

Drug- and Virus- or Bacteria-induced Exanthems: The Role of Immunohistochemical Staining for Cytokines in Differential Diagnosis

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Background: The differential clinical diagnosis between drug-induced exanthema (DIE) and virus- or bacteria-induced exanthema (VBIE) is frequently not easy because the serologic analysis for virus and bacteria and skin tests are not always exhaustive. In these cases, only the oral challenge test is nullifying.

Objectives: This study wants to identify 1 or more structural changes and/or cytokine markers that might be helpful in discriminating the etiology and the possible correlation with the clinical features, type of the involved drug, blood and skin eosinophilia, and time of skin biopsy.

Methods: Involved non-sun-exposed skin biopsy specimens were obtained from 36 patients with DIE and 30 patients with VBIE. Blood investigations, skin tests, and oral rechallenge tests were carried out in all subjects. The histopathologic features and the immunohistochemical expression of a cytokine panel [fatty acid synthase–ligand, granzyme B, interleukin (IL) 2, IL-4, IL-5, IL-10, IL-13, interferon γ , perforin, tumor necrosis factor α] were analyzed.

Conclusions: Finally, DIE and VBIE have distinct skin cytokine profile (IL-5 alone or in combination with granzyme B and perforin in DIEs was statistically more frequent than in VBIEs, mainly when skin biopsy was carried out within 2 days from clinical onset), which might be helpful in discriminating the etiology.

ERYTHEMATOUS MACULOPAPULAR eruptions are the most common cutaneous adverse drug reactions, but their frequency could be overestimated because the differential clinical diagnosis from viral and bacterial exanthemas is not always easy. However, the not-uniform morphology and size of lesions, their wine red color and symmetrical distribution, the acute onset on the trunk, the subsequent spread to the superior extremities and skin folds, the saving of the face, severe pruritus, and lesion resolution with desquamation are in favor of an exanthematous drug eruption, whereas enanthema, palms and soles involvement, fever, and lymphadenopathies are fundamental but not exclusive indicators of infectious etiology.¹ The diagnosis is also based on anamnestic data (ie, pharmacologic history, length of latency period), some laboratory tests (ie, liver enzymes, eosinophilia, activated lymphocytes, antiviral antibodies, and inflammatory

markers), and skin tests (patch test, intradermal test).¹ The histopathologic features of skin biopsies are believed less useful.^{2–4}

Although the pathomechanism of maculopapular drug rashes remains still incompletely understood, a delayed cell-mediated immune mechanism involving drug-specific T lymphocytes is thought to be important.^{5,6} Therefore, drug-induced exanthems have been included in type IV of Coombs and Gell classification⁷ and, recently, in subtypes IVb and IVc according to functional differences in the effector phase of T lymphocytes and in production pattern of cytokines and chemokines.^{8,9} In particular, the type IVb reaction is mainly due to the activity of T-helper type 2 cells, which secrete interleukin (IL) 4, IL-13, and IL-5. Type IVc reaction has been investigated mainly in severe cutaneous drug reactions, such as Stevens-Johnson syndrome and toxic epidermal necrolysis.¹⁰

It is possible, however, that also the type IVa and IVd reactions are involved in the drug-induced exanthemas. The first is a T_H1 immune reaction characterized by the releasing of large amounts of interferon (INF- γ), which activates monocytes and macrophages, induces production of antibody isotypes (IgG1, IgG3), and favors tissue inflammation and CD8 lymphocyte response by means of tumor necrosis factor α (TNF- α) and IL-12 production.⁹ Tuberculin skin test and contact dermatitis are clinical examples of this reaction. In the IVd reaction, the releasing of IL-8 and granulocyte macrophage colony-stimulating factor by T cells activates and recruits neutrophils and prevents their apoptosis,

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respectively. This type predominates in acute generalized exanthematous pustulosis.¹¹

Based on these data and the lack of studies of the in situ expression of cytokines in acute drug-induced rashes, we carried out histopathologic and immunohistochemical investigations on skin biopsies from patients having drug- or virus- and bacteria-induced maculopapular exanthemas to identify 1 or more structural changes and/or cytokine markers that might be helpful in discriminating the etiology. The secondary aim of this study was to evaluate whether in patients with drug reactions the cytokine profile is correlated with the clinical features, type of the involved drug, number of blood and skin eosinophils, and time of skin biopsy.

