*This is the peer reviewed version of the following article: [**Posadas SJ, Padial A, Torres MJ, Mayorga C, Leyva L, Sanchez E, Alvarez J, Romano A, Juarez C, Blanca M. Delayed reactions to drugs show levels of perforin, granzyme B, and Fas-L to be related to disease severity. J Allergy Clin Immunol. 2002 Jan;109(1):155-61. PMID:11799383**], which has been published in final form at [**doi: 10.1067/mai.2002.120563**].*

Title: Delayed reactions to drugs show levels of perforin, granzyme B, and Fas-L to be related to disease severity.

Authors: Sinforiano J. Posadas, PhD,[a] Antonia Padial, MD,[a] Maria J.Torres, PhD, MD,[b] Cristobalina Mayorga, PhD,[b] Laura Leyva, PhD, MD,[b] Elena Sanchez, MD,[a] Javier Alvarez, PhD,[a] Antonino Romano, MD,[c] Carlos Juarez, PhD, MD,[b] and Miguel Blanca, PhD, MD[a]

Affiliation

a. Allergy Service, "La Paz" Hospital, Madrid, Spain;

b. Research Unit for Allergy Diseases, "Carlos Haya" Hospital, Málaga, Spain;

c. IRCCS Oasi Maria S S, Troina, Italia.

Corresponding author and reprint requests:

Miguel Blanca, PhD, MD,

Servicio de Alergia,

Hospital Universitario La Paz,

Paseo de La Castellana 261,

28046 Madrid, Spain.

Support

This work was supported in part by FIS Grants 00/0838 and 98/0681.

Acknowledgments

We thank Ian Johnstone for his help with the English language version of this paper.

Abstract

Background Drugs can induce different immunologic reactions; T-cell mediated responses produce the most severe reactions. Although in vitro studies show that T cells recognize drugs or their metabolites and induce an effector cytotoxic response, direct in vivo evidence of involvement is lacking. T lymphocytes produce cytotoxic markers that are responsible for 2 major pathways to cell death: granule-mediated exocytosis (perforin and granzyme B) and Fas/FasL interaction.

Objective: The purpose of this investigation was to establish the role of proinflammatory TNF-α and cytotoxic markers in subjects with delayed responses to drugs.

Methods: We assessed expression levels by quantitative-competitive PCR of TNF-α, perforin, granzyme B, and FasL in mononuclear cells from peripheral blood and blister fluid from subjects with delayed reactions to drugs. Samples were obtained within 24 hours of the reaction and 30 days later. Fifteen patients were included and classified according to severity of the reaction, as follows: (A) maculopapular exanthema, (B) desquamative exanthema, (C) Stevens-Johnson syndrome, (D) toxic epidermal necrolysis.

Results: At the acute stage, there was a large increase in TNF-α (9-fold), perforin (6-fold), and GrB (7-fold) in patients in comparison with control subjects. FasL was expressed in PBMCs only in Stevens-Johnson syndrome and toxic epidermal necrolysis. A high association between cytotoxic markers and disease severity was seen ($P < .001$).

Conclusions. Our data show that TNF-α, perforin, GrB, and FasL are increased in the early stage of disease, suggesting that a cytotoxic mechanism might be taking part. These findings support the role of T cells in allergic drug reactions and provide further clues pertaining to therapeutic interventions.

Key words: *Drugs, allergy, perforin, Fas-L, granzyme B*

Introduction

Drug allergies are a significant problem in public health and represent an important element in iatrogenic pathology, being responsible for an underestimated number of deaths, frequent morbidity, and high cost.[1] Drugs are capable of inducing any of the 4 types of immunologic reactions described by Gell et al.[2] IgE-mediated allergy, the most common form, results from the interaction between the drug and specific IgE antibodies on the surfaces of the mast cells and basophils.[3] Drugs or their metabolites can also directly activate T lymphocytes, induce proliferation, and produce an effector response,[4,5] inducing delayed allergic reactions (type IV in the system of Gell et al[2]), such as maculopapular eruptions, contact dermatitis, toxic epidermal necrolysis (TEN), and Stevens-Johnson syndrome. Of these, bullous skin eruptions are the most severe.[6]

T lymphocytes in PBMCs from patients with delayed reactions to drugs express increased levels of the activation markers CD69, CD25, and DR, as well as of the cutaneous lymphocyte antigen, during the acute response; these levels are normalized when the clinical symptoms subside.[7-9] Immunohistochemical studies have demonstrated that in drug-induced diseases such as Stevens-Johnson syndrome and TEN, an infiltrate composed of CD8+ T cells is present in the epidermis and an infiltrate composed of CD4+ T cells is present in the dermis.[6,7]

Recent evidence shows that immunologic reactions to drugs follow the classical T_H1/T_H2 paradigm in vivo.[10] On the other hand, most *in vitro* studies have shown a heterogeneous profile of cytokine production, there being no agreement with the clinical diagnosis.[11,12]

Despite these considerations, the precise role of cytotoxic T cells in allergic drug reactions remains unclear. Two major pathways lead to apoptosis of target cells by cytotoxic T lymphocytes: granule-mediated exocytosis and Fas/FasL interaction.[13-15]

The presence of activated T cells expressing granzyme B (GrB) and perforin has been observed in various immunologic diseases, including acute heart, lung, and kidney allograft rejection.[16-18] This mechanism has also been seen in adverse cutaneous drug reactions.[19] When FasL binds to its cognate receptor Fas, apoptotic signals are delivered to the cells, and FasL expression is present at the transcription and protein level in T cells.[20,21]

In this investigation, a well-characterized group of subjects with delayed reactions to drugs was studied to determine the degree of involvement in PBMCs of the 2 cytotoxic pathways just mentioned, both during the acute response and after recovery from the disease. A competitive RT-PCR was used to quantitate the specific transcription of TNF-α, perforin, GrB, and FasL, markers that are thought to play a relevant role in the reactions studied.

