

Original Article

Patients Taking Amoxicillin-Clavulanic Can Become Simultaneously Sensitized to Both Drugs

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What is already known about this topic? Patients taking a combination of amoxicillin and clavulanic acid can have selective reactions to either amoxicillin or clavulanic acid.

What does this article add to our knowledge? Reactions, immediate or nonimmediate, to both amoxicillin and clavulanic acid can appear in the same patient.

How does this study impact current management guidelines? We need to include different β -lactam determinants in the allergological workup, because the presence of hypersensitivity to one β -lactam does not refute the existence of allergy to others, even if cross-reactivity can be discounted.

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BACKGROUND: Patients can react to amoxicillin (AX) and clavulanic acid (CLV) taken in combination because of selective reactions to either drug. However, scant information exists concerning patients who react simultaneously to both compounds.

OBJECTIVE: To analyze the mechanisms involved in 4 patients who developed allergic reactions to AX-CLV administration (3 with immediate IgE-mediated reaction and 1 with nonimmediate T-cell-mediated reaction) and who responded specifically to both AX and CLV.

METHODS: Skin tests with benzylpenicillin (BP), AX, and CLV were done and, if necessary, drug provocation tests with BP/penicillin V, AX, and AX-CLV were carried out. In immediate reactors, serum specific IgE to benzylpenicilloyl and amoxicilloyl was determined by using the CAP-FEIA system (Pharmacia Diagnostics, Uppsala, Sweden), and basophil activation test to BP, AX, CLV, and AX-CLV was done. In nonimmediate reactors, immunohistochemistry of skin biopsy and analysis of dendritic cell maturation and T-cell-specific response to BP, AX, CLV, and AX-CLV at both acute and resolution phases of the reaction were conducted.

RESULTS: All patients with immediate reactions (N = 3) had good tolerance to BP and penicillin V. Two cases also had specific IgE to AX and all had a basophil activation test positive to AX, CLV, and AX-CLV. The patient with a nonimmediate reaction exhibited dendritic cell and T-lymphocyte responses specific to both AX and CLV. Finally, the analysis of the cells infiltrating the skin and peripheral blood during the acute phase indicated a T_H1 pattern response.

CONCLUSIONS: Our study provides evidence that reactions to both AX and CLV can appear in the same patient. © 2017 American Academy of Allergy, Asthma & Immunology (J Allergy Clin Immunol Pract 2017;■■:■-■)

Key words: Amoxicillin; Clavulanic acid; Allergy; IgE; T cells

Abbreviations used

AX- Amoxicillin
 BAT- Basophil activation test
 BL- β -lactam antibiotics
 BP- Benzylpenicillin
 BPO- Benzylpenicilloyl
 BPOL- Benzylpenicilloyl octa-L-lysine
 CLV- Clavulanic acid
 DC- Dendritic cell
 DHR- Drug hypersensitivity reaction
 DM- Minor determinant
 DPT- Drug provocation test
 PV- Penicillin V
 SI- Stimulation index
 ST- Skin test
 WTM- Wortmannin

β -Lactams (BLs) are the most frequently prescribed antibiotics to treat and prevent infections.¹ However, they are also the most common cause of drug hypersensitivity reactions (DHRs) mediated by a specific immunological mechanism.² These reactions are commonly classified as immediate or nonimmediate depending on their onset during treatment, with the former mainly induced by an IgE-mediated mechanism and typically occurring within 1 hour after the first dose of the last drug administration and the latter occurring at least 1 hour after the initial drug administration, but more typically multiple hours to days later and are often associated with a T-cell-dependent mechanism.³

Although all BLs can induce DHRs, some are more frequently involved than others, and this has changed over time and across countries, probably because of the changes in prescription patterns.² Benzylpenicillin (BP) was initially responsible for the largest number of DHRs; however, it has been gradually replaced as the most common culprit by amoxicillin (AX), which was originally prescribed alone. However, because of the increase in bacterial resistance,⁴ AX is now often prescribed alongside a β -lactamase inhibitor, such as clavulanic acid (CLV).²

Initial studies performed in animal models and with few human subjects indicated a lack of immunogenicity of CLV.⁵ However, since the first description of 2 patients in 1995,⁶ selective IgE-mediated reactions to CLV are increasingly being reported.⁷⁻⁹ In fact, in Spain, reactions to CLV have actually been reported as more common than for BP.⁸ Nevertheless, to our knowledge, few cases of nonimmediate reactions to CLV have been described,^{7,10} and these have been confirmed by *in vivo* tests without *in vitro* tests. In spite of the fact that AX and CLV are BLs, cross-reactivity between them has not been reported, which may be due to differences in their chemical structures and the determinants produced during their metabolism.⁹ Furthermore, it is unclear why some subjects treated with a combination of AX and CLV develop a selective allergy to CLV while tolerating AX, and vice versa.⁷⁻⁹ Further studies are needed to clarify this issue.

An accurate diagnosis of patients who suffer a DHR after AX-CLV intake is crucial to avoid the use of inappropriate alternative antibiotics, which are often more expensive and can potentially lead to more adverse effects.¹¹ In addition, patients selective to CLV can use all the other BLs, including AX, increasing the therapeutical arsenal of these patients. However, to our knowledge, there are no reports of patients with reactions to AX-CLV who respond to both drugs, either in *in vivo* or in

in vitro tests. In this article, we present for the first time the findings of 4 patients who developed DHRs after AX-CLV administration and who responded specifically to both AX and CLV. Out of these 4 patients, 3 had immediate IgE-mediated reactions and 1 a nonimmediate T-cell-mediated reaction.

METHODS**Patients and controls**

Three patients (P1, P2, and P3) who developed immediate reactions and 1 patient (P4) who developed a nonimmediate reaction to AX-CLV were included in the study. Two control groups of patients with immediate reactions after AX-CLV intake diagnosed by history and positive skin test (ST) results were included: group A consisted of 5 patients with selective reactions to AX (ie, they tolerated BP administration), and group B had 5 patients with selective reactions to CLV (ie, they tolerated BP and AX administration).

The study was conducted according to the Declaration of Helsinki. All patients and controls participating in the study gave their informed consent and protocols were approved by an institutional ethics committee (Ethical Committee of Málaga). Samples from patients were processed following standard procedures and frozen immediately after they were received. These were provided by the Málaga Regional Hospital Biobank, as part of Andalusian Public Health System Biobank.