MATERIALS AND METHODS

Patient Selection

Over a period of 4 years (2006–2009), patients hospitalized in our department section with maculopapular eruptions were included in this study but only when they were older than 18 years and were not treated with systemic antihistamines and/or corticosteroids. All patients gave written informed consent for all diagnostic procedures and a possible scientific use of the obtained data. The study was reviewed and approved by the local ethical committee and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practices.

Subdivision of Patients

The enrolled patients were subdivided in 2 groups: one with drug-induced exanthemas (DIEs) and another with virus- or bacteria-induced exanthemas (VBIEs). The patients with positive skin test or a positive challenge test and negative viral and bacterial serology were included in the DIE group, whereas those with presence of specific IgM or seroconversion of IgG titers at the successive evaluations after the first were enclosed in the VBIE group.

Blood Sample Collection and Serological Analysis

Blood samples were taken from every patient at 3 different times: the first (T0) during the eruption acute phase (within 48–72 hours after the onset of rash), and the second (T1) and the third (T2) at intervals of 2 weeks to verify the increase of blood antibody levels. A peripheral blood cell count, routine blood chemistry investigations (including inflammatory markers, hepatic and renal profiles), and total serum IgE were performed at the admission moment; IgM and IgG antibodies for Epstein-Barr virus, cytomegalovirus, respiratory syncytial virus, herpes simplex virus type I and II, parvovirus B19, *Chlamydia trachomatis*, *Mycoplasma pneumoniae*, rubella virus, measles virus, and *Bordetella pertussis* were detected at T0, T1, and T2.

Skin Samples: Histologic and Immunohistochemical Evaluation

Skin biopsy specimens were obtained from involved skin of non-sun-exposed areas under local anesthesia, fixed in 4%

formalin, routinely processed, and embedded in paraffin. Four-micrometer-thick sections were cut and routinely stained with hematoxylin and eosin, Giemsa, azure A, and periodic acid-Schiff.

Other sections were stained with monoclonal or polyclonal antibodies (Table 1) using standardized automated procedures on a BOND MAX-automated slide stainer (Vision Biosystems, Mount Waverley, Australia). Immunostaining was performed using avidin-biotin complex/alkaline phosphatase method.

Both hematoxylin-eosin and immunohistochemically stained sections were evaluated in a blinded fashion with a light microscope (Nikon Y-THM, Tokyo, Japan) by 2 independent observers.

Cells with a strong cytoplasmic staining were considered positive cells. These were counted using an eyepiece square grid of 100 squares and a $\times 400$ magnification.

The sections presenting a number of stained cells per squared millimeter above the cutoff of each tested primary antibody were considered positive (Table 1). The cutoff normal range was calculated using the 95th percentile of distribution of stained cells per squared millimeter detected on paraffin sections of healthy skin of 75 subjects (both sexes; mean age, 57.8 years; range, 35–72 years). The same method was used to calculate the cutoff of the skin eosinophilia (skin section with eosinophilia, >2.11 eosinophils/mm²).

Statistical analysis was performed using the χ^2 test. A value equal or less than 0.05 was considered statistically significant.

