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Transfection of Drug-Specific T-Cell Receptors into Hybridoma Cells: Tools to Monitor Drug Interaction with T-Cell Receptors and Evaluate Cross-Reactivity to Related Compounds[S]

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ABSTRACT

In the context of drug hypersensitivity, our group has recently proposed a new model based on the structural features of drugs (pharmacological interaction with immune receptors; p-i concept) to explain their recognition by T cells. According to this concept, even chemically inert drugs can stimulate T cells because certain drugs interact in a direct way with T-cell receptors (TCR) and possibly major histocompatibility complex molecules without the need for metabolism and covalent binding to a carrier. In this study, we investigated whether mouse T-cell hybridomas transfected with drug-specific human TCR can be used as an alternative to drug-specific T-cell clones (TCC). Indeed, they behaved like TCC and, in accordance with the p-i concept, the TCR recognize their specific drugs in a direct, processing-independent, and dose-dependent way. The presence of antigen-presenting cells was a prerequisite for interleukin-2 production by the TCR-transfected cells. The analysis of cross-reactivity confirmed the fine specificity of the TCR and also showed that TCR transfectants might provide a tool to evaluate the potential of new drugs to cause hypersensitivity due to cross-reactivity. Recombining the α- and β-chains of sulfanilamide- and quinolone-specific TCR abrogated drug reactivity, suggesting that both original α- and β-chains were involved in drug binding. The TCR-transfected hybridoma system showed that the recognition of two important classes of drugs (sulfanilamides and quinolones) by TCR occured according to the p-i concept and provides an interesting tool to study drug-TCR interactions and their biological consequences and to evaluate the cross-reactivity potential of new drugs of the same class.

Adverse reactions to drugs are encountered frequently, occurring in up to 5% of patients, and are a major problem in the clinic and during drug development (Naisbitt et al., 2003c). Most reactions are caused by the pharmacological or toxicological actions of the drug (type A reactions) and are generally predictable (Roujeau and Stern, 1994; Naisbitt et al., 2000; Pichler, 2003). However, approximately 20% of the reactions are unpredictable and still poorly understood (type B reactions), although several potential mechanisms exist (Pichler, 2003; Manfredi et al., 2004). In general, they are considered to be immune-mediated: different immune mechanisms (IgE, IgG, immune complexes, and T cells) are involved and can lead to many clinically distinct diseases (Pichler, 2003). Severe delayed drug-induced hypersensitivity reactions have led to the withdrawal of drugs from the market as such reactions can cause hepatitis, severe bullous skin diseases (Steven Johnson's syndrome and toxic epidermal necrolysis), autoimmune diseases, and so on, resulting in hospitalization, or, in the worst cases, death.

Patients with delayed drug-induced hypersensitivity harbor drug-specific T cells in their peripheral blood and also in the affected tissues (Brander et al., 1995; Mauri-Hellweg et al., 1995; Yawalkar et al., 2000; Naisbitt et al., 2003a,b). To uncover the role of drug-specific T cells, it is important to understand how drugs stimulate T cells. During the past years, our group has investigated this issue and reached some startling conclusions. In particular, we proposed a new model for drug recognition by T cells termed pharmacological interaction with immune receptors, or p-i concept (Pichler,

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ABBREVIATIONS: p-i, pharmacological interaction with immune receptors; TCR, T-cell receptor(s); MHC, major histocompatibility complex; TCC, T-cell clone(s); rIL, recombinant interleukin; IL, interleukin; SMX, sulfamethoxazole; SMT, sulfamethizole; STH, sulfamethiazole; SPD, sulfapyridine; SMP, sulfamethoxypyridazine; SID, sulfisomidine; SDM, sulfadimethoxine; SDX, sulfadoxine; CPFX, ciprofloxacin; SEA, staphylococcal enterotoxin A; SEB, staphylococcal enterotoxin B; HLA human leukocyte antigen; mAb, monoclonal antibodies; PBMC, peripheral blood mononuclear cells; APC, antigen presenting cell; HBSS, Hanks' balanced salt solution; NRFX, norfloxacin.

2002). It states that certain drugs would bind to some of the highly variable antigen-specific T-cell receptor (TCR) and major histocompatibility complex (MHC) molecules in a direct way. This interaction would not be based on a covalent binding of the drug to a carrier molecule and would not depend on a metabolism or processing step; rather, it would be similar to drug interactions with other, nonimmunological receptors and suffice to stimulate the T cell if a MHC interaction were provided. This stimulation would depend only on the structural features of the drug to fit into TCR and not on its immunogenicity. If true, this concept could explain some peculiar features of drug allergies and the failure to predict adverse immune responses by toxicological tests (Pichler, 2001; Bala et al., 2005), because they are designed to recognize hapten-like features of drugs only (Pichler, 2005).

The p-i concept first arose from the analysis of T-cell clones (TCC) specific for sulfanilamides (Schnyder et al., 1997) and local anesthetics (Zanni et al., 1998a) and was further analyzed by the study of TCC specific for antiepileptics (Naisbitt et al., 2003a,b), quinolones (Schmid et al., 2006), and other drugs (Sieben et al., 2002). There is a need to further prove and confirm this model as well as to facilitate its investigation.

However, T-cell reactions are complicated and difficult to monitor because they rely on the proliferation of the TCC themselves, which are derived from patients presenting drug allergies. Indeed, these TCC can be expanded in vitro only to a limited extent and often loose their specificity over time (Effros and Pawelec, 1997; Migliaccio et al., 2005). For these reasons, we looked for alternatives able to provide a tool to investigate drug-TCR interactions. Here, we present data on mouse hybridoma T cells transfected with drug-specific TCR showing that this system is a stable and reliable tool that can be used to analyze drug-TCR interactions. It can be used to further study the p-i concept and to screen for cross-reactivity with newly generated compounds of a same drug family.

Materials and Methods

Culture Media. Human T cells were cultured in RPMI 1640 medium (Invitrogen, Basel, Switzerland) supplemented with 10% pooled, heat-inactivated human AB serum (Swiss Red Cross, Bern, Switzerland), 25 mM HEPES buffer (Biochrom AG, Rupperswil Switzerland), 2 mM L-glutamine (Biochrom AG), 100 μg/ml streptomycin (Amimed Products, Allschwill, Switzerland), 100 U/ml penicillin (Amimed Products), and 25 μg/ml transferrin (Sigma Chemical Co., Buchs, Switzerland) and enriched with 100 U/ml human recombinant interleukin (rIL)-2 (Roche Pharma AG, Basel, Switzerland). Epstein-Barr virus-transformed B-lymphoblastoid cell lines (B-LCL) were cultured in RPMI 1640 medium supplemented with 10% pooled, heat-inactivated fetal calf serum (Invitrogen), 25 mM HEPES buffer, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin. The IL-2 sensitive CTLL cells and T-cell transfectants were cultured in the same media as B-LCL but additionally complemented with 50 μM 2-mercaptoethanol. Human rIL-2 at 50 U/ml served as source of IL-2 to maintain CTLL cells.

