 ± 15,949 |
| 5 | $CD4^+/CD8^+$ purification | $CD4^+$ | 4 | 1781 ± 464 | 11,926 ± 8064 | 10,328 ± 8024 |
| | | $CD8^+$ | 4 | 12,434 ± 8658 | 19,434 ± 12,426 | 15,760 ± 11,834 |
| | | $CD4^+CD8^+$ | 2 | 7486 ± 453 | 16,068 ± 2166 | 19,983 ± 3639 |

*CBZ*, Carbamazepine.
*If SI less than 2, antigen specificity confirmed by using cytotoxicity assay.
†Data presented as means ± SDs of all clones with a given phenotype (coefficient of variation consistently less than 20% for individual clones).

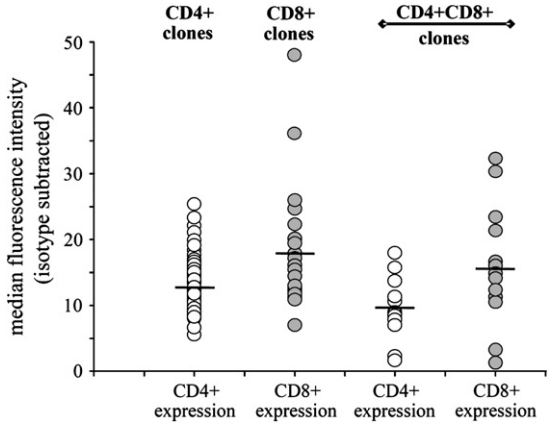


**FIG 1.** Expression of $CD4^+$ and $CD8^+$ receptors on T-cell clones generated from hypersensitive patients.

140; Table III). There was no correlation between $CD8^+$ expression and the strength of the proliferative response or cytotoxicity. $CD4^+CD8^+$ T-cell clones proliferated strongly after carbamazepine stimulation, but exhibited only low levels of cytotoxicity (Tables III and IV).

$CD4^+$, $CD8^+$, and $CD4^+CD8^+$ mediated carbamazepine-specific killing of B-LCL (32.2 ± 19.4% killing; n = 6 clones) was inhibited with concanamycin A (3.2 ± 3.4% killing), but not brefeldin A (28.8 ± 17.8% killing), which is indicative of perforin-mediated cytotoxicity.

**TABLE III.** Characteristics of carbamazepine-specific T-cell clones

| Clone ID | CD | Mean proliferative response (cpm) Carbamazepine (μg/mL) 0 | 5 | 10 | 25 | 50 | 100 |
|---|---|---|---|---|---|---|---|
| 45 | 8 | 1061 | 31,353 | 32,775 | 30,630 | 23,773 | 15,438 |
| 49 | 8 | 507 | | 793 | 528 | 765 | |
| 80 | 8 | 1054 | 3226 | 3178 | 2934 | 2948 | 1600 |
| 140 | 8 | 446 | | 424 | 574 | 482 | |
| 52 | 8 | 1106 | | 3335 | 3506 | 2783 | 945 |
| 77 | 8 | 1025 | 1864 | 1786 | 927 | 762 | |
| 50 | 8 | 294 | | 2844 | 4040 | 5838 | |
| 44 | 4 | 810 | | 1658 | 1910 | 2086 | |
| 7 | 4 | 875 | | 13,096 | 12,901 | 9322 | 4742 |
| 135 | 4 | 334 | 614 | 966 | 1188 | 1271 | 666 |
| 25 | 4 | 974 | 2943 | 6046 | 10,545 | 15,520 | 11,935 |
| 56 | 4 | 884 | 774 | 1979 | 3759 | 4208 | 1017 |
| 21 | 4 | 815 | 2936 | 3684 | 3562 | 3006 | 126 |
| 108 | 4 | 626 | | 2183 | 3432 | 5236 | |
| 198 | 4 | 509 | | 1366 | 1992 | 3002 | |
| 91 | Dual | 1565 | 15,939 | 14,179 | 14,096 | 6009 | 8073 |
| 21b | Dual | 979 | | 1929 | 3106 | 5173 | 4812 |
| 20 | Dual | 7034 | | 13,902 | 23,826 | 16,444 | 6945 |
| 7b | Dual | 1499 | 5148 | 3418 | 2817 | 1817 | 2167 |

