T Cells Isolated from Positive Epicutaneous Test Reactions to Amoxicillin and Ceftriaxone are Drug Specific and Cytotoxic

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In order to investigate the function of T cells in cutaneous adverse drug reactions, skin-derived T cells were analyzed in two patients with a drug-induced exanthem. Skin biopsy specimens were obtained from positive epicutaneous test reactions to amoxicillin and ceftriaxone. Immunohistochemical analysis revealed that the majority of the cell infiltrate in both biopsy specimens was composed of activated T cells, of which some expressed perforin. By limiting dilution 36 amoxicillin-specific and 10 ceftriaxone-specific T cell clones were raised. All of these T cell clones expressed CD4/T cell receptor αβ. Cytokine analysis after antigen stimulation of the seven best proliferating T cell clones (four specific for amoxicillin and three for ceftriaxone) revealed that these cells secrete high amounts of interleukin-5 and mostly lower or no amounts of tumor necrosis factor α, interleukin-4, and interferon-γ. A part of these CD4+ T cell clones were cytotoxic, i.e., two selected ceftriaxone-specific T cell clones killed target cells after antigen stimulation. The amoxicillin-specific T cell clones failed to show drug-specific cytotoxicity, but killed target cells in the presence of concanavalin A, indicating a principal ability to be cytolytic. In correlation with the *in situ* expression of perforin on T cells, the ceftriaxone-specific T cell clones also expressed perforin *in vitro*. In conclusion, a substantial part of the T cells in drug-induced epicutaneous test reactions are drug specific and are composed of a heterogeneous cell population. Drug-specific T cells producing interleukin-5 may contribute to eosinophilia, whereas cytotoxic CD4+ T cells may account for tissue damage. These data underline the role of T cells in delayed-type cutaneous adverse drug eruptions and drug-induced epicutaneous test reactions. ***Key words: cytokines/cytotoxicity/drug allergy/immunochemistry/skin/T lymphocytes. J Invest Dermatol 115:647–652, 2000***

Drugs can elicit various cutaneous adverse reactions, which may partly be of immunologic origin. The diagnosis of drug allergy has primarily been focused on the detection of IgE-mediated reactions. The pathogenesis of these reactions is relatively well understood and their diagnosis is based on *in vivo* and *in vitro* tests, namely prick or intradermal tests and detection of drug-specific serum IgE, respectively (Breathnach, 1995a, b). Increasing evidence, however, suggests that T-cell-mediated immune responses are mainly involved in certain cutaneous adverse drug reactions (ADR), i.e., in maculopapular exanthemas, fixed drug eruptions, as well as more severe bullous reactions like erythema exsudativum multiforme, Steven Johnson syndrome, and toxic epidermal necrolysis (Breathnach, 1995a, b; Barbaud *et al*, 1997). This has partly been inferred from epicutaneous testing and lymphocyte transformation tests (LTTs), which have been reported to be useful in identifying a T-cell-mediated sensitization to certain drugs (Osawa *et al*, 1990; Calkin and Maibach, 1993; Nyfeler and Pichler, 1997; Barbaud *et al*, 1998). Moreover, the analysis of drug-specific T cell lines (TCL) and T cell clones (TCC) has revealed new data on the recognition of drugs by T cells and their functional capacity, including cytokine production and cytotoxic potential (Hertl and Merk, 1995; Mauri-Hellweg *et al*, 1995, 1996; Schnyder *et al*, 1997, 1998; Zanni *et al*, 1997, 1998; Pichler *et al*, 1998; Von Greyerz *et al*, 1999). Notwithstanding such evidence for an involvement of T cells in certain ADR, the significance of epicutaneous testing in the assessment of drug allergies has still remained controversial. Reasons for this are the lack of standardized topical preparations as well as the uncertain sensitivity and specificity of such test procedures. Furthermore, data on specificity and functional capacity of drug-specific T cells derived from positive epicutaneous test reactions to drugs have so far remained scarce (Hertl *et al*, 1993).

In order to improve our understanding of the function of T cells in cutaneous ADR, positive epicutaneous test reactions to amoxicillin and ceftriaxone were investigated in two patients with the respective drug allergies. Immunohistochemical analysis was performed to determine the nature of the inflammatory cell infiltrate *in vivo*. Drug-specific TCC were generated from the skin biopsy specimens and analyzed with regard to their phenotype, specificity, cytokine profile, and cytotoxic capacity *in vitro*. Our data demonstrate a great functional heterogeneity of skin-infiltrating T cells in drug-induced epicutaneous test reactions and provide further evidence for a role of drug-specific T cells in cutaneous ADR.

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Abbreviations: ADR, adverse drug eruptions; B-LCL, B-lymphoblastoid cell lines; CM, culture medium; LTT, lymphocyte transformation test; SI, stimulation index; TCL, T cell lines; TCC, T cell clones.