Drugs and Antigens. The sulfanilamides [sulfamethoxazole (SMX), sulfamethizole (SMT), sulfamethiazole, (STH) sulfapyridine (SPD), sulfamethoxypyridazine (SMP), sulfisomidine (SID), sulfadimethoxine (SDM), and sulfadoxine (SDX)] and quinolones [except ciprofloxacin (CPFX; Bayer AG, Wuppertal, Germany), moxifloxacin (Bayer AG), and levofloxacin (Sanofi-Aventis, Frankfurt, Germany)] were purchased form Sigma (Fluka Holdings AG, Buchs, Switzerland). The CPFX metabolites (desethylene-, sulfo-, oxo-, and formyl-CPFX) were kindly provided by Dr. P. Seiler (Bayer AG). Their chemical structures and abbreviations are depicted in Supplemental Fig. 1. Superantigens [staphylococcal enterotoxin A (SEA) and B (SEB)], used as positive controls at 100 ng/ml, were purchased from Sigma.

Antibodies and Flow Cytometry. The following monoclonal antibodies were used for flow cytometry: hamster anti-murine CD3ε (145-2C11) (Leo et al., 1987) or phycoerythrin-conjugated hamster anti-mouse CD3ε (BD Biosciences PharMingen, Basel, Switzerland), fluorescein isothiocyanate-conjugated anti-hamster IgG cocktail (BD Biosciences PharMingen), phycoerythrin-conjugated anti-human CD4 (Dako Denmark A/S, Glostrup, Denmark). For flow cytometric analyses, aliquots of 10^5 cells were stained either directly with fluorochrome-conjugated monoclonal antibodies (mAb) or with unlabeled primary mAb, followed by staining with fluorescein isothiocyanate-conjugated secondary mAb for 30 min at 4°C and then analyzed on a Coulter EPICS XL-MCL flow cytometer (Beckman Coulter, Nyon, Switzerland) or on a FACSCanto (BD Biosciences, San Jose, CA).

T-Cell Clones and Cell Lines. The CPFX-specific human $CD4^+$ TCC RuBa1, RuBa2, and RuBa11 were generated from the CPFX-allergic donor RuBa and have been partly described previously (patient 1 in Schmid et al., 2006). The SMX-specific human $CD4^+$ TCC UNO2, UNO3, and H13 were generated from the SMX-allergic donor UNO and have been described previously (Zanni et al., 1998a; Schnyder et al., 2000). To generate TCC, peripheral blood mononuclear cells (PBMC) of the patients were stimulated with the drug responsible for the hypersensitivity reaction for 7 days. Human rIL-2 (40 U/ml; Roche Pharma AG) was then added to the culture, and the T cells (T-cell line) were restimulated with the drug and autologous PBMC on day 14 as well as tested for their specificity. Two weeks later, the drug-activated cells were cloned by limiting dilution (Zanni et al., 1997). Growing TCC were expanded in culture medium (as described above) with 100 U/ml rIL-2 and restimulated every 14 days with allogeneic, irradiated PBMC and 1 μg/ml phytohemagglutinin (Difco, Detroit, MI) (Zanni et al., 1997; von Greyerz et al., 1999). Supplemental Fig. 2 depicts the different steps leading to TCC. The murine T-cell hybridoma 54ζ17 was a gift of O. Acuto (Institut Pasteur, Paris, France) and has been described previously (Blank et al., 1993). This hybridoma is a variant of the TCR-negative CD4-negative T-cell hybridoma 58 $\alpha^-\beta^-$ (Letourneur and Malissen, 1989) transfected with vectors for the human CD4 molecule and the murine CD3ζ chain. As antigen-presenting cells (APC), we used either autologous B-LCL generated as described previously (Wyss-Coray et al., 1993) (donor UNO: HLA-DRB1*01/10, donor RuBa: HLA-DRB1*01/08) or allogenic HLA partly matched B-LCL (HLA-DRB1*1/x). The study was approved by the ethics committee of the university, and informed consent was received from the donors.

Transfection of the Vα- and Vβ-Chains of the TCR into Mouse T-Cell Hybridoma. The 54ζ 17 mouse T-cell hybridomas were transfected as described previously (Depta et al., 2004) according to the method of Vollmer et al. (1999). In brief, rearranged human variable TCR α- and β-genes isolated from human, SMX-, and CPFX-reactive TCC (Table 1) were amplified by polymerase chain reaction and cloned into expression vectors containing the mouse constant and regulatory TCR sequences. The resulting vectors were cotransfected by electroporation into the TCR-negative murine T-cell hybridomas 54ζ 17, which also express the human CD4 coreceptor. Supplemental Fig. 3 shows schematically the steps leading to the creation of those transfectant cells. The oligonucleotide primers used in this study were described previously for the transfection of the TCR of TCC, UNO2, and UNO3 from patient UNO (Depta et al., 2004). The oligonucleotide primers used to amplify the variable TCR α- and β-genes from the TCC H13, RuBa2, RuBa11, and Ruba11 are shown in Table 2.

The TCR-transfected clones outgrowing in selective medium were picked. Expression of the correct TCR α and β was confirmed by DNA sequencing using a primer for the human variable region together

with a primer for the mouse constant region (Vollmer et al., 2000). Homogeneous expression of CD4 and TCR were assessed by flow cytometry as described above, and the transfected clones with the highest TCR expression were selected for assessment of reactivity and specificity, which was measured by TCR-induced IL-2 production.

IL-2 Secretion Assay. Transfectants (5×10^4 cells) were cocultured in triplicates in 200 μl of medium with 2.5×10^4 B-LCL as APC and indicated antigen concentrations. After a 24-h incubation at 37°C, 100 μl of the supernatants was used for a CTLL assay as described by Grabstein et al. (1986): 100 μl (5×10^3 cells) of IL-2-sensitive CTLL was added to the supernatant for 30 h at 37°C. [^{3}H]Thymidine was added, cells were harvested 14 to 16 h later (96-well cell harvester; Inotech, Wohlen, Switzerland), and the incorporated radioactivity was measured using a scintillation, liquid-free beta-counter (Trace96; Inotech).

