

Original article

Cytotoxic mechanisms in different forms of T-cell-mediated drug allergies

Background: Cytotoxic mechanisms are involved in different forms of drug induced exanthems.

Methods: Here we compare the killing pathways of CD4⁺, CD8⁺ and CD4/CD8⁺ T-cell lines (TCL) and clones derived from patients suffering from maculopapular, bullous and pustular drug eruptions. *In vitro*, perforin and Fas-mediated killing was analysed in cytotoxicity assays against autologous Epstein–Barr virus (EBV)-transformed B-cell lines, Fas-transfected mouse lymphoblasts and natural killer (NK)-target cells. In addition, affected skin lesions and the TCL and clones were stained for perforin and FasL-expression.

Results: We detected perforin and some FasL-mediated killing in all three types of exanthems. Some of the drug-specific T-cell clones analysed exerted mainly perforin-, other more FasL-mediated killing showing no strict relationship between their perforin- and Fas-mediated cytotoxic capacity. Using a cell culture method focusing on the generation of cytotoxic T cells, we detected drug-specific CD8⁺, TCR $\alpha\beta$ ⁺ T cells, which failed to proliferate to drug presentation by antigen presenting cells but killed in a drug dependent way. Interestingly, these cells had substantial natural killer-like T cell(s) like features as they were CD56⁺ and CD94⁺ and had the ability to kill the NK-sensitive cell line K562.

Conclusion: Our data underline the important role of cytotoxic mechanisms in different forms of drug induced exanthems and suggest that even some T cells with NK-like characteristics may be involved in drug hypersensitivity.

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T-cell-mediated cutaneous drug allergies manifest themselves in distinct clinical pictures, which are caused by different immune mechanisms. These differences can be attributed to distinct T-cell functions, primarily because of a different cytokine pattern (1). For e.g. in maculopapular exanthem (MPE), drug-specific T cells secrete various cytokines and chemokines, in particular high levels of interleukin-5 (IL-5), which is thought to be responsible for blood and tissue eosinophilia, which are typical clinical hallmarks of drug reactions (2–4). In acute generalized exanthematous pustulosis (AGEP), a high IL-8 production may be responsible for polymorphonuclear neutrophils (PMN) recruitment, while in bullous skin reactions interferon- γ (IFN- γ) production by T cells may be dominant.

A common feature of these different drug exanthems appears to be the involvement of cytotoxic T cells: in MPE, the most frequent cutaneous drug reaction (5), drug-specific CD4⁺ T cells are found to be cytotoxic and able to kill IFN- γ pretreated keratinocytes (6). In bullous skin disease, a relatively high number of cytotoxic CD8⁺

T cells emigrate into the epidermis and may contribute to the formation of bullae (7, 8) and recently it was shown that cytotoxic CD4⁺ and CD8⁺ T cells are also present in the early phase of AGEP (9). These data are based on both the *in vitro* analysis of drug-specific T-cell clones (TCC) and T-cell lines (TCL) as well as on *ex vivo* data, namely an immunohistochemical analysis, where perforin positive T-cell infiltrations were found in close apposition to keratinocytes showing signs of cell death (2, 4, 7–9).

T cells can kill target cells via the perforin/granzyme B mechanism, which relies on granule exocytosis upon cell contact and specific activation of cytotoxic T-lymphocytes (CTL). In addition, induction of apoptosis may occur by the surface expression of FasL which binds to Fas on target cells, or on the release of tumour necrosis factor- α (TNF- α). Most data indicated that drug-specific T cells kill target cells mainly by granule-exocytosis (6, 9). This was supported by the finding that perforin/granzyme B-positive T cells were documented in allergic skin lesions by immunohistochemistry (8), and that *in vitro* cytotoxic drug-specific T cells were perforin/granzyme B positive (6, 10). Moreover, the killing of drug-presenting target cells in a 4-h ⁵¹Cr-release assay was blocked by concanamycin A (CMA), a specific inhibitor of the perforin-pathway (6).

Abbreviations: AGEP, acute generalized exanthematous pustulosis; MPE, maculopapular exanthem; TEN, toxic epidermal necrolysis; APC, antigen presenting cells; NK-T, natural killer-like T cell(s).

The role of FasL mediated killing was until now less well documented in drug-induced exanthems: FasL levels (like perforin/granzyme B), measured by quantitative PCR in peripheral blood cells, was found to correlate to the severity of drug allergic reactions during the acute phase (11). In addition, FasL-mediated killing was reported to be important in toxic epidermal necrolysis (TEN), the most severe drug allergic skin reaction (12).

In this study we compare drug-specific T cells obtained from MPE, bullous exanthem and AGEF using standard cytotoxicity assays and immunohistochemical analysis. The data suggest that simultaneous involvement of both killing-pathways perforin/granzyme B as well as Fas/FasL can occur together in these different cutaneous drug reactions. Moreover, we describe a TCR $\alpha\beta$ +, CD8+ T-cell population, which failed to proliferate to drug presentation by antigen presenting cells (APC), but was detected because it was cytotoxic, as it killed drug-presenting target cells in an drug dependent mechanism. Moreover, this T-cell subset had natural killer-like T cell(s) (NK-T)-like features as it expressed CD56 and CD94 and killed the NK-susceptible cell line K562.

Materials and methods

Patients characteristics

The history of the five patients having suffered from a drug-elicited allergic reaction of the skin patients has been described previously (2, 13–15). Briefly, patient UNO suffered from a generalized MPE after treatment with co-trimoxazole [sulfamethoxazole (SMX), plus trimethoprim; Hoffman La-Roche, Basel, Switzerland) (15). Patients AB and MBZ developed a bullous skin eruption after oral administration of ceftriaxone (Roche Pharma, Reinach, Switzerland) (13, 14). Patients AP and JS were classified as having AGEF after oral administration of AugmentinTM (amoxicillin and clavulanic acid; SmithKline Beecham Pharmaceuticals, Irvine, UK) according to the criteria of Roujeau (16) and the AGEF Euro-SCARE validation score (2, 17).