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MATERIALS AND METHODS

Patients Three days after intravenous administration of Augmentin (amoxicillin and clavulanic acid), which was given as a prophylaxis against infection after hysteroscopy and laparoscopy, a 22-y-old female (patient AB) developed erythrodermia with some bullae on her legs, fever (38°C–40°C), and blood eosinophilia (1.3×10^9 per liter). Sensitization to amoxicillin was confirmed by epicutaneous testing (described below) and an LTT [stimulation index (SI) for amoxicillin 500 μg per ml, 10.5]. After discontinuation of Augmentin and treatment with systemic and topical corticosteroids the patient recovered within 2 wk.

The detailed history of the second patient has been described previously (Yawalkar *et al*, 1999). Briefly, an 80-y-old female (patient MBZ) developed a drug-induced linear IgA bullous dermatosis during treatment with ceftriaxone, metronidazole, furosemid, cinnarizine, lorazepam, acetaminophen, and codeinphosphate. Laboratory results showed peripheral leukocytosis (18.5×10^9 per liter) with eosinophilia (3.8×10^9 per liter). A sensitization to ceftriaxone and metronidazole was detected by epicutaneous testing and an LTT (SI for ceftriaxone 100 μg per ml, 21.4; for metronidazole 10 μg per ml, 5.5). After discontinuation of the drugs and subsequent treatment with systemic corticosteroids for 8 d the bullous eruptions subsided and erosions healed within 6 wk.

Epicutaneous testing Epicutaneous testing (scratch-patch) was performed 2 mo after recovery with phosphate-buffered saline (PBS) (negative control), ceftriaxone (125 mg per 0.5 ml PBS), and amoxicillin (375 mg per 0.5 ml PBS), as described previously in detail (Yawalkar *et al*, 1999). Briefly, after scarification of the epidermis freshly prepared drug solutions were applied on the upper back using Al-test patch test disks (IMAL Pharmaceutica, Zürich, Switzerland). The test reactions were read at 24 and 48 h, and scored as recommended by the International Contact Dermatitis Research Group (Wilkinson *et al*, 1970). No positive reactions to ceftriaxone and amoxicillin were found in 10 controls, confirming the specificity of the scratch-patch test.

Culture media The culture medium (CM) consisted of RPMI 1640 supplemented with 10% pooled heat-inactivated human AB serum (Swiss Red Cross, Bern, Switzerland), 25 mM HEPES buffer, 2 mM L-glutamine, 10 mg per ml streptomycin, and 100 U per ml penicillin. CM^+ used to culture TCL and TCC was enriched with 20 U per ml recombinant interleukin-2 (IL-2) (Dr. A. Cerny, Inselspital, Switzerland). The medium for culture of Epstein-Barr virus (EBV) transformed B-lymphoblastoid cell lines (B-LCL) was RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Paisley, Scotland), 25 mM HEPES buffer, but no L-glutamine and no antibiotics. The B-LCL were generated by transformation of freshly isolated peripheral blood mononuclear cells (PBMC) with supernatant of the EBV-producing cell line B95-8. Cyclosporin A (1 μg per ml; Novartis, Basel, Switzerland) was added to prevent EBV-induced T cell growth.

Immunohistochemistry of skin biopsy specimens Five millimeter punch biopsy specimens were obtained from the positive epicutaneous test reaction to amoxicillin and ceftriaxone after 48 h and were divided in two equal parts. One half of the skin biopsy was snap-frozen in tissue embedding medium using isopentane precooled in liquid nitrogen, and was stored at −70°C. The following monoclonal antibodies were used for single immunostaining: anti-CD4 (clone MT310; concentration 2.3 μg per ml; Dako, Glostrup, Denmark), anti-CD8 (clone DK25, 1.5 μg per ml; Dako), anti-CD25 (clone ACT-1, 3.8 μg per ml; Dako), anti-HLA-DR (clone CR3/43, 2.7 μg per ml; Dako), and antiperforin antibodies (clone δG9, 33 μg per ml; Ancell, Bayport, MN). Substitution of the primary antibody with isotype-matched IgG and omission of the primary antibody served as negative controls. Immunostaining was performed using the avidin-biotin-complex/alkaline phosphatase (ABC/AP) method. Five micron sections from the snap-frozen tissue blocks were cut on a cryostat, air-dried, and fixed in 2% formaldehyde (for perforin) or acetone (for the remaining antibodies). Slides were then incubated with the primary antibody, followed by a biotinylated rabbit antimouse IgG (dilution 1:50 for perforin, 1:100 for the remaining antibodies; E0413; Dako) and thereafter with ABC/AP (K0376; Dako). Finally, all sections were developed in new fuchsin-naphtol (Sigma, St. Louis, MO) and counterstained with hematoxylin.

Double immunostaining was performed by combining two indirect staining methods with two unlabeled primary antibodies of mouse (antiperforin or antigranzyme B) and rat (anti-CD4, clone YNB46.1.8, Serotec, Oxford, U.K., or anti-CD8, clone YTC 141.1HL, Serotec) origin as described previously (Yawalkar *et al*, 2000). Briefly, slides were initially incubated with the primary antibodies, followed by alkaline phosphatase-conjugated goat antimouse immunoglobulin (Dako, D0486) and peroxidase-conjugated goat antirat immunoglobulin (Jackson ImmunoResearch Laboratories, West Grove, PA). Sections were then developed using new fuchsin-naphtol and diaminobenzidine, which revealed a red and brown staining color, respectively, and were finally counterstained with hematoxylin. The specificity of the reaction was confirmed by omitting the primary antibodies and using appropriate isotype-matched antibodies as negative controls.