None of the drugs at the used concentrations interfered with the proliferation of the CTLL (data not shown). Detection of IL-2 by enzyme-linked immunosorbent assay was also assessed, but the method was less sensitive than the use of CTLL. The CTLL yielded cpm of [^{3}H]thymidine incorporation between 3600 and 49,600 cpm at optimal drug concentrations. This corresponds to an IL-2 production between 0.4 and 53 U/ml. To distinguish between the response to covalently and noncovalently presented drugs (direct, processing-independent drug presentation), B-LCL were pulsed or fixed with glutaraldehyde, respectively, as described previously (Shimonkevitz et al., 1983). In brief, B-LCL were incubated with or without the drug for 2 h at the given concentration in Hanks' balanced salt solution (HBSS). The pulsed B-LCL were then washed twice with HBSS, and 2.5×10^4 cells were added to the transfectants. APC (2×10^6) in 0.5 ml of HBSS were fixed by addition of 0.05% glutaraldehyde for 30 s at room temperature. This glutaraldehyde concentration has been used to evaluate tetanus-specific responses, which where regularly completely suppressed, because they relied on the uptake and processing of tetanus-toxoid. This treatment might reduce the antigen-presenting function of the cells, which explains the diminished, but not abolished, response of the TCR-transfected cells observed in some experiments. The reaction was stopped by adding 500 μl of 0.2 M L-glycine for 45 s. The cells were then washed twice with RPMI 1640 medium before being added to the transfectants.

Results

Characterization of the T-Cell Clones and Transfectants. The human CD4$^+$ RuBa1, RuBa2, and RuBa11 were generated from the peripheral blood mononuclear cells of the CPFX-allergic RuBa (Schmid et al., 2006). All three clones were reactive to the inert and noncovalently binding antibacterial drugs CPFX. RuBa1 and RuBa11 were additionally reactive to norfloxacin (NRFX) (Table 1). The human CD4$^+$ TCC UNO2, UNO3, and H13 were generated from the peripheral blood mononuclear cells of the SMX-allergic patient UNO (Zanni et al., 1998a; Schnyder et al., 2000; Depta et al., 2004). They were reactive to the chemically inert and noncovalently binding antibiotic SMX. UNO2 reacted additionally to other drugs of the same family (SMT, STH, SPD, SMP,

TABLE 1

Characterization of the original TCC

T-cell receptor nomenclature according to Lefranc (2001). This nomenclature defines the TRAV (5*01) and TRBV (5-4*04) families, each of which contain many subfamilies (5*01).

TCC	Specificity	Cross-Reactivity	TRAV Expression	TRBV Expression	Transfectants
RuBa1[a]	PFX	NRFX	5*01	5-4*04	T1.84
RuBa2[a]	CPFX		26-1*01	20-1*02	T2.18
RuBa11[a]	CPFX	NRFX	8-4*01	19*01	T11.1
UNO2[b]	SMX	SMT, STH, SPD, SMP, SID, SDM, SDX	3*01	2*01	T25
UNO3[b]	SMX		3*01	25-1*01	T327
H13[c]	SMX	SPD	9-2*02	5-1*01	T13

TRAV, T-cell receptor α gene for the variable region; TRBV, T-cell receptor β gene for the variable region.
[a] TCC described in Schmid et al. (2006).
[b] TCC described in Depta et al. (2004).
[c] TCC described in von Greyerz et al. (2001).

TABLE 2

Oligonucleotide primers used to amplify the TRAV and TRBV of the TCC

Restriction sites for BamHI and SalI are in bold, and splice sites are indicated by lowercase letters. The TCR gene segments and numbering were defined according to Lefranc (2001).

TCC and Primers	Primer Sequence
PrLAV9-2*02	TTGAATTCGGAATGGACTATTCTCCAGGC
AJ15*01splice-BamH1	GATC**GGATCC**acttacTGGAACTCACTGATAAGGTG
PrLBV5-1*01	TTGAATTCGCCATGGGCTCCAGGCTGCTC
BJ1-1*01splice-SalI	GATCG**TCGAC**tcttacCTAGGATGGAGAGTTCGAGTC
RuBa1	
PrLAV5*01	ACTCCAGTGGCTCAGAAAATGAAGACATTTGCTGG
AJ33*01splice-BamHI	GATC**GGATCC**acttacCTGGCTTTATAATTAGC
PrLBV5-4*01	ACCTGCCTTGGTCCCAAGATGGGCCCTGGGCTCC
BJ2-7*01splice-SalI	GATCG**TCGAC**tcttacCTGTGACCGTGAGCCTG
RuBa2	
PrLAV26-1*01	GATCGGATCCACTTACAGGGCTGGATGATTAG
AJ45*01splice-BamH1	GATC**GGATCC**acttacAGGGCTGGATGATTAG
PrLBV20-1*02	ACCTGCCTTGGTCCCAAGATGCTGCTGCTTC
BJ1-6*02splice-SalI	GATCG**TCGAC**tcttacCTGTCACAGTGAGC
RuBa11	
PrLAV8-4*01	ACTCCAGTGGCTCAGAAAATGCTCCTGCTGC
AJ31*01splice-BamHI	GATCG**GATCC**acttacTGGGCTTCACCAC
PrLBV19*01	ACCTGCCTTGGTCCCAAGATGAGCAACCAGGTGC
BJ1-3*01splice-SalI	GATCG**TCGAC**tcttacCTACAACAGTGAGC

SID, SDM, and SDX) (Table 1; Supplemental Fig. 1). All clones used expressed $\alpha\beta$-TCR and used different Vα- and Vβ-chains.

The rearranged V(D)J-regions of the functional TCR α- and β-chains were amplified by polymerase chain reaction, cloned into expression vectors, and transfected into the TCR$^-$ mouse T-cell hybridoma 54ζ17 (Supplemental Data 3). Expression of TCR on the cell surface of transfectants was assessed by DNA sequencing and flow cytometry using monoclonal antibodies against the human Vβ-chain or the mouse CD3ε as described under *Materials and Methods*. The functionality of the expressed TCR was assessed either by stimulation with anti-mouse CD3ε and determination of IL-2 secretion or by stimulation with the drug as evaluated below. Figures 1 to 6 show the results for two quinolone- (T1.84 and T11.1) and two sulfanilamide-specific transfectants (T25 and T327). T2.18 and T13 showed a lower and less consistent responsiveness to the drugs; therefore, the results are not shown.

