

Original article

Expression of the skin-homing receptor in peripheral blood lymphocytes from subjects with nonimmediate cutaneous allergic drug reactions

Background: In nonimmediate cutaneous reactions to drugs, the skin is the organ most frequently involved, and T cells may play a relevant role. T cells related to skin immune responses express the cutaneous lymphocyte-associated antigen (CLA), the skin-homing receptor.

Methods: We studied the expression of the CLA in peripheral blood T cells from nine subjects with exanthematous reactions induced by β -lactams (4), phenytoin (2), propyphenazone (1), spiramycin plus metronidazol (1), and captopril plus tiazide (1). The cutaneous symptoms appeared at least 6 h after drug intake. CLA expression was evaluated by flow cytometry at the time of the reaction (T1) and 1 month later (T2). HLA-DR activation marker expression was also evaluated at T1. In four patients, it was necessary to readminister the culprit drug to establish a causal relationship, and sequential estimation of the markers was performed. Two control groups were included: healthy controls and subjects exposed to the culprit drugs with good tolerance. Values were compared by nonparametric statistics.

Results: The expression of circulating CLA + T cells at T1 was increased compared to healthy controls (median = 20.4 vs 9.4) ($P < 0.001$), and the patients also expressed increased levels of HLA-DR (median = 3.8) ($P < 0.005$). Comparison between T1 and T2 (median = 11.2) also showed differences in levels of CLA + T cells ($P < 0.01$). The patients re-exposed to the culprit drug showed an increase followed by a decrease of circulating CLA + T cells ($P < 0.05$) and CLA + HLA-DR + ($P < 0.05$) paralleling the symptoms.

Conclusions: These data support the immunologic nature of delayed skin reactions to drugs, and suggest that these CLA + T cells parallel the disease evolution and may participate in the pathophysiologic mechanisms.

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One of the most frequent causes of adverse reactions to drugs is an allergic response in which different immunologic mechanisms may be involved. Among the clinical manifestations reported are anaphylaxis, urticaria, exanthema, desquamative lesions, and Steven-Johnson syndrome (1). The appearance of symptoms may vary from minutes to days or even weeks after drug intake, depending on the mechanisms involved. In general, the anaphylactic reactions occurring immediately after drug intake are due to activation of mast cells and basophils by specific IgE antibodies that recognize the drug as a hapten (2). However, the great majority of drug reactions with skin involvement occur hours or days after drug intake, and IgE mechanisms do not seem to take part (3). Although the mechanisms that induce delayed cutaneous reac-

tions to drugs are complex, the participation of activated T cells has been suggested (4, 5). In some drug-allergic patients, peripheral lymphocytes proliferate in the presence of the specific drug (6), and activated T cells are the primary component of the skin inflammatory infiltrate in these reactions (7).

T cells related to the cutaneous immune response can be identified because most skin-infiltrating T cells express the cutaneous lymphocyte-associated antigen (CLA) (8). Moreover, 10–15% of circulating CD3+ cells are CLA+, and CLA+ T cells bind and migrate through endothelial cells by adhesive interaction with E-selectin (9, 10). Leukocyte extravasation into tissue is a multistep process (11), and the molecules involved in the migration of CLA+ T cells include VCAM-1, ICAM-1, and the interleukin-8 receptor B (12, 13).

However, the exact extravasation mechanism has not been completely characterized (14, 15). A recent study using a hu-SCID mouse grafted with human skin and human T cells provided direct evidence of the role of the CLA receptor in directing lymphocyte traffic into the skin (16). Moreover, the response to skin-associated antigens has been shown to be restricted to the circulating CLA+ T-cell population. In atopic dermatitis, CLA+ T cells preferentially respond to extract of house-dust mite; in contact dermatitis, the skin-homing population responds to nickel (17). Since these and other reports have underlined the importance of the CLA antigen as the human skin-homing receptor, we studied the CLA+ T-cell population in patients affected by delayed cutaneous allergic reactions to drugs. Our results indicate the involvement of CLA+ T cells in this type of reaction.