Isolation of drug-specific T cells from epicutaneous test reactions The second half of the biopsy specimen was mechanically disaggregated and kept overnight in a 0.25% trypsin solution at 4°C. A single-cell suspension was obtained by extensively resuspending the skin pieces in CM with a Pasteur pipette before filtrating the supernatant over a 70 μm nylon cell strainer (Falcon, Franklin Lakes, NJ). The isolated cells were cultured with 25,000 irradiated allogeneic PBMC plus 1 μg per ml phytohemagglutinin (PHA) (Boehringer, Mannheim, Germany) in a final volume of 100 μl CM per well at 1 cell per well for patient AB and 10 cells per well for patient MBZ in 5–8 96 well round-bottomed plates (Falcon). Growing T cell lines or clones were expanded in CM^+ and restimulated every 14 d with allogeneic PBMC and PHA as described above.

Proliferation of skin-derived TCL and TCC to amoxicillin and ceftriaxone To measure the antigen-specific proliferation of TCL and TCC, 2.5×10^4 cells were incubated with the same number of autologous irradiated PBMC (3000 rad) or 5×10^3 B-LCL (6000 rad) in the presence or absence of amoxicillin (500 μg per ml) or ceftriaxone (100 μg per ml) in a final volume of 200 μl CM in a 96 well round-bottomed plate (Falcon). After 48 h, [^{3}H]thymidine (0.5 mCi) was added for 12 h, and cells were harvested on glass fiber disks and counted in a microplate beta counter (Inotech Filter Counting System INB-384; Inotech, Dottikon, Switzerland).

Flow cytometry analysis of TCC The phenotype and T cell receptor (TCR) Vβ chain distribution of the TCC was analyzed by double-color cytofluorometry as described previously (Zanni *et al*, 1997). The following fluorochrome-conjugated antibodies were used: anti-CD3 (Dako), anti-CD4 (Dako), anti-CD8 (Dako). Anti-TCR Vβ5.1 (clone LC4), Vβ 5.2/5.3 (clone 1C1), Vβ5.3 (clone W112), Vβ6.7 (clone OT145), and Vβ12 (clone S511) were obtained from T Cell Science (Cambridge, MA), and Vβ1 (clone BL37.2), Vβ2 (clone E22E7), Vβ3 (clone IMMU222), Vβ7 (clone ZOE), Vβ9 (clone FIN9), Vβ13.3 (clone JU74.3), Vβ14 (clone CAS1.1.3), Vβ16 (clone TAM-MAYA1.2), Vβb17 (clone E17.5F31513), Vβ18 (clone BA62), Vβ20 (clone ELL1.4), Vβ21.3 (clone IG125), Vβ22 (clone IMMU546), and Vβ23 (clone AF23) from Immunotech (Marseille, France).

Briefly, aliquots containing 1.0×10^5 cells were stained with fluorochrome-conjugated antibodies in PBS, 1% FBS, and 0.02% NaN_3 for 30 min at 4°C. After two washings with the above mentioned buffer, cells were fixed with 0.5% paraformaldehyde and then analyzed on an EPICS profile II flow cytometer (Coulter, Hialeah, FL).

Immunofluorescence analysis for perforin on TCL and TCC Expression of perforin on selected TCC (DB3, FD3, FD11) was investigated by indirect immunofluorescence analysis using a mouse antihuman antiperforin antibody (clone δG9, 33 μg per ml, Ancell). Cytospots with approximately 2×10^4 cells each were prepared, air-dried, and fixed in 2% formaldehyde. After washing, cytospots were incubated with antiperforin antibodies for 3 h. Substitution of the primary antibody with isotype-specific IgG and omission of the primary antibody served as negative controls. Cell smears were then washed and incubated with a fluorescein-isothiocyanate-labeled antimouse antibody (dilution 1:200, Sigma). Staining was evaluated by two investigators. More than 200 cells were counted and the mean percentage of perforin positive cells ± SD were expressed.

Cytotoxicity assay Cytotoxicity of the TCC was tested at days 8–14 after restimulation by a standard 4 h cytotoxicity assay as described previously in detail (Schnyder *et al*, 1997, 1998). Briefly, autologous B-LCL were generated by transformation of freshly isolated PBMC with supernatant of the EBV-producing cell line B95-8. Alternatively, PBMC were also used as targets in some experiments. ^{51}Cr labeling was performed by incubation with 50 μCi per well ^{51}Cr sodium chromate solution (Amersham International) for 60 min. A total of 5×10^4 cells were then used as targets in the presence or absence of amoxicillin (500 μg per ml) and ceftriaxone (100 μg per ml). In addition, mitogen-induced cytotoxicity was assessed by using concanavalin A (Con A) (2.5 μg per ml). For APC-pulsing

experiments, autologous B-LCL or PBMC were incubated with and without the drugs overnight in CM. The cells were then washed twice with Hanks' balanced salt solution and resuspended in CM. E/T ratios were 10:1, 3:1, 1:1. Specific lysis was calculated as (lysis in the presence of the drug or Con A) – (lysis without the drug or Con A). Spontaneous ^{51}Cr release from different target cells ranged between 55 and 210 cpm.