The Specificity of the Transfectant Was Due to the TCR. The specificity of the TCC can be transferred into hybridoma cells by transfecting their TCR. Indeed, the TCR transfectants produced IL-2 in a dose-dependent manner in answer to the drugs to which the original TCC reacted (Fig. 1). IL-2 was detected by proliferation of the CTLL line. Detection of IL-2 by enzyme-linked immunosorbent assay was also assessed, but the method was not sensitive enough. T1.84 reacted to CPFX and NRFX (CPFX is shown), T11.1 reacted to CPFX and NRFX (NRFX is shown), T25 reacted to six different sulfanilamides (SMT is shown), and T327 reacted to SMX only (cross-reactivity shown in Fig. 2). The concentration for maximal IL-2 production was 20 μg/ml for quinolones and ranged between 500 and 1000 μg/ml for sulfanilamides. Higher concentrations inhibited IL-2 production (Forsgren et al., 1987). The chemical structures of the drugs used are shown in the online Supplemental Data 1.

Cross-Reactivity of the Quinolone and Sulfanilamide Transfectants. The transfectants showed cross-reactivity patterns similar to those of their parent TCCs: T2.18 was highly specific to CPFX (Table 1; data not shown), whereas T1.84 and T11.1 were cross-reactive to CPFX and NRFX. T25 reacted to SMX, SMT, SDM, SDX, and to a lesser extent to SMP and STH. T13 and T327 were highly specific to SMX (Fig. 2; data not shown). The quinolone transfectants were specific to the chemically inert original compound and did not show reactivity to any CPFX metabolites (desethylene-, sulfo-, oxo-, and formyl-CPFX). Although the transfectants showed some degree of cross-reactivity, they were still exclusively specific to chemically similar compounds because they could not be stimulated by drugs with other core structures: the sulfanilamide-specific transfectants did not cross-react with quinolones (20 μg/ml), and the quinolone-specific transfectants did not cross-react with sulfanilamides (800 μg/ml) or with compounds of other unrelated drug families (data not shown).

T-Cell Receptor Recognition of Quinolones and Sulfanilamides Was APC-Dependent. T-cell stimulation is a complex process involving TCR, MHC molecules, and a 9- to 16-amino acid peptide to which the TCR reacts. In drug responses, the peptide might be eluted from the MHC molecule without affecting the drug-specific T-cell response (Burkhart et al., 2002), but the MHC molecule seems to be required (Zanni et al., 1998a). Thus, to investigate whether this trimolecular complex of TCR, drug/peptide and MHC, is

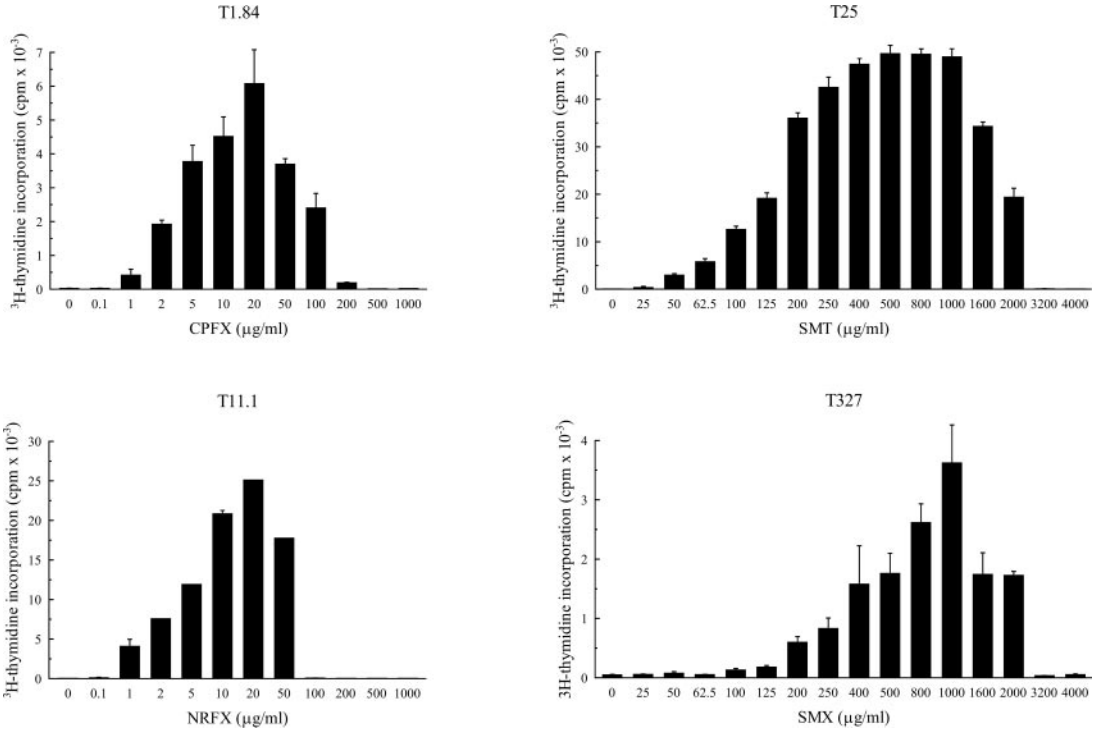


Fig. 1. The TCR transfectants responded to the drug in a dose-dependent manner. The transfectants were incubated in the presence of B-LCL (2.5×10^4 cells/well) with increasing concentrations of the drug, and the resulting IL-2 secretion was determined by [^{3}H]thymidine incorporation of CTLL cells. The results (mean ± S.D.) are representative of three independent experiments, each performed in triplicate. Similar dose-response curves were obtained with T11.1 for CPFX and with T25 for SMX.

needed for the activation of TCR-transfected cells, the different transfectants were incubated with the drug and increasing numbers of autologous B-LCL (Fig. 3). There was no production of IL-2 when the transfectants were stimulated with the drug in the absence of B-LCL. This indicates that the transfectants, which do not express human MHC molecules, could not present the drug to the TCR (which bear human α- and β-chains) and that APC expressing human MHC-II molecules were needed for optimal stimulation of the TCR transfected. The IL-2 concentration increased with in-

