

Delayed Allergic Reaction to Suxamethonium Driven by Oligoclonal Th1-Skewed CD4+CCR4+IFN- γ + Memory T Cells

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Key Words

Adhesion molecule • CC chemokine receptor 4 • Cutaneous lymphocyte antigen • Cytometry • Delayed-type hypersensitivity • Drug allergy • Suxamethonium • T helper 1 cell • T cell allergy • T cell receptor repertoire

Abstract

Background: Muscle relaxants represent the drugs most frequently involved in intraoperative anaphylaxis during surgical procedures. Our aim was to report the case of a delayed reaction to suxamethonium and analyze specific T cell lines with regard to their specificity, phenotype and cytokine profile. **Methods:** We generated a drug-specific T cell line from a biopsy at the site of positive intradermal reactions and analyzed the immunophenotype, T cell receptor V β domain expression and cytokine profile. **Results:** T cells isolated from positive intradermal test reactions to suxamethonium showed a strict dose-dependent proliferation in response to drug-pulsed autologous antigen-presenting cells. The drug-specific CD4+ T cells were oligoclonal memory CD3+CD4+ T cells and expressed the skin homing receptors cutaneous lymphocyte antigen (CLA) and CCR4. Furthermore CD4+ suxamethonium-reactive T cell lines were IFN- γ -positive and synthesized high levels of IFN- γ and TNF- α . **Conclusion:**

The study describes a delayed hypersensitivity to suxamethonium, driven by an oligoclonal T helper cell 1-skewed CD4+ memory T cell population, expressing the skin homing receptors CLA and CCR4.

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Introduction

On the basis of the time interval between drug administration and the onset of the symptoms, an adverse reaction to a drug can be classified as immediate (within 1 h), accelerated (between 1 and 72 h) and delayed (after 72 h) [1–3]. The allergic reaction to drugs comprises both IgE- and T cell-mediated mechanisms [4]. The drug allergy diagnostic workup comprises both in vivo (skin prick, intradermal, epicutaneous and provocation tests) and in vitro tests (detection of drug-specific IgE and lymphocyte transformation test) [3, 5]. T cell-mediated immune responses play an important role in both immediate and delayed types of adverse drug reactions [6, 7]. Studies on T cell lines (TCL) and/or T cell clones have provided useful information about the characteristics and the molecular mechanisms involved in drug recognition by specific T cells [6, 8–11].

Several agents are responsible for the intraoperative anaphylaxis in the course of general anesthesia [12]. Muscle relaxants represent the drugs most frequently involved in immunologically mediated adverse drug reactions during surgical procedures [12–16]. Muscle relaxant reactions are almost exclusively IgE-mediated, whereas cases of delayed reaction due to suxamethonium have been rarely described [17–19].

In this study we report the immunological evaluations in a patient with a delayed reactivity to suxamethonium.

Methods

Case Report

A 71-year-old non-atopic woman, who underwent total hysterectomy under general anesthesia, developed a diffuse morbiliform eruption after 2 days. She had no angioedema, respiratory problems, hypotension or any other relevant symptom. The drugs used during surgery were propofol (Diprivan®, AstraZeneca, Italy), atropine (Atropina®, Farmigee, Italy) and suxamethonium (Midarine®, GlaxoSmithKline, Italy).

One year later the patient was referred to our allergy clinic for the etiological diagnosis. After obtaining the patient's written consent, an allergy study was carried out. The patient had no personal or family history of allergy or adverse drug reactions, and previous general anesthetic procedures had been performed without adverse reaction. She was not undergoing any concomitant therapy at the time of her adverse reaction. However, sensitization to common respiratory allergens and foods was ruled out on the basis of a negative immediate hypersensitivity test. The IgE levels were 58 kU/l, and the blood eosinophil count was 72 cells/ μ l.

