

T-cell-mediated cytotoxicity against keratinocytes in sulfamethoxazol-induced skin reaction

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Summary

Background The incidence of skin rashes or erythema multiforme to sulfamethoxazole in exposed patients is about 3%. Among patients with acquired immunodeficiency syndrome the risk is ≈ 10 times higher. The pathogenesis of these reactions and the reason for the increased frequency in HIV infections are not understood.

Objective To investigate drug specific T-cell-mediated cytotoxicity in sulfamethoxazole-induced skin reactions.

Methods Specific T-cell lines and T-cell clones generated from a donor who developed a skin rash to sulfamethoxazole were assessed with a standard 4 h ^{51}Cr cytotoxicity assay in the presence or absence of soluble sulfamethoxazole. B lymphoblasts and keratinocytes with and without interferon gamma pretreatment were used as target cells. Selective blockers of FasL/Fas and perforin-mediated killing and immunostaining for perforin were used to evaluate the involvement of the different cytolytic pathways.

Results CD4^+ and CD8^+ sulfamethoxazole specific T-cell clones showed a drug-specific and MHC-restricted cytotoxicity against autologous B lymphoblasts in the presence of soluble sulfamethoxazole. Keratinocytes, if pretreated with interferon gamma, were specifically killed predominantly by CD4^+ T-cell clones. Specific T-cell clones of both CD4^+ and CD8^+ phenotype showed a strong immunoreactivity for perforin and the cytotoxicity was blocked by concanamycin A which suggests a perforin-mediated killing.

Conclusion Perforin-mediated killing of autologous keratinocytes in the presence of soluble sulfamethoxazole by drug-specific CD4^+ lymphocytes may be a pathway for generalized drug-induced delayed skin reactions. The requirement of interferon gamma pretreatment of keratinocytes for efficient specific killing might explain the increased frequency of drug allergies in generalized viral infections like HIV, when interferon gamma levels are elevated.

Keywords: drug eruptions, HIV, hypersensitivity, interferon gamma, sulfamethoxazole, T lymphocyte

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Introduction

The incidence of hypersensitivity reactions to sulfamethoxazole (SMX) in exposed patients with normal immunity is

between 3 and 6% [1]. Among patients with HIV infection, which frequently use trimethoprim-sulfamethoxazol against *Pneumocystis carinii* pneumonia, the incidence of SMX hypersensitivity is ≈ 10 times higher [2,3]. The reasons for the increased frequency during HIV infections are not known, and higher doses, altered drug metabolism and immune-dysregulation have been proposed as possible

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causes [4]. The great majority of these reactions comprise morbilliform, cutaneous eruptions [3,4] and do not represent any of the four principal types of immune-mediated hypersensitivity responses as classified by Gell and Coombs [5]. Because of immunohistological findings and the correlation of the frequency of hypersensitivity to the total T-lymphocyte count in advanced HIV infection [6,7], T lymphocytes are suggested to be directly involved in these reactions.

Like most drugs, the SMX molecule is too small to represent a complete antigen. It is chemically non-reactive and thus unable to form a hapten-carrier complex. It is usually thought that SMX gains its immunogenicity by drug metabolism in the liver with consecutive covalent binding to a carrier protein [8]. Our previous investigations with SMX specific T-cell clones (TCC) revealed that T cells can specifically recognize autologous antigen presenting cells which are reversibly modified only by the continuous presence of SMX. This recognition was MHC dependent and may be best explained by an unstable presentation of SMX without requirement of processing or drug metabolism [9]. We suggested that such a recognition by presensitized T cells might be an important feature for eliciting allergic manifestations particularly in organs without known SMX metabolism (e.g. skin). In this study we evaluated whether the T-cell mediated, SMX specific cytotoxicity can be directed against keratinocytes and which killing mechanism might be involved.

Materials and methods

Culture media and reagents

The culture medium used for peripheral blood mononuclear cells (PBMC) and lymphocytes was RPMI-1640 supplemented with 10% pooled, heat-inactivated human AB serum (Swiss Red Cross, Bern, Switzerland), 25 mmol/L HEPES buffer, 2 mmol/L L-glutamine (Seromed, Fakola, Basel, Switzerland), 25 µg/mL transferrin (Biotest). T-cell clones were cultured using medium enriched with 20 U/mL recombinant IL2 (obtained from Dr D. Wrann, Sandoz Research Institute, Vienna, Austria). Growth medium enriched with bovine pituitary extract, 4 mL/L (KGM, PromoCell, Heidelberg, Germany) was used for keratinocytes in secondary cultures.