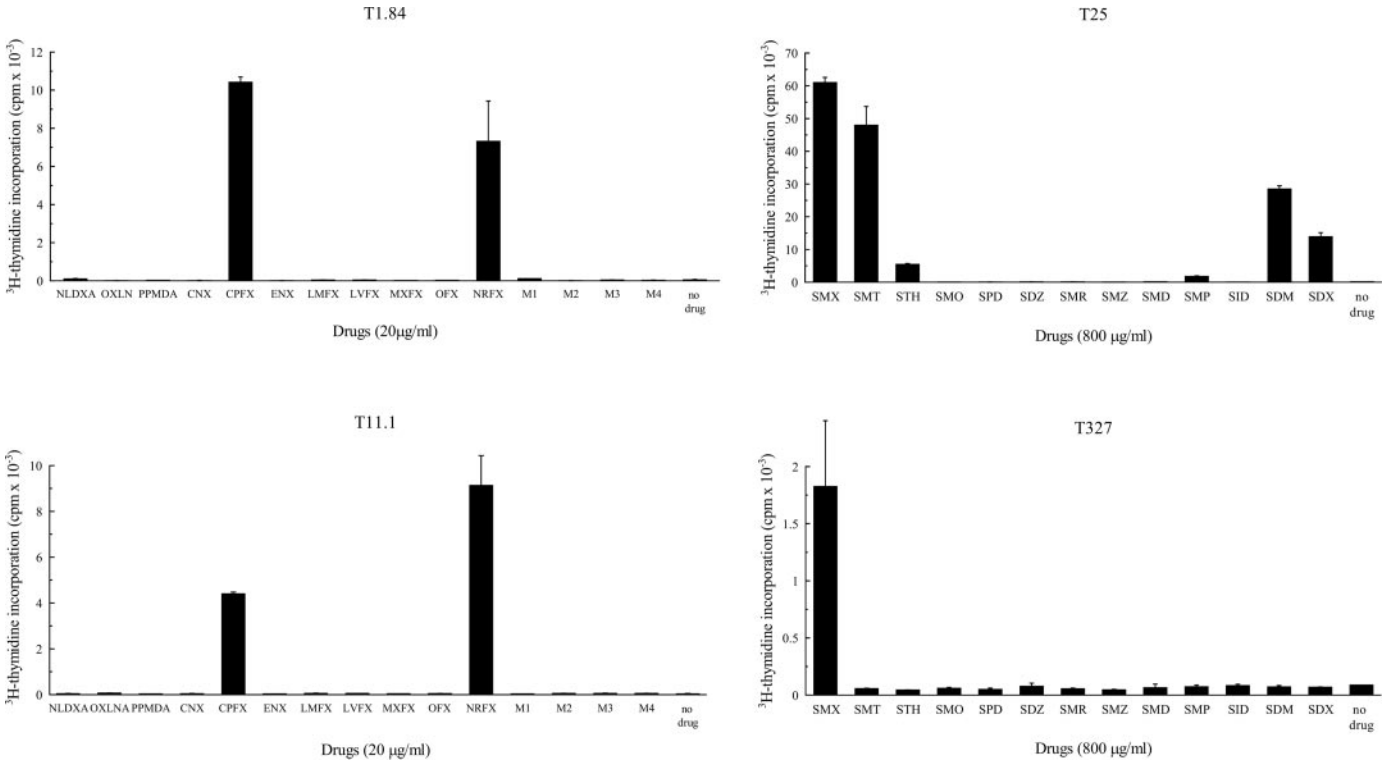


Fig. 2. Cross-reactivity of the TCR transfectants. The transfectants were incubated in the presence of B-LCL (2.5×10^4 cells/well) and different quinolones (20 μg/ml) or sulfanilamides (800 μg/ml), and their IL-2 production was detected by [^{3}H]thymidine incorporation by CTLL. Results (mean $\pm$ S.D.) are representative of three independent experiments performed in triplicate. M1, desethylene-CPFX; M2, sulfo-CPFX; M3, oxo-CPFX; and M4, fomyl-CPFX.

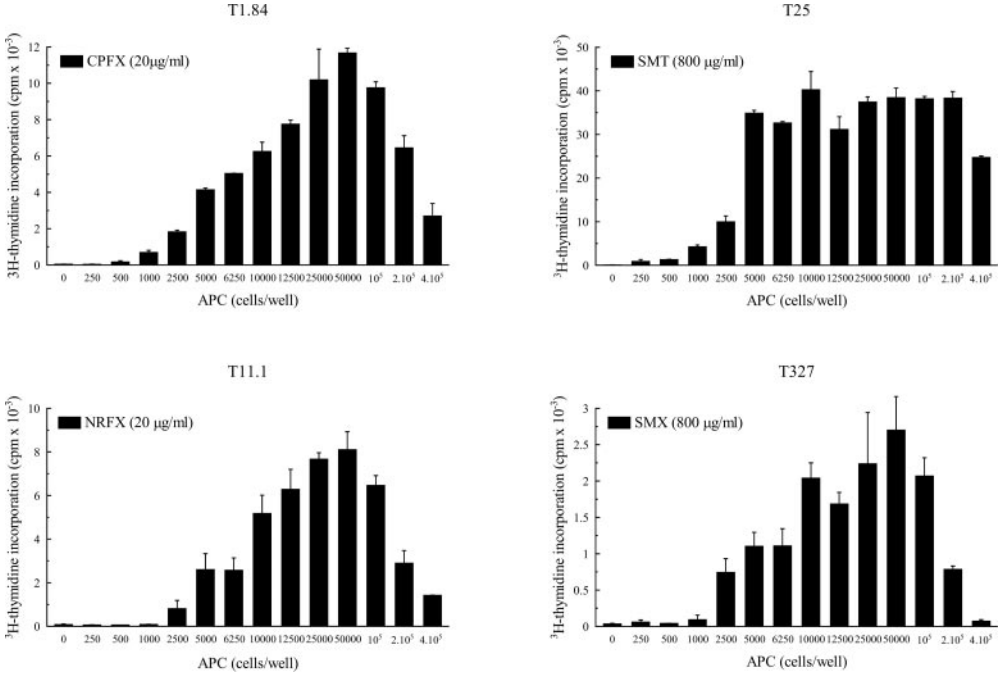


Fig. 3. TCR recognition of the drug was APC-dependent. The transfectants were incubated with the drug and increasing numbers of B-LCL. IL-2 production was detected by [^{3}H]thymidine incorporation by CTLL. Results (mean $\pm$ S.D.) are representative of three independent experiments performed in triplicate.

creasing number of B-LCL present (Fig. 3), with a maximum concentration achieved in the presence of 5×10^4 B-LCL for quinolone-specific transfectants, and in presence of up to 2×10^5 B-LCL for sulfanilamide-specific transfectants. The reduced IL-2 production at maximum cell concentrations was probably due to the culture conditions, because the cell expansion was limited in 200-μl microwells. The B-LCL used as APC could be either autologous or MHC-matched: concerning the TCR transfectants analyzed in this study, HLA-DR1-matched B-LCL were sufficient to activate the transfectant cells and induce IL-2 production (data not shown). However, if B-LCL with unmatched HLA-DR (e.g., HLA-DR7/13) were used, no IL-2 production was seen.

T-Cell Receptor Stimulation by Quinolones and Sulfanilamides Was Time-Dependent. In this study, the reactivity of the transfectants was measured by IL-2 secretion. To investigate how much contact time was needed to induce this IL-2 production, the transfectants were incubated with the drug in presence of APC for different times. Coincubation for less than 6 h did not lead to detectable amounts of IL-2 (Fig. 4). Significant IL-2 production was observed after 24-h coincubation of quinolone- or sulfanilamide-specific transfectants with their respective drugs (Fig. 4). A plateau was reached after 48 to 70 h of coincubation of the drugs with the transfectant cells (Fig. 4).