The diagnostic procedures consisted of skin prick and intracutaneous tests with suxamethonium, atracurium (Tracrium®, GlaxoSmithKline), vecuronium (Norcuron®, Organon, Italy), propofol, sodium thiopental (Pentotal sodium®, Hospira, Italy), neostigmine methylsulfate (Intrastigmina®, Lusofarmaco, Italy), atropine, diazepam (Valium®, Roche, Italy) and latex extract (ALK-Abellò, Madrid, Spain). The skin prick tests were performed using undiluted commercial preparations. The intracutaneous tests were carried out in dilutions ranging from 1:100,000 to 1:100, as stated in several papers [20–23]. The saline solution produced a negative result, and the histamine 1 mg/ml epicutaneous prick test resulted in an 8-mm wheal with a flare. No immediate reactions were observed with general anesthetic drugs. However, 24 h after the test, itching and erythematous patches developed at the injection site of suxamethonium (0.05 mg/ml), while no reaction was observed at the injection sites of the other drugs.

Generation of TCL

A 5-mm punch biopsy was obtained at the site of the positive delayed reactions of the intracutaneous test with suxamethonium to isolate drug-specific TCL from skin-infiltrating lymphocytes (SIL). The skin specimens were washed with PBS and then placed in culture in antibiotic-free RPMI 1640 medium supplemented

with 2 mM of L-glutamine (Life Technologies, Chagrin Falls, Ohio, USA; complete RPMI), 10% heat-inactivated FCS (HyClone, Logan, Utah, USA) and 60 U/ml of rIL-2 (Boehringer Mannheim, Germany) for 2 weeks, with fresh medium and IL-2 replaced every 3–4 days. Peripheral mononuclear cells were isolated from 20 ml of heparinized blood via density gradient centrifugation (Ficoll/Hypaque). After 2 wash cycles the peripheral mononuclear cells were incubated both with soluble antigen (Ag) as described above or with Ag-pulsed antigen-presenting cells (APC).

The TCL obtained were assayed for Ag specificity after extensive washing to remove IL-2. Different stimulation procedures were chosen, as previously described [24]. Briefly, both a direct stimulation of peripheral blood mononuclear cells (PBMC) and TCL with different doses of suxamethonium (from 0.01 to 1 mM/l) and PBMC and TCL stimulation with drug-modified autologous PBMC, named Ag-pulsed APC, were performed. Modification of APC was achieved by incubation of APC with 0.01, 0.1 and 1 mM/l of suxamethonium. After washing with complete antibiotic-free RPMI 1640 medium and irradiation (6,000 rad), Ag-pulsed stimulatory cells were added to 1×10^6 cells/well of the corresponding PBMC or TCL. After 48 h the cells were resuspended, and a 200- μ l aliquot was transferred into 96-well U-bottom microtiter plates. The proliferation of these cells was determined by overnight incubation with 0.5 μ Ci/ml of [^{3} H]thymidine (Amersham, Little Chalfont, UK). The cells were then harvested onto fiber-coated 96-well plates (Packard Instruments, Groningen, the Netherlands), and incorporated radioactivity was measured on a scintillation liquid-free β -counter (Topcount, Packard Instruments).

Immunophenotyping of TCL and Analysis of V β Domains of Expressed T Cell Receptor β Chains

A 4-color flow cytometry analysis was performed as previously described [25]. Briefly, 2×10^5 lymphocytes from PBMC were delivered into the test tube containing 20 μ l of each monoclonal antibody [MAb; FITC, phycoerythrin (PE), phencyclidine (PcP), PE-Cy5 or APC-conjugated] and placed in an ice water bath for 20 min. After 2 washes with PBS containing 2% FCS and 0.02% NaN₃, the cells were resuspended in 300 μ l of PBS containing FCS and NaN₃ for immediate flow cytometry analysis. The following MAb were used: CD3, CD4, CD8, cutaneous lymphocyte antigen (CLA; Becton Dickinson Immunocytometry system, Mountain View, Calif., USA), CXCR3, CXCR4, CCR5 (Pharmin-gen, Becton Dickinson), CCR1, 2 and 4–8, and CXCR1, 2, 5 and 6 (R&D System, Minneapolis, Minn., USA). An irrelevant mouse IgG1 MAb (Becton Dickinson) was used as a negative control.