Enzyme-linked immunosorbent assay (ELISA) Human IL-4, IL-5, tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ) were measured 48 h after stimulation with the corresponding drug in the supernatant of TCL and TCC with a cytokine-specific sandwich ELISA using monoclonal antibody pairs (native capture monoclonal antibodies and biotinylated detecting monoclonal antibodies, all obtained from Pharmingen, San Diego, CA) as described previously (Zanni *et al*, 1997). Samples were measured in duplicate. The detection limit of the assays performed was 4 pg per ml.

RESULTS

Perforin immunoreactivity is enhanced in positive epicutaneous test reactions to amoxicillin and ceftriaxone Epicutaneous testing (scratch-patch) revealed a strong allergic reaction with erythema, papules, and some vesicles to amoxicillin and ceftriaxone in patient AB and patient MBZ, respectively. Skin biopsy specimens showed a dense, superficial, predominantly mononuclear cell infiltrate with some eosinophils. A marked exocytosis and spongiosis were seen in the epidermis. Furthermore, focal interface changes with vacuolar alteration in the basal cell layer were observed.

Immunohistochemical staining revealed predominantly a T cell infiltration consisting of approximately two-thirds of CD4+, CD45RO+ and one-third of CD8+, CD45RO+ cells in both biopsy specimens. Approximately 90%–100% of the mononuclear cell infiltrate was strongly stained for HLA-DR. CD25 (IL-2R) was detected on about 15%–20% of the mononuclear cell infiltrate, especially on cells located at the dermoepidermal junction. Furthermore, perforin immunoreactivity was observed in the cytoplasm of lymphocytic cells scattered throughout the dermis and in particular at the dermoepidermal junction and in the epidermis. A representative section from the skin biopsy specimen of patient MBZ is shown in **Fig 1**. In addition, double immunostaining experiments demonstrated that both CD4+ and CD8+ cells express perforin (not shown). Expression of perforin was observed on 10%–15% and 14%–18% of the CD4+ and CD8+ T cells, respectively.

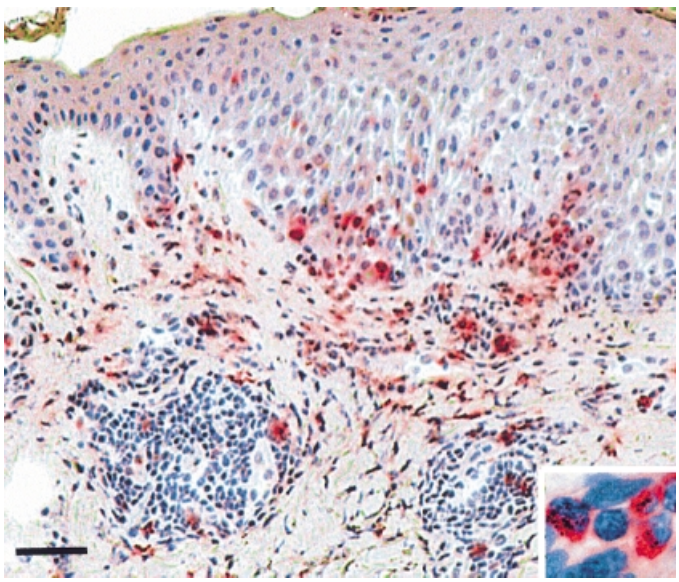


Figure 1. Localization of perforin in a positive epicutaneous test reaction to ceftriaxone. Perforin immunoreactivity was found on lymphocytic cells (insert) infiltrating the dermis and in particular the dermoepidermal junction. *Scale bar:* 50 μm (insert 10 μm).

T cells isolated from positive epicutaneous test reactions to amoxicillin and ceftriaxone are drug specific Amoxicillin-specific and ceftriaxone-specific TCC were generated from the positive epicutaneous test reaction to amoxicillin and ceftriaxone from patient AB and patient MBZ, respectively. A total of 104 TCC were directly gained from patient AB, of which 36 were specific for amoxicillin (SI = 3–70.9). All TCC of patient AB were only able to recognize the drug with autologous PBMC but not with B-LCL as antigen-presenting cells (APC). Selected TCC (18 of 36) were analyzed with regard to their phenotype (T cell subset, TCR specificity) by cytofluorometry. All of these TCC expressed CD4/TCRαβ. Six TCC reaching the highest SI were further characterized with regard to their TCR Vβ chain. As shown in **Fig 2**(*a*), three TCC expressed Vβ 3, one TCC Vβ 17, one TCC Vβ 22, and another Vβ 13.1, indicating no preferential use of a certain TCR.