**TABLE IV.** Characteristics of carbamazepine-specific T-cell clones

| | | | | Cytotoxicity (%) | | | |
|---|---|---|---|---|---|---|---|
| | | Phenotype | | Carbamazepine (25 μg/mL) | | | |
| Clone ID | CD | CD4* expression | CD8* expression | 5:1† | 10:1 | 25:1 | MHC restriction |
| 45 | 8 | <0.3 | 11.9 | 60.9 | 86.3 | 85.2 | II |
| 49 | 8 | <0.3 | 12.2 | 65.0 | 77.9 | 83.6 | NP |
| 80 | 8 | <0.3 | 11.4 | 47.9 | 54.5 | 70.9 | I |
| 140 | 8 | <0.3 | 47.9 | 22.3 | 38.4 | 57.9 | I |
| 52 | 8 | <0.3 | 24.7 | 32.4 | 39.7 | 55.3 | NP |
| 77 | 8 | <0.3 | 6.6 | 6.7 | 11.3 | 13.0 | I |
| 50 | 8 | <0.3 | 16.9 | 10.9 | 23.5 | 19.8 | II |
| 44 | 4 | 6.8 | <0.3 | 0 | 0 | 0 | II |
| 7 | 4 | 12.5 | <0.3 | 0 | 0 | 2.3 | NP |
| 135 | 4 | 18.0 | <0.3 | 5.9 | 5.1 | 10.1 | NP |
| 25 | 4 | 14.2 | <0.3 | 5.3 | 3.0 | 6.7 | NP |
| 56 | 4 | 14.9 | <0.3 | 1.6 | 0 | 2.0 | II |
| 21 | 4 | 18.8 | <0.3 | 5.5 | 10.1 | 13.5 | II |
| 108 | 4 | 19.5 | <0.3 | 0 | 9.3 | 33.4 | II |
| 198 | 4 | 15.6 | <0.3 | 3.0 | 14.5 | 19.5 | II |
| 91 | Dual | 7.6 | 14.6 | 15.1 | 14.7 | 28.0 | Neither |
| 21b | Dual | 2.1 | 32.2 | 5.9 | 1.1 | 8.7 | Neither |
| 20 | Dual | 17.9 | 3.3 | 2.7 | 1 | 0.1 | NP |
| 7b | Dual | 10.0 | 13.2 | 0.3 | 2.4 | 3.5 | NP |

*NP*, Not performed.
*$CD4^+$/$CD8^+$ data expressed as median fluorescence intensity with isotype control subtracted.
†Effector:target cell ratio.

A panel of T-cell clones was found differentially to express 6 chemokine receptors (CXCR4, CCR4, CCR5, CCR8, CCR9, CCR10; minimum fold change 1.4 compared with isotype control; Table V). All clones expressed high levels of CCR8, CD11a, and CD18. CCR4, CCR9, and CCR10 were expressed at lower levels on most clones, whereas the expression of CXCR4 and CCR5 was more variable (Table V). Expression levels of CXCR6, CCR3, CCR7, cutaneous lymphocyte antigen, and CD27 were not distinguishable from isotype controls. Furthermore, no clear chemokine receptor pattern was discernible when $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ clones were compared.

As reported previously with $CD4^+$ clones,[14] stimulation of T-cell clones resulted in the secretion of IFN-γ ($CD4^+$, 361.9 ± 384.8 pg/mL [n = 4]; $CD8^+$, 435.0 ± 581.9 pg/mL [n = 4]; $CD4^+CD8^+$, 1320 pg/mL [n = 1]) and/or IL-5 ($CD4^+$, 398.5 ± 700.5 pg/mL [n = 4]; $CD8^+$, 493.5 ± 706.9 pg/mL [n = 4]; $CD4^+CD8^+$, 1335 pg/mL [n = 1]). IL-4 and IL10 were not detected in culture supernatant.