Skin testing

Skin prick and intradermal tests were carried out as described elsewhere,¹² using 0.03 mL of solution prepared daily. The reagents were benzylpenicilloyl octa-L-lysine (BPOL) (0.04 mg/mL with a molar concentration of the benzylpenicilloyl [BPO] moiety of 8.64×10^{-5} mol), minor determinant (DM; 0.5 mg/mL with a molar concentration of sodium benzylpenilloate of 1.5×10^{-3} mol), AX (20 mg/mL), and CLV (20 mg/mL) (all from Diater-Ferrer, Madrid, Spain).^{9,12} In skin prick testing, a wheal larger than 3 mm surrounded by erythema, with a negative response to the control saline, was considered positive. In intradermal tests, the wheal area was marked initially and 20 minutes after testing, and an increase in diameter greater than 3 mm surrounded by erythema was considered positive. In those cases with a history of severe anaphylaxis, we performed the test with increasing dilutions before reaching the maximum concentration as described. In those cases with a history of nonimmediate reactions, a delayed reading was also done at 24, 48, and 72 hours. Patch testing was also performed in P4 using AX and CLV (5% wt/wt in petrolatum).¹³

Drug provocation test

A single-blind placebo-controlled drug provocation test (DPT) was carried out using BP, AX, and AX-CLV at regular intervals and with increasing doses under strict hospital surveillance.¹² In subjects with negative ST results to BPOL and DM, a DPT with BP was performed by parenteral route with increasing doses until reaching a 10^6 IU cumulative dose and this was followed by a 2-day therapeutic course with penicillin V (PV) of 500 mg/8 h. If the DPT with BP and PV and the ST result with AX were both negative, a DPT with AX was done using incremental doses with 30-minute time intervals until reaching cumulative doses of 500 mg. This was followed by a 2-day therapeutic course of AX of 500 mg/8 h. If good tolerance to AX was demonstrated and the ST result with CLV was negative, a DPT with AX-CLV was performed following the same procedure described earlier for the DPT with AX.

Determinations in patients with an immediate reaction

Specific IgE determination. Specific IgE was determined by using the CAP-FEIA system (Pharmacia Diagnostics) following the manufacturer's instructions.

Basophil activation by flow cytometry. A basophil activation test (BAT) was performed as described elsewhere,¹⁴ with some modifications. The whole blood was incubated with AX (Clamoxyl, GlaxoSmithKline, Madrid, Spain), AX-CLV (Augmentine, GlaxoSmithKline), and CLV (Sigma Aldrich, St Louis, Mo). The flow cytometry strategy is shown in Figure E1 in this article's Online Repository at www.jaci-inpractice.org.

Determinations in patients with a nonimmediate reaction

Immunohistochemistry. A 4-mm punch skin biopsy was done during the acute phase of the reaction and processed for hematoxylin-eosin and immunohistochemical staining as described elsewhere¹⁵ with the polyclonal antibodies to CD3, CD4, CD8 (Ventana Medical Systems, Tucson, Ariz), CD45RO, CD57, CD68, tryptase (Dako, Ely, UK), CXCR3 (Abcam, Cambridge, UK), and CLA (BD Pharmingen, San Diego, Calif).

Phenotyping by flow cytometry. The frequencies of different T-cell subpopulations (CD4, CD8, CD56, and regulatory T cells), cytokines (IFN- γ , TNF- α , IL-12, and IL-10), chemokines (CCR3, CCR4, CCR6, and CLA), and cytotoxic markers (granzyme and perforin) were assessed in PBMCs at acute (T1) and resolution phases of the reaction (ie, 60 days later; T2).¹⁶

Generation of monocyte-derived dendritic cells and dendritic cell maturation assays. Monocytes were purified from PBMCs and differentiated into immature dendritic cells (imDCs). For maturation analysis, imDCs were stimulated with AX, AX-CLV, and CLV at 250 and 50 μ g/mL, and the expression of CD80, CD86, and CD83 was assessed by flow cytometry.

Lymphocyte proliferation. Autologous lymphocytes were cocultured with drug-prematured DCs as antigen-presenting cells, and the percentage of CD3⁺, CD4⁺, and CD8⁺ proliferating cells was assessed by flow cytometry.

A detailed description of the *in vitro* methods can be found in this article's Online Repository at www.jaci-inpractice.org.

RESULTS

Patient 1 (P1) was a 48-year-old woman who, 2 months before performing the diagnostic workup, developed a generalized severe pruritus, hypotension, shortness of breath, difficulty in swallowing, hands edema, and loss of consciousness 5 minutes after the administration of a tablet of AX-CLV for an odontologic infection. This reaction required treatment with epinephrine. The patient did not take any other drug in the 24 hours before the development of the reaction. Three weeks before the reaction, she had also experienced mild generalized urticaria and angioedema 30 minutes after AX-CLV administration for an odontologic infection.

Patient 2 (P2) was a 43-year-old woman who, 3 months before the diagnostic workup, developed generalized urticaria and angioedema with intense pruritus 20 minutes after the intake of the first tablet of AX-CLV for an acute pharyngotonsillitis.

Three hours before the reaction, the patient also took ibuprofen, which was tolerated later at the allergological workup.

Patient 3 (P3) was a 44-year-old man who, 2 months before the diagnostic workup, developed generalized erythema, throat pruritus, and difficulty in swallowing 30 minutes after the intake of the first tablet of AX-CLV for an odontologic infection. This reaction required treatment with epinephrine. The patient took paracetamol 1 hour before the reaction, a drug that was tolerated afterward during the allergological workup.

Patient 4 (P4) was a 65-year-old man who, 4 months before the diagnostic workup, developed a generalized maculopapular exanthema and facial angioedema, with symptoms resolving completely in 1 month. His symptoms appeared 72 hours after starting AX-CLV treatment for bronchitis. Although diagnosis was performed 4 months after the reaction, skin biopsies and blood samples were taken from P4 both during the acute phase and 1 month after resolution.

In the 3 patients with immediate reactions (P1, P2, and P3), we performed STs, DPTs, and BATs as well as determined the specific IgE. Considering the *in vivo* studies, we found that P1 and P2 had positive ST results to both AX and CLV (P1 had a positive result to AX by skin prick test and to CLV by intradermal ST, and P2 had a positive result to AX and to CLV by intradermal ST [Figure 1, A]) and negative ST results to both BPOL and DM (data not shown in figure). In both patients, DPT with BP and PV showed good tolerance. Finally, P3 gave a positive intradermal ST result only to CLV, being negative to BPOL, DM, and AX, and the DPT showed good tolerance with BP and PV; however, after DPT with AX, the patient developed generalized pruritus and facial erythema 30 minutes after taking 125 mg of AX. The reaction was resolved in 45 minutes after the administration of intramuscular dexchlorfeniramine.