Culture media and reagents

Culture medium (CM) for T cells consisted of RPMI 1640 (Sigma Chemical, St Louis, MO, USA) supplemented with 2 mM L-glutamine (No. 663.710; Biotest, Heidelberg, Germany), 100 µg/ml streptomycin and 100 U/ml penicillin (No.4-01F00-H; Amimed, BioConcept, Allschwil, Switzerland). Either 10% pooled heat-inactivated human AB-serum (Swiss Red Cross; Bern, Switzerland), 25 mM HEPES buffer (No. L1613; Seromed, Basel, Switzerland) for CD4+ or 10% foetal calf serum or 5 mM HEPES buffer for CD8+ T cells was added.

The EBV-transformed B-lymphoblastoid cell lines (B-LCL) were generated and cultured as described (18). The following drugs were used: amoxicillin 0.5 mg/ml (SmithKline Beecham), ceftriaxone 0.1 mg/ml (Roche), and SMX 0.25 mg/ml (Hoffman La-Roche). For T-cell stimulation stock solutions of each drug were always freshly prepared in CM just before use.

Concanamycin A (purity grade 95%, product number 27689), brefeldin A and concanavalin A (ConA) were purchased from Sigma.

Generation of drug-specific cytotoxic T cells

Peripheral blood mononuclear cells (PBMC) were obtained either from peripheral blood after the acute phase (patient UNO: UNO-SMX 18.53, P26B, S211E, S23E, UNO 1.3; patient AP: AP 8.6, AP B8; patient JS: JS B7) (2, 15) or from positive epicutaneous skin test biopsy specimens (patient AB: AB 39; patient MBZ: MBZ DB3, MBZ FD3) (14).

As previous cloning procedures yielded a low frequency of CD8+ T cells, we used a separate protocol to propagate CD8+ T cells from patients UNO and AP (CD8+ CTLs UNO-SMX 18.53 and AP 8.6). Briefly, freshly isolated PBMC were depleted from CD4+ T cells using immunomagnetic negative selection (19) (Dynabead, No. 111.08; Dynal SA, Oslo, Norway) and 2×10^4 cells per well were seeded in 96-well U-bottomed microtitre-plates in presence of 1×10^6 /ml irradiated (4500 rads) autologous PBMC and the respective drug in CM containing 10% AB serum. After the addition of 40 U/ml of IL-2 on day 3, drug-specific cultures were weekly selected for their cytotoxic activity in a standard 4-h ^{51}Cr release assay. Cultures with a specific lysis >20% were pooled and weekly restimulated as before. After several rounds, cultures with a drug-specific lysis >80% were further propagated using 10^6 cells/ml irradiated allogenic PBMC, 10^5 cells/ml irradiated (6000 rad) autologous B-LCL, 60 U/ml IL-2 and 2 µg/ml PHA, and soluble drug. Generation of the other drug-specific TCL and TCC following a selection for proliferative capacity was performed as described previously (15, 20).

Flow cytometry analysis of T cells

Aliquots containing 1×10^5 T cells were stained with fluorochrome-labelled monoclonal antibodies (mAb) as described before (9). The analysis was performed with a Coulter EPICS XL-MCL Flow Cytometer (Beckman Coulter Inc., Hialeah, FL). The following fluorochrome-labelled mAb were used for phenotypical analysis: anti-CD3, anti-CD4, anti-CD8, anti-CD16, anti-CD56, and anti-CD94 mAb (all from PharMingen/BD, Mountain View, CA). The TCR V β chain distribution of the TCC was analysed using a panel of 22 fluorochrome-labelled mAb detecting ~75% of all V β families as described before (21). CD16, CD56 and CD94 were measured upon activation with anti-CD3 (OKT3, Ortho-Clinical Diagnostics; Johnson & Johnson, Neckargrund, Germany) and anti-CD28 (from BD) mAb.

T cell proliferation assays

Interleukin-2 deprived TCLs and TCCs (5×10^4 cells/well) were incubated in 96-well U-bottom plates together with 1×10^4 autologous B-LCL in 0.2 ml medium in the presence of indicated concentrations of drug. ^3H -thymidine was added to the culture and the incorporated radioactivity was counted as described before (2). Stimulation indices (SI) were calculated as cpm in culture with antigen divided by cpm in culture without antigen.

HLA-restriction of drug presentation

The human leucocyte antigen (HLA)-class I or II restriction of established TCC and TCL was carried out using blocking mAb anti-HLA class I (W6-32, American Type Culture Collection, Rockville, MD), anti-DP (SPV-L3, anti-DQ (B7-21), or anti-DR (L 243) (a kind gift of Dr E. Padovan, Bern) in proliferation- or cytotoxicity-assays. The HLA-allele restriction was assessed with partially matched allogenic B-LCL in proliferation assays or by cytotoxicity assays, respectively as described earlier (22).

Perforin killing

Cytotoxicity of T cells was assessed in a standard 4-h cytotoxicity assay in CM for B-LCL, containing neither IL-2 nor PHA as described (6, 19). Briefly, after 1-h labelling of 1×10^6 autologous B-LCL as target cells with 50 μCi (^{51}Cr -sodium chromate solution, Amersham Pharmacia Biotech, Cardiff, UK) at 37°C , triplicate cultures of 2.5×10^3 of target cells were coincubated in 96-well microtitre U-bottom plates with drug-specific T cells at mentioned E : T ratios in presence or absence of soluble drug or ConA.