MATERIAL AND METHODS

Patient selection

Fifteen subjects (mean age, 44.06 ± 10.24 years) who developed delayed responses after drug intake were included in the study. The criterion for diagnosis was the development of cutaneous lesions 24 hours or more after drug intake. The reactions were classified in 4 categories according to severity, as described previously[22]:

A. (+) Maculopapular exanthema, consisting of maculopapules of varying sizes on different parts of the body

B. (++) Desquamative maculopapular exanthema, consisting of maculopapules of varying sizes on different parts of the body followed by skin desquamation but without formation of blister

C. (+++) Stevens-Johnson syndrome, consisting of erythema and maculopapules followed by vesicles and bullae with mucosal involvement

D. (++++) TEN, consisting of widespread erythema with formation of bullae and detachment of the epidermis resembling scalding.

The clinical reactions are shown in Table I. Skin testing was done through use of intradermal or patch methods, and 8 cases proved to be positive (Table I). Intradermal testing was carried out only for β-lactams, as previously described.[23] In brief, 0.2 mL of a 20-mg/mL solution of the β-lactam involved (Table I) was injected in the volar surface of the forearm. Readings were made after 48 hours, and results were considered positive when an indurated area with a diameter greater than 5 mm was present. Patch testing, conducted according to international standard procedures,24 was undertaken in patients with phenytoin, amiodarone, sulfamethoxazole, allopurinol, or carbamazepine, all drugs being prepared 5% to 10% in petrolatum. The occlusion time was 48 hours, and readings were made as described previously.[25]

Challenge testing was done through oral administration of the culprit drug. Patients 1, 2, 4, 5, 6, 9, 10, 11, 12, 14, and 15 developed positive responses at the doses indicated in Table I. Patients 5, 6, 10, and 11 developed exanthema of the trunk 24 hours after challenge; this reached maximum expression at 72 hours. Patients 1 and 12 developed exanthema of the trunk, abdomen, and legs 48 hours after challenge, with a maximum expression after 4 days. Patients 9 and 15 had symptoms similar to those described for patients 1 and 12, but at 5 days their exanthema was followed by skin desquamation. Patients 2, 4, and 14 developed exanthema 48 hours after challenge but without the desquamation that occurred with the original reaction. In all instances, responses were induced with doses lower than the recommended therapeutic concentrations. Because of the risk of inducing severe reactions, 4 patients (3, 7, 8, and 13) were not challenged.

Controls

To assess whether the drug itself could induce any change in TNF-α and cytotoxic marker expression as a result of pharmacologic or other mechanisms, the same drugs that were involved in the reactions were administered to a group of 21 control subjects.Because a total of 7 different drugs were involved, 5 control subjects took amoxicillin, 5 ampicillin, 4 sulphametoxazole, and 4 phenytoin. None of the control subjects had a previous history of allergic drug reactions or presented with any cutaneous or immunologic disease, including atopy, at the moment of selection. The concentrations used for intradermal testing and patch testing were the same as those for the patients. Responses were negative in all of the tests.

The study was approved by our institutional review board, and informed consent for all diagnostic procedures was obtained from all patients and control subjects.

Sample collection

Two blood samples were obtained in each case, the first 24 hours after initiation of the reaction and the second 30 days later. In each sample, 20 mL of peripheral blood was obtained. Ten milliliters in EDTA were processed immediately for RNA extraction, cDNA synthesis, and competitive RT-PCR analysis. In patients 3, 7, 8, and 13, blisters were present during the course of the disease and 2 to 8 mL of blister fluid containing mononuclear cells (MNCs) was obtained (at the time when the first blood sample was obtained) for RNA extraction, cDNA synthesis, and competitive PCR analysis. Samples from the controls were taken at the same time.

PBMC isolation, RNA isolation, and cDNA synthesis

PBMCs and MNCs were isolated on Ficoll-Paque gradients (Pharmacia Biotech Inc, Piscataway, NJ). Total RNA was isolated from cells by the method of Chomczynski and Sacchi.[26] Total RNA was incubated for 15 minutes at 37°C with Dnase I (Boehringer Mannheim, Indianapolis, Ind). Rnase inhibitor was present during all enzymatic manipulations of RNA. Concentrations of total RNA were determined in a spectrophotometer (Ultrospec III, Pharmacia LKB, Uppsala, Sweden); the OD260/280 ratio was between 1.8 and 2.2 in all samples. The cDNA template for RT-PCR was synthesized from RNA by reverse transcription through use of a first-strand cDNA Synthesis Kit (CLONTECH Laboratories, Palo Alto, Calif). In every sample, the quantity of total RNA that was retrotranscribed was 1 μg per reaction. For comparison of TNF-α and cytotoxic markers, mRNA and cDNA concentrations were normalized to yield equivalent β-actin products (housekeeping gene). Results with this control amplification can be used to adjust the amount of the cDNA used in the PCR to use equivalent amounts of cDNA in each reaction.