Ninety-six TCL were gained from the positive epicutaneous test reaction to ceftriaxone from patient MBZ. Eight of them showed an enhanced proliferation in the presence of ceftriaxone either with PBMC or with B-LCL as APC (SI = 3–28.4). For further proliferation tests B-LCL were used as APC. Subcloning of the two best proliferating TCL 37D (SI 16.07) and 37F (SI 28.42) was performed by limiting dilution. Eight of 18 TCC (DB1, DB2, DB3, DB4, DB6, DB7, DC6, DD9) from TCL 37D and two of 19 TCC (FD3, FD11) from TCL 37F were found to be specific to

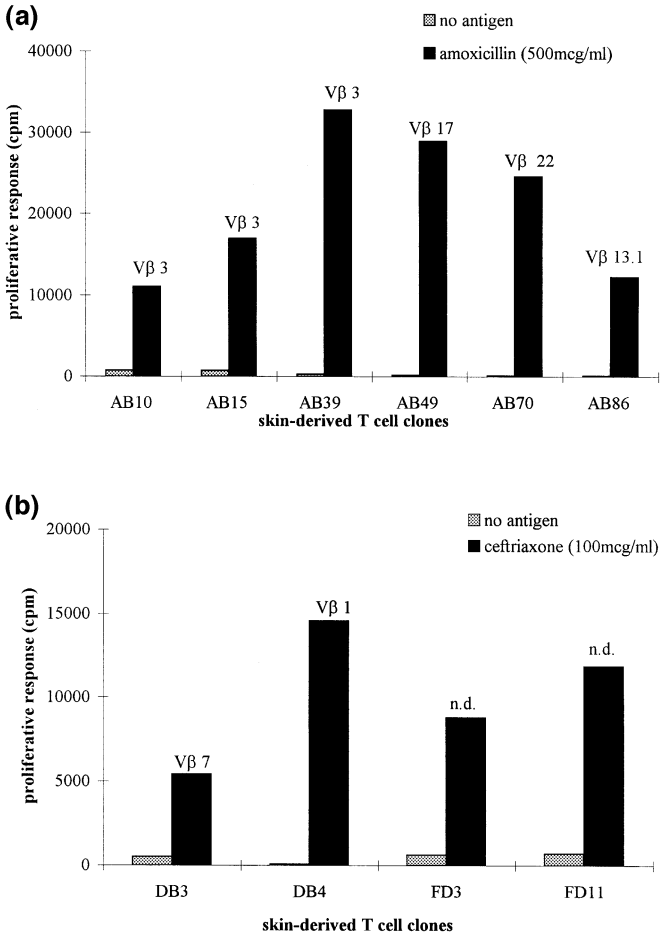


Figure 2. Specificity of amoxicillin-specific and ceftriaxone-specific TCC. The specificity and TCR Vβ chain distribution of selected skin-derived TCC from patient AB (*a*) and patient MBZ (*b*) are shown. Clone cells (5×10^4) were incubated with the same number of autologous APC (PBMC or B-LCL) with or without amoxicillin (500 μg per ml) or ceftriaxone (100 μg per ml). [^{3}H]thymidine was measured after 48 h. TCR Vβ chain distribution was analyzed by flow cytometry. Representative results from one of three experiments are demonstrated. n.d., not detectable.

ceftriaxone. All of these TCC were CD4+/αβ+. Four of these TCC, reaching the highest SI, were also characterized with regard to their TCR Vβ chain. Vβ staining revealed Vβ 1 for TCC DB4 and Vβ 7 for DB3 (**Fig 2*b***). The TCR Vβ chain was not detectable in the TCC FD3 and FD11.

Furthermore, proliferation assays with different concentrations of amoxicillin or ceftriaxone (10–5000 μg per ml) were performed with one TCC of each specificity reaching the highest SI (AB39, DB4). As shown in **Fig 3**, a dose-dependent proliferation was found for both TCC. No proliferation was found for penicillin G (10–10,000 μg per ml) or tetanus toxoid (5 μg per ml) (data not shown) confirming the specificity of the TCC.

Drug-specific TCC predominantly produce IL-5 To evaluate the cytokine profile of these skin-derived, drug-specific TCC, cytokine production was measured in the supernatant of seven selected TCC (four specific for amoxicillin and three for ceftriaxone) by a sandwich ELISA 48 h after stimulation with the respective drug. As shown in **Fig 4**, IL-5 reached the highest levels in most cultures (33.6–930 pg per ml). Furthermore, considerable amounts of TNF-α (40–333.9 pg per ml) were also secreted, whereas only lower or no amounts of IL-4 (12.9–39.6 pg per ml) and IFN-γ (5.8–31 pg) were detected.

Cytolytic response of TCC Cytolytic assays were performed in the continuous presence or absence of amoxicillin (500 μg per ml) and ceftriaxone (100 μg per ml). Alternatively, target cells were also pulsed with amoxicillin (500 μg per ml) or ceftriaxone (100 μg per ml) overnight in CM. In patient AB neither pulsing nor the continuous presence of amoxicillin led to cytotoxic response against PBMC (data not shown). Cytotoxic assays with B-LCL as targets were not feasible as TCC from patient AB were unable to recognize the drug presented by B-LCL. As shown in **Fig 5(*a*)**, however, mitogen-induced cytotoxicity with Con A (2.5 μg per ml) and PBMC as targets could be detected in four of five TCC, indicating the principal ability of some TCC to kill.