In vitro specific IgE using the CAP-FEIA system showed negative results to BPO for all the 3 patients and positive results to amoxicilloyl in P1 (2.06 kUI/L) and P2 (3.64 kUI/L), whereas P3 showed negative results.

The basophil activation was analyzed by flow cytometry in the 3 patients stimulating the cells with BP, AX, AX-CLV, and CLV. For all 3 patients, BAT was negative to BP and positive to AX (P1, 19.4; P2, 7.15; P3, 2.10), AX-CLV (P1, 18.57; P2, 5.32; P3, 3.10), and CLV (P1, 18.28; P2, 6.57; P3, 2.87). To validate these results, BAT was carried out in 2 control groups: group A (AX-selective patients) and group B (CLV-selective patients). We found that group A was negative to BP and CLV and positive to AX (mean, 7.97; SEM, 3.48) and AX-CLV (mean, 8.15; SEM, 3.62), and that group B was negative to BP and AX and positive to AX-CLV (mean, 8.68; SEM, 6.48) and CLV (mean, 8.458; SEM, 5.623) (Figure 2, A). To prove that this activation was IgE-mediated, BAT was carried out using wortmannin (WTM). The BAT (stimulation index [SI]) values were reduced for all 3 patients after WTM inhibition, showing a mean decrease of 79.6% for AX, 80.7% for AX-CLV, and 83.4% for CLV (Figure 2, B). The individual SI values of the 3 patients before and after WTM treatment are presented in Table 1.

In the patient with the nonimmediate reaction (P4), intradermal ST with delayed reading was negative for BPOL, DM, AX, and CLV and patch testing was negative with AX. However, he had a positive patch test to CLV (Figure 1, B). DPT was negative with BP and PV and it was positive with AX, developing a generalized exanthema and pruritus 8 hours after 250 mg of AX intake.

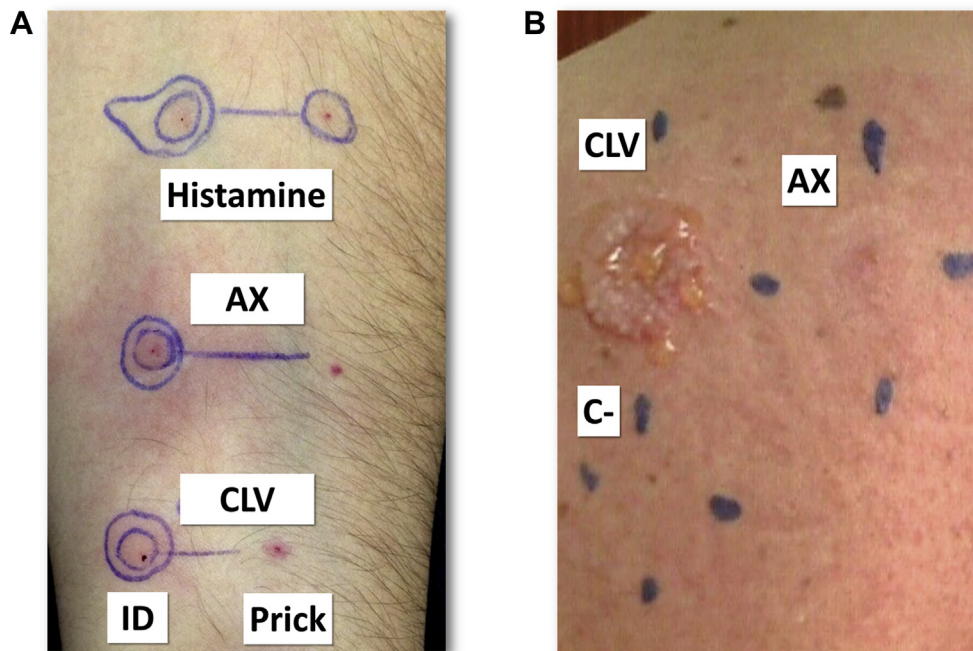


FIGURE 1. A, Intradermal (ID) positive results with AX and CLV in the immediate reading from P2. B, Patch positive result with CLV in the delayed reading from P4.

The immunohistochemical staining of the skin biopsy taken during the acute phase of the reaction showed a perivascular infiltrate, with similar levels of CD4 and CD8 cells and expression of the memory marker CD45RO. The high expression of CXCR3⁺ confirmed a T_H1 phenotype, and the absence of tryptase release refuted the existence of an IgE-mediated mechanism (Figure 3).

We also analyzed the peripheral blood cell subpopulations after the reaction from T1 (acute phase) to T2 (resolution phase), and we observed a decrease in the percentage of CD4, CD8, and regulatory T-cell subpopulations during the resolution phase (Figure 4, A and D). Moreover, CD3 population showed a decrease in the production of cytokines (IFN- γ , TNF- α , and IL-12) and slightly cytotoxic markers, and a reduced expression of cutaneous homing chemokine receptors (CLA, CCR3, CCR6, and CCR4) (Figure 4, B). Although the CD56 population did not display changes in cell frequencies (Figure 4, A), a decrease in perforin, granzyme, and, although at a lower level, IFN- γ expression by these cells was observed (Figure 4, C). We also observed a decrease in IL-10 production during the resolution phase (Figure 4, D).

We finally performed 2 different analyses to assess the cellular response to different drugs. For these, DCs were incubated with AX-CLV, AX, and CLV, and a positive maturation, measured by an increased expression of CD86 and CD83 markers, was observed (Figure 5, A). In the lymphocyte transformation test, we found a positive proliferation of CD3 and CD4 lymphocytes after incubation with the 3 drugs (Figure 5, B). No proliferative responses to any drug were observed in CD8 cells.