Material and methods

Study population

Subjects attending the emergency department of Carlos Haya Hospital (Spain) with a generalized cutaneous reaction after drug intake were evaluated. For patients to be included in the study, symptoms had to appear at least 6 h after drug intake. In all the patients studied, symptoms disappeared over a period of time varying from 5 days to 2 weeks after onset of the reaction. We excluded from the study those patients with an IgE-mediated reaction (patients having urticaria/angioedema or anaphylaxis/anaphylactic shock within a period of 30 min after drug intake, those with immediate positive skin tests, or those with positive RAST), viral infections (positive viral serology), or disorders with organ involvement such as hepatitis or nephritis.

Two control groups were included. One group of 10 healthy unexposed controls and the other of eight subjects undergoing standardized treatment with the same drugs as those eliciting the reaction in the patients: three taking amoxicillin (one for 5 days and two for 7 days), one penicillin V for 4 days, two phenytoin for more than 1 month, one propyphenazone for 1 day, and one spiramycin plus metronidazole for 7 days. All controls were sex- and age-matched with the patient group and had normal skin at the moment they were evaluated for the study, and none had a history of allergy to drugs. Informed consent was obtained from all patients and controls before entry into the study.

Patients were evaluated at two different times: at the time of the reaction (T1) and 1 month later (T2) when they were free of symptoms. Controls were also evaluated at two different times with an interval of 1 month.

Monoclonal antibodies (mAbs)

The HECA-452 (a rat IgM) mAb against CLA was kindly provided by Picker (18). The anti-CD3 conjugated to FITC and PE was obtained from Ortho Diagnostic Systems (Raritan, NJ, USA), and anti-HLA-DR conjugated to PE-Cy5 from Immunotech (Marseille, France). Anti-L-selectin conjugated to FITC was obtained from Serotec (Oxford, UK). IgG1 conjugated to FITC (Ortho Diagnostic Systems) and IgG1 conjugated to PE-Cy5 (Immunotech) were used as isotype controls. Goat anti-rat IgM conjugated to PE and rat IgM used as isotype control for the HECA-452 were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

Cell staining and flow cytometry analysis

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll density gradient (Nycomed As, Oslo, Norway), washed, and then processed for immunofluorescence staining, as previously described (19). Briefly, 5×10^5 cells were sequentially stained with HECA-452, goat anti-rat PE-conjugated, CD3-FITC, and HLA-DR-PE-Cy5. All steps were performed at 4°C. Stained cells were fixed in 1% paraformaldehyde in PBS and stored at 4°C until analysis.

Five-parameter analysis was performed in an Ortho Cyturon Absolute flow cytometer (Ortho Diagnostic Systems) equipped with a 488-nm argon laser, with FITC, PE, and PE-Cy5 as the three fluorescence parameters. FITC emissions were measured with a 510/550 band pass filter, PE with one of 550/560, and PE-Cy5 with one of >600. Cells were analyzed by Ortho Immunocount System Software. Isotype control mAbs were used in all analyses to verify nonspecific fluorescence and also as a guide to determine positive and negative populations.

Quantification of specific IgE antibodies

For exclusion of IgE-mediated reactions, skin testing was performed and specific IgE antibodies were determined in those patients with suspected β -lactam allergy. The following determinants were used for skin testing: benzylpenicilloyl (BPO) 5×10^{-5} mM/l, minor determinant mixture (MDM) 2×10^{-2} mM/l (both provided by Allergopharma, Merck ([Darmstadt, Germany]), amoxicillin (AX) 20 mg/ml from Beecham (Toledo, Spain), ampicillin (AMP) 20 mg/ml from Antibiotic SA (León, Spain), and the culprit drug at a concentration proven to be nonirritant in a control group of 10 patients. These were tested by skin prick, and if the tests were negative, intradermal tests were carried out. Classification as positive or negative was made as previously described (20).

Specific IgE antibodies to benzylpenicilloyl-poly-L-

lysine (BPO-PLL) and amoxicilloyl-poly-L-lysine (AXO-PLL) were determined *in vitro* by RAST, as previously described (20, 21), at the moment patients were evaluated, and the levels were considered positive if they were higher than 2.5, which is more than the mean + 2 SD.