Skin Tests and Oral Rechallenge Test

Patch test and intradermal test were performed 2 months after recovery in all patients who took drugs in the days before maculopapular eruption, irrespective of viral serology results. Patch test with suspected drugs 10% in petrolatum (FIRMA Diagent, Florence, Italy) was carried out on the upper back with Haye's Test Chambers (Haye's Service B.V., Alphen aan der Rijn, The Netherlands) on Soffix tape (Artsana, Grandate, Italy) and was removed after 2 days. The readings and scores were made as recommended by the

TABLE 1. The Type of Primary Antibody Tested and the Cutoff of Cells Per Squared Millimeter Calculated for Each Antibody in the Healthy Skin of 75 Subjects

Antibodies	Percentiles			
	25th	50th	75th	95th
FAS-L (200 μ g/mL)*	3.0	7.0	11.0	15.0
GrB (200 μ g/mL)*	1.0	1.0	3.0	10.0
IL-2 (500 μ g/mL)†	0.0	0.0	1.0	2.2
IL-4 (100 μ g/mL)*	0.0	0.0	1.0	11.2
IL-5 (500 μ g/mL)†	0.0	1.0	3.0	13.2
IL-10 (500 μ g/mL)†	3.0	7.0	9.0	13.0
IL-13 (200 μ g/mL)*	0.0	0.0	0.0	2.0
INF- γ (200 μ g/mL)*	1.0	3.0	6.0	9.2
Perforin (200 μ g/mL)‡	0.0	7.0	11.0	17.2
TNF- α (200 μ g/mL)*	1.0	5.0	10.0	14.0

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†R&D System, Mich.

‡Neo Markers, Fremont, Calif.

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Intradermal testing was performed only for β -lactams and when the patch test was negative.¹³

If skin tests were negative, an oral challenge test was carried out with increasing doses of the suspected drug in an environment with emergency equipment.

RESULTS

Clinical Features and Etiology of Maculopapular Eruptions

Drug-induced exanthemas were diagnosed in 36 patients (21 females, 15 males; mean age, 63.6 years; range, 18–92 years). The clinical feature of eruption was morbilliform in 18, scarlatiniform in 13, and erythema multiforme-like in 5. β -Lactams (14 patients: amoxicillin in 6, amoxicillin/clavulanic acid in 5, ceftriaxone in 2, and cephixime in 1), allopurinol (5 patients), and nonsteroidal antiinflammatory drugs (3 patients: nimesulide in 2, morniflumate in 1) were the most involved drugs. Ciprofloxacin, rifampicin, acetaminophen, acetazolamide, bactolise, carbamazepine, hydroxychloroquine, imatinib mesylate, methylprednisolone sodium succinate, myotan, phenobarbital, seleparin, terbinafine, and topotecan were involved in 1 case, respectively. Antibiotics (and in particular amoxicillin) and allopurinol were the most involved drugs.

Virus- or bacteria-induced exanthemas were diagnosed in 30 patients (16 males, 14 females; mean age, 59.8 years; range, 16–77 years). The clinical feature of eruption was morbilliform in 21, scarlatiniform in 8, and erythema multiforme-like in 1.

Histological Features

The histopathologic features of the maculopapular eruptions are reported in Table 2. There were not any significant qualitative and

quantitative differences of the epidermal, dermal, and vascular changes in the 2 examined patient groups (Fig. 1).

Cytokines Expression and Its Correlation With Etiology and Clinical Features

The immunohistochemical evaluation of the cutaneous cytokine expression in maculopapular eruptions (Table 3) showed a higher number of IL-5-, perforin-, and granzyme B (GrB)-positive cells in DIEs than in VBIEs (Fig. 2), but only the IL-5 expression was significantly increased (52.8%, $P < 0.05$). Fatty acid synthase-ligand (FAS-L), IL-10, and INF- γ , instead, were expressed in approximately 40% of subjects, independently from the etiology of exanthemas. The statistical analysis, however, revealed that the concomitant enhancement of IL-5, perforin, and GrB was significant in patients with DIE as compared with patients with VBIE (Table 4).

The relationship between cutaneous expression of cytokines and involved drugs is shown in Table 5. Of the 11 amoxicillin-induced exanthemas, 9 (81.8%) and 5 (45.5%) showed significant positivity for FAS-L ($P = 0.05$) and TNF- α ($P = 0.05$), respectively. On the contrary, the cytokine expression was not correlated with the clinical feature of rashes.