TCL were also studied for T cell receptor (TCR)-V β domain expression using an IOTest® Beta Mark panel (Immunotech, Marseille, France) of 24 V β mAbs: V β 1 (BL37.2), V β 2 (MPB2D5), V β 3 (CH92), V β 4 (WJF24), V β 5.1 (IMMU157), V β 5.2 (36213), V β 5.3 (3D11), V β 7.1 (ZOE), V β 7.2 (ZIZOU4), V β 8 (56C5.2), V β 9 (FIN9), V β 11 (C21), V β 12 (VER2.32), V β 13.1 (IMMU222), V β 13.2 (H132), V β 13.6 (JU-74), V β 14 (CAS1.1.3), V β 16 (TAMAYA 1.2), V β 17 (E17.5F3), V β 18 (BA62), V β 20 (ELL 1.4), V β 21.3 (IG125), V β 22 (IMMU546) and V β 23 (AF 23). Data acquisition and analysis of the TCR-V β immunofluorescence stainings were performed in triple staining. The test combines 3 TCR-V β -specific reagents in a single test tube employing only 2 colors. One TCR-V β antibody is conjugated to FITC, another one to PE

and the third to both FITC and PE. The third TCR-V β -stained population shows up in the diagonal of quadrant 2 in a FL1/FL2 histogram. The first incubation with 20 μ l of TCR-V β mAb mixtures (A: TCR-V β 5.3, 3 and 7.1; B: TCR-V β 9, 16 and 17; C: TCR-V β 18, 5.1 and 20; D: TCR-V β 13.1, 8 and 13.6; E: TCR-V β 5.2, 12 and 2; F: TCR-V β 23, 21.3 and 1; G: TCR-V β 11, 14 and 22, and finally H: TCR-V β 13.2, 7.2 and 4) was followed, after washing, by incubation with CD4-PcP and CD8-APC. To define single TCR-V β expression, the mean normal TCR-V β value was used.

For the detection of intracellular cytokines, TCL were restimulated for 6 h with 10 ng/ml of PMA and 1 mg/ml of ionomycin in the presence of either 3 mM of monensin or 10 mg/ml of brefeldin A (all from Sigma). After restimulation the cells were surface-stained with CD4-PcP and CD8-APC as described above. The cells were then washed twice with complete PBS and fixed in ice-cold PBS containing 0.4% paraformaldehyde for 10 min. After 2 additional washes in cold PBS, the cells were resuspended in 300 ml of PBS containing 0.1% saponin, 10% human AB serum, 100 mg goat IgG and 0.01 M of HEPES buffer. The cells were spun down after 10 min, and FITC- or PE-conjugated anti-IFN- γ or anti-IL-4 Abs (Becton Dickinson) that had been diluted in 1 mg/ml of PBS containing 0.1% saponin and 0.1 M of HEPES buffer were added for 20 min at room temperature. After 2 additional washes the cells were resuspended in PBS and analyzed by flow cytometry.

The quantitative analysis of the 4-color flow cytometry was carried out using a FAC-Scan instrument (Becton Dickinson). At least 10,000 events were acquired in list mode, and all the data were analyzed by the CellQuest software (Becton Dickinson). Live lymphomonocytes were electronically gated by forward and right-angle scatters.