Quinolone and Sulfanilamide Recognition Was Processing-Independent. The quinolones and sulfanilamide derivatives used were considered chemically nonreactive compounds but able to bind covalently after metabolism into reactive metabolites (like SMX-NO).

However, both groups of drugs evaluated here were directly stimulatory: B-LCL fixed with glutaraldehyde (which prevents antigen uptake and processing) before incubation with the TCR transfectants and the drugs were still able to induce IL-2 production by the TCR transfectants (Fig. 5). This suggests that the quinolones and sulfanilamides tested did not need to be processed to stimulate the transfectants. Moreover, pulsing B-LCL with the drugs followed by a washing step resulted in no IL-2 secretion, indicating a labile binding of quinolones and sulfanilamides to APC (Fig. 5). This, as well as the failure to react to CPFX metabolites (Fig. 2), suggested a direct, processing-independent and labile/reversible binding of the tested drugs to the MHC and TCR.

T-Cell Receptor Recognition of Quinolones and Sulfanilamides Was α- and β-Chain-Dependent. Peptides are recognized by both the α- and β-chains of the TCR, whereas superantigens (such as SEA or SEB) stimulate T cells by binding simultaneously to the MHC class II molecules and to the variable segments of the β-chain (Vβ) (Fleischer and Schrezenmeier, 1988). To define the α- and β-chain contribution to drug specificity, we combined the RuBa1 α-chain with the UNO2 β-chain and the RuBa1 β-chain with the UNO2 α-chain. The same combinations were made with the α- and β-chains of RuBa1 and UNO3. The resulting

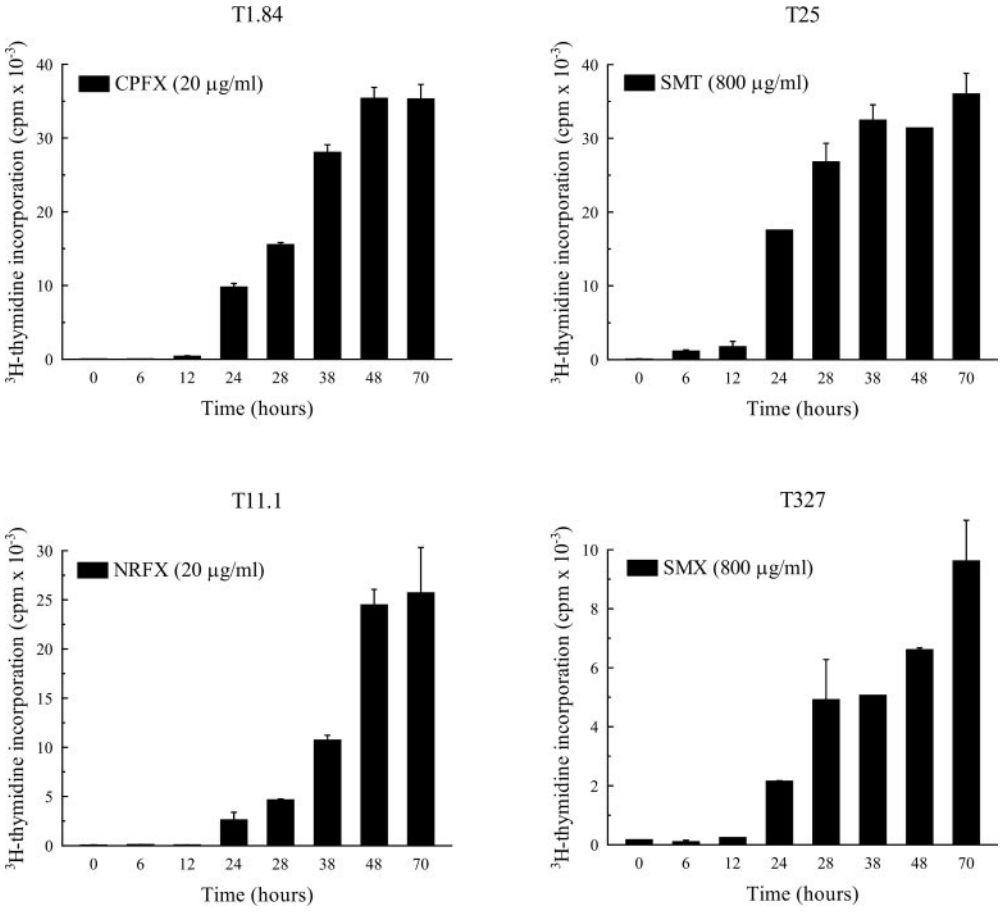


Fig. 4. TCR recognition of the drug was time-dependent. The transfectants were incubated over different time periods with B-LCL (2.5×10^4 cells/well) and the drug. IL-2 production was detected by [^{3}H]thymidine incorporation by CTLL. Results (mean ± S.D.) are representative of three independent experiments performed in triplicate.

selected transfectants expressed the TCR at the cell surface, and the TCR signaling was confirmed by stimulation with the corresponding Vβ-reactive superantigen (Fig. 6; flow cytometry data not shown). However, the transfectants resulting from the combination of the RuBa1 β-chain and the UNO3 α-chain expressed no TCR at the surface. Figure 6 shows the results for the combination of the α- and β-chains of RuBa1 and UNO2 and the β-chains of RuBa1 with the UNO3 α-chain. The RuBa1α/UNO2β transfectants responded to the TRBV19-specific superantigen SEB, the RuBa1β/UNO2α transfectants responded to the TRBV2-specific superantigen SEA, and the RuBa1β/UNO3α responded to the TRBV25-specific superantigen SEB. Nevertheless, all chain combinations failed to react to CPFX and SMX in the presence of autologous B-LCL of both patients, even though they were HLA-DR1-matched. A mix of B-LCL from both patients were used as APC as well, but it also failed to lead to a stimulation of the transfectants. This implies that both the α- and β-chain contributed to the drug specificity of these TCR.