Cytotoxic activity of TCC AB39, MBZ DB3 and MBZ FD3 was tested with 2.5 $\mu\text{g}/\text{ml}$ of the unspecific mitogen concanavalin (ConA), all other drug-specific T cells were specifically stimulated with their corresponding drug. After 4 h, 100 μl of supernatant per well were collected and ^{51}Cr -release was determined in a γ -counter (GAMMAMatic Basic, Kontron Analytical, Münchenstein, Switzerland). Specific lysis of target cells was proportional to the radioactivity released and calculated as $100 \times [\text{experimental release with Ag} - \text{experimental release without Ag}] / (\text{maximal release} - \text{spontaneous release})$.

FasL-mediated killing

To evaluate the FasL-mediated cytotoxicity, we used autologous B-LCL as APCs, ^{51}Cr -labelled Fas-transfected L-1210 mouse lymphoblasts as target cells, and untransfected L-1210 cells as negative control (23). After labelling of the mouse-lymphoblasts for 90 min, some 2×10^3 target cells and nonlabelled B-LCL were co-incubated for 18 h at 37°C in duplicate or triplicate cultures with drug-specific cytotoxic T lymphocytes at mentioned E : T ratios in presence or absence of drug or ConA. Specific lysis was calculated as difference between lysis of targets with drug or ConA and targets without drug as described above.

Specific blocking of cytotoxic pathways

Blocking experiments with 10 $\mu\text{M}/\text{ml}$ brefeldin A or 100 nM/ml CMA were performed as described before (6, 24). In short, effector T cells were preincubated with brefeldin A and/or CMA for 2 h at 37°C and subsequently a standard cytotoxicity assay was performed as described above.

In all cytotoxicity assays, maximum release was determined by lysis of targets with 2 M HCl, spontaneous release by lysis of targets

in medium, both in presence or absence of drug, ConA or the blocking agents. Spontaneous release was always below 20% of maximum release.

Immunohistochemical analysis of cytopins and skin biopsy

For preparation of cytopins, CD8+ TCL AP 8.6 and UNO 18.53 were incubated for 4 h (perforin) or 18 h (FasL) with soluble drug and autologous B-LCL as APCs at an E : T ratio of 3 : 1 as described above. The cells were subsequently centrifuged to the carrier (Cytospin 2; Shandon, Pittsburgh, PA) and stained with anti-perforin-mAb or with anti-FasL mAb (9).

Immunohistochemistry of cutaneous punch-biopsy specimens from an acute MPE-lesion was performed using the avidin-biotin complex/alkaline phosphatase method (Dako, Glostrup, Denmark) as described earlier (8, 9).

NK-like cytotoxicity

The NK-like cytotoxicity of T cells was assessed in a 4-h cytotoxicity assay (25). Briefly, 1×10^6 K562 target-cells (NK-sensitive human erythroleukaemic cell line) were labelled for 1.5 h at 37°C with 50 μCi ^{51}Cr -sodium chromate solution (Amersham Pharmacia Biotech, Cardiff, UK). Triplicate cultures of 2.5×10^3 labelled target cells were coincubated in 96-well microtitre U-bottom plates at 37°C with T cells at mentioned E : T ratios without addition of the specific drug or ConA. The NK-like cytotoxicity was calculated as $100 \times [(\text{experimental release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})]$. Lysis of K562 target-cells was calculated as difference between lysis of targets with effector T cells and targets in medium.

Results

Characterisations of drug-specific T-cell clones and lines

Phenotypic analysis of 11 drug-specific TCL and TCC was performed using FACS-analysis, while drug-specificity and MHC-class restriction was shown in cytotoxicity assays and/or proliferation assays (Table 1). T cells expressed TCR $\alpha\beta$ and were CD4+, CD8+, or CD4/

Table 1. Characterization of drug-specific T cells

Drug allergy	TCL or TCC		TCR $\alpha\beta$	TCR V β	CD3 (%)	CD56 (%)	CD16 (%)	CD94 (%)	SI	Drug	Restriction
MPE (patient UNO)	UNO-SMX 18.53	CD8+	+	†	97	24	0	19	<1*	SMX	MHC class II
	P26B	CD4+8+	+	Not done	95	0	0	1	213	SMX	MHC class II
	S211E	CD8+	+	16	97	Not done	Not done	Not done	136	SMX	MHC class I
	S23E	CD4+	+	13.2	97	0	8	8	51.7	SMX	MHC class II
	UNO 1.3	CD4+8+	+	Not done	95	0	0	0	32.3	SMX	Not done
Bullous Exanthem (patients MBZ, AB)	MBZ DB3	CD4+	+	7	99	0	0	0	8	Ceftriaxone	Not done
	MBZ FD3	CD4+	+	†	98	13	0	0	9.9	Ceftriaxone	Not done
	AB 39	CD4+	+	3	98	0	0	0	67.3	Amoxicillin	Not done
	AP8.6	CD8+	+	°21.3	95	86	0	86	<1*	Amoxicillin	MHC class I
AGEP (patients AP, JS)	AP B8	CD8+	+	Not done	98	Not done	Not done	Not done	Not done*	Amoxicillin	MHC class II
	JS B7	CD4+	+	†	96	Not done	Not done	Not done	3.9	Amoxicillin	MHC class II

* Drug-specificity was shown in cytotoxicity assays.

† No TCR-V β -family was detectable.

T-cell lines (TCL) are written in *italics*, T-cell clones (TCC) in standard type face. UNO-SMX 18.53 had no selected TCR V β expression, while 43% of AP 8.6 expressed V β 21.3 SI, Stimulation Index of specific drug-induced proliferation, calculated as cpm with drug/cpm without drug.