Measurement of Cytokines

Supernatants from the drug-specific TCL and PBMC cultures were collected after 48 h (10^6 cells/ml in 24 wells) in complete RPMI supplemented with 5% human serum in the presence of 10 ng/ml of PMA and 1 μ g/ml of ionomycin (Sigma), in order to provide a maximal stimulus, filtered and then stored at -80°C . TNF- α , IFN- γ , IL-4, IL-6 and IL-18 content was measured with commercially available sandwich ELISA kits (Quantikine, R&D Systems, Minneapolis, Minn., USA) following the manufacturer's instructions, and the results were interpolated from the standard reference curve provided with each kit. The same cytokines were also measured in patient's serum.

Results

T Cells Isolated from Positive Intradermal Test Reactions to Suxamethonium Are Drug-Specific

We took advantage of the presence of a delayed hypersensitivity to suxamethonium to perform a punch biopsy at the site of the delayed reaction on the forearms in order to isolate drug-specific TCL. We tested the TCL obtained for drug specificity, using drug-pulsed autologous APCs and adding the Ag directly to the cell cultures.

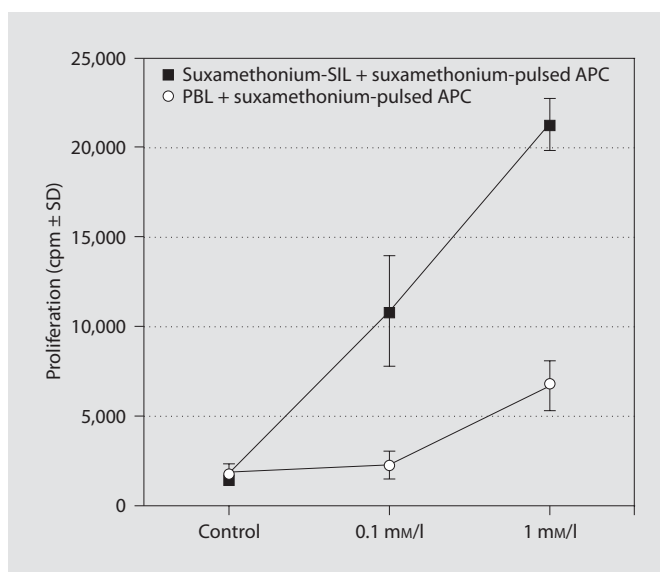


Fig. 1. Dose-dependent proliferative response of SIL and PBMC to suxamethonium.

The stimulation of suxamethonium skin-derived TCL with suxamethonium-pulsed APC resulted in a strong proliferation to 0.1 and 1 mM/l with a stimulation index (ratio of counts per minute with and without Ag in triplicate culture) of 7 and 14, respectively. In a similar way we also tested the response of PBMC to Ag-pulsed APC. A slight response at 1 mM/l of suxamethonium-pulsed APC was achieved (stimulation index of 4; fig. 1).

TCL and PBMC were finally stimulated by adding suxamethonium directly to the cell cultures in the absence of any APC. Neither TCL nor PBMC were stimulated by the continuous presence of soluble suxamethonium, showing that classical Ag processing was required for the presentation of the drug in the case of TCL, while the unresponsiveness of peripheral blood lymphocyte (PBL) to soluble suxamethonium can be explained by a scant presence of suxamethonium-reactive cells in the peripheral blood.

PBMC from 3 nonallergic donors did not respond to either Ag stimulation.

Flow Cytometry Analysis

The suxamethonium-specific TCL were CD3+CD4+ T cells (95%), which express the CD45R0 isoform, CLA and CCR4, a typical immunophenotype of memory skin-committed cells (table 1). As previously reported, drugs can trigger an oligoclonal expansion of CD4+ T cells bearing certain TCR-V β chains [26]. Then we sought to