DISCUSSION

The combination of AX and CLV is widely used in Europe to control and prevent infections¹; however, nowadays it is also the

most frequent cause of allergic drug reactions.² Most patients suffering a hypersensitivity reaction after AX-CLV intake present selective sensitization to either AX or CLV.⁷⁻⁹ In fact, although they belong to the same family (BLs), the 2 drugs do not show cross-reactivity.⁹ This has important clinical implications for antibiotic prescription, particularly with regard to bacterial resistance, because CLV-allergic subjects can tolerate other BLs, including AX, but AX-allergic subjects can develop allergic reactions to other penicillins and some cephalosporins.¹⁷ It has been widely demonstrated that AX can induce both immediate and nonimmediate reactions,³ and although immediate reactions to CLV have also been described,⁶⁻⁹ there are only a few reports of nonimmediate reactions.^{7,10} Our study provides evidence that sensitization to both AX and CLV can appear in the same patient and potentially lead to both immediate and nonimmediate reactions.

In the patients with immediate reactions, the detection of specific IgE to AX in sera was very clear, with values of antibody concentration much higher than the positive reference ones. These values have helped to prove an IgE-mediated mechanism and the presence of AX-specific IgE together with the results observed in ST and DPT,¹² and the positive BAT results to AX allowed us to diagnose these patients as allergic to AX.¹⁴ However, regarding CLV, there is a lack of direct methods that allow us to determine the presence of specific IgE. Because of this, the positive results of this work for CLV in BAT constitute an indirect way to assess the presence of specific IgE to this drug.⁸ A final confirmation that the basophil activation was mediated via specific IgE was the decrease in CD63 expression observed after the stimulation with both haptens, when the cells were previously treated with WTM. This molecule is a potent inhibitor of phosphoinositide 3-kinase enzymes, which are part of the IgE signaling pathway,¹⁸ and has been shown to be one of the important kinases activated by the high-affinity IgE receptor, Fc ϵ RI, cross-linking.¹⁹

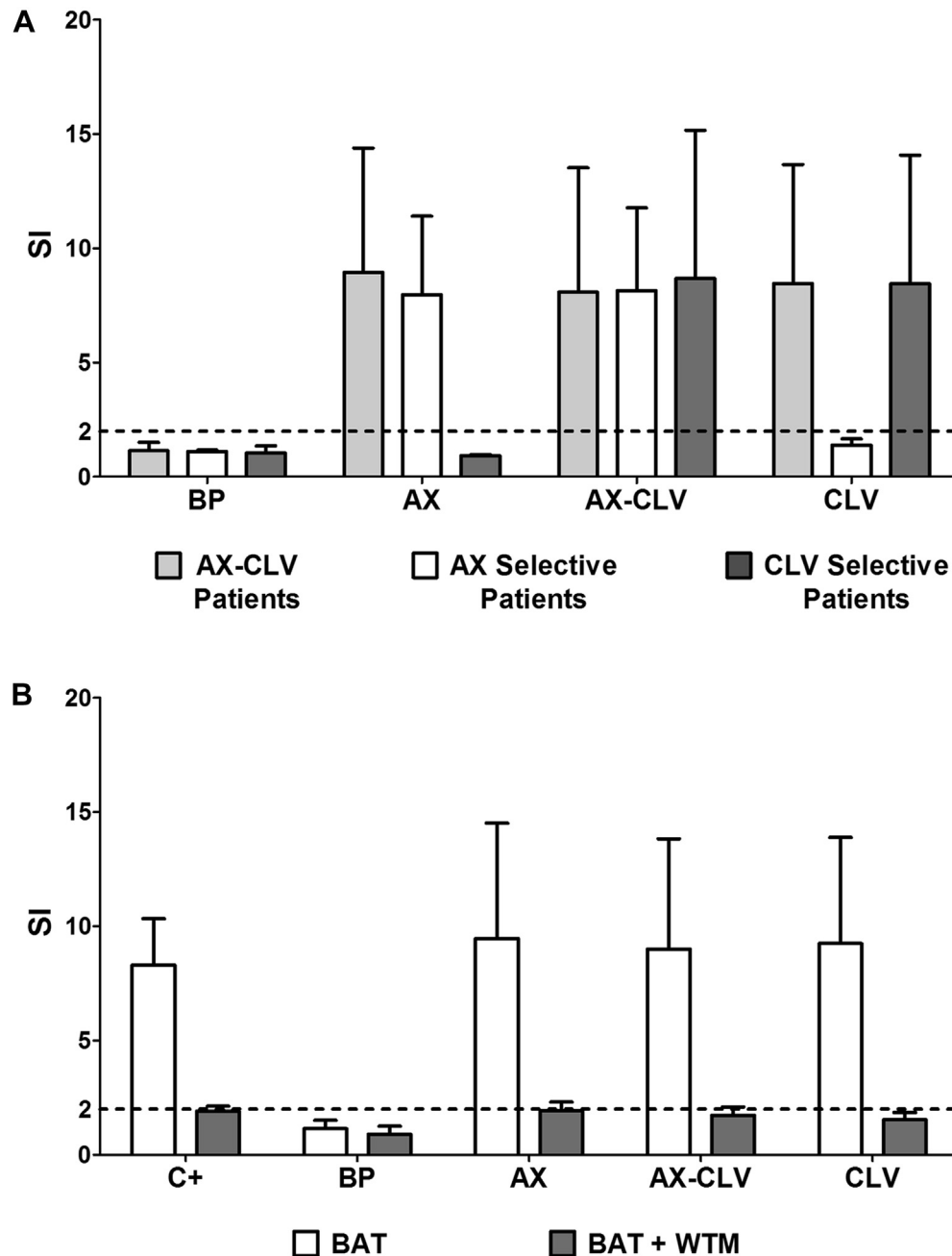


FIGURE 2. **A**, Bars represent mean and SEM of BAT with different BLs in P1, P2, and P3 and 2 control groups. **B**, Mean and SEM of BAT with different BLs in P1, P2, and P3 without and with WTM inhibition.

TABLE I. Results of BAT expressed as SI in P1, P2, and P3 before and after WTM treatment

Patient	AX		AX-CLV		CLV	
	BAT	BAT + WTM	BAT	BAT + WTM	BAT	BAT + WTM
P1	19.40	2.60	18.57	2.40	18.28	2.10
P2	7.15	1.90	5.32	1.20	6.57	1.50
P3	2.10	1.30	3.10	1.60	2.87	1.00

P1, P2, P3, Patient 1, 2, and 3.