CD8 double positive. As observed previously, some TCC were CD8+ but MHC class II restricted (10). The TCL UNO-SMX 18.53 and AP 8.6 were generated from CD4+ T-cell depleted PBMC, following a protocol favouring the outgrowth of CD8+ T cells. These cell lines did not proliferate well *in vitro*, which limited the analysis and several attempts to generate clones failed. They expressed some NK-like surface molecules (CD56, CD94) (26), but none of the analysed TCL and TCC was positive for CD16, another typical marker for NK-T cells.

Drug-specific T cells kill autologous target cells in a perforin/granzyme mediated pathway

Recently it has been shown that in cutaneous drug reactions drug-specific T cells kill keratinocytes in a perforin-dependent mechanism (6, 14). Figure 1 shows the perforin-mediated killing of autologous target cells by drug-specific CD4+, CD8+ and CD4+/CD8+ T cells.

Perforin-mediated killing was observed in TCC and TCL obtained from MPE (Fig. 1A), bullous exanthem (Fig. 1B) and AGEP (Fig. 1C). There were, however, some TCC which had only moderate killing potential in a 4-h ^{51}Cr release-assay, namely JS B7 (AGEP) and UNO 1.3, which showed a killing potential between 10% (Fig. 1A) and 23% (Fig. 3A).

Drug-specific T cells can also kill target cells in a Fas/FasL-mediated pathway

To evaluate the role of Fas/FasL-mediated killing, we tested drug-specific TCC and TCL for their cytotoxic activity against a mouse lymphoblastoma cell line transfected with Fas (L1210-Fas) (Fig. 2). Fas/FasL-mediated cytotoxicity was clearly dependent on stimulation, either by the drug or by ConA, as no killing was observed in absence of T-cell activation (data not shown). The two CD8+ TCL, which had also NK-like killing capacity (UNO-SMX 18.53, AP 8.6) had a relatively high background killing but showed some additional Fas-killing, suggesting that also FasL-mediated killing may be involved. As the cell line L1210-Fas does not express human MHC-surface molecules, and because only Brefeldin A blocked the cytotoxicity, an accompanying perforin killing was not involved (see below). Fas/FasL-mediated cytotoxicity was not detected after 4 h because in all cases specific lysis was below 5% (data not shown). After 18 h, however, eight of 10 analysed TCL and TCC showed a cytotoxic activity against the Fas-expressing cell line (Fig. 2). Two of the cell lines showed either a very low specific killing (<15%) or Fas/FasL-mediated activity was completely absent (Fig. 2A and C). Interestingly, the cell lines and clones that were highly cytotoxic in a perforin dependent pathway were not necessarily potent FasL-killers (e.g.

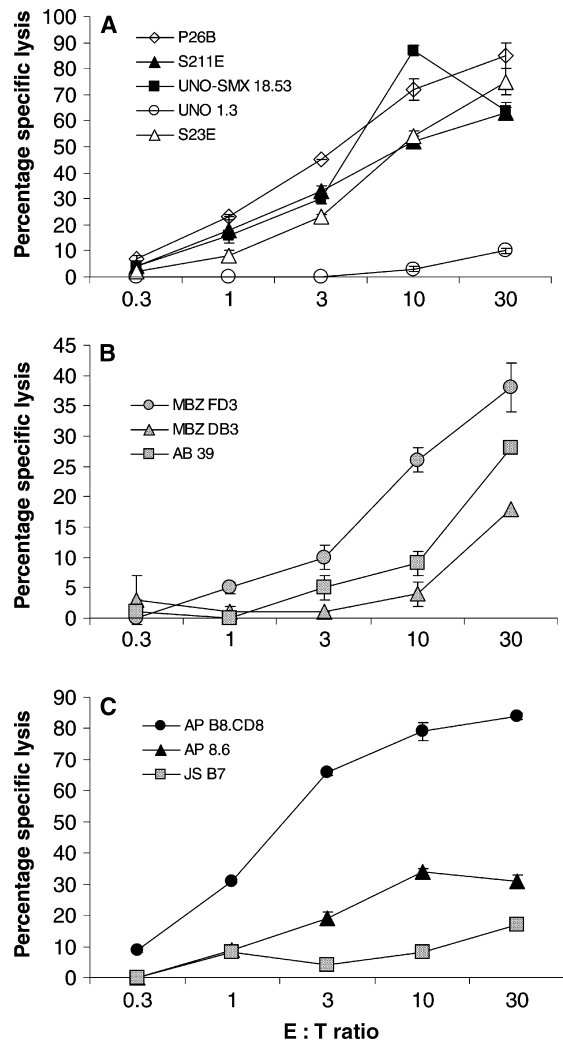


Figure 1. Perforin/granzyme-mediated cytotoxicity of drug-specific T cells obtained from maculopapular exanthem (A), bullous exanthem (B) and acute generalized exanthematous pustulosis (C) was evaluated in a 4-h ^{51}Cr release assay with autologous B-lymphoblastoid cell lines as targets. CD4+ (grey symbols), CD8+ (filled symbols), CD4/CD8+ T cells (open symbols). The data of TCL AP B8 and TCC JS B7 was already shown previously (9). All assays are performed in duplicate or triplicate culture with SEM.

P26B), and on the contrary, certain cells with low perforin killing capacity showed a strong FasL-killing activity (e.g. UNO1.3). These data indicate that there is no strict correlation between Fas/FasL- and perforin-mediated cytotoxic potential in an individual TCC or TCL. Both killing pathways appear to coexist independently of each other.

The FasL-mediated cytotoxicity increased proportionally with the E : T ratio, but even at a very low E : T ratio with an threefold excess of APC and target cells compared with effector cells, a significant specific lysis was observed for TCC S23E (Fig. 2A).