Interleukin 5, Perforin, and GrB Correlation With Eosinophilia and Time Skin Biopsy

An increased cutaneous expression of IL-5 was found in 6 of the 13 DIE patients with skin eosinophilia (46.1%) and in 12 of the 16 with blood eosinophilia (75.0%). In contrast, perforin and GrB were positive only in a few patients (in 3, respectively).

Finally, cutaneous expression of IL-5 alone or together with perforin and GrB seems to be correlated with the time of skin biopsy. The distribution of cytokines positivity gradually decreased with the increase of days between the onset of the exanthem and

TABLE 2. Histopathologic Skin Changes in 36 DIEs and in 30 VBIEs

Histologic Features	DIEs, n (%)	VBIEs, n (%)
Epidermis		
Hyperkeratosis	26 (72.2)	19 (63.3)
Orthokeratosis	23 (63.9)	18 (60.0)
Parakeratosis (focal)	3 (8.3)	1 (3.3)
Acanthosis (mild)	9 (25.0)	6 (20.0)
Spongiosis	22 (61.1)	19 (63.3)
Hydropic degeneration	25 (69.4)	20 (66.7)
Lymphocyte exocytosis	17 (47.2)	9 (30.0)
Neutrophil exocytosis	7 (19.4)	4 (13.3)
Vacuolar interface dermatitis	11 (30.5)	10 (33.3)
Papillary and reticular dermis		
Edema	32 (88.9)	26 (86.7)
Vascular dilatation	34 (94.4)	28 (93.3)
Plump endothelial cells	16 (44.4)	14 (46.7)
Eosinophils and neutrophils in the lumina of blood vessels	7 (19.4)	5 (16.7)
Perivascular infiltrate (lymphocytes, mast cells, occasional eosinophils)	33 (91.7)	28 (93.3)
Eosinophils	13 (36.1)	6 (20.0)

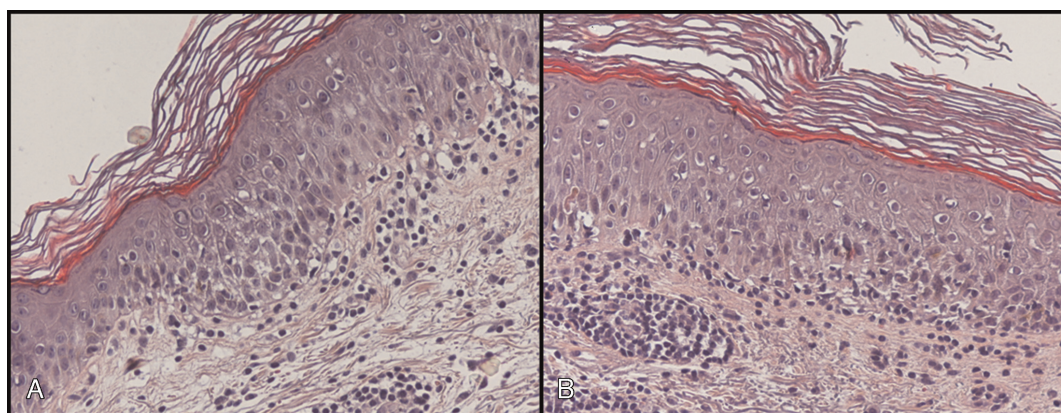


Figure 1.

the time of biopsy. In fact, the cutaneous expression of IL-5 was increased in 4 (40.0%) of 10 patients biopsied in the first or second day after the onset of the exanthem, in 4 (28.6%) of 14 biopsied between the third and fifth day, and in 2 (16.7%) of 12 biopsied after the fifth day. In addition, the concomitant enhancement of IL-5, perforin, and GrB decreased in the time [7/10 (70.0%) between the first or second day, 6/14 (42.9%) between the third and fifth day, and 6/12 (50.0%) after the fifth day].