In the single patient with a nonimmediate reaction, data obtained during the acute phase at both skin and peripheral blood helped us to confirm the involvement of T cells in the reaction. The immunohistochemical staining of the tissue obtained with the biopsy showed the presence of a T-cell infiltrate expressing CXCR3 responsible for the skin exanthema.^{15,20-22} Moreover, the possibility of an IgE-mediated response was refuted by the presence of tryptase inside the cells, excluding this mediator as the cause of the patient's symptoms.²³ The T-cell

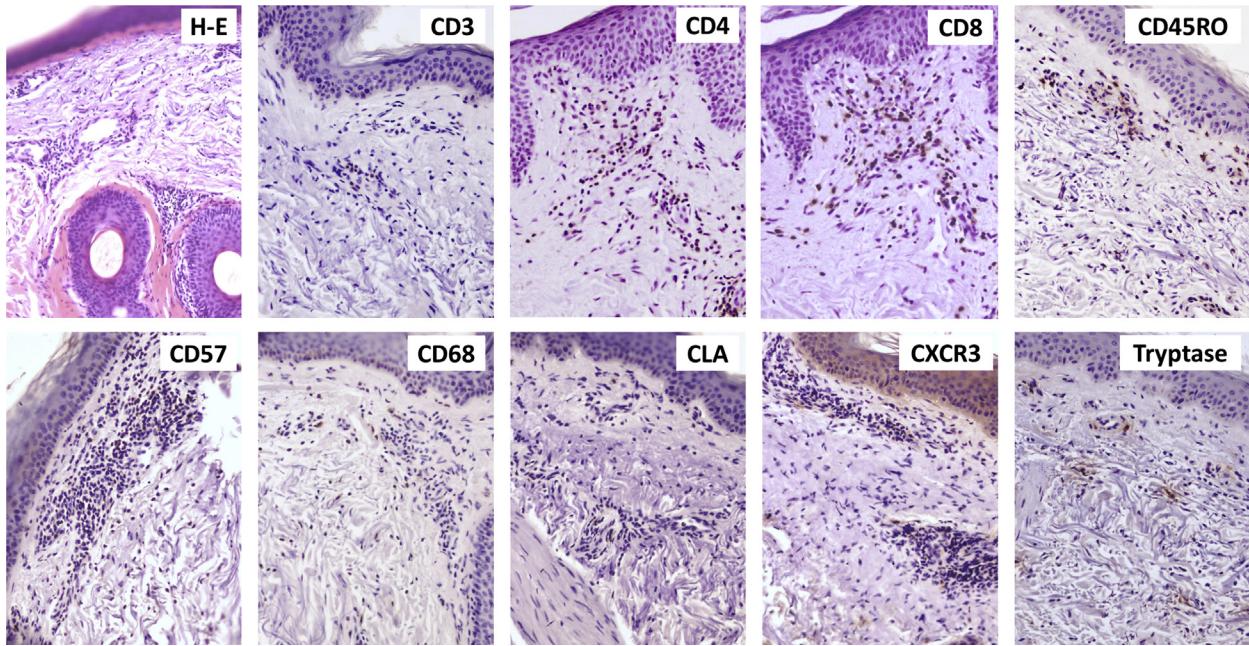


FIGURE 3. Immunohistochemical staining in the skin biopsy from P4 obtained during the acute phase. Hematoxylin-eosin (H-E) staining showed a dermic perivascular infiltrate of lymphocytes.

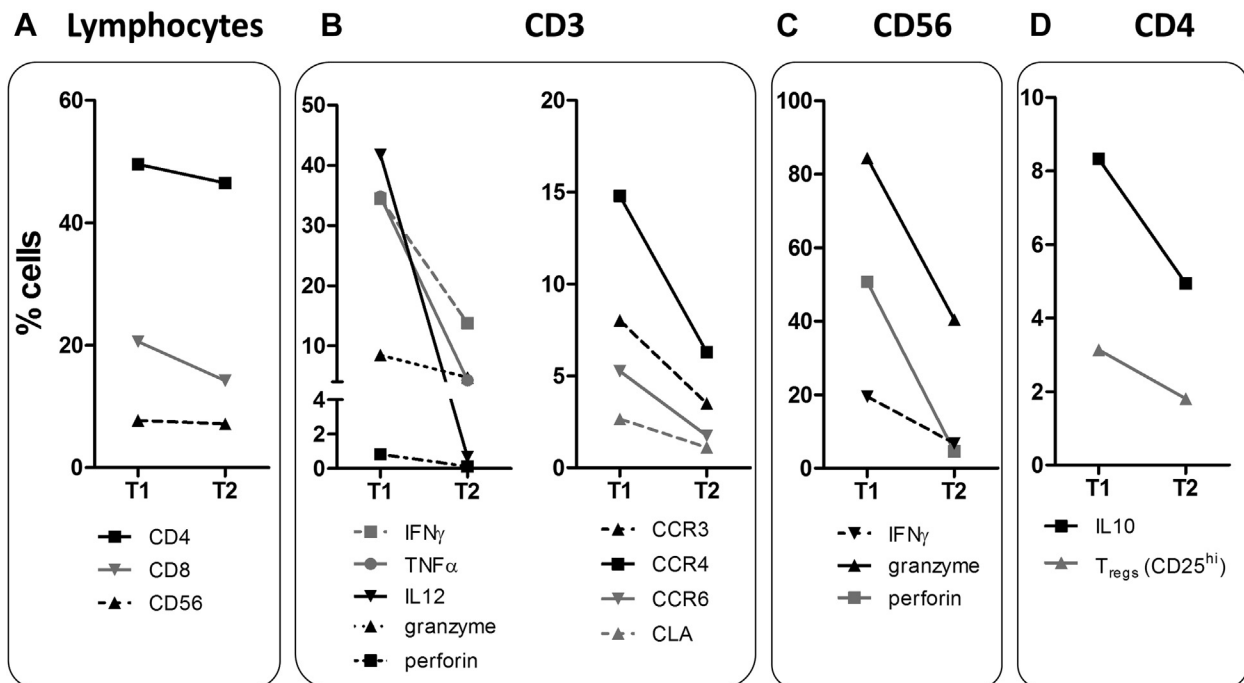


FIGURE 4. Percentage of different cell subpopulations and cytokine expression at T1 (acute phase) and T2 (resolution phase). **A**, Lymphocyte subpopulations. **B**, Different cytokines, cytotoxic markers, and homing receptors in CD3⁺ cells. **C**, Cytotoxic markers and IFN- γ expression in CD56⁺ cells. **D**, IL-10 expression in CD4⁺ cells and regulatory T-cell frequencies.