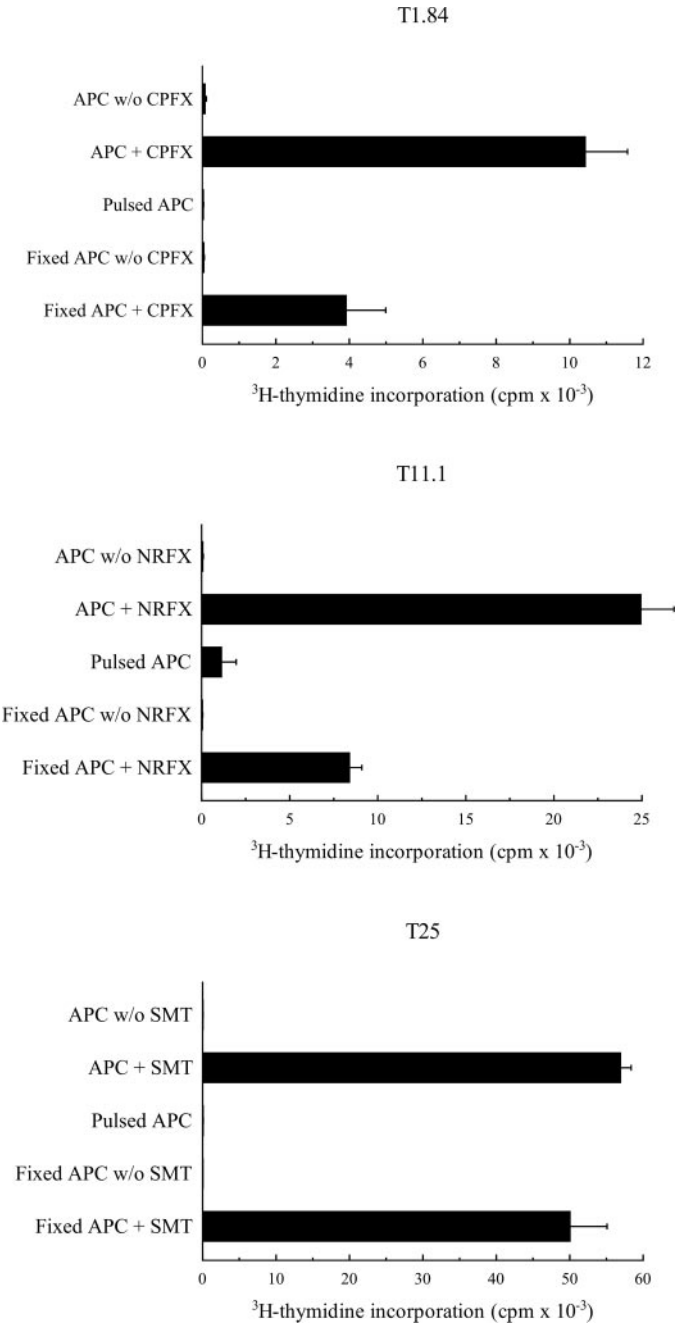


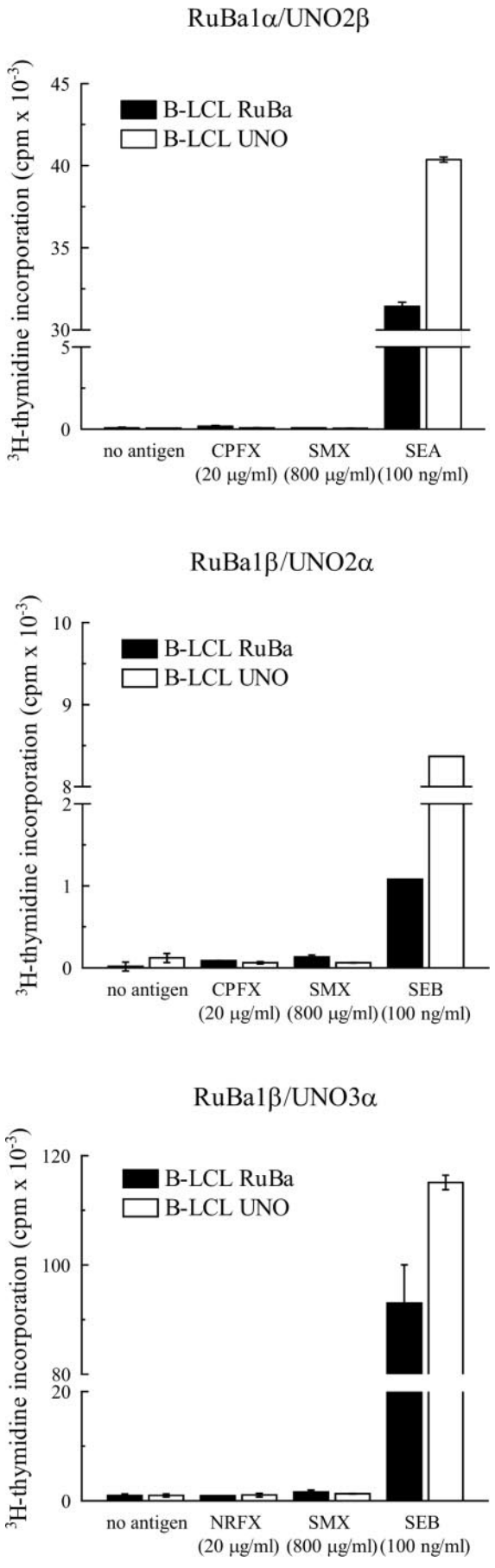
Fig. 5. TCR recognition of the drug, presented in a labile way, was processing-independent. Quinolone- and sulfanilamide-pulsed APC failed to stimulate quinolone- and sulfanilamide-specific transfectants. Coincubation of the drug (20 μg/ml for quinolones and 800 μg/ml for sulfanilamides) with APC and transfectants was required to elicit proliferation. Glutaraldehyde fixation of APC did not interfere with the presentation of quinolones and sulfanilamides to quinolone, or sulfanilamide-specific transfectants. Data from transfectant T327 are not shown because they needed relatively high concentrations of SMX to react and were less reliable and in a very low range of cpm after manipulations of the cells (e.g., fixation, washings). The IL-2 production was assessed by [^{3}H]thymidine incorporation by CTLL. One of two experiments performed with similar results is shown. Mean values with S.E.s of triplicate cultures are presented.

Discussion

Immune responses, triggered by stimulation of T cells by antigens like hapten-carrier complexes, are intricate, involving immunoglobulins and different cell types. These responses underlie hapten-specific allergic reactions, such as contact dermatitis or anaphylaxis to penicillin as well. However, many hypersensitivity reactions are possibly caused by the structural and chemical properties of the eliciting antigen, which triggers the immune cells by interacting with and stimulating immune receptors (p-i concept). Indeed, in adverse drug reactions, many compounds are believed to act according to the p-i concept: the inert parent drug and not the reactive metabolites triggers T cells, even in reactions for which the hapten concept has been accepted as explanation so far (e.g., *p*-phenylenediamine; Sieben et al., 2002). The resulting severe, even life-threatening diseases, such as drug rash with eosinophilia and systemic symptoms, Steven Johnson's syndrome, and toxic epidermal necrolysis, emphasize the need of test systems to unravel the mechanisms of drug-T-cell interactions.