Concanamycin A (CMA) and Brefeldin A were purchased from Sigma (St Louis, MI, USA), INFγ from Boehringer Mannheim (Mannheim, Germany). SMX was obtained from Hoffmann La Roche (Basel, Switzerland) and freshly prepared before use.

Patients characteristics

The blood cells as well as keratinocytes were obtained from

a HIV-negative patient, who experienced drug allergic symptoms the first time 9 years ago: he developed an erythematous exanthema after therapy with cotrimoxazol (= SMX plus trimethoprim). Two years later he experienced a generalized exanthema within 1 day after oral intake of cotrimoxazol (infection of the upper respiratory tract) which persisted several days. No specific IgE and IgG antibodies against the SMX or trimethoprim could be detected (RAST, Pharmacia). Skin tests (scratch and scratch/patch) with trimethoprim and sulfamethoxazol were negative (results recorded 20 min, respectively, 48 and 72 h after testing), whereas the lymphocyte transformation test demonstrated a strong proliferation to SMX.

Generation and characterization of drug-specific human T-cell lines and T-cell clones (TCC)

Freshly isolated PBMC were stimulated with SMX 0.1 mg/mL in culture medium at a cell-density of 1.5×10^6 per well of a 24-well culture plate (Falcon 3047). After 10 days two T-cell lines were used for cytotoxicity assay. The TCC shown in this study were the same as described earlier [9,10]. The cells were restimulated every 10–16 days with allogenic irradiated PBMC plus PHA (10 µg/mL, Boehringer, Mannheim, Germany). The phenotype was checked by immunofluorescence periodically 5–7 days after restimulation as described [10].

Assessment of cytotoxic activity and target cells

Cytotoxicity was tested 8–14 d after restimulation of the T cells by a standard 4-h-cytotoxicity assay [11] in presence or absence of SMX at 0.1 mg/mL or the indicated concentrations. Specific lysis was calculated as: (lysis in the presence of SMX) – (lysis without SMX). Spontaneous ^{51}Cr release from different target cells ranged between 115 and 384 c.p.m. The ratio between maximal release and spontaneous release was between 4 and 5. To discriminate perforin- or FAS-mediated killing, we performed blocking experiments with brefeldin A and concanamycin A (CMA) as described [12]. EBV-transformed lymphoblastoid B-cell lines (B-LCL targets) were generated by transformation of freshly isolated PBMC with supernatant of the EBV producing cell line B95–8. MHC restriction was evaluated using B-LCL from donors, which were partly matched in certain HLA-alleles [9]. Autologous human follicular keratinocytes were generated as described [13]. They were subcultured in 96-well U-bottom plates (Falcon 3227) in growth medium with and without INFγ (150 U/mL) at a density of 5×10^3 cells/well for 72 h. ^{51}Cr labelling was performed in these cells by incubation with 4 µCi/well ^{51}Cr sodium chromate solution (Amersham International) for 90 min.

Immunohistochemistry

Immunostaining of the SMX specific TCC (8–14 d after restimulation) and of autologous B-LCL as negative controls was performed according to the APAAP method as described previously [14]. Perforin was stained with mouse anti-human perforin antibodies (clone δ G9, IgG2b isotype; Pharmingen, San Diego, CA, USA); an unspecific IgG2b antibody served as isotype control.

Results

Specificity of TCC

Specificity of the TCC has been described earlier [10]. In addition, we tested the newly generated two T-cell lines (PBMC which are only once restimulated with SMX), which also showed a clear cytotoxicity against autologous B-LCL (data not shown). Several attempts to generate SMX-specific clones from two HLA-matched donors who were exposed to SMX without clinical reactions and who could present SMX to the SMX specific TCC were without any success.

Dose dependence of the cytolytic response

Cytolytic assays in the presence of various concentrations of soluble SMX in a effector: target ratio of 10:1 showed a dose-dependent response against autologous B-LCL. MHC restriction (no cytotoxicity occurred against B-LCL without the appropriate MHC match) confirms an immune-mediated pathway and rules out an unspecific cytotoxic effect. A clear response was observed already at a dosage of 0.1–0.2 mg/mL, which corresponds with the plasma concentration during a standard therapy with SMX (Fig. 1).