PCR

PCR was performed with a mixture containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP, 0.4 micromolar 5′ and 3′ oligonucleotide specific primers, and 2 U of Taq polymerase (Boehringer Mannheim). Aliquots were then amplified by 35 cycles in a GeneAmp 9600 thermocycler (Perkin Elmer, Irvine, Calif). Each cycle consisted of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds, and extension at 72°C for 2 minutes for TNF-α. For perforin and FasL, we used 30 cycles, each consisting of denaturation at 94°C for 2 minutes, annealing at 55°C for 2 minutes, and extension at 72°C for 1 minute; for GrB, we used 30 cycles, each consisting of denaturation at 94°C for 2 minutes, annealing at 67°C for 1 minute, and extension at 72°C for 2 minutes. Aliquots of PCR products were electrophoresed in 1.8% agarose gels and visualized by means of ethidium bromide staining. The sequences of 5′ and 3′ TNF-α–specific primer pairs were provided by CLONTECH Laboratories.[27,28]

Competitive PCR for TNF-α and cytotoxic markers

To determine the number of molecules expressing TNF-α, perforin, GrB, and FasL, we used competitive PCR experiments. In this method, a DNA competitor containing the same primer template sequences as the target cDNA competes for primer binding and amplification. To quantitate the relative amounts of gene transcript present in various samples, the individual PCRs were performed by coamplifying the cDNA of interest with an internal PCR control. Differences as low as 2-fold in cytokine gene expression can be detected through use of this competitive template technique. The competitor DNA fragment (higher MW) was derived from the v-erb B gene (CLONTECH Laboratories) to which the primer templates had been added. The

amplification product of each synthetic competitor differs in size from the original cDNA product. Amplification of the competitor fragment as an internal standard in RT-PCR allows amplification of both the wild-type original DNA and the competitor fragment DNA in the same reaction through use of gene-specific primers and separation of the products on the basis of size. At similar concentrations of the PCR product, comparable band intensities will occur in the ethidium bromide gel electrophoresis. The target cDNA was adjusted to equal concentrations by competitive PCR between β-actin cDNA and competitor fragment.[29] Evaluation of competitive RT-PCR experiments was performed on digitalized agarose gels through use of image analysis software (1D Manager, TDI, Technology for Research and Diagnosis, Madrid, Spain). The detection limit of this method was determined through use of serial dilutions of a known concentration of positive control cDNA; after PCR optimal amplification, this was equivalent to 200 molecules of competitor fragment.

Statistical analysis

The values obtained by competitive PCR of TNF-α, perforin, GrB, and FasL determined in the assay were analyzed by means of the Pearson correlation coefficient. The differences in TNF-α and cytotoxic marker expression between the subjects with drug allergy and the controls were assessed through use of the nonparametric Mann-Whitney test. Data are presented in terms of means and SDs unless otherwise indicated.

To study the association between the cytokine and cytotoxic mediators and the severity of the disease, this was classified as mild (+; category A), intermediate (++; category B), severe (+++; category C), or extremely severe (++++; category D). These categories were related through use of the Spearman rank correlation coefficient.

RESULTS

Correlation between TNF-α and cytotoxic markers during the acute phase

To ascertain the relationship between TNF-α and the cytotoxic markers perforin and GrB, we used a Pearson correlation coefficient (data not shown). No significant correlation was observed between TNF-α and perforin or GrB.

Correlation between cytotoxic markers during the acute phase

Comparison of the relationship between the cytotoxic markers perforin and GrB at 24 hours gave a very high correlation, the *R* value being 0.904 ($P < .01$; Fig 1). Because FasL was expressed in only 4 patients (in categories C and D), no comparison was made between this and expression of perforin or GrB**.**

Analysis of TNF-α and cytotoxic marker expression levels between patients and controls during the acute and postreaction phases

Levels of TNF-α and cytotoxic markers were compared between subjects and controls (Fig 2). The TNF-α expression levels in patients and controls are presented in Fig 2, *A*. Although TNF-α expression was observed in the patients and most of the controls at the 24-hour measurement, there were very significant differences in the expression levels, the mean values being 9106 ± 1596 for the patients and 1158 ± 553 for the controls ($P < .006$). Comparison of mean values at the 30-day measurement showed no significant differences (1233 ± 271 for the patients and 1276 ± 467 for the controls).

Comparison of the cytotoxic markers perforin and GrB in the patients and controls is presented in Fig 2, *B* and *C*. The perforin levels showed significant differences between patients and controls at the 24-hour measurement, the mean values being 6260 ± 1914 for the patients and 1042 ± 87 for the controls ($P < .001$). Comparison of mean values at the 30-day measurement showed no significant differences (2273 ± 887 for the

patients and 1564 ± 584 for the controls). A similar pattern was observed when we looked at GrB expression in the patients and the controls; the mean values at the 24-hour measurement were 7453 ± 2553 for the patients and 1264 ± 1136 for the controls ($P < .006$).