The immense heterogeneity of TCR prevents the use of T-cell populations as a whole, because only few T cells react. These specific T cells need to be expanded to be useful. Indeed, so far, most of the findings on T-cell-drug interaction rely on TCC. However, T-cell culture is tedious and cumbersome, and T cells often lose their specificity over time and have only a limited life span. To overcome these difficulties and enable a more detailed analysis of the trimolecular interactions between TCR, drug, and peptide-MHC molecules, we selected some well characterized TCC specific for sulfanilamides and quinolones for transfection of their TCR into mouse hybridoma cells. In this study, two T-cell transfectants specific for quinolones and two T-cell transfectants specific for sulfanilamides were examined more closely.

The transfection of the TCR resulted in cells expressing functional receptors: the TCR could be detected at the cell surface and the TCR transfectants could be stimulated by the specific drug. These transfected hybridoma cells showed the same specificity as the original TCC and did not cross-react with unrelated compounds; i.e., quinolone transfectants did not react with sulfanilamides and vice versa. This implies that the TCR is necessary for the stimulation of the TCR-transfectant cells. The reactivity for both quinolone- and sulfanilamide-specific transfectants was dose-dependent,



with sulfanilamide-specific transfectants needing far higher doses to show detectable IL-2 production. This difference is probably related to the drug class and the TCC selected because the original TCC reacted to similar high drug concentrations. The responses of the transfectants to their specific drugs were similar to those observed by Vollmer et al. (2000) and Gamerdinger et al. (2003) evaluating $NiSO_4$ responses.

For a drug-induced IL-2 response, the presence of APC is needed, but these APC can be exchanged as long as the individual's restricting element (HLA-DR1 for the TCR transfectants of this study) is present. This proves the necessity of a fitting MHC molecule to supplement the TCR-mediated stimulation of the TCR-transfected cells. A role of other molecules on APC in the drug-peptide complex presentation to TCR is therefore ruled out. No processing or metabolism seems to be required for the drugs to stimulate the TCR-transfectants as the APC were still functional after fixation (which abrogates uptake and subsequent processing) (Schnyder et al., 1997). The drugs bind in a labile way to the immune receptors (TCR and possibly also MHC), because washing abrogated the stimulation. All the TCR-transfected cells reacted only if the compound was present during the entire reaction time. A closer analysis revealed that an exposure time of over 6 h is required for the TCR transfectants to secrete enough IL-2 to be detected. In theory, assays detecting drug binding to a soluble TCR could also be used alone (without reactive cell). However, they would detect not only stimulatory bindings, but also inactive or inhibitory bindings. The biological system used has the advantage that the trimolecular complex (peptide-MHC, drug, and TCR) is provided, which is a prerequisite for optimal stimulation of the cells. Thus, the biological relevance of the drug-TCR interaction in the presence of APC can immediately be demonstrated, as stimulation leads to an enhanced IL-2 production.

At present, the p-i concept implies that chemically nonreactive drugs can interact in a reversible, noncovalent way with the TCR and/or MHC, leading to a stimulation of the specific T cell. Which of those two molecules (or both) the drug is actually interacting with is not yet clear. However, that the HLA-bound peptides and the HLA-molecules can be exchanged without affecting the stimulation of the TCC- and TCR-transfected cells, as well as ongoing work, suggest a primary interaction of the drug with the TCR (Zanni et al., 1998b; Burkhart et al., 2002). The HLA molecules would play only an accessory role.

The p-i concept has some similarities to superantigen stimulations, such as the binding of antigens from the outside, the simultaneous stimulation of CD4 and CD8 T cells, and the possibility to exchange the peptide within the MHC mol-

Fig. 6. Both α- and β-chain of the TCR were needed for drug recognition. The α- and β-chain of RuBa1 and UNO2 or UNO3 were transfected in different combinations: the α-chain of RuBa1 together with the β-chain of UNO2, the β-chain of RuBa1 together with the α-chain of UNO2, the α-chain of RuBa1 together with the β-chain of UNO3, and the β-chain of RuBa1 together with the α-chain of UNO3. The transfectants were incubated with B-LCL (2.5×10^4 cells/well), and the drug and the resulting IL-2 production was assessed by [^{3}H]thymidine incorporation by CTLL. One of two experiments performed with similar results is shown. Mean values with S.E.s of triplicate cultures are presented. The transfection of the RuBa1 α-chain and UNO3 β-chain did not yield cells with detectable TCR at the cell surface.

ecule (p-i concept reviewed in Pichler, 2002). Superantigens bind simultaneously to MHC class II molecules and to a certain TCR Vβ family, which is sufficient to stimulate the T cells (Fleischer and Schrezenmeier, 1988; Fleischer et al., 1996). In contrast, both α- and β-chains of the original specific TCR were needed to allow stimulation by the drugs, because exchange of the TCR α- or β-chains with other chains abrogated stimulation. This suggests that, at least for the TCR analyzed, the drug stimulation occurs by interacting with both chains.

This reductionist system not only facilitates the study of drug-TCR interactions but also may already have an immediate use: the TCR were obtained from patients with T-cell-mediated hypersensitivity reactions, some of which might show a cross-reactivity to related new compounds as well. Indeed, the TCC- as well as the TCR-transfected hybridoma cells reacted to related compounds to which the patient was not previously sensitized: some of the sulfanilamides tested in vitro (and inducing an IL-2 production) were not on the market or only available for veterinary medicine. The level of cross-reactivity is surely dependent on the structural relationship between the priming drug (causing an allergy) and the related drugs tested. However, this cross-reactivity cannot be predicted based on the structure alone, as revealed by analyzing TCC specific for sulfanilamides, local anesthetics, as well as quinolones (Zanni et al., 1997; von Greyerz et al., 1999; Schmid et al., 2006). Thus, a test system demonstrating cross-reactivity might be informative. However, it is clear that the more transfectants with different TCR are used, the more reliable the information regarding the cross-reactivity will be. For this purpose, it is essential to transfect TCR with broad specificity/cross-reactivity.

In conclusion, TCR-transfected hybridoma cells provide an alternative to the use of T cells to study drug-TCR. Indeed, they share the same characteristics as the original TCC in terms of specificity, cross-reactivity, and drug recognition. Therefore, TCR-transfected hybridoma cells are interesting tools to study a new drug target, namely, the human TCR and the biological consequence of the interaction of the drug to the TCR. These new tools are providing insights into the mechanism of drug stimulation of T cells via TCR and might allow confirmation of cross-reactivity between established and new drugs.

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