mechanism was confirmed in the analysis performed in PBMCs obtained during the acute phase and 2 months later in the resolution phase, with a pattern of cytokines and cytotoxic markers that have been usually associated with the development of a nonimmediate clinical entity with a T_H1 pattern such as the

maculopapular exanthema,^{15,20,22,24} corroborating the results found in the biopsy. The involvement of both AX and CLV in the development of the reaction was confirmed by the results obtained in the cellular cultures. Specific DC maturation was shown with both drugs, indicating a specific recognition of these

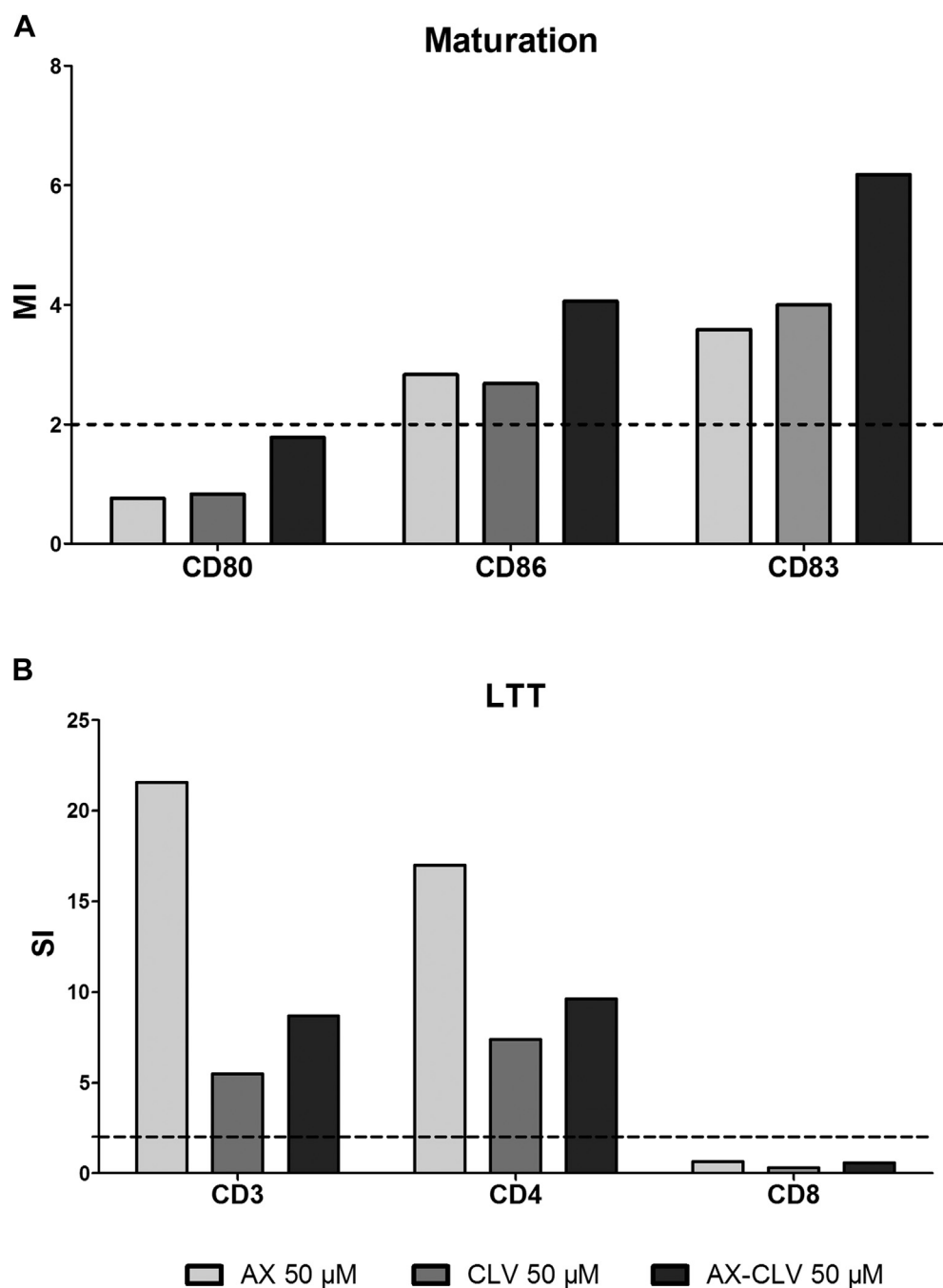


FIGURE 5. **A**, Bars represent maturation index of DCs with 50 μ M of AX (light-gray bars), CLV (dark-gray bars), and AX-CLV (black bars). **B**, Bars represent proliferation index of CD3⁺, CD4⁺, and CD8⁺ lymphocytes with 50 μ M of AX (light-gray bars), CLV (dark-gray bars), and AX-CLV (black bars). Dashed lines represent the cutoff point for considering positive values. *LTT*, lymphocyte transformation test.

haptens, as has been previously demonstrated in other studies with patients allergic to AX.²⁵⁻²⁷ Moreover, these drug-activated DCs were able to induce specific proliferation of T lymphocytes, specifically CD4 lymphocytes, demonstrating the T-cell recognition of both drugs.^{28,29}

Although simultaneous allergy to AX and CLV has been demonstrated in this study, it is not clear why most patients develop AX allergy while tolerating CLV, whereas for others the opposite occurs.⁷⁻⁹ Although a case of a patient suffering an

immediate reaction to CLV and a few days later a nonimmediate reaction to AX has been described,³⁰ to our knowledge this is the first study showing that the same type of reaction, IgE- or T-cell-mediated, can occur in the same patient with specific responses to both drugs. This finding has important implications for the diagnosis and the treatment of patients with allergic reactions to AX-CLV, demonstrating the need to include different BL determinants in the allergological workup, because the presence of hypersensitivity to 1 BL does not directly refute