Table 1. Immunophenotype and TCR-V β analysis of PBMC and skin-derived TCL

	PBMC %	TCL %
CD3+	75	99
CD3+/CD4+	43	96
CD3+/CD8+	28	2
CD3-/CD16+	10	<0.1
CD19+	10	<0.1
CD4+/CLA+	5	85
CD4+/CD45R0+	65	99
CD4+CD45R0+/CXCR3+	58	0.8
CD4+CD45R0+/CXCR4+	57	0.4
CD4+CD45R0+/CCR1+	1.8	<0.1
CD4+CD45R0+/CCR2+	0.5	0.4
CD4+CD45R0+/CCR3+	0.4	0.7
CD4+CD45R0+/CCR4+	27	59.8
CD4+CD45R0+/CCR5+	34	0.2
CD4+CD45R0+/CCR6+	17	0.5
CD4+CD45R0+/CCR7+	71	0.2
CD4+CD45R0+/CCR8+	2	0.2
CD3+/CD4+/TCR-V β 1+	3.2	0.7
CD3+/CD4+/TCR-V β 2+	9.78	4.1
CD3+/CD4+/TCR-V β 3+	4.89	15.9
CD3+/CD4+/TCR-V β 4+	2.1	<0.1
CD3+/CD4+/TCR-V β 5.1+	6.9	13.3
CD3+/CD4+/TCR-V β 5.2+	1.1	0.2
CD3+/CD4+/TCR-V β 5.3+	1.2	0.1
CD3+/CD4+/TCR-V β 7.1+	1.8	1.4
CD3+/CD4+/TCR-V β 7.2+	1.8	0.6
CD3+/CD4+/TCR-V β 8+	4.8	18.1
CD3+/CD4+/TCR-V β 9+	4.2	0.7
CD3+/CD4+/TCR-V β 11+	0.6	0.1
CD3+/CD4+/TCR-V β 12+	2.5	12.1
CD3+/CD4+/TCR-V β 13.1+	3.5	0.3
CD3+/CD4+/TCR-V β 13.2+	1.09	0.3
CD3+/CD4+/TCR-V β 13.6+	1.5	0.3
CD3+/CD4+/TCR-V β 14+	5.4	2
CD3+/CD4+/TCR-V β 16+	1.24	0.3
CD3+/CD4+/TCR-V β 17+	7.2	1.6
CD3+/CD4+/TCR-V β 18+	0.7	<0.1
CD3+/CD4+/TCR-V β 20+	2.5	2.6
CD3+/CD4+/TCR-V β 21+	3.4	1.4
CD3+/CD4+/TCR-V β 22+	4.56	1.3
CD3+/CD4+/TCR-V β 23+	0.2	<0.1

evaluate if any modification could be detected in the V β repertoire in the course of a delayed reaction to general anesthesia drugs. As shown in figure 2, a FACS analysis revealed an oligoclonal expansion of TCR-V β 3, 5.1, 8 and 12 in the SIL.

We further determined that about 75% of the suxamethonium-specific TCLs were IFN- γ -positive, while very few were IL-4-positive (below 5%; fig. 3), thus suggesting the presence of a T helper cell 1 (Th1)-like phenotype.

Cytokine Pattern Released by Suxamethonium-Specific TCL

Since we found a delayed reactivity to suxamethonium driven by an oligoclonal population of CD4+ T cells, we sought to investigate the cytokine pattern of these reactive cells. The cytokine pattern of the drug-specific TCL generated from our patient was assayed before and after 48 h of stimulation with PMA/ionomycin. The values of TNF- α , IFN- γ , IL-4, IL-6 and IL-18 are presented in table 2. We found a Th1-skewed picture of released cytokines. In fact, PMA/ionomycin stimulation tended to increase the secreted levels of IFN- γ and TNF- α and to decrease the levels of IL-6, while poor levels of IL-4 were observed in both the stimulated and the unstimulated conditions.

