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Title Page

T-cell activation in two cases of Stevens-Johnson syndrome after receiving amoxicillin-clavulanic acid

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To the Editor:

Stevens-Johnson syndrome (SJS) is a rare life-threatening condition attributed almost exclusively to drug exposure (1). The main etiologic factor for SJS is treatment with drugs, primarily sulfonamides and anticonvulsants, followed by penicillins and non-steroidal anti-inflammatory drugs (1). Among penicillins, amoxicillin/clavulanic acid (AMX/CLV), despite generally being a well-tolerated antimicrobial, has been reported as a risk factor for SJS in adults and children in several publications (2, 3). The pathomechanisms of SJS are not fully understood, but immunologic mechanisms, reactive drug metabolites, and/or interactions between these two have been described in several reviews (4, 5). In this paper, two children with SJS due to AMX/CLV treatment were investigated using *in vitro* testing by studying the

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expansion of T lymphocytes. The first case (patient #1) is a child treated with AMX/CLV during EBV infection, and the second case (patient #2) is a child treated with AMX/CLV where other possible diseases were ruled out.

Patient #1 is a Caucasian boy who had already used oral AMX/CLV to treat common upper airway infectious diseases. At three years of age, he was treated with oral AMX/CLV syrup for an acute febrile pharyngitis (39.8°C) and lymphadenopathy that was later diagnosed as EBV infection by serological tests. On the third day, he was evaluated at the emergency department of Anna Meyer Children Hospital, where he appeared in poor general condition with fever (38.5°C), malaise, generalized maculopapular exanthema (MPE) with purpuric lesions covering the entire body surface plus both plantar and palmar regions, redness of the eyes, oral erosions of the lips, throat hyperemia, and cervical lymphadenopathy. According to these clinical symptoms, he was diagnosed as being affected by SJS due to the treatment with AMX/CLV during EBV infection. The drug was stopped and he received supportive therapies, including a short course of corticosteroids, fluid replacement with electrolyte solution and analgesic therapy, and recovered within 10 days. Six months later, he was referred to the Allergy Unit of Anna Meyer children's hospital for an allergologic work-up.

According to the European Network for Drug Allergy (ENDA) diagnostic algorithm for *in vivo* allergologic evaluation of non-immediate reaction to β -lactams, patch tests and skin intradermal tests (ID) with AMX/CLV were consecutively performed (6). Patch tests at 5 and 20% in petrolatum were tested, with negative readings 15 minutes after removal of the strips and 24-48 hours later (7). Intradermal testing with AMX/CLV at 2-20 mg/ml concentrations was also negative (including delayed readings at 24, 48 and 72 hours). Positive and negative controls for SPT and ID were obtained using histamine (ALK-Abellò, Milan, Italy: 10 and 1 mg/mL) and normal saline. A positive Patch test was defined as when an infiltrate was

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detected. Skin testing was considered positive either when the ID was greater than 3 mm from the initial wheal or when an increase in the diameter of the initial wheal was associated with a flare, alongside a negative saline control result, within 15 minutes and at 24, 48, and 72 hours (6, 7).

Despite the negative results of *in vivo* testing, we advised his parents to absolutely avoid AMX/CLV. One year and a half later, he came to our Allergy Unit for a follow-up visit. At that time, we were able to perform the Lymphocyte transformation tests (LTT) and the induction of hapten-specific short-term T-cell lines (TCLs) (8).

PBMC were isolated using a Lymphoprep (Sentinel Diagnostic, Milan, Italy) density gradient followed by two washings with PBS at pH 7.2. Groups of 2×10^5 cells were cultured in triplicate in round-bottomed 96-well plates in complete medium plus 5% heat-inactivated autologous serum in a final volume of 0.2 ml for 5 days in the presence of 4 increasing doses of each β -lactam antibiotic (PEN 2.5-0.5-0.1-0.02 mg/ml, AMP 2.5-0.5-0.1-0.02 mg/ml, AMX 1-0.5-0.1-0.02 mg/ml, AMX/CLV 0.5-0.1-0.02-0.004 mg/ml) or medium alone as a negative control at 37°C in a 5% CO₂ humidified atmosphere. As positive controls, 2500 UI/ml recall antigen streptokinase (SK) and 1% vol/vol polyclonal activator phytohemagglutinin (PHA) were used. After 16 h pulsing with 0.5 μ Ci ³HTdR per well (PerkinElmer, Waltham, MA, USA), cultures were harvested, and radionuclide uptake was measured by scintillation counting. The stimulation index (SI) was calculated as the ratio between the radioactivity from stimulated cultures and that from unstimulated cultures.⁶ An SI \geq 3 was considered to be positive. TCLs were generated as previously described⁶ by culturing 1×10^6 PBMCs in the presence of single drugs (PEN 0.5 mg/ml, AMP 0.5 mg/ml, AMX 0.5 mg/ml and AMX/CLV 0.1 mg/ml) for 6 days. Activated T cells were then