DISCUSSION

The histopathologic changes in erythematous maculopapular drug eruptions, which may be synthesized as a superficial perivascular and spongiotic dermatitis, appear nonspecific. Epidermal (orthohyperkeratosis, hydropic degeneration of keratinocytes, more or less severe spongiosis but without blistering, and lymphocyte exocytosis) and papillary dermis changes (edema, vascular dilatation with intraluminal clusters of leukocytes, plump endothelial cells, and mild perivascular infiltrate composed of small lymphocytes, mast cells, and occasional eosinophils) are similar to those observed in VBIEs (Table 2). The hydropic degeneration of keratinocytes at the basal layer (the so-called vacuolar interface dermatitis) and small foci of spongiosis in the lower epidermis have been considered the 2 most typical histopathologic features of DIEs.^{14–16} Nevertheless, these alterations are not a constant finding and are not diagnostic because they were seen in 30.5% and 40% of VBIEs investigated by us and Wang et al,¹⁷ respectively. It is possible that the primary electron microscopic changes of DIEs (intercellular and intracellular edema and disruption of epidermal basal cells with some pyknotic nuclei) observed by Fellner and Prutkin¹⁸ in 4 patients with morbilliform eruption due to penicillin are present also in infectious exanthems.

According to our results, the currently emphasized presence of eosinophils in the skin of drug eruptions is not specific because it is only more frequent (Table 2) and perhaps more marked¹⁷ in DIEs than in VBIEs. In conclusion, there is not a single histopathologic feature that reliably differentiates a DIE from a VBIE (Fig. 1).

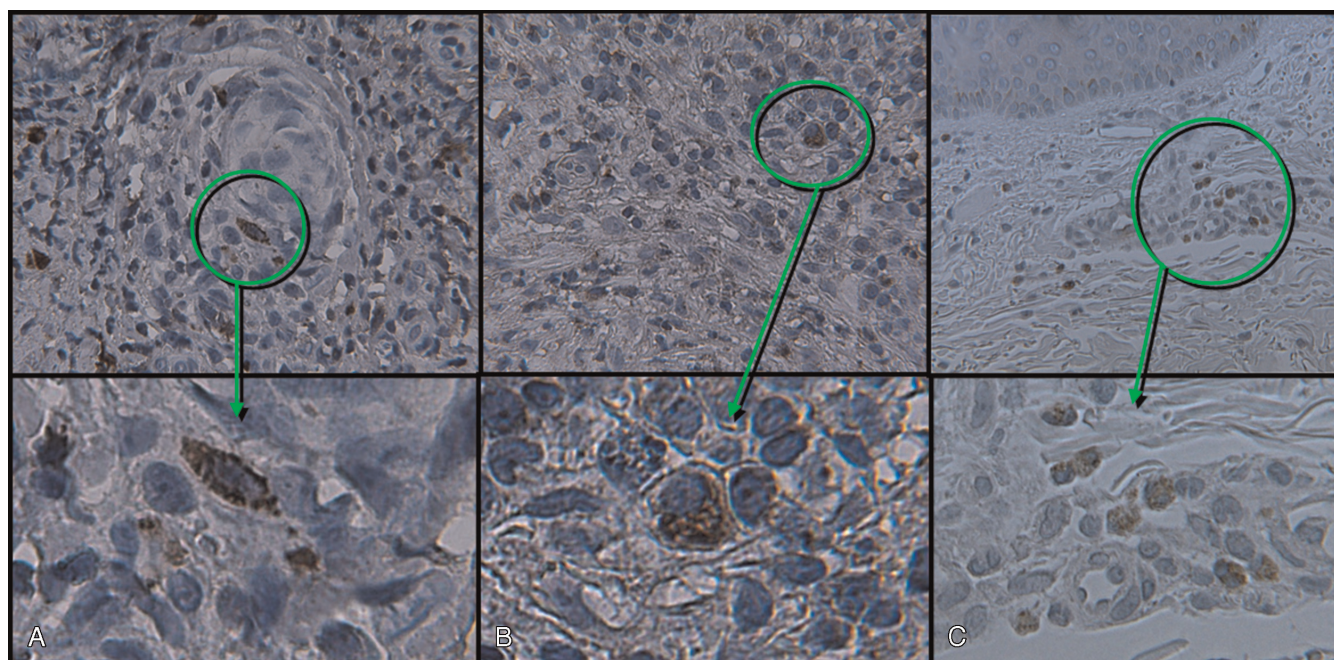
Many cytokines are up-regulated in DIEs,^{2,14,19} but our immunohistochemical investigations showed that only the increased IL-5 production and the concomitant enhancement of IL-5, perforin, and GrB expression are significantly increased and so seem to have value in the differential diagnosis with VBIEs (Fig. 2). The increased cutaneous expression of IL-5 in our patients with DIE was correlated with skin biopsy time, and it is associated with skin and blood eosinophilia in 46.1% and 75.0%, respectively. Because IL-5 is a key factor in regulating the growth, differentiation, and activation of eosinophils^{20,21} and because a significant correlation between IL-5 serum levels and eosinophil counts in the blood of drug-induced exanthema patients was found also by Hari et al,² it is possible that skin eosinophilia is more frequent because the standard histologic stainings (hematoxylin and eosin, Giemsa) are not able to reveal eosinophils after the release of their toxic granule proteins (such as eosinophilic cationic protein, major basic protein, and eosinophil peroxidase), which induce tissue damage. In fact, the most specific methods to identify these proteins are the direct immunofluorescence for major basic protein and the radioimmunoassay method for eosinophilic cationic protein.²²