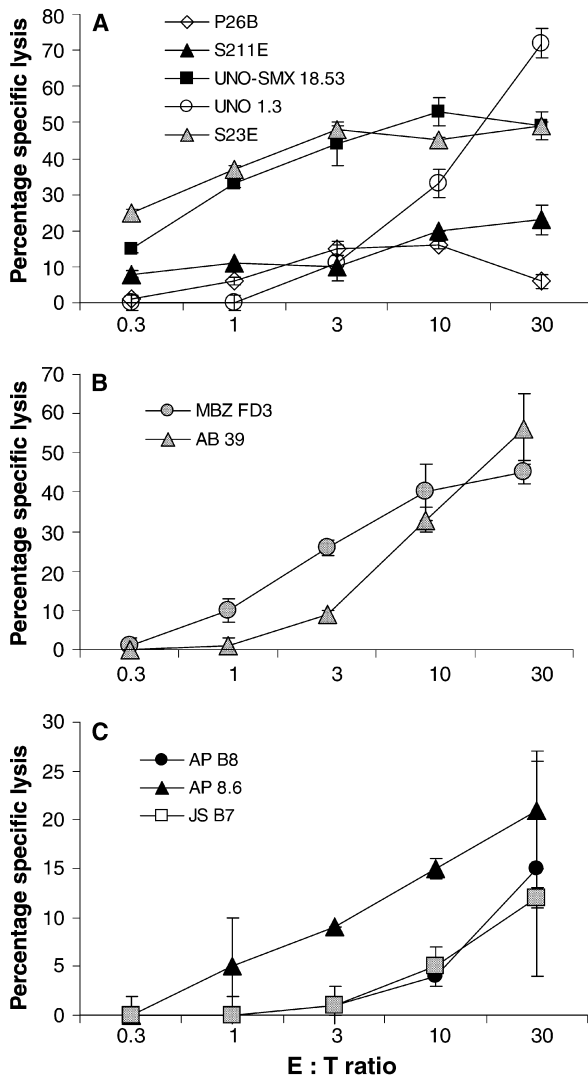


Figure 2. Fas/FasL-mediated cytotoxicity of drug-specific T cells obtained from maculopapular exanthem (A), bullous exanthem (B) and acute generalized exanthematous pustulosis (C) was assessed using ^{51}Cr labelled mouse lymphoblasts L-1210-Fas in presence or absence of drug or ConA, respectively. CD4⁺ (grey symbols), CD8⁺ (filled symbols), CD4/CD8⁺ T cells (open symbols). The data of TCL AP 8.6 was already shown previously (9). All assays are performed in duplicate or triplicate culture with SEM.

Discrimination of cytolytic pathways

Concanamycin A is a potent inhibitor that blocks perforin-based killing, while brefeldin A selectively inhibits the release of FasL (24). Perforin-mediated cytotoxicity was confirmed for S23E, AP8.6 and UNO1.3 in a 4-h assay against autologous B-LCL, while in a corresponding experiment perforin-mediated cytotoxicity was effectively blocked by CMA (Fig. 3A). The Fas-mediated cytotoxicity was inhibited by brefeldin A in an 18-h assay using L-1210-Fas as targets and S23E and UNO 1.3 as effector cells (Fig. 3B).

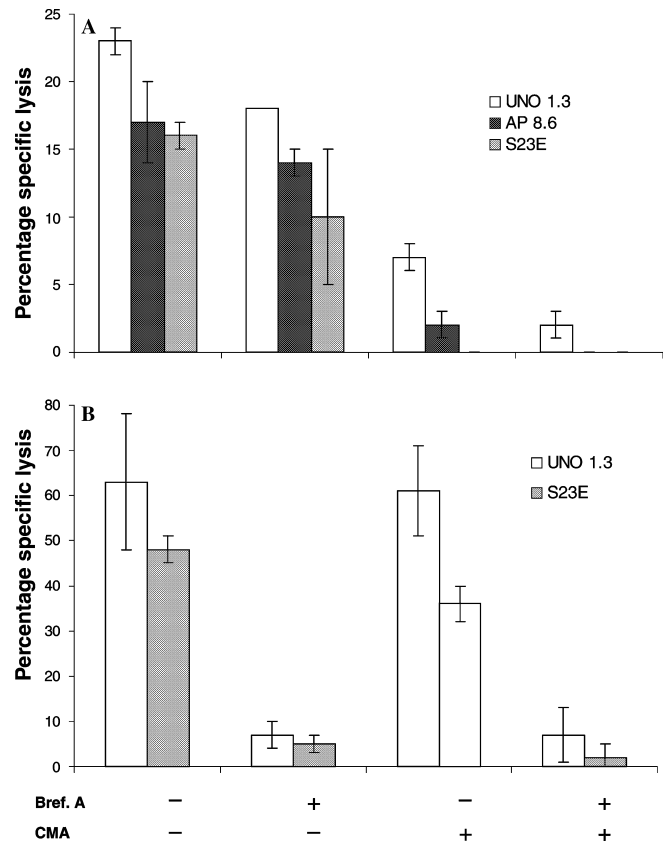


Figure 3. Selective blocking of cytolytic pathways for representing drug-specific T cells UNO1.3, AP 8.6, S23E. E : T ratio was 10 : 1. Positive control (—) shows cytotoxicity without the blocking agents. All assays are performed in duplicate or triplicate culture with SEM. (A) Blocking of the perforin/granzyme mediated killing in 4-h cytotoxicity assay with autologous B-lymphoblastoid cell lines (B-LCL) as targets. (B) Blocking of the Fas/FasL mediated killing 18 h cytotoxicity assay with L-1210-Fas as targets and autologous B-LCLs as antigen presenting cells.