In patient MBZ two of the selected three TCC were only able to lyze the target cells when they were first pulsed overnight with ceftriaxone (**Fig 5*b***). No cytotoxicity was detected when soluble ceftriaxone was continuously present during the assay (not shown). The reason for this lack of cytotoxicity is unclear. Previous reports have indicated that β-lactams may have an inhibitory effect on cytokine production by T cells, however, which subsequently could influence the cytotoxic potential of these cells (Padovan *et al*, 1999).

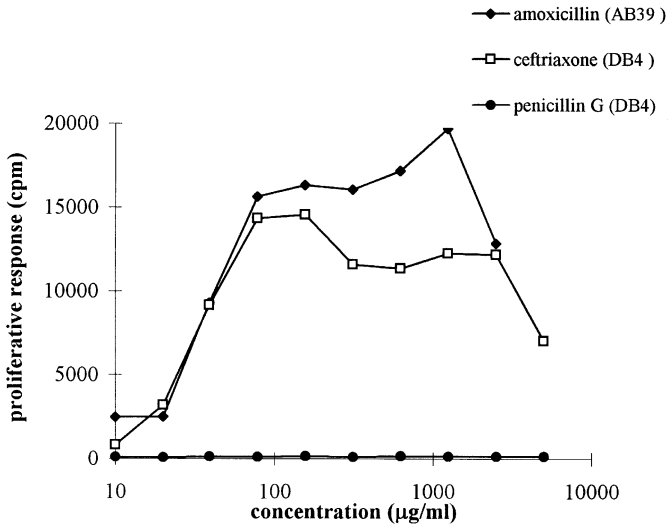


Figure 3. Dose response of TCC to amoxicillin and ceftriaxone. Dose-dependent proliferative response of one selected TCC to amoxicillin (patient AB) and ceftriaxone (patient MBZ) is shown. No proliferation was detectable for penicillin G, confirming the specificity of the clones (shown for DB4).

Perforin is expressed in skin-derived ceftriaxone-specific TCC In order to investigate the involvement of perforin in drug-induced cytotoxicity reactions *in vitro*, three selected ceftriaxone-specific TCC were stained for perforin by indirect immunofluorescence. A representative staining of a T cell (from TCC FD3) is shown in **Fig 6(*a*)**. Strong granular intracellular staining for perforin was found on 42.5%, 26.9%, and 12.3% of TCC DB3, FD3, and FD11, respectively. No staining was seen in the negative controls using an isotype-matched antibody (**Fig 6*b***) or in B-LCL (not shown).

DISCUSSION

In this study drug-specific T cells were isolated from positive epicutaneous test reactions to amoxicillin and ceftriaxone in two patients with the respective drug allergy and analyzed with regard to their phenotype, specificity, cytokine profile, and cytotoxic capacity. Our data suggest that the positive epicutaneous test reactions to these drugs, which had caused systemic reactions after oral administration, are not a toxic or irritative phenomenon. In particular, we demonstrate that such reactions are infiltrated by drug-specific T cells, which may orchestrate inflammation in the skin and subsequently lead to delayed-type skin test reactions. The distinct morphology between the eczematous reaction after epicutaneous testing and the acute exanthem may reflect the differences in how these drugs were applied, namely via the skin surface and not systemically as during the acute allergic reaction.

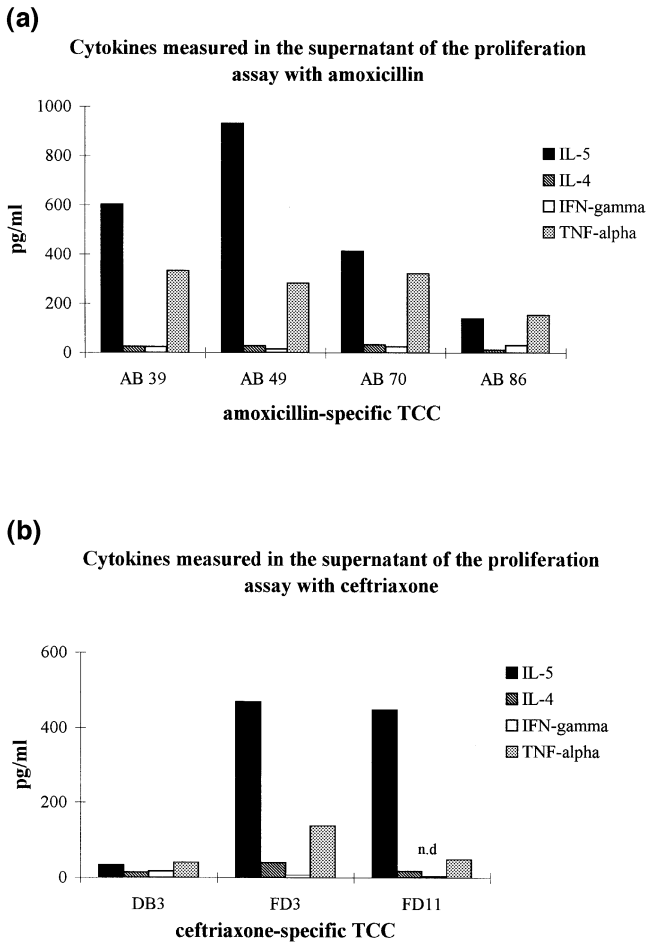
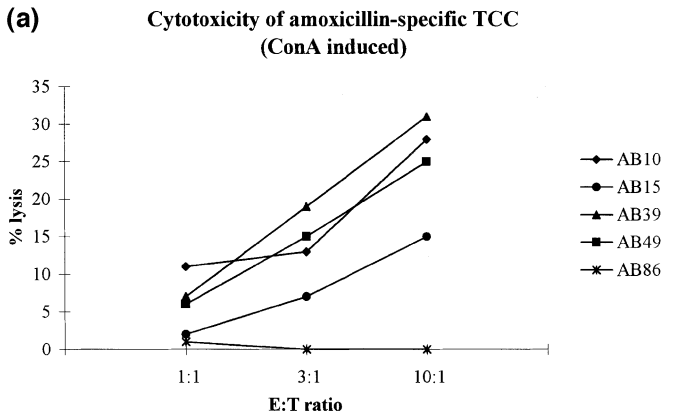