Lytic susceptibility of IFN γ pretreated keratinocytes

IFN gamma up-regulates MHC class I and II and is expected to render keratinocytes more susceptible to T-cell-mediated lysis [15]. Since keratinocytes are potential target cells for SMX specific cytotoxicity *in vivo*, we used autologous keratinocytes pretreated with and without IFN γ as targets and assessed the drug-specific cytotoxicity. As shown in Fig. 2, keratinocytes are susceptible to SMX-specific CD4-mediated cytotoxicity after pre-incubation with IFN γ . Repeated attempts to demonstrate a CD8-mediated, SMX-specific cytotoxicity showed only a small (3–5%) but constant increment of lysis of INF γ pretreated keratinocytes in the presence of SMX (data not shown).

Perforin-dependent cytotoxicity

Perforin- and Fas-based cytolytic pathways are the two

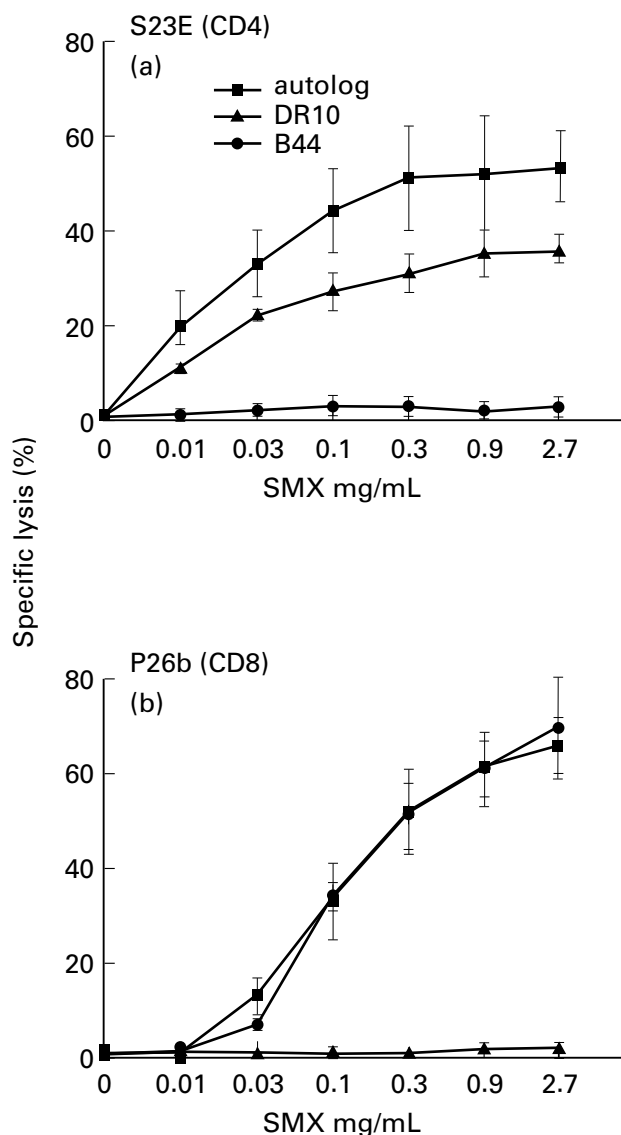


Fig. 1. Dose-dependence and MHC restriction of specific cytotoxicity. The drug-specific cytotoxicity was evaluated at different concentrations of SMX at an effector:target ratio of 10:1. The assay was performed with autologous and partially matched heterologous target cells (B-LCL). Only target cells with the appropriate MHC allele (HLA DR10 for CD4 and HLA B44 for CD8) were killed in the presence of SMX, which confirms an immune-mediated pathway. The values represent the mean of three independently performed assays $\pm$ SD.

major mechanisms of cell-mediated cytotoxicity. The perforin-based pathway is mediated by secretion of a pore-forming protein by cytotoxic T cells, whereas the Fas-based pathway relies on the interaction of the Fas ligand which is upregulated during T-cell activation with the apoptosis-inducing Fas molecule on the target cell [16]. Brefeldin A is known as a selective and potent inhibitor of