Expression of perforin and FasL by drug-specific T cells

Drug-specific TCLs derived from MPE (UNO-SMX 18.53) and AGEP (AP 8.6) were stained for perforin (Fig. 4A and C) and FasL (Fig. 4B and D). Both TCL had an almost 100% perforin-positive staining. Over 30% of TCL AP 8.6 showed a positive staining for FasL (Fig. 4C), while much less FasL-upregulation was found in TCL UNO-SMX 18.53 (Fig. 4B), although this TCC showed a substantial FasL-killing in the functional assay (Fig. 2A).

Perforin/granzyme and Fas/FasL-mediated killing is detectable at site of acute lesion

Previous studies have already documented the involvement of perforin- and FasL-killing in AGEP (2, 9). Here we show that also in acute MPE-lesions expression of FasL on T cells infiltrating the dermis can be detected by immunohistochemical stainings (Fig. 5C) of a skin

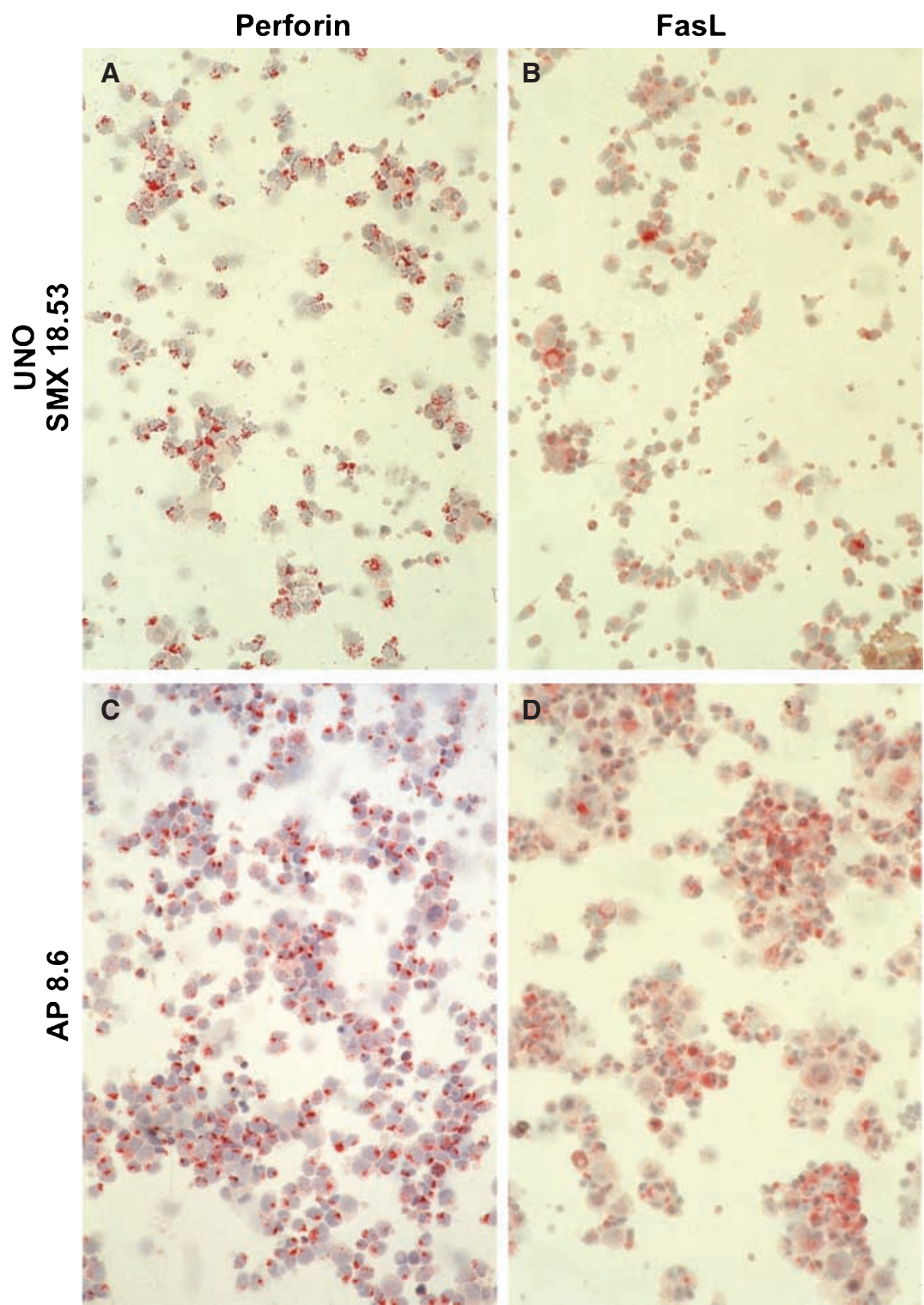


Figure 4. Cytospin preparations of T-cell lines UNO-SMX 18.53 (A, B) and AP 8.6 (C, D) with anti-perforin monoclonal antibodies (mAb) (A, C) and anti-FasL mAb (B, C) following specific stimulation with the corresponding drug. Control staining of autologous B-lymphoblastoid cell lines was negative for both perforin/granzyme B and FasL (data not shown). Original magnification $\times 200$.