expanded for a further 8 days by the addition of rIL-2 (25 U/ml) every three days. At the end of the culture period, T cell blasts were recovered, washed, counted, adjusted to 1×10^6 /ml and assessed for specificity by thymidine incorporation in the presence of the respective drug (PEN, AMP and AMX 0.5 mg/ml, AMX/CLV 0.1 mg/ml) and autologous irradiated PBMC (1:1 ratio) for 3 days. An SI ≥ 3 was considered to be positive.

LTT was negative for both AMX and AMX/CLV, whereas Penicillin (PEN)-specific T-cell lines could be obtained (data not shown). Six months later (2 years since the SJS episode), LTT, induction of hapten-specific TCLs and intracellular fluorescent labeling with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) to track proliferating T-cells were performed (8). First, 10×10^6 /ml PBMCs were labeled for 4 minutes with 5 μ M CFDA-SE (Invitrogen, Carlsbad, CA, USA) in PBS; then the cells were extensively washed, and 2×10^5 labeled cells were cultured in quadruplicate in round-bottomed 96-well plates in the presence of 3 scalar doses of each β -lactam (PEN 2.5-0.25-0.025 mg/ml, AMP 2.5-0.25-0.025 mg/ml, AMX 1-0.25-0.025 mg/ml, AMX/CLV 0.25-0.025-0.0025 mg/ml) at 37°. Medium was used as a negative control. SK and PHA were used as positive controls, as described above. On day 7, total rabbit IgG was added to each tube to saturate non-specific binding sites, and the cultures were then incubated with specific (PB anti-CD3, APC-Cy7 anti-CD8, APC anti-CD4) or isotype control mAbs (Becton Dickinson) for 20 minutes at 4°C. Cells were analyzed using a FACSCalibur cytofluorimeter (Becton Dickinson) and gated as CD3+CD4+, acquiring a total of 10^4 events for each sample. The results are expressed as the stimulation index, calculated as the ratio of the geometric Mean Fluorescence Intensity of proliferating cells between stimulated and unstimulated cultures. LTT remained negative and TCL induction confirmed the previous results (Fig. 1A). – CFDA-SE - assay towards PEN, Ampicillin (AMP), AMX, and AMX/CLV was positive on

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day 7 of incubation, with proliferation of specific T-cells of the CD4+ (Fig. 1B) and CD8+ (data not shown) phenotype. Clinical history and positive *in vitro* tests, together with positive serologic testing for EBV, indicated that our patient had experienced SJS due to AMX/CLV and EBV infection.

Patient #2 is a 16 year old Moroccan boy admitted to Anna Meyer Children hospital because of SJS.

Two days after finishing a six-day treatment with oral AMX/CLV, he developed bullous lesions at the upper arms, hard palate, gingivae and lips without fever. Itchy scabs and flaccid blisters filled with serous liquid were located on genital mucosae (Fig. 2). Multiple bilateral lymphadenopathies (< 1 cm diameter) were detected in the neck. Laboratory investigation showed the following: an increased white blood cell count ($13.5 \times 10^9/L$; normal range $4-10 \times 10^9/L$) without eosinophilia; increased erythrocyte sedimentation rate (ESR) (53 mg/dL), CRP (6.09 mg/dL) and fibrinogen (683 mg/dL, normal range: 200-400 mg/dl); elevated liver aspartate aminotransferase (98 IU/L, normal range 3-40 IU/L); and normal prothrombin time and renal function tests. All infectious screening tests, including viruses (HSV 1 and 2, HCV, HIV, Adenovirus, Enterovirus, EBV, CMV), bacteria (Streptococcus pyogenes, Salmonella typhi and paratyphi, Brucella, Staphylococcus aureus, Syphilis) and other pathogens (including Strongyloides spp., Toxocara canis, Mycobacterium tuberculosis, Fusobacterium necrophorum and Mycoplasma pneumonia) were negative. Blood cultures were also negative. Therefore, AMX/CLV-induced SJS was suspected. Intravenous corticosteroids and skin care were started with a favorable outcome. In one week, lesions had progressively improved, and the patient was discharged with advice to avoid AMX/CLV. Six months later, he was referred to the Allergy Unit of Anna Meyer Children's Hospital where he was investigated for AMX/CLV hypersensitivity. *In vivo* tests were performed according to the European

Network for Drug Allergy (6,7) recommendations in the following sequence: patch tests with AMX/CLV at 5 and 20% in petrolatum with readings at 24-48 hours and intradermal (ID) testing with 1/100 and 1/10 dilutions of the full-strength (200 mg/mL) AMX/CLV concentration at 20- to 30-minute intervals. ID tests were also read after 24, 48, and 72 hours for delayed T-cell-mediated reactions. *In vivo* test results were all negative. *In vitro* studies including LTT, induction of hapten-specific short-term TCLs and CFDA-SE assay were subsequently performed. The LTT results were negative (data not shown), AMP-specific TCLs proliferated against PEN (Fig. 1A) and, of note, – CFDA-SE - assay results were positive for AMX in CD4+ cells exclusively (Fig. 1B) (8).