Re-exposure

For confirmation of a causal relationship, the drug was re-administered to those patients having an exanthematous reaction. If any patient reported more than one episode involving the same drug, it was not re-administered, nor was a controlled administration made in cases of desquamative reactions.

The re-exposure consisted of giving incremental doses of the drug at 1-week intervals, as previously described (22). This was performed between 2 and 4 months after the onset of the reaction once the patients were asymptomatic. Values of peripheral T cells were determined before re-exposure (A), and 24 h (B) and 1 week (C) after. The study was approved by the Institutional Review Board.

Statistical analysis

Data were analyzed with the Mann–Whitney U test. For sequential values, we used the Wilcoxon matched-pairs signed-rank test, and for re-exposure study the Friedman test. Values are presented as medians and 25–75th percentiles.

Results

Clinical data

A total of nine patients were included in the study (Table 1), three men and six women. The median age was 49 years (range 19–69). The clinical manifestations were exanthema in six cases and desquamative exanthema in three. The drugs most often involved belonged to the β -lactam group of antibiotics (patients 2, 4, 8, and 9); in these four patients, immediate skin tests to all penicillin determinants used were negative and no specific IgE antibodies were detected *in vitro*. Patients 2 and 8 had a positive delayed intradermal test

to amoxicillin (patient 2 had an increase of 5 mm in two diameters and patient 8 had an increase of 4 mm 24 h later), and patient 4 to cloxacillin (an increase of 5 mm in two diameters 24 h later). Patients 4, 5, 6, and 8 were re-exposed after the disappearance of symptoms and the normalization of peripheral T-cell parameters (68 days in patient 4, 72 in patient 5, 90 in patient 6, and 83 in patient 8). Since the reaction had occurred previously in patients 2 and 9 on two and three occasions, respectively, no re-exposure test was done. Patients 1, 3, and 7 were not readministered the drug because they developed desquamative exanthema.

CLA data

The expression of the CLA in lymphocytes from peripheral blood of the subjects, studied at the moment of the allergic drug reaction (T1), is presented in Fig. 1. These values were increased compared to the mean of the normal controls, presented in the last bar of the figure. The median CD3+ CLA+ value for the study group was 20.4 and for the healthy control group 9.4 ($P < 0.001$). As CD3+ CLA+ levels in the control group of subjects taking drugs but with no reactions (median = 8.9) were not statistically different from those of unexposed controls, we used only the healthy control group for comparisons. When we compared the results in patients and controls 1 month later (T2), the median CD3+ CLA+ values were 11.2 and 10.9, respectively, and this difference was not significant ($P < 0.1$) (Fig. 1). The evaluation of the sequential values of CLA expression in CD3+ lymphocytes (T1 and T2) in each individual case by the Wilcoxon matched-pairs signed-rank test showed a significant decrease in the patient group ($P < 0.01$), but no differences were observed in the control group ($P = 0.113$).

The median of the expression of the activation marker HLA-DR (Fig. 2) in the CD3+ CLA+ subpopulation was higher in the patient group at the moment of the reaction than the control group (3.8 vs 1.4) ($P < 0.005$). However, when we compared the HLA-DR expression in the CD3+ CLA– subpopulation, there were no significant differences in the median between patients and controls (1.4 vs 1.0). The

Table 1. Clinical data and culprit drug in nine patients studied. Interval is time elapsed (in hours) between appearance of reaction and evaluation of subject

Patient no.	Symptoms	Interval (h)	Drugs involved	Age (years)	Sex
1	Desquamative exanthema	24	Propyphenazone	49	F
2	Exanthema	18	Amoxicillin	57	F
3	Desquamative exanthema	12	Phenytoin	27	M
4	Exanthema	24	Cloxacillin	55	F
5	Exanthema	24	Spiramycin plus metronidazole	45	M
6	Exanthema	16	Phenytoin	19	M
7	Desquamative exanthema	24	Captopril plus tiazide	49	F
8	Exanthema	24	Amoxicillin plus clavulanic acid	69	F
9	Exanthema	12	Penicillin V	29	F