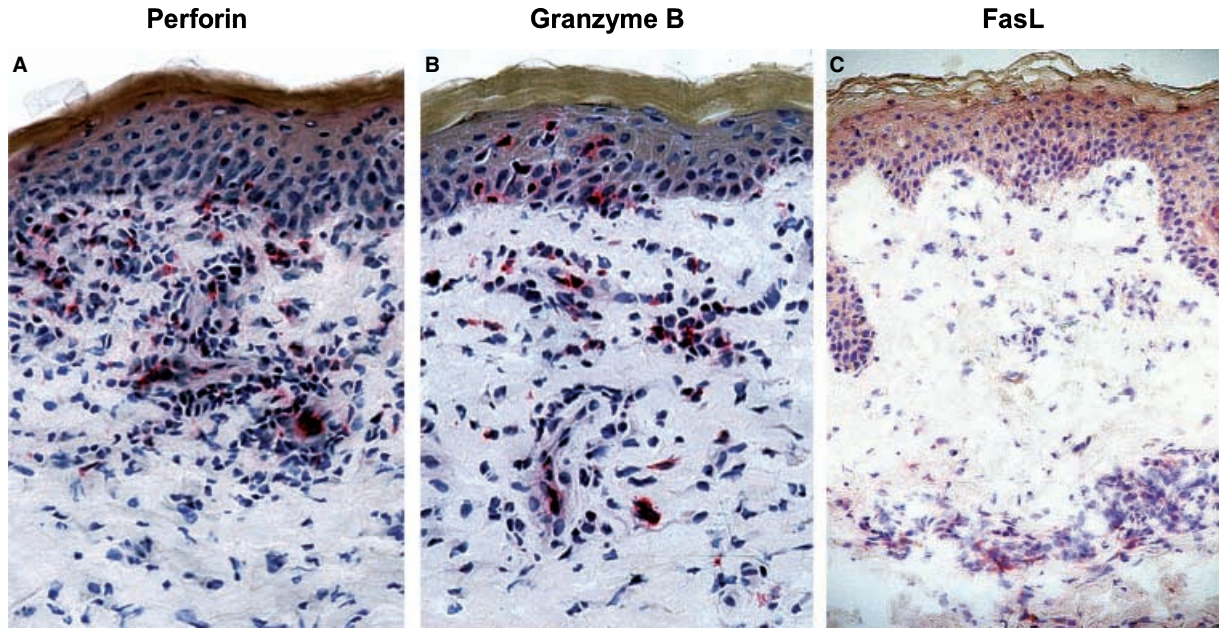


Figure 5. Immunohistochemical staining of acute skin lesions in maculopapular exanthem (MPE) documents the presence of perforin (A) and granzyme B (B) at site of local tissue destruction. (C) FasL was detectable mainly in the dermis, but not epidermis of this MPE-lesion. Original magnification $\times 250$.

biopsy, although in a substantially lower amount than perforin and granzyme B (Fig. 5A and B).

CD8⁺ TCL with a poor proliferative response show NK-like cytotoxicity

Following a protocol for the generation of HCV-peptide-specific CD8⁺ T cells (19), we succeeded in establishing two drug-specific CD8⁺ T cell lines UNO-SMX 18.53 and AP 8.6 from two patients. These T cell lines proliferated weakly in the presence of their specific drug and fitting APC; however they were clearly drug specific, as revealed by a drug-specific cytotoxicity (Figs 1 and 2). These rather peculiar T cell lines showed various expression of CD56, a marker of natural killer cells and of highly cytotoxic CD8⁺ T cells (27) and of CD94, a marker for NK-T cells: in T cell line UNO-SMX 18.53 24% were CD56⁺, and 19% were CD94⁺, while in T cell line AP 8.6 86% were CD56⁺ and 86% were CD94⁺. Both T cell lines were CD16 negative (Table 1).

The HLA-restriction of drug presentation was evaluated by blocking experiments with mAb against HLA class I and II as well as with partially matched APC in cytotoxicity assays. UNO-SMX 18.53 is MHC class II restricted (DR 10), whereas AP 8.6 is MHC class I restricted (HLA-A33, HLA-B51). Both T cell lines revealed a drug-dose dependant killing in a 4-h cytotoxicity assay (data not shown).

The peculiar features and phenotype of these cells prompted us to investigate their ability to kill K562, the classical NK-target cell: Indeed, in addition to a drug-specific killing, these T cell lines mediated a nonspecific NK-like

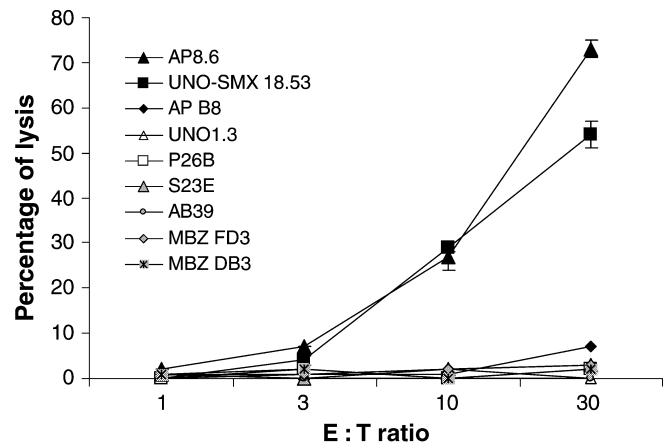


Figure 6. Cytotoxicity of drug-specific T-cell lines and clones against the NK-sensitive cell line K562 evaluated in a 4-h ^{51}Cr release assay in absence of drug or ConA. Assays at several E : T ratios were carried out in duplicates and SEM was calculated.

killing. None of the other analysed T cell lines showed such a NK-like killing pattern or phenotype (Fig. 6, Table 1). Interestingly none of these T cell lines expressed the TCR $\text{V}\alpha 24/\text{V}\beta 11$, which is associated with NK-T-like T cells (28).

Discussion

Previous studies have already shown that cytotoxic mechanisms are involved in certain types of exanthems (6, 7). The cytotoxic mechanism was first attributed to

perforin/granzyme B (6), but later data suggested that FasL mediated killing is also or even more important: in severe bullous drug reactions such as TEN, FasL expressing T cells were found in blister fluid (25). Posadas et al. (11) showed that patients with severe drug exanthem have not only higher levels of perforin expressing T cells, but also higher levels of FasL, measured by mRNA expression in circulating cells during the acute phase. Another study documented that tissue damage in TEN might be inhibited using blocking antibodies against the apoptosis-receptor Fas (CD95) (12).