SJS frequently requires hospitalization in childhood and is due to a Type IV hypersensitivity reaction towards drugs. The role of pathogens in SJS is still undefined. The clinical diagnosis of severe, delayed drug-hypersensitivity reactions is challenging, and the diagnostic value of current allergologic tests is not well defined. In our patients, according to ENDA (6), patch tests were used as part of the first line investigation. These are reported as safer than intradermal (ID) tests that can be avoided in cases of positive results. Patch tests are, however, less sensitive (albeit more specific) than delayed-reading ID tests (7), especially when performed alone. Sensitivity is reported as ranging between 10.8 and 37.5%, depending on the publication, and this variability depends on the vehicle used, the type of eruption and the involved drug. It has been reported that the negative predictive value of drug skin testing is approximately 90% (9). Our results stand in contrast with the literature (10), as *in vivo* testing was of no clinical utility for the diagnosis of SJS to AMX/CLV. Provocation tests, the gold standard of a negative allergologic work-up, are contraindicated in SJS/TEN because re-exposure can elicit a new episode with increased severity (6). However, there are currently no reliable laboratory tests to determine the offending drug in these cases. In this paper, we show

that the extension of the *in vitro* work-up to less conventional tests was extraordinarily useful for identifying the etiology of the severe reaction. AMX/CLV was the suspected drug for SJS in both patients, but the lymphocyte transformation test was negative even though all β -lactams were screened. It has been reported that the range of sensitivity for LTT is highly variable, but in particular, it is less than 30% in SJS/TEN (11). In our hands, the induction of short-term T cell lines (TCLs), and particularly cytofluorometric assessment of lymphocyte proliferation by CFDA-SE assay, resulted in the demonstration of circulating β -lactam-specific T lymphocyte (Fig. 1) with a specific response to the combination of AMX and CLV in patient #- 1. Unfortunately, the role of CLV in the sensitization cannot be exploited as CLV was not individually tested. Although skin biopsies were not performed, clinical manifestations were highly suggestive for SJS (Fig. 2) The role played by concurrent infections was unclear. In patient #1, cellular-mediated hypersensitivity towards AMX/CLV persisted two years after the reaction, and we suggest that EBV may have additionally contributed to the development of SJS. The hypothesis describing facilitation of drug sensitization by EBV is still controversial. However, the hypothesis that medications and pathogens such as EBV could contribute to drug reactions remains very popular, particularly given the high frequency of skin rashes resulting from aminopenicillin treatment during infectious mononucleosis (12). This hypothesis has never been supported by clear evidence in SJS/TEN, where a complete screening for infections, in particular for primary EBV infection (12), should be performed in all SJS cases without any imputable medication. Our results suggest that clinicians should be aware of the risk of AMX/CLV-induced SJS in children and that identification of the culprit drug may be aided by *in vitro* tests. These latter tests may be of importance for the growing patient to avoid recurrence, particularly given the concrete risk of a fatal outcome.

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Legend to the Figures

Figure 1 Specificity of hapten-specific T-cell lines and proliferation of CFDA-SE-labeled PBMCs towards beta-lactams.

Δ: Short term hapten-specific TCLs were generated from PBMCs of patients #1 and #2 in the presence of PEN, AMP, AMX or AMX/CLV as described in the text. Specificity was

assessed under MHC-restricted conditions against each β -lactam by ^3H thymidine incorporation. The results are expressed as stimulation index and the dotted line represents the cut-off of positivity ($\text{SI} \geq 3$).

B: CFDA-SE-labeled PBMCs from patients #1 and #2 were cultured in the presence of scalar concentrations (dose 1 > dose 2 > dose 3) of PEN, AMP, AMX or AMX/CLV or positive controls (SK and PHA) and proliferating CD3+CD4+ cells were analyzed by FACS as described. The results are expressed as stimulation index and the dotted line represents the cut-off of positivity ($\text{SI} \geq 3$). Representative plots of CD3+CD4+ gated cells for each patient are also shown (AMX dose 1).

Figure 2 Patient #2 “target” lesions and extensive mucous membrane involvement with flaccid blisters affecting the hands.