Relationship of TNF-α and cytotoxic marker levels with disease severity during the acute phase

There were positive correlations between disease severity and levels of TNF-α expression ($R_s = 0.55$; $P < .05$), perforin ($R_s = 0.987$; $P < .01$), and GrB ($R_s = 0.911$; $P < .01$; Fig 3, *A-C*, respectively). No statistical comparison was made for FasL, because it was expressed in only 4 cases, corresponding to the patients with the most severe reactions—two each in categories C and D.

Results in MNCs from blister fluid

For the 4 patients with blisters (3, 7, 8, and 13), only perforin and GrB were found in PBMCs and MNCs (Table II). It can be seen that their expression in blister fluid MNCs was almost always at least twice the expression in PBMCs.

DISCUSSION

Although there have been many in vitro studies focusing on the lymphocyte recognition and effector response in immunologic reactions induced by drugs,[11,30] these do not always mimic the in vivo situation.[31] Thus, complementary in vivo studies might be necessary for a better understanding of the immunologic response. In this work we monitored disease activity and/or severity and the related immunologic effector response by measuring expression levels of the proinflammatory cytokine (TNF-α) and the cytotoxic markers perforin, GrB, and FasL in PBMCs and MNCs during delayed drug reactions. The advantage of working with PBMCs is the ease of obtaining samples at different time intervals both during the acute phase and after resolution of the disease, as well as the ease of obtaining adequate specimens in control subjects. Although obtaining parallel samples from peripheral blood and the skin is difficult to achieve, we were able to obtain samples from blister fluid and to measure the same cytotoxic markers as those measured in blood in the patients with blisters.

The data showed an increase in levels of TNF-α, GrB, and perforin during the acute phase of the reaction, the levels being normal after resolution of the reaction. This increase was not observed in the controls exposed to the same drugs, suggesting a T-cell mediated effector response. Our data confirm previous studies carried out in bullous skin reactions to drugs in which cytotoxic activity was shown,[32] and they provide additional evidence of direct involvement of perforin and GrB. The determination made at 30 days, after the symptoms had resolved, showed a decrease in their expression, such that their levels were similar to those in the control group. To our knowledge, this increase in the expression of cytotoxic makers during the acute phase of a delayed druginduced reaction has not been previously reported in immunologic reactions to drugs.

The tissue response during the acute phase of the reaction was studied in the skin blister fluid in the patients in whom bullae were present (categories C and D). The PCR study results showed a higher expression of perforin and GrB in blister fluid than in peripheral blood samples drawn simultaneously (Table II). These results indicate that in both PBMCs and MNCs, the activation pattern reflects the pathophysiologic mechanism occurring in the target tissue, as has been suggested in other diseases.[33,34]

Although perforin and GrB levels can be constitutively expressed in lymphocytes of healthy donors,[35,36] the increased values in the patients studied—the highest expression being seen in the most severe reactions—indicates that a cytotoxic mechanism was involved.[37] Direct participation of FasL has been shown in severe diseases induced by drugs, confirming the role of this cytotoxic mediator in disease activity.[38] There was also a significant association between TNF-α and disease severity in the study group. This cytokine has been reported to induce adhesion and activation of T cells and monocytes[39,40] and also to participate in apoptosis independently of perforin.[41] We therefore, believe that this is a relevant cytokine contributing to the skin lesions.[42] The association between disease severity and perforin or GrB was high and significant ($P < .001$), being preferentially expressed in the most severe cases. This difference between severe and mild drug reactions is relevant because it can be used for predicting and monitoring disease activity and/or severity. The observations made in PBMCs constitute the predominant tendency of a number of clones that contribute to the pathologic phenomena in patients with delayed reactions to drugs. However, although we did not determine which T-cell subpopulations were involved, it can be assumed that CD4 and CD8 take part in this response, as has been reported by others.[34] Despite the differences seen in disease severity and cutaneous reactions, there seems to be a common mechanism of T-cell activation, the clinical response being related to the

levels of the proinflammatory and cytotoxic markers studied. The reasons for these differences are being investigated by our group.

In conclusion, this is the first in vivo evidence suggesting the functional role of T cells with cytotoxic activity in delayed reactions to drugs, indicating that the clinical and laboratory findings are in agreement with the pathophysiologic mechanism involved in these diseases. Further studies of lymphocyte subpopulations, in addition to parallel monitoring of the skin lesion response, should be carried out for the sake of gaining a better understanding of the immunologic mechanisms involved, leading to improved therapeutic interventions.

REFERENCES

1. Bates DW, Cullen DJ, Laird N. Incidence of adverse drug events and potential adverse drug events. JAMA 1995;274:29-34.

2. Gell PGH, Coombs RRA, Lachmann PJ. Clinical aspects of immunology. 3rd ed. Oxford: Blackwell; 1975.

3. Amos H. Antigenic determinants of the penicillin molecule. In: Amos, H. E editors. Allergic drugs reactions. Oxford: Edward Arnold; 1976. p.64.

4. Hertl M, Merck HF. Lymphocyte activation in cutaneous drug reactions. J Invest Dermatol 1995;105:95S-98S.

5. Nyfeler B, Pichler WJ. The lymphocyte transformation test for the diagnosis of drug allergy: sensitivity and specificity. Clin Exp Allergy 1996;27:175-81.