The increased skin expression of perforin and GrB, but not of FAS-L, in drug maculopapular eruptions is in agreement with the

TABLE 3. Overexpression of Cytokines in the Skin of 36 DIEs and in 30 VBIEs

Cytokines	DIEs, n (%)	VBIEs, n (%)
FAS-L	16 (44.4)	13 (43.3)
GrB	18 (50.0)	12 (40.0)
IL-2	6 (16.7)	7 (23.3)
IL-4	11 (30.6)	9 (30.0)
IL-5	19 (52.8)*	7 (23.3)
IL-10	15 (41.7)	12 (40.0)
IL-13	10 (27.8)	8 (26.7)
INF- γ	14 (38.9)	12 (40.0)
Perforin	21 (58.3)	12 (40.0)
TNF- α	8 (26.7)	6 (16.7)

* $P < 0.05$.

**Figure 2.**

results of Posadas et al,²³ which investigated IL-2, IL-4, IFN- γ , TNF- α , perforin, GrB, and FAS-L messenger RNA expression levels in peripheral blood mononuclear cells by competitive reverse transcriptase–polymerase chain reaction. Perforin and GrB have an essential role in cell-mediated cytotoxic reactions^{24,25} and contribute to trigger cell death by forming pores in the target cell membrane and inducing degradation of their DNA.¹⁴ Because these cytokines have been found in both CD4 and CD8 T cells in the skin,^{14,26} our data provide further evidence for an involvement of these cytotoxic cells in the pathogenesis of DIEs. In particular, CD4⁺ T lymphocytes would be involved mainly in keratinocyte damage of drug maculopapular eruptions,²⁶ whereas CD8⁺ T cells, in more severe tissue destruction of bullous cutaneous reactions caused by drugs.^{27,28}

Fatty acid synthase–ligand, a type-II transmembrane protein that belongs to the TNF family, is responsible for keratinocyte death.^{29,30} Its serum titer has been found increased in toxic epidermal necrolysis and Stevens-Johnson syndrome,^{30,31} but elevated levels of FAS-L were detected also in serum and lesional skin of patients with DIEs by Stur et al.³² Nevertheless, we observed a FAS-L expression in suprabasal and basal keratinocytes in 16 (44.4%) of 36 subjects with DIEs and, in particular, in 9 (81.8%) of 11 patients who had developed a maculopapular eruption after intake of amoxicillin. No traces of FAS-L were detected in 5 patients who had received allopurinol. In contrast to Stur et al,³² who did not find elevated titers of soluble FAS-L in sera of patients with VBIEs, we observed an increased staining intensity of FAS-L in 13 (43.3%) of 30 skin biopsies carried out in subjects having acute infectious exanthems. Therefore, the overexpression of FAS-L seems

to represent a discriminating tool only for amoxicillin-induced rashes, but the data must be verified on larger sample of patients.