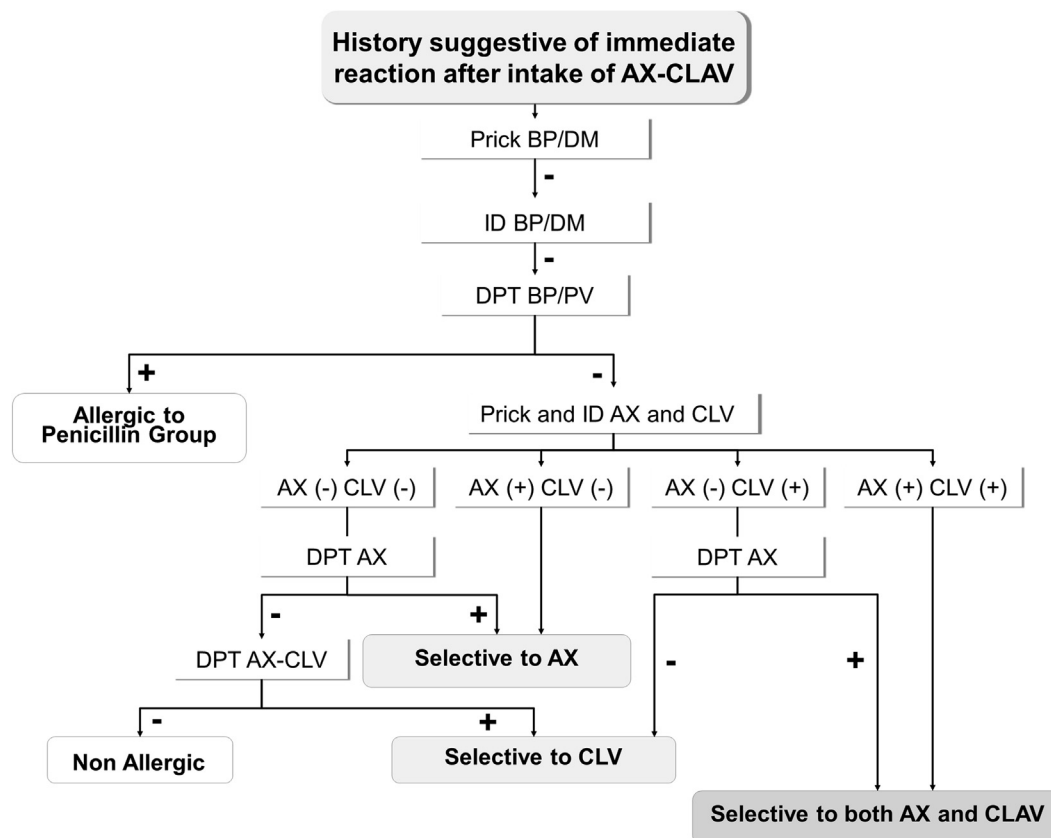


FIGURE 6. Flowchart of the suggested allergological workup to evaluate patients who developed a reaction after AX-CLV administration.

the existence of allergy to the other, even if cross-reactivity can be discounted. These results have important implications because patients reacting to AX-CLV must be diagnosed carefully, taking into account the possibility that they may react to AX or CLV, or even both.

Therefore, in patients who develop a reaction after AX-CLV administration and with positive ST result to CLV, it is necessary to administer AX alone to rule out a coexisting sensitization to both AX and CLV. Because CLV cannot be administered alone, it is not necessary to evaluate allergy to this compound in patients allergic to AX (Figure 6). Furthermore, care must be taken when choosing alternative antibiotics, and cross-reactivity with other BLs must be ruled out.

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APPENDIX**Methods****Determinations in patients with an immediate reaction**

Specific IgE determination. This was performed by using the CAP-FEIA system (Pharmacia Diagnostics), using c1 (BPO) and c6 (amoxicilloyl), following the manufacturer's instructions. The results were obtained by direct comparison with standards run in parallel, considering a value higher than 0.35 kUA/L as positive.^{E1}

Basophil activation by flow cytometry. BAT was performed as described elsewhere,^{E2} with some modifications. The whole blood was incubated with AX (Clamoxyl, GlaxoSmithKline) at 4, 2, and 0.4 mg/mL, AX-CLV (Augmentine, GlaxoSmithKline) at 2.5, 1.25, and 0.25 mg/mL, and CLV (Sigma Aldrich) at 1.25, 0.25, and 0.05 mg/mL. These concentrations were chosen on the basis of dose-response curves and cytotoxicity studies.^{E3,E4}

Cells were stained with mAbs, anti-CD63-FITC, -CD203c-PE, and -CCR3-APC (Caltag Laboratories, Burlingame, Calif), and acquired in a FACSCalibur flow cytometer (Becton-Dickinson Bioscience, San Jose, Calif) by acquiring at least 500 to 1000 basophils per sample, selected as CCR3⁺CD203c⁺ cells (see Figure E1). Results were analyzed with FlowJo software (Tree Star, Inc., Ashland, Ore) and activation expressed as SI, which was calculated as the ratio between the percentage of activated basophils (CCR3⁺CD203c⁺CD63⁺ cells) in samples stimulated with the different haptens and the unstimulated sample. The percentage of spontaneously activated basophils was required to be equal to or greater than 5% to calculate the SI, as described elsewhere.^{E4} We considered a positive result when SI was greater than 2.^{E2-E4} To confirm that basophil activation was IgE-mediated, we analyzed the WTM (an IgE pathway inhibitor) effect at 1 μ M.^{E5,E6}

Determinations in patients with a nonimmediate reaction

Immunohistochemistry. A 4-mm punch skin biopsy was done during the acute phase of the reaction and fixed in 10% formalin, routinely processed, and paraffin-embedded. Microtome sections (8- μ m) were processed for hematoxylin-eosin and immunohistochemical staining as described elsewhere^{E7} with polyclonal antibodies to CD3, CD4, CD8 (Ventana Medical Systems, Tucson, Ariz), CD45RO, CD57, CD68, tryptase (Dako), CXCR3 (Abcam), and CLA (BD Pharmingen). The binding of these primary antibodies (rabbit and goat IgG) was detected using a broad-spectrum super picture horseradish peroxidase polymer conjugate (Invitrogen, Thermo Fisher Scientific, Carlsbad, Calif). The visualization of the signal was carried out by using the 3-3'-diaminobenzidine substrate kit (Sigma Aldrich) in an optical microscope.