Figure 4. Cytokine secretion pattern of drug-specific skin-derived TCC. Human IL-4, IL-5, TNF-α, and IFN-γ were measured 48 h after stimulation with the corresponding drug in the supernatant of TCC with an ELISA assay. Both amoxicillin-specific (*a*) and ceftriaxone-specific (*b*) TCC secreted high amounts of IL-5 and mostly lower or no amounts of TNF-α, IL-4, and IFN-γ. n.d., not detectable.



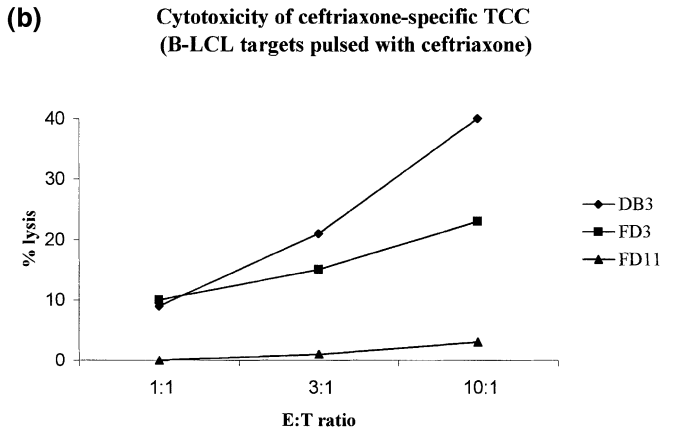


Figure 5. Cytotoxic response of amoxicillin-specific and ceftriaxone-specific TCC. Cytotoxicity of selected CD4+ TCC of patient AB was evaluated in a 4 h Cr release assay in the presence of Con A (2.5 μg per ml) and autologous PBMC as targets (*a*). Four-fifths of these TCC killed target cells in a dose-dependent manner in the presence of Con A, indicating a principal ability of these TCC to kill. In patient MBZ cytotoxicity of selected CD4+ TCC was evaluated after preincubation of autologous B-LCL with ceftriaxone (100 μg per ml) overnight (pulsing) (*b*). Specific lysis was found in two-thirds of these TCC. E/T ratios were 10:1, 3:1, 1:1. Percentage lysis was calculated as (lysis in the presence of the drug or Con A) – (lysis without the drug or Con A).

Immunophenotyping of the inflammatory cell infiltrate of both epicutaneous test reactions showed a predominance of CD4+ cells. These cells were mainly scattered throughout the dermis, but particularly also infiltrated the epidermis at sites of marked spongiosis. Interestingly, all the drug-specific TCC in both of our patients were exclusively of the CD4 phenotype. In contrast to these findings, Hertl *et al* were only able to generate CD8+ TCC from epicutaneous test reactions to ampicillin and benzylpenicillin (Hertl *et al*, 1993). These discrepancies may be due to differences in the isolation methods used, as T cells were only eluted from the epidermis by Hertl *et al* and not, as in our case, from the dermis as well. Another explanation might be that some drug-induced reactions are preferentially mediated by CD4+ T cells and others by CD8+ T cells. A selective generation of CD4+ TCC as an artifact of the cloning procedure seems unlikely, as we were previously able to establish both CD4+ and CD8+ TCC from the peripheral blood in other patients with drug allergies (Zanni *et al*, 1997). The marked predominance of CD4+ T cells in the epicutaneous test reaction at 48 h, however, could have exclusively favored the selection of drug-specific CD4+ TCC and we cannot exclude that isolation at a later time point or sequential analysis may have revealed some CD8+ T cells. Thus, although some cytotoxic CD8+ T cells may also be involved in eliciting tissue damage *in vivo*, the exclusive