6. Rojeau JC, Stern RS. Severe cutaneous adverse reactions to drugs. N Engl J Med 1994; 331:1272-85.

7. Leyva L, Torres MJ, Posadas S, et al. Anticonvulsantinduced toxic epidermal necrolysis. Monitoring of the immunologic response. J Allergy Clin Immunol 2000;105:157-65.

8. González FJ, Carvajal MJ, Leyva L, Blanca M. Expression of the cutaneous lymphocytes-associated antigen in circulating T cells in drug-allergic reactions. Int Arch Allergy Immunol 1997;113:345-7.

9. González FJ, Carvajal MJ, Del Pozo V, Blanca M. Erythema multiforme to phenobarbital: involvement of eosinophils and T cells expressing the skin homing receptor. J Allergy Clin Immunol 1997;100:135-7.

10. Posadas SJ, Leyva L, Torres MJ, et al. Subjects with allergic reactions to drugs show in vivo polarized patterns of cytokine expression depending on the chronology of the clinical reaction. J Allergy Clin Immunol 2000;106:769-76.

11. Brander C, Mauri-Helweg D, Rolli HP, Goldman M, Pichler WJ. Heterogeneous T cell response to beta-lactam-modified self structures in penicillin allergic individuals. J Immunol 1995;155:2670-8.

12. Mauri-Helweg D, Bettens F, Mauri, D, Brander C, Hunziker T, Pichler WJ. Activation of drug-specific CD4+ and CD8+ T cells in individuals allergic to sulfonamides, phenytoin and carbamazepine. J Immunol 1995;155:462-72.

13. Stenger S, Rosat JP, Bloom BR, Krensky AM, Modlin RL. Granulysin: a lethal weapon of cytolitic T cells. Immunol Today 1999;20:390-4.

14. Podack E, Young J, Cohn Z. Isolation and biochemical and functional characterization of perforin 1 from cytolytic T-cell granules. Proc Natl Acad Sci U S A 1985;2:8629-33.

15. Shi L, Kam CM, Powers JC, Aebersold R, Greenberg, AH. Purification of three cytotoxic lymphocyte granule serine proteases that induce apoptosis through distinct substrate and target cell interactions. J Exp Med 1992;176:1521-9.

16. Clement MV, Haddad P, Soulie A, et al. Perforin and granzyme B as markers for acute rejection in heart transplantation. Int Immunol 1991;3:1175-81.

17. Clement MV, Haddad P, Ring GH, Pruna, A, Sasportes M. Granzyme Bgene expression: a marker of human activated in vitro or in renal allografts. Hum Immunol 1990;28:159-66.

18. Strehlau J, Pavlakis M, Lipman M, et al. Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation. Proc Natl Acad Sci U S A 1997;94:695-700.

19. Yawalkar N, Egli F, Hari Y, et al. Infiltration of cytotoxic T cells in druginduced cutaneous eruptions. Clin Exp Allergy 2000;30:847-55.

20. Sloand EM, Young, NS, Kumar P, et al. Role of Fas ligand and receptor in the mechanism of T-cell depletion in acquired immunodeficiency syndrome: effect on CD4+ lymphocyte depletion and human immunodeficiency virus replication. Blood 1997;89:1357-63.

21. Spinozi, F, Fizzoti M, Agea E, et al. Defective expression of Fas messenger RNA and Fas receptor on pulmonary T cells from patients with asthma. Ann Intern Med 1998;128:363-9.

22. Elias PM, Fritsch PO. Erythema and toxic epidermal necrolysis. In Fitzpatric TB, Eisen AZ, Wolf K, Freedberg IM, Austen KI. Dermatology in general practice. 3rd ed. New York: McGraw-Hill; 1987. p. 555-67.

23. Terrados S, Blanca M, García J, et al. Non-immmediate reactions to betalactams, prevalence and the role of different pennicillins. Allergy 1995;50:563-7.

24. Fisher AA. Contact dermatitis. 3rd ed. Philadelphia: Lea & Febiger; 1988. p. 954.

25. Wilkinson DS, Fregert S, Magnuson B, et al. Terminology of contact dermatitis. Acta Derm Venereol 1970;50:287-92.

26. Chomczynski P, Sacchi N. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156-9.

27. Sugita Y, Miyamoto M, Koseki M, Ishii N,Nakajima H. Supression of tumour necrosis factor-(alpha) expression in leprosy skin lesions during treatment for leprosy. Br J Dermatol 1997;136:393-7.

28. Ikura S, Terao K, Matsuzaki I, Inoue-Murayama M, Murayama Y. U5 monoclonal antibody identifies a novel lymphocyte surface antigen preferentially expressed in human circulating natural killer cells with high cytotoxic activity. Immunology 1999; 96:485-90.

29. Siebert PD, Larrick J. Competitive PCR. Nature 1992;359:557-8.

30. Padovan E, Mauri-Helweg D, Pichler WJ, Weltzien HU. T cell recognition of penicillin G: structural features determining antigenic specificity. Eur J Immunol 1996;26:42-8.

31. Katagiri K, Itami S, Hatano Y, Takayasu S. Increased levels of IL-13 mRNA, but not IL-4, are found in vivo in peripheral blood mononuclear cells (PBMC) of patients with atopic dermatitis (AD). Clin Exp Immunol 1997;108:289-94.