Phenotyping by flow cytometry. The frequencies of different T-cell subpopulations, cytokines, chemokines, and cytotoxic markers were assessed in PBMCs at acute phase (T1) and resolution phase of the reaction (ie, 60 days later; T2).^{E8} Surface and intracellular characterizations were carried out with specific monoclonal antibodies and proper buffers following the manufacturer's instructions. For intracellular staining, cells were fixed with the BD Cytofix/CytoPerm Fixation/Permeabilization solution kit (BD Biosciences, Milpitas, Calif). Cells were washed and immediately phenotyped with a BD FACSCanto II flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc). We assessed the frequencies of CD4, CD8, and CD56 subpopulations; the expression of cutaneous homing receptors (CCR3, CCR4, CCR6, and CLA), cytokines (IFN- γ , TNF- α , and IL-12), and cytotoxic markers (granzyme and perforin) in CD3⁺ cells; and the presence of regulatory T cells (CD4⁺CD127^{low}CD25^{hi}) and their expression of IL-10.

Generation of monocyte-derived DC and DC maturation assays. Monocytes were purified from PBMCs with anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) following the manufacturer's protocol. The CD14⁺ fraction was frozen for a later lymphocyte transformation test. CD14⁺ cells were differentiated into imDCs by incubating with rhGM-CSF (200 ng/mL) and rhIL-4 (100 ng/mL) (both from R&D Systems, Inc, Minneapolis, Minn) for 5 days at 37°C.^{E9}

For maturation analysis, imDCs (2×10^5 cells/well) were stimulated with AX, AX-CLV, and CLV at 250 and 50 μ g/mL. After 48 hours, the DCs from different conditions were incubated with mAbs (CD80, CD86, and CD83) (BD Pharmingen) and acquired in a FACSCanto II. Data were analyzed with FlowJo software and expressed as percentage of cells expressing the different markers.^{E9} Results were considered positive when the maturation index, calculated as the ratio between the percentages of drug-stimulated cells compared with nonstimulated cells, was higher than 2.

Lymphocyte proliferation. Carboxyfluorescein succinimidyl ester (CFSE) (Invitrogen, Thermo Fisher Scientific)-labeled autologous lymphocytes (1.5×10^5) were cocultured with 1.5×10^4 of drug-prematured DCs as antigen-presenting cells. After 7 days, the percentage of CD3⁺, CD4⁺, and CD8⁺ expressing CFSE^{low} was assessed by flow cytometry. The results were considered positive when the proliferation index was higher than 2. The proliferation index was calculated as follows:

$$PI = \frac{\%CFSE^{low}ly + DC_{stimulated} - \%CFSE^{low}ly + DC_{unstimulated}}{\%CFSE^{low}ly}$$

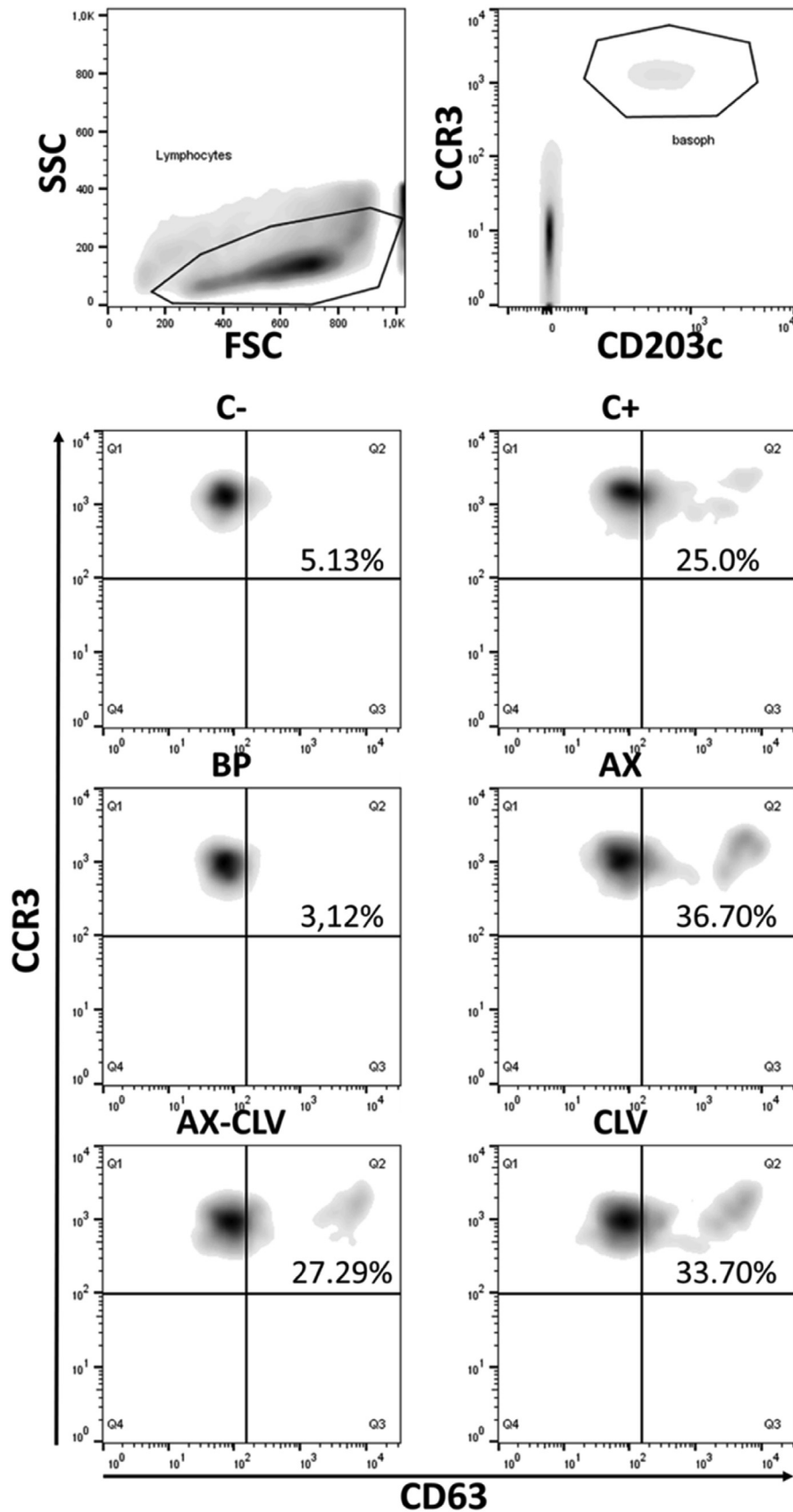


FIGURE E1. Representative strategy of basophil selection and determination of activation of P2. Basophils were selected as CCR3⁺CD203c⁺ cells from the lymphocyte population. Activation was measured as the percentage of CD63⁺ basophils.

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