**TABLE V.** Receptor expression on the surface of carbamazepine-specific T-cell clones

| | $CD4^+$ | | $CD8^+$ | | $CD4^+CD8^+$ | |
|---|---|---|---|---|---|---|
| Receptor | 21 | 45 | 77 | 80 | 21b | 91 |
| CXCR4* | 2.8 | 1.0 | 1.6 | 1.7 | 1.2 | 1.3 |
| CXCR6 | 1.0 | 0.9 | 0.9 | 1.1 | 1.0 | 0.9 |
| CCR3 | 1.2 | 1.0 | 1.1 | 1.2 | 1.2 | 1.1 |
| CCR4 | 1.2 | 1.6 | 1.7 | 1.6 | 1.0 | 1.8 |
| CCR5 | 0.9 | 7.1 | 1.1 | 16.5 | 1.1 | 13.4 |
| CCR7 | 1.4 | 0.8 | 1.0 | 1.1 | 0.8 | 0.9 |
| CCR8 | 3.5 | 10.4 | 1.4 | 10.6 | 3.7 | 2.4 |
| CCR9 | 1.1 | 1.4 | 1.9 | 1.8 | 1.8 | 1.8 |
| CCR10 | 1.4 | 2.9 | 1.4 | 1.1 | 1.1 | 1.6 |
| CLA | 1.0 | 1.1 | 1.1 | 1.0 | 1.1 | 1.0 |
| CD11a | 21.4 | 126.8 | 202.3 | 147.0 | 150.0 | 232.0 |
| CD18 | 11.7 | 27.5 | 15.1 | 11.6 | 11.7 | 16.5 |
| CD27 | 1.1 | 1.0 | 1.1 | 1.3 | 1.0 | 1.2 |

*Receptor expression expressed as fold change in MFI.

## Drug presentation to carbamazepine-specific $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T-cell clones

The importance of MHC in carbamazepine presentation to clones was assayed in antibody blocking experiments. First, carbamazepine stimulation of $CD4^+$ T-cell clones was MHC class II restricted; second, carbamazepine was presented to some $CD8^+$ T-cell clones in the context of MHC class I and others in the context of MHC class II; and third, carbamazepine stimulation of certain $CD4^+CD8^+$ T-cell clones was not inhibited by antibodies against MHC class I or II, or when both antibodies were added together (Table IV). These data are indicative of direct presentation of carbamazepine to T cells. To explore this possibility further, a panel of T-cell clones were stimulated with carbamazepine directly in the absence of B-LCL. Carbamazepine did not directly stimulate $CD4^+$ or $CD8^+$ T-cell clones; however, $CD4^+CD8^+$ T-cell proliferation was detectable with carbamazepine in the absence of B-LCL (Fig 2, *A*).

Carbamazepine-specific $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T-cell clones did not proliferate in the presence of B-LCLs pulsed with carbamazepine. Furthermore, B-LCLs fixed with glutaraldehyde were able to present carbamazepine to