32. Hertl M, Bohlen H, Jugert F, et al. Predominance of epidermal CD8+ T lymphocytes in bullous cutaneous reactions caused by b-lactam antibiotics. J Invest Dermatol 1993;101:794-9.

33. Kivisákk P, Stawiarz L, Matusevicius D, et al. High number of perforin mRNA expressing CSF cells in multiple sclerosis patients with gadolinium-enhancing brain MRI lesions. Acta Neurol Scand 1999;100:18-24.

34. Vasconcellos LM, Asher F, Schachter D, et al. Cytotoxic lymphocyte gene expression in peripheral blood leukocytes correlates with rejecting renal allografts. Transplantation 1998;66:562-6.

35. Spaeny-Dekking EHA, Hahna WL, Wolbink AM, et al. Extracellular garnzymes A and B in humans: detection of naïve species during CTL responses in vitro and in vivo. J Immunol 1998;160:3610-6.

36. Rukavina D, Laskarin G, Rubesa G, et al. Age related decline of perforin expression in human cytotoxic T lymphocytes and natural killer cells. Blood 1998;92:2410-20.

37. Inachi S, Mizutani H, Shimizu M. Epidermal apoptotic cell death in erythema multiforme and Steven-Johnson syndrome. Contribution of perforin-positive cell infiltration. Arch Dermatol 1997;133:845-9.

38. Viard I, Wehrli P, Bullani R, et al. Inhibition of toxic epidermal necrolysis by blockade of CD95 with human intravenous immunoglobulin. Science 1998;282:490-3.

39. Hershkoviz R, Cahalon L, Miron S, et al. TNF-αa associated with fibronectin enhances phorbol-miristate acetate or antigen-mediated Integrin-dependent adhesion of CD4+ T cells via protein tyrosine phosphorylation. J Immunol 1994;153:554-65.

40. Eierman D, Johnson CE, Haskill S. Human monocyte inflammatory mediator gene expression is selectively regulated by adherence substrates. J Immunol 1989;142:1970-6.

41. Spaner D, Raju K, Radvani L, Lin Y, Miller RG. A role for perforin in activation-induced cell death. J Immunol 1998;160:2655-64.

42. Schneider P, Holler N, Bodmer JL, et al. Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss liver toxicity. J Exp Med 1998;187:1205-13.

TABLE I. Clinical characteristics in subjects with delayed reactions.

Pat	Sex	Age (y)	Clinical entity	Body extension (%)	Severity*	Drug involved	Skin test†	Challenge‡
1	F	42	MPE	30	+	Ceftriaxone	+ID	+ (100 mg)
2	M	47	DMPE	30	++	Phenytoin	–P	+ (50 mg)
3	M	60	SJ	40	+++	Phenytoin	–P	ND
4	M	47	DMPE	30	++	Amiodarone	–P	+ (100 mg)
5	F	49	MPE	40	+	Amoxicillin	+ID	+ (250 mg)
6	M	32	MPE	60	+	Amoxicillin	+ID	+ (250 mg)
7	F	21	TEN	80	++++	Carbamazepine	–P	ND
8	M	53	SJ	30	+++	Phenytoin	–P	ND
9	M	37	DMPE	40	++	Ampicillin	+ID	+ (250 mg)
10	F	43	MPE	60	0	Amoxicillin	+ID	+ (250 mg)
11	M	35	MPE	30	+	Amoxicillin	+ID	+ (250 mg)
12	F	59	MPE	40	+	Amoxicillin	+ID	+ (250 mg)
13	M	50	TEN	50	++++	Allopurinol	–P	ND
14	F	40	DMPE	60	++	Sulfamethoxazole	–P	+ (200 mg)
15	M	46	DMPE	40	++	Amoxicillin	+ID	+ (250 mg)

F, Female; M, male; ID, intradermal; P, patch; MPE, maculopapular exanthema; DMPE, desquamative maculopapular exanthema; SJ, Stevens-Johnson; TEN, toxic epidermal necrolysis; ND, not done.

*Disease severity was scored in 4 categories.

†Plus sign (+) indicates that the patient exhibited a positive response after testing.

‡The dose used in the challenge is given in parentheses.

TABLE II. Cytotoxic marker expression levels in cells from peripheral blood and blisters

Patient nº	Perforin		Granzyme B	
	Blood	Blister	Blood	Blister
3	M	60	SJ	40
7	F	21	TEN	80
8	M	53	SJ	30
13	M	50	TEN	50

Results are expressed as numbers of molecules per microgram of total RNA.