In this study we present *in vitro* and *in vivo* data indicating that both killing pathways, perforin/granzyme B and Fas/FasL, are involved in distinct forms of drug-induced, T cell-mediated cutaneous drug reactions, namely MPE, bullous exanthem or AGEP. This is based on immunohistology as well as on generation of CD4+ and/or CD8+, TCR $\alpha\beta$ + TCC and TCL derived from patients suffering from such cutaneous drug reactions.

In agreement with previous data, some drug-specific T cells derived from blood or the skin (after positive patch-test reactions), TCL and TCC from MPE, bullous exanthema and AGEP are able to exert perforin-mediated killing against drug presenting APC (Fig. 1) (8, 14, 15). Our study confirms the implication of a perforin/granzyme B-dependent killing pathway in cutaneous drug eruptions, as the killing activity of TCC was blocked by CMA only (Fig. 3A), and not by brefeldin A, which selectively blocks Fas/FasL-mediated cell death in three, two representing TCL and TCC respectively (Fig. 3B) (6, 24). Moreover, effector cells stained positive for perforin and granzyme B (Fig. 4). These apoptosis activating molecules were also found in cell-infiltration of skin biopsies of MPE (Fig. 5) and were previously described in bullous skin-diseases (8) and, as shown recently, for AGEP (9). This rules out that a culture artefact accounts for this perforin and FasL detection in TCL and TCC.

The FasL-dependent killing was detected in most of the tested drug-specific T cells (Fig. 2). The FasL involvement was confirmed by the inhibitory effect of brefeldin A (Fig. 3B) and the finding that only Fas-transfected, but not the untransfected target-cells were killed. Importantly, this FasL-mechanism is not associated with the perforin mechanism, as T cells with a high perforin-mediated killing potential were not necessarily the best Fas/FasL-mediated killer cells and vice versa, high FasL killing was not associated with perforin killing. Activation of both, perforin/granzyme B- and Fas/FasL-mediated killing was found to be dependent on the recognition of the relevant drug. Thus, a combined cytotoxicity by a perforin/granzyme- and Fas/FasL-mediated pathways may occur in different forms of exanthems. Such a combination has been described previously in acute contact dermatitis, emphasizing the similarities between contact dermatitis and drug-induced exanthema (29, 30).

The cytotoxic activity observed *in vitro* was similar in drug-specific T cells from the three types of exanthems (Figs 1 and 2). This does not mean that the two mechanisms are equally important in these exanthems *in vivo*. Our data do actually not allow to make quantitative conclusions regarding the contributions of the different killing pathways. This is difficult if not impossible, as the time kinetic might be important for the *in vivo* evaluation (e.g. biopsy) and as our data rely on the *in vitro* generation of TCL and TCC. Nevertheless, the immunohistochemical investigations – with rather low FasL expression in MPE (Fig. 5C) and the correlation of severity of drug reactions to perforin-mRNA levels suggests that perforin/granzyme B is the principal cytotoxic mechanism (6, 9, 11), and that FasL may be involved in selected reactions only.

On the contrary, it might be that not the functional capacity and amount of T cells with cytotoxic potential but the antigen restriction of FasL- and perforin/granzyme B-expressing cells is decisive for the severity of the disease. FasL+ or perforin+ CD8+ T cells may be more damaging, if they recognize the drug presented on MHC-class I and can be activated by drug presenting keratinocytes or other body cells, while the activity of killer cells recognizing the drug in association with MHC-class II may be more limited, even if they are more abundant. This is consistent with the findings that in cutaneous drug eruptions a high number of skin infiltrating CD8+ T cells correlates with an increased risk for severe clinical symptoms such as bullous disease or hepatic damage (4). Indeed, in bullous skin diseases a higher proportion of CD8+ T cells is found in the epidermis (4, 7).

By changing *in vitro* culture conditions we were able to generate a peculiar subset of drug-specific CD8+ T cells, which were TCR $\alpha\beta$ +, exerted perforin and Fas/FasL-mediated killing and killed NK-sensitive targets K562 in a 4-h ^{51}Cr -release assay without drug stimulation (Fig. 6). These T cells were generated from patients with MPE and AGEP. We cannot exclude the possibility that these T cells featuring NK-like characteristics actually developed because of our cell culture conditions and reflect a primary *in vitro* stimulation and not an *in vivo* priming. Nevertheless, the finding of such cells is interesting in various respects: (a) They were only detected by the cytotoxicity assay, as they were negative in proliferation tests, which is the usual way to detect drug-specific T cells. It illustrates, that a broader approach using cytotoxicity assays might be appropriate in certain diseases where killing is more important than proliferation. Indeed, the lymphocyte transformation tests is thought to be not very reliable in TEN, a disease thought to be due to killer cells (31). (b) Similar T cells have been found in blister fluid in TEN (25, 32). The presence of such drug-specific T cells with NK-like characteristics within the cell pool of patients with relatively *mild* drug allergies suggests that the described NK-like T-cells are controlled *in vivo* during mild drug allergies. However,

altering the immune status as it happens e.g. in HIV infection, may also activate such T cells exerting an NK-like killing-pattern, which might contribute to the increased incidence of severe drug reactions in HIV-infections (33).

In conclusion, cytotoxic T cells which use the perforin/granzyme B- and/or the Fas/FasL-mediated killing pathway are involved in various forms of drug hypersensitivity of the delayed type. Thereby, the *in vitro* data suggest that the cytotoxicity is induced in CD4⁺ and CD8⁺ T

cells by drug presentation, but that there exist also a subset of T cells with NK-T like features, which kill both in a drug dependant and independent (NK-like) way.

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