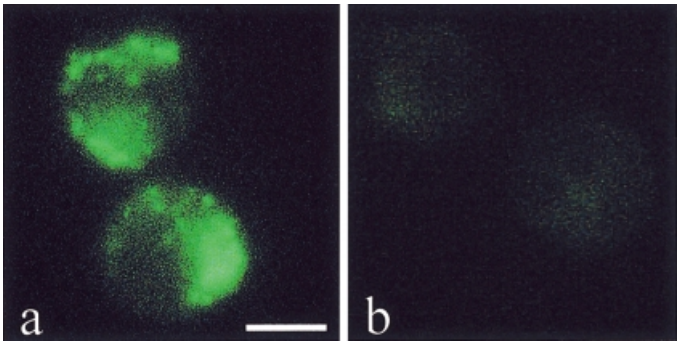


Figure 6. Expression of perforin on a ceftriaxone-specific TCC. Intracellular expression of perforin was visualized by indirect immunofluorescence. A representative staining from one ceftriaxone-specific TCC (FD3) is shown in (*a*). Negative control using an isotype-matched antibody is demonstrated in (*b*). *Scale bar:* 6 μm.

isolation of drug-specific CD4+ T cells strongly suggests that these cells mainly contribute to the inflammatory skin reaction at the time point of the skin biopsy (i.e., at 48 h).

In this study T cells were cloned by using PHA and irradiated allogenic PBMC as feeder cells in order to provide a maximal stimulus to all T cells at the start and to obtain a high yield of TCC. Autologous PBMC together with the drugs were not used as the drug-specific T cells, which had already been highly stimulated *in vivo,* may be refractory to further antigen-dependent stimulation *in vitro*. A further reason in favor of the method used in our study is that it allows the cloning frequency to be analyzed, as an unspecific stimulus to all cells (drug-specific and unspecific) is provided. In patient AB we succeeded in directly generating TCC from the positive epicutaneous test reaction to ampicillin, which allowed an estimate of drug-specific T cells in the skin infiltrate. About a third of the clones obtained were specific for amoxicillin; thus, a relatively high proportion of the infiltrating cells *in situ* appears to be drug specific. A similar frequency could also be obtained from the ceftriaxone-induced epicutaneous test reaction, although this estimate is indirect, as TCL were generated first. The remaining T cells (about two-thirds) may be either specific to another form of the drug (i.e., to a certain drug-peptide complex) or represent bystander activation of T cells with other specificities (e.g., to autoantigens) in the course of the inflammatory reaction.

A major finding of this study was the heterogeneity of the drug-specific CD4+ T cells, with regard to both TCR Vβ usage and their function. Although some drug-specific reactions can be oligoclonal, as previously demonstrated by a preferential usage of a certain TCR Vβ family (Mauri-Hellweg *et al*, 1996; Zanni *et al*, 1997), most drug-specific TCC generated from the peripheral blood bear heterogeneous TCRs (Von Greyerz *et al*, 1999). Particularly interesting was the heterogeneity of the T cell functions observed in this study. A part of the infiltrating CD4+ T cells were cytotoxic, as revealed by antigen specific and unspecific cytotoxicity tests. Together with the *in vivo* and *in vitro* expression of perforin on T cells, our data reemphasize the concept that cytotoxic T cells may contribute to tissue damage in cutaneous ADR. In particular, our previous results and these findings suggest that drug-specific cytotoxic CD4+ T cells may be substantially responsible for some typical histopathologic features such as vacuolar alteration and necrosis of keratinocytes as well as spongiosis in acute cutaneous ADR and positive epicutaneous test reactions to drugs (Schnyder *et al*, 1998; Yawalkar *et al*, 2000).

Interestingly, however, not all infiltrating drug-specific T cells were cytotoxic. Whereas some only secreted various cytokines, others secreted cytokines and had some cytotoxic capacity as well. As in previous studies from the peripheral blood (Mauri-Hellweg *et al*, 1995; Zanni *et al*, 1997; Pichler *et al*, 1997), IL-5 was the most abundantly produced cytokine. The enhanced production of IL-5 may explain the frequently observed eosinophilia in patients with drug allergies, as IL-5 is an important differentiation and activation

factor for these cells. On the other hand, the production of TNF-α and IFN-γ by some of the drug-specific T cells may again reflect the presence of cytotoxic cells and promote T-cell-mediated cytotoxicity. Taken together, the functional capacity of the T cells eluted from the skin was found to be similar to the function of drug-specific T cells from the peripheral blood (Mauri-Hellweg *et al*, 1995, 1996; Zanni *et al*, 1997, 1998; Pichler *et al*, 1998; Schnyder *et al*, 1998). The presence of such T cells with different functional capacities, i.e., activation of T cells producing different cytokines and/or cytotoxic T cells, may explain the variability and complex clinical picture of cutaneous ADR.

In conclusion, our data demonstrate that a high proportion of drug-specific CD4+ T cells are found in drug-induced epicutaneous test reactions. These findings provide further evidence that such reactions are mediated by an underlying immunologic mechanism and emphasize that epicutaneous testing may be helpful in establishing the cause of delayed-type drug allergies.

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