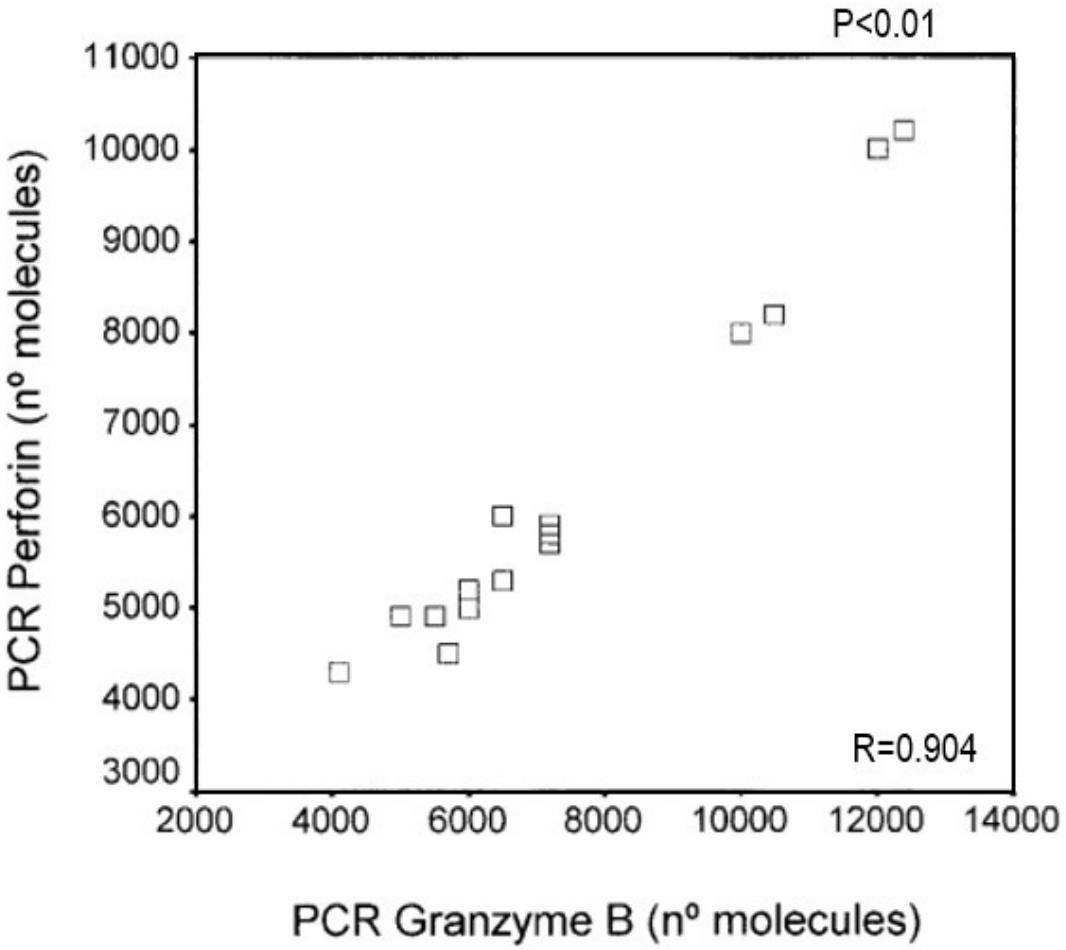

FIG 1. Comparison by Pearson correlation coefficient between the expression of perforin and the expression of GrB in the 15 cases studied.