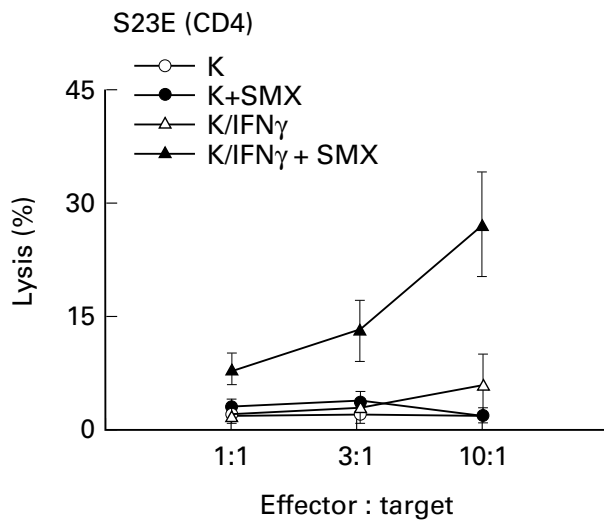


Fig. 2. Cytotoxicity against keratinocytes. CD4-mediated lysis against keratinocytes (K) and keratinocytes pretreated with IFN γ (K/IFN γ) with or without SMX. The values represent the mean of three independently performed assays $\pm$ SD.

Fas-based cytotoxicity whereas CMA inhibits the perforin-mediated but not the Fas-mediated cytotoxicity [12]. The killing of B-LCL targets by TCC of both CD4⁺ and CD8⁺ phenotype was abrogated by CMA but not by brefeldin A, demonstrating a predominantly perforin-mediated killing (Fig. 3). Moreover, using immunohistochemistry, intense cytoplasmic staining for perforin was observed in these drug specific TCC (Fig. 4).

Discussion

We describe a drug-specific cytotoxicity of T-cell lines and TCC generated from a patient with SMX-induced skin rash. The cytotoxicity was HLA restricted, which confirms an immune-mediated (and not toxic) mechanism. Specific cytotoxicity against autologous B-LCL was mediated by either CD4⁺ and CD8⁺ TCC and was predominantly perforin dependent. Cytotoxicity required the continuous presence of soluble SMX and was dose dependent, which might explain the clinical observation that a low prophylactic dose is often tolerated in patients who are hypersensitive to high doses [17]. In addition, we found a mainly CD4-mediated specific killing directed to autologous, IFN γ preincubated keratinocytes in the presence of SMX.

Previously, we could demonstrate a drug-specific and MHC-dependent T-cell recognition of autologous antigen-presenting cells that were reversibly modified by the continuous presence of SMX, independent of processing or metabolism [10]. Based on this finding the data presented suggest the following pathway for SMX-induced skin rash in the patient described: if the plasma concentration of the