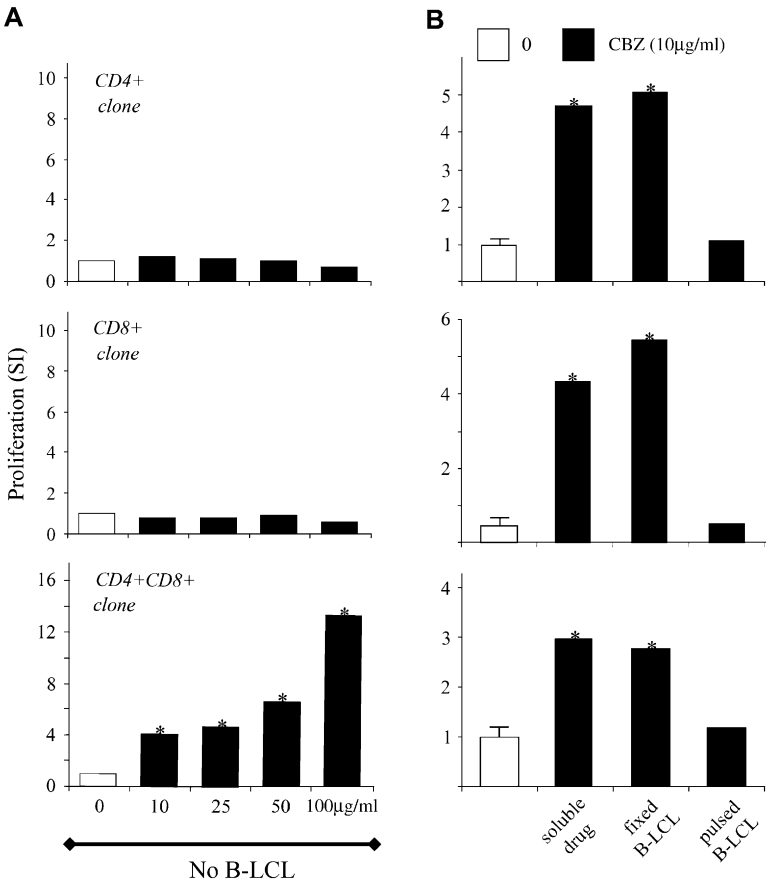


**FIG 2. A,** Carbamazepine *(CBZ)* stimulates proliferation of $CD4^+CD8^+$ T-cell clones in the absence of B-LCL. **B,** CBZ is presented to $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T-cell clones directly, in the absence of processing. Proliferation was determined by incorporation of [$^3$H]-thymidine. Statistical analysis compares incubations in the presence and absence of drug (*$P < .05$). Coefficient of variation was consistently less than 20%.

$CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T-cell clones, which is indicative of processing-independent drug presentation (Fig 2, *B*).

## DISCUSSION

Carbamazepine hypersensitivity syndrome is a severe delayed-type immune-mediated adverse drug reaction. Patients usually present with skin rash and fever, and may also manifest a multitude of phenomena including eosinophilia, lymphadenopathy, viral reactivation, and internal organ involvement. Data presented herein, using T-cell clones generated with traditional cloning methods from the blood of 5 patients with hypersensitivity and a positive lymphocyte transformation test to carbamazepine (Table I), show that the vast majority of carbamazepine-specific clones preferentially express the $CD4^+$ coreceptor (Table II). Approximately 10% of clones expressed high levels of $CD8^+$, which is in agreement with our previous findings.[14,15] Nevertheless, carbamazepine-specific $CD8^+$ T-cell clones were generated in high numbers by depleting $CD4^+$ T cells before serial dilution. Approximately 50% of carbamazepine-specific T-cell clones generated by using this revised methodology preferentially expressed the $CD8^+$ coreceptor (patients 4 and 5; Table II). Importantly, by monitoring [$^3$H] thymidine incorporation and immune-mediated killing of [$^{51}$Cr] loaded B-LCL as markers of antigen specificity simultaneously, certain $CD8^+$ clones were identified as antigen-specific exclusively on the basis of killing of target cells (Table III). These data draw attention to the fact that previous studies that have used T-cell clones generated by serial dilution are most likely to have underestimated the frequency and importance of $CD8^+$ T-cells.

Antigen-specific immune tolerance or anergy is a feature of repeatedly stimulating cloned $CD8^+$ T cells. Approximately 25% of $CD8^+$ clones were found to convert to this nonresponsive state rapidly and/or were resistant to mitogen-mediated expansion *in vitro*. Therefore, only minimal functional analyses could be performed on these clones. An additional atypical fraction of $CD8^+$ clones (approximately 10% of all $CD8^+$ clones) showed expansion in high numbers in the presence of only IL-2 for as long as 6 months. Such clones retained antigen specificity throughout this culture period (eg, clone 45; Tables III and IV).