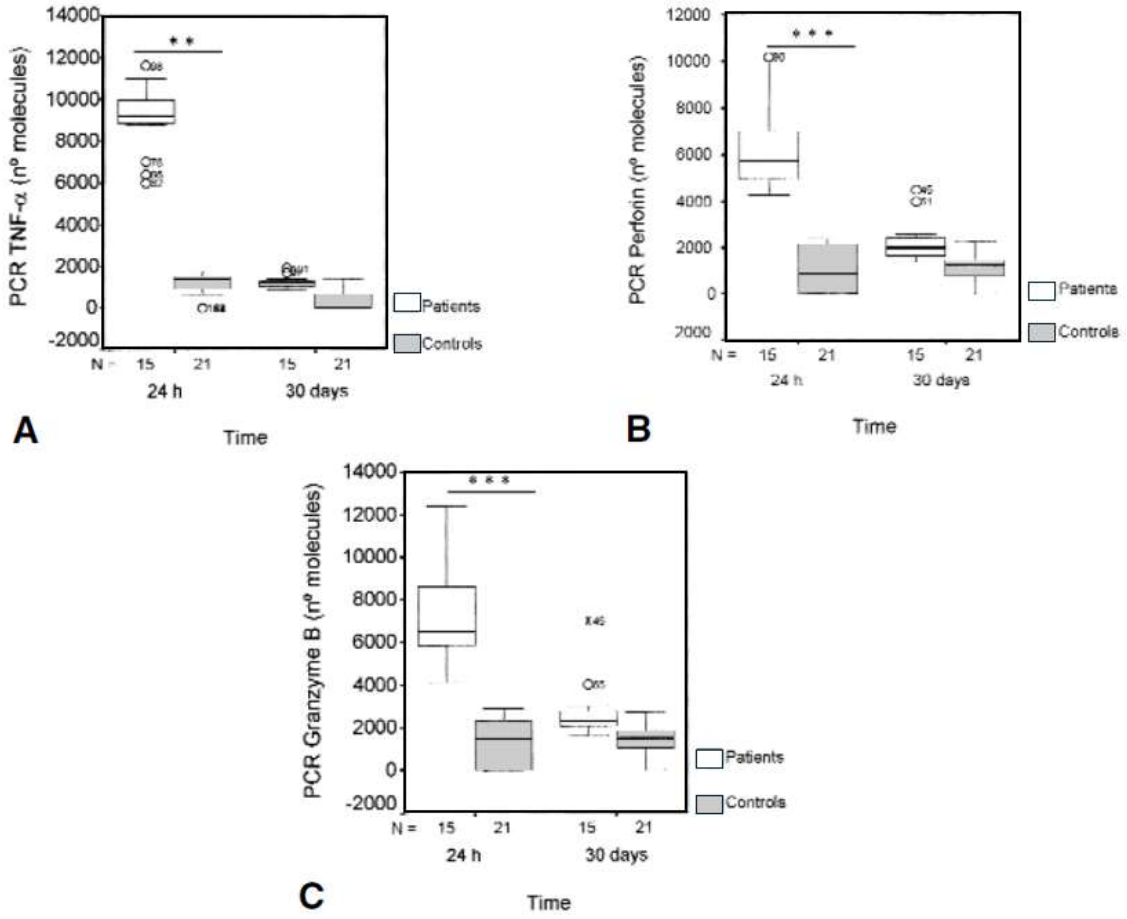

FIG 2. mRNA determination of TNF-α (A), perforin (B), and GrB (C) in 15 patients (white boxes) and 21 controls (gray boxes) 24 hours and 30 days after the initiation of the reaction. Data were analyzed by MannWhitney test. ***P < .001. **P < .006.

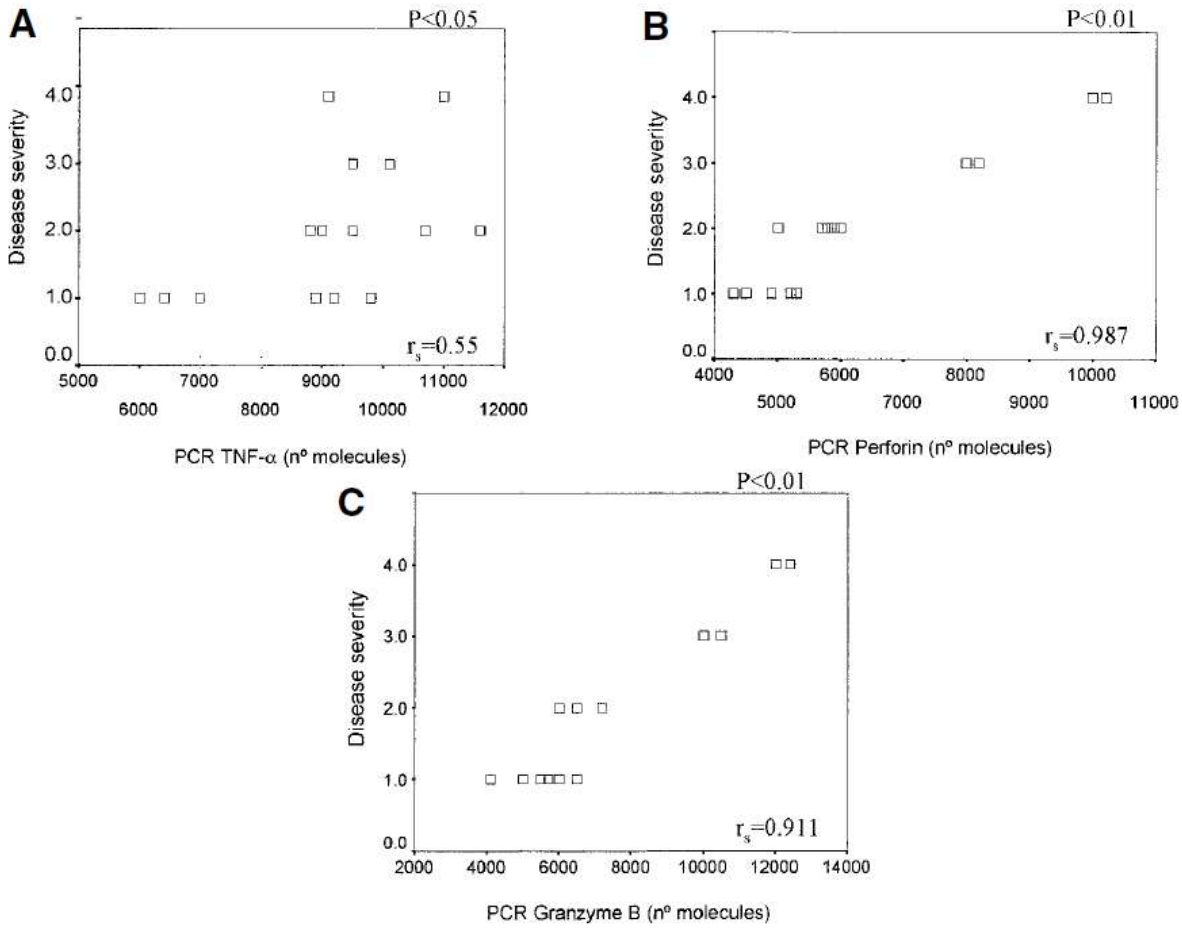

FIG 3. Comparison by Spearman correlation coefficient of disease severity and mRNA expression of TNF-α (A), perforin (B), and GrB (C). Disease severity was classified in 4 categories—A, B, C, and D (of increasing severity in that order), corresponding to 1, 2, 3, and 4 on the y axis. The association was significant in all cases—more so for the 2 cytotoxic markers perforin and GrB.