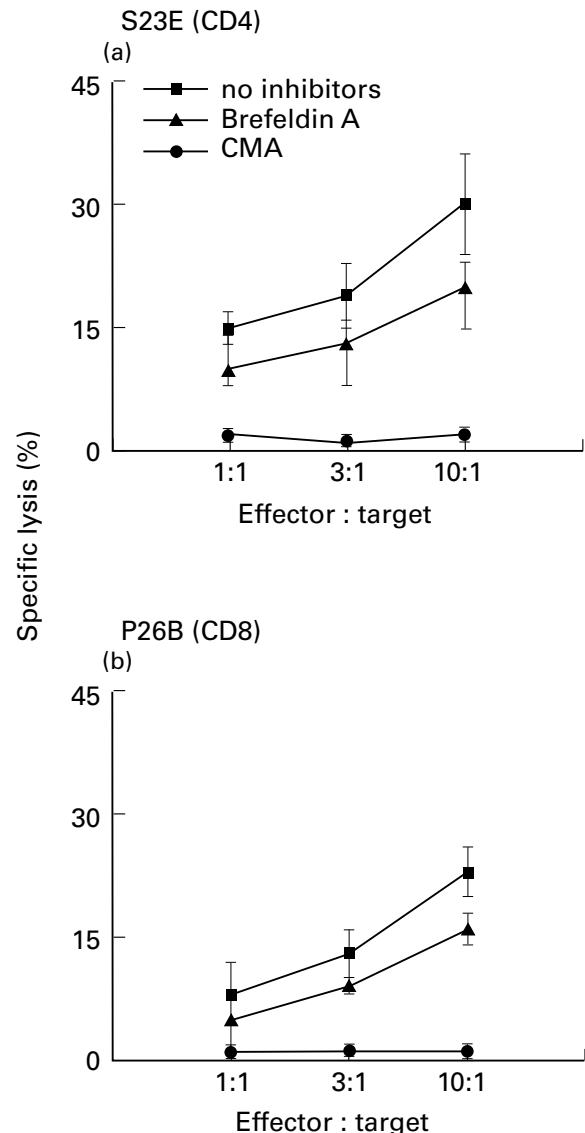


Fig. 3. Cytolytic pathway. Specific lysis by CD4 and CD8 TCC against B-LCL was inhibited by addition of CMA but not brefeldin A, suggesting a predominantly perforin-based cytolytic pathway. Specific lysis was calculated as: (lysis in the presence of SMX) – (lysis without SMX). The values represent the mean of three independently performed assays $\pm$ SD.

drug is high enough, MHC–peptide complexes of autologous skin cells are reversibly modified by SMX. Consecutively, these target cells are recognized and killed mainly by presensitized, drug specific CD4⁺ T cells. Such a pathway may be common in generalized drug-induced delayed skin reactions. It might explain manifestations of drug hypersensitivity in the skin, where SMX metabolism is poor since a functional expression of SMX-related phenotypes of cytochrome P450 isoforms in the skin has not yet been shown. It is consistent with the immunohistological findings in

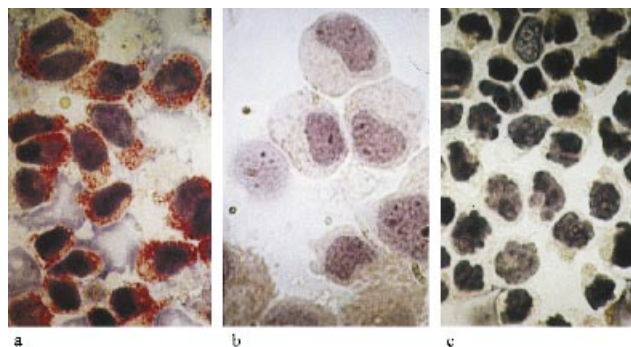


Fig. 4. Immunostaining for perforin. Strong immunoreactivity for perforin was detected in specific TCC (shown for the CD4⁺ TCC S23E). (a) No staining was found in B-LCL (b) or the isotype-specific negative control (c).

morbilloform cutaneous eruptions of patients with drug hypersensitivity, where a hydropic degeneration of the basal layer and infiltration by T lymphocytes [18] was observed. In addition we detected recently perforin expressing lymphocytes infiltrating the skin in a biopsy of a patient with a skin rash to carbamazepim and we could generate drug-specific cytotoxic T-cell lines from the skin of a patient with a hypersensitivity reaction to ceftriaxon (own observation, in preparation).

IFN γ induces MHC up-regulation on keratinocytes and renders these cells more susceptible to T-cell-mediated lysis [15,19]. In agreement with these findings, we observed CD4⁺ T-cell-mediated specific lysis against keratinocytes only if the keratinocytes were preincubated with IFN γ . Therefore, a constellation of increased IFN γ levels, as described in HIV patients [20], and treatment with high SMX doses may promote hypersensitivity reactions.

As we failed to detect a strong SMX specific CD8-mediated killing of keratinocytes, we assume that cytotoxic CD4⁺ T cells with cytotoxic potential are of relevance in causing skin rash in this patient. Cytotoxic CD4⁺ T cells have been previously implicated in viral infections and cytotoxicity against keratinocytes was also predominantly CD4-mediated [19]. A CD4-mediated mechanism would also fit with the observation that the dermis and epidermis infiltrating lymphocytes during cutaneous skin eruptions in non-HIV-infected patients bear frequently the CD4 phenotype (own observation, in preparation). However, in patients with bullous exanthems and epidermal necrolysis the infiltrating lymphocytes were predominantly of the CD8 phenotype [21,22], which suggests that CD8 killing might be important in certain skin eruptions as well.

In conclusion, our results indicate cytolytic T-lymphocyte response as a possible pathological pathway for drug-induced, generalized non-urticarial morbilliforme-like skin reactions.

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