In contrast with $CD4^+$ clones that proliferate readily but display limited cytotoxicity after carbamazepine

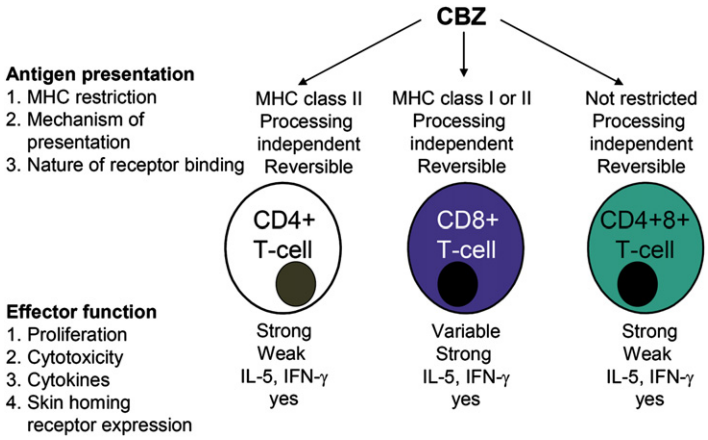


**FIG 3.** Scheme depicting functionality and mechanisms of CBZ presentation to $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T-cell clones. *CBZ,* Carbamazepine.

stimulation, $CD8^+$ clones were found to kill autologous target cells (Table IV), often at very low effector:target cell ratios (0.05:1-1:1; results not shown). Cytotoxicity was inhibited with concanamycin A, which suggests a perforin-mediated killing. Although these data were derived from a small cohort of carbamazepine-hypersensitive patients, identification of $CD8^+$ clones was not patient-specific and did not seem to be related to the severity of disease (Table I). This provides evidence that carbamazepine-specific $CD8^+$ T cells are the mediators of the cytotoxic response in hypersensitive patients. Previously, carbamazepine-specific $CD4^+$ clones have been shown to secrete IFN-γ and variable levels of IL-5.[14] To compare cytokine secretion from $CD4^+$ and $CD8^+$ clones, levels of $T_H1$-type and $T_H2$-type cytokines (IFN-γ, IL-4, IL-5, and IL-10) were compared after carbamazepine stimulation. $CD4^+$ and $CD8^+$ secreted similar levels of IFN-γ and IL-5, but IL-4 and IL-10 secretion was not observed. IFN-γ is a cytokine that drives cell-mediated immune responses by increasing basal keratinocyte expression of MHC class I and II, rendering them more susceptible to T-cell–mediated cytotoxicity.[5] IL-5 secretion is thought to mediate the prominent eosinophilia associated with carbamazepine hypersensitivity.

$CD4^+$ T-cell clones were stimulated by carbamazepine exclusively in an MHC class II restricted manner (Table IV).[14] In contrast, carbamazepine was presented to individual $CD8^+$ T-cell clones in the context of either MHC class I or MHC class II. MHC class I and class II restricted drug presentation to $CD8^+$ T-cell clones has been reported previously with sulfamethoxazole and amoxicillin.[4,9] Keuchler et al[9] suggest that MHC class I restricted drug presentation to $CD8^+$ clones might be more damaging, whereas killing by $CD8^+$ T cells recognizing drugs in association with MHC class II might be more limited. Data presented herein do not support this hypothesis (carbamazepine presentation in the context of MHC class I and II stimulated similar levels of cytotoxicity); however, the importance of these 2 different forms of drug presentation to $CD8^+$ T cells requires further investigation.

An unforeseen observation of our study was the frequent outgrowth of $CD4^+CD8^+$ carbamazepine-specific T-cell clones (Fig 1; Table III). Such T cells are distinguished and characterized on the basis of levels of receptor expression. T cells expressing high levels of one receptor and low levels of the other are thought to be produced as a consequence of repetitive stimulation and long-term *in vitro* expansion,[21] and as such, their relevance to human disease appears limited. However, T cells expressing high levels of both coreceptors are more interesting because they exist *in vivo*, presumably by evading thymic deletion, and their frequency is increased in pathogenic conditions such as rheumatoid arthritis and atopic dermatitis.[22,23] The majority of carbamazepine-specific $CD4^+CD8^+$ T-cell clones described in our patients expressed moderate to high levels of $CD4^+$ and $CD8^+$; these levels were comparable to those seen in single positive clones (Fig 1). They exhibited functional characteristics similar to $CD4^+$ T cells in that antigen stimulation was associated with a strong proliferative response and IFN-γ and IL-5 secretion, but only low levels of cytotoxicity (Table IV). Interestingly, stimulation of certain $CD4^+CD8^+$ T-cell clones was not inhibited by anti-MHC class I and II blocking antibodies, and antigen-stimulated proliferation was detected when carbamazepine was incubated with T cells alone, in the absence of APCs. Previous studies have shown that prenyl pyrophosphate antigens can be presented to γδ T cells through a direct pathway that requires neither processing nor known antigen-presenting molecules;[24-26] however, to the best of our knowledge, the results outlined are the first to show drug antigen-specific proliferation of αβ T cells in the absence of APCs and MHC.