Determination of IFN- γ serum level has been considered informative in differentiating the etiology of skin eruptions because this cytokine was increased in patients with measles, rubella, and parvovirus infection, but not in those with drug allergy.² Our data are in disagreement with this assertion because IFN- γ was overexpressed in the skin of DIEs and VBIEs in the same percentage (38.9% vs 40.0%). It is possible that a T_H1 immune reaction, which is typical of contact dermatitis, is involved in some cases of drug maculopapular reactions.

Finally, the expression of other examined cytokines (IL-2, IL-4, IL-10, IL-13, and TNF- α) is similar in DIEs and VBIEs, and therefore, it is not discriminant in differential diagnosis.

In conclusion, despite clinical and histopathologic similarities of erythematous maculopapular eruptions, DIEs and VBIEs have

TABLE 4. The Trend of Associations Among IL-5, Perforin, and GrB Expression

Cytokines	DIEs, n (%)	VBIEs, n (%)
IL-5+ Perforin+ GrB+	10 (27.8)*	2 (6.7)
IL-5– Perforin– GrB–	7 (19.4)	11 (36.7)
IL-5+ Perforin+ GrB–	5 (13.9)	2 (6.7)
IL-5– Perforin+ GrB–	5 (13.9)	4 (13.3)
IL-5– Perforin– GrB+	4 (11.1)	4 (13.3)
IL-5+ Perforin– GrB+	3 (8.3)	2 (6.7)
IL-5+ Perforin– GrB–	1 (2.8)	1 (3.3)
IL-5– Perforin+ GrB+	1 (2.8)	4 (13.3)

* $P < 0.05$.

TABLE 5. Cutaneous Expression of Cytokines in 36 Exanthemas Induced by Amoxicillin in 11 Patients, Allopurinol in 5, and Other Drugs in 20

Cytokines	DIEs		
	Amoxicillin, n (%)	Allopurinol, n (%)	Other Drugs, n (%)
FAS-L	9 (81.8%)*	0*	7 (35.0)*
GrB	6 (54.5)	4 (80.0)	8 (40.0)
IL-2	2 (18.2)	1 (20.0)	3 (15.0)
IL-4	5 (45.5)	1 (20.0)	5 (25.0)
IL-5	8 (72.7)	1 (20.0)	10 (50.0)
IL-10	6 (54.5)	3 (60.0)	6 (30.0)
IL-13	5 (45.5)	1 (20.0)	4 (20.0)
INF- γ	7 (63.6)	3 (60.0)	4 (20.0)
Perforin	8 (72.7)	3 (60.0)	10 (50.0)
TNF- α	5 (45.5)†	1 (20.0)	2 (10.0)†

*FAS-L expression in DIEs: amoxicillin versus allopurinol, $P < 0.05$; versus other drugs, $P < 0.05$.

†TNF- α expression in DIEs: amoxicillin versus other drugs, $P < 0.05$.

distinct skin cytokine profiles that might be helpful in discriminating the etiology, especially during the acute phase of rashes. Our immunohistochemical investigations have demonstrated an elevated expression of IL-5, perforin, and GRB in DIEs, independently of clinical features and the type of the involved drug. The first cytokine may contribute to generate tissue and blood eosinophilia, whereas the other 2 may support vacuolar degeneration and death of keratinocytes, not only of the basal layer. The FAS-L overexpression, however, seems to be connected with the causative drug (amoxicillin).

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