Discussion

Muscle relaxants represent the most common cause of preoperative anaphylactic reaction, followed by latex, whose incidence seems to decrease [27]. We report a case of delayed reaction to suxamethonium. We isolated drug-specific TCL from skin biopsy at the site of the positive intradermal test reactions and analyzed them with regard to their specificity, phenotype and cytokine profile. The specificity was demonstrated by the proliferation obtained in response to drug-pulsed autologous APC (fig. 1). A FACS analysis revealed a predominance of memory CD4+CD45R0+ cells in the inflammatory cell infiltrate of the intradermal test (table 1). Interestingly CLA and CCR4 were expressed by the totality of CD4+CD45R0+ cells in the inflammatory cell infiltrate. It is widely known that homing to the skin is mediated by the adhesion receptor CLA and leukocyte function-associated antigen (LFA)-1, and by the thymus and activation-regulated (TARC) and macrophage-derived chemokines (MDC) that recruit CCR4-positive memory cells. CCR4 and its ligands therefore mediate the preferential recruitment of α 4 β 7- systemic memory T cells, particularly the skin homing population [28].

When we observed the TCR-V β repertoire of the reactive infiltrate, we found that suxamethonium-specific CD4+ T cells were oligoclonal with regard to TCR-V β usage (fig. 2). Mauri-Hellweg et al. [26] described similar

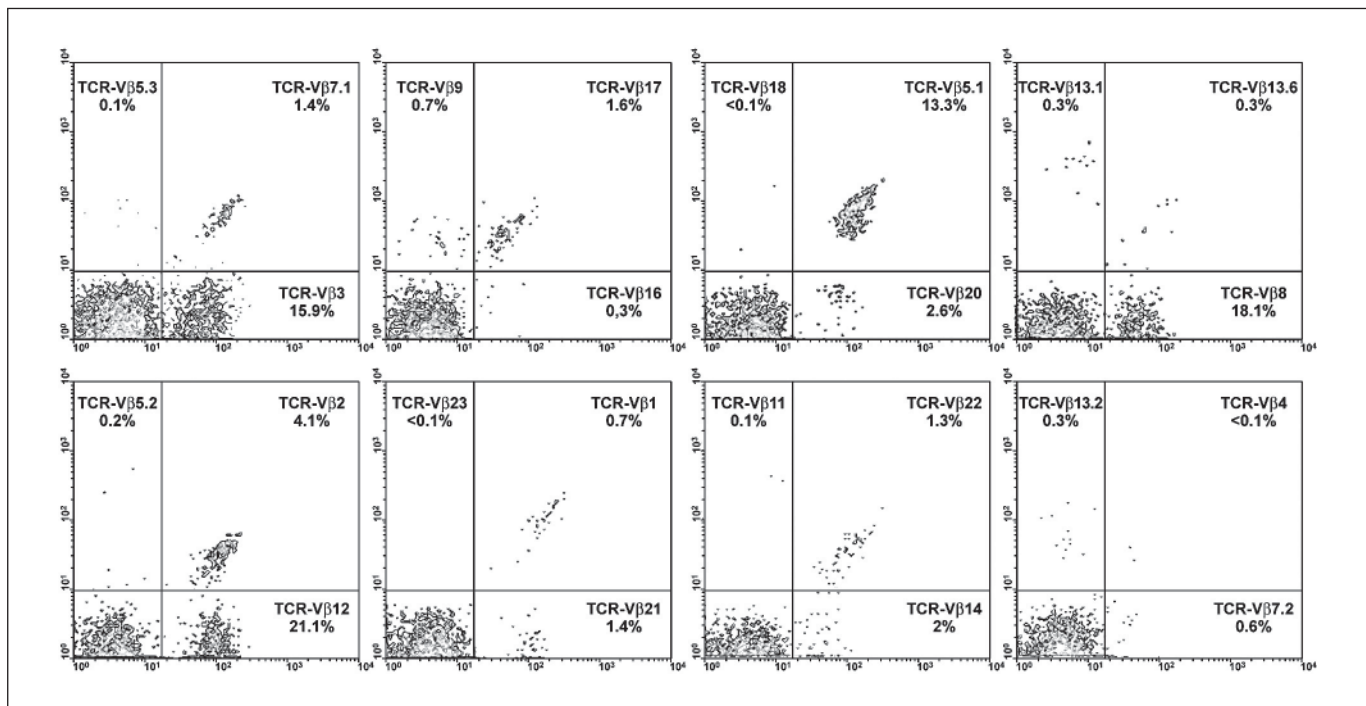


Fig. 2. TCR-V β analysis of skin-derived suxamethonium-reactive TCL.