Carbamazepine stimulates $CD4^+$ and additionally $CD4^+CD8^+$ and $CD8^+$ clones in the absence of any apparent covalent binding or antigen processing (Fig 2), which adds to the growing body of evidence that certain drugs/chemicals can mimic the binding interaction of peptides with specific T-cell receptors via a series of thus far undefined weak reversible binding interactions.[7,20,27-30]

The migration of immune cells to sites of inflammation is known to be a critical determinant of allergic disease, including drug hypersensitivity. Leyva et al[12] previously reported increased T-cell expression of the skin homing receptor, cutaneous lymphocyte antigen, in skin and blood of patients with anticonvulsant hypersensitivity, which paralleled the severity of the disease. We have also shown high expression of cutaneous lymphocyte antigen on T-cell clones from a patient hypersensitive to the anticonvulsant lamotrigine.[31] Somewhat surprisingly, a panel of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T-cell clones expressing $CD11a^+CD27^-$ and thus referred to as *effector memory T cells* according to Hamann et al[32] were found not to express cutaneous lymphocyte antigen. Additional pathways may also explain cell selective cutaneous migration, particularly expression of chemokine receptors such as CCR4, CCR8, and CCR10. CCR8 is responsible for the steady-state trafficking of memory T cells into healthy skin,[33,34] whereas chemokine receptors CCR4 and CCR10 orchestrate selective entry of T cells into inflamed skin. Increased expression of CCR4 and CCR10 is associated with susceptibility to allergic diseases such as contact dermatitis, atopic dermatitis, and psoriasis.[35-39] We found high expression of CCR8 on all carbamazepine-specific $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ clones (Table V), indicating a potential pathway for cutaneous migration of carbamazepine-specific T cells. Interestingly, the ligand for CCR8, I-309, is constitutively expressed in skin, whereas stimulated $CCR8^+$ T cells secrete significant quantities of I-309. This will lead to further recruitment of $CCR8^+$ T cells through a positive feedback mechanism.[33] Indeed, we have previously shown increased expression of I-309 mRNA in activated T-cell clones isolated from patients with lamotrigine hypersensitivity.[31] Carbamazepine-specific T cells were also found to express detectable levels of CCR4 and/or CCR10, which highlights that multiple pathways are involved in cutaneous T-cell infiltration. These data also agree with previous studies showing elevated CCR10 mRNA levels in lesional skin during the acute phase of a drug hypersensitivity reaction.[40] Certain T-cell clones were found differentially to express additional chemokine receptors (eg, CXCR4, CCR5, and CCR9) not associated with cutaneous migration. Whether these homing receptors are involved in the migration of carbamazepine-specific T cells to other internal organs is not known and is an area that warrants further investigation.

In conclusion, data presented herein show that carbamazepine-specific $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells displaying different effector functions (Fig 3) persist in the circulation of hypersensitive patients' blood for many years after resolution of clinical symptoms. Additional studies are planned to clone T cells directly from inflamed skin of carbamazepine-hypersensitive patients. We intend to compare the phenotype, function, and expression of skin homing receptors on cutaneous and blood-derived T cells, focusing particularly on (1) differences in proliferation and cytotoxicity; (2) migration of T cells toward chemokines released from inflamed skin; and (3) differential cytokine secretion by analysis of a wider array of cytokines.

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