Table 2. Cytokine profile of suxamethonium-specific TCL

	Before stimulus	After stimulus
IFN- γ , pg/ml	49	436
TNF- α , pg/ml	4.3	53
IL-4, pg/ml	8.5	15.2
IL-6, pg/ml	660	493
IL-18, pg/ml	9	6

data in penicillin-allergic patients, suggesting that the presence of T cells bearing a certain TCR-V β may be a sign of a limited crossreactivity. Furthermore oligoclonal expansion of T cells might be associated with a restricted hapten specificity [29]. Therefore we can hypothesize that a selective use of TCR-V β 3, 5.1, 8 and 12, into which suxamethonium fits better than other muscle relaxants, occurs.

Contrasting data have been reported about the cytokine profile of drug-reactive TCL [30–34]. Penicillin G-specific T cell clones were found to produce high amounts of IFN- γ , showing a prevalent Th1-like pattern [31], while

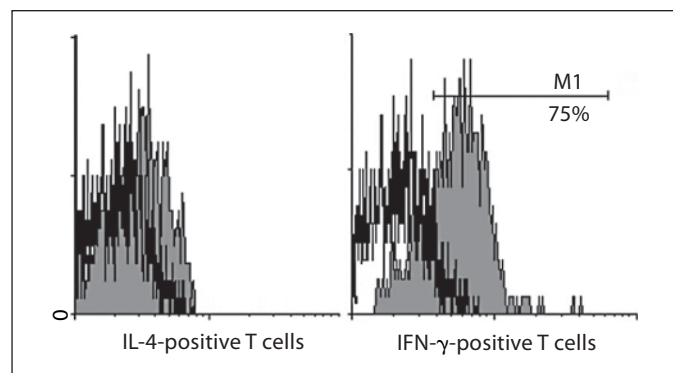


Fig. 3. IL-4- and IFN- γ -positive cells within suxamethonium-reactive TCL.

in other studies TCL derived from peripheral blood of patients with hypersensitivity to penicillin G were predominantly CD4⁺ cells with a Th2 profile of cytokine production [4]. In contrast a heterogeneous cytokine pattern was found by other authors [31, 33]. We demonstrated the coexistence in the same patient of a delayed hypersensitivity to both penicillin G and betamethasone, driv-

en by penicillin G-specific Th2-skewed CD8+ and betamethasone Th0 CD4+ T cells, respectively [24].

No data are actually available for muscle relaxant-specific TCL. In our hands suxamethonium TCL displayed a preferential Th1 profile, as demonstrated both by the prominent release of TNF- α and IFN- γ and the low levels of IL-4 synthesis (table 2). The presence of about 75% of IFN- γ -positive cells within suxamethonium-specific TCL adds emphasis to this scenario (fig. 3).

Different types of clinical manifestation seem to be driven by different T cell subsets. Indeed in β -lactam-allergic patients, bullous exanthemas are associated with

the presence of CD8 Th1-like T cells, while urticarial exanthemas are associated with a CD4+ Th2-like population [4, 35]. Maculopapular reactions were observed in the presence of a predominant activation of CD4+ cells [36]. According with these reports our patient complained of a severe CD4 Th1-driven reaction after general anesthesia.

In conclusion, we demonstrated that in a patient with a delayed reactivity to suxamethonium, the sensitivity was driven by an oligoclonal, Th1-skewed CD4+ memory β T cell population, expressing the skin homing receptors CLA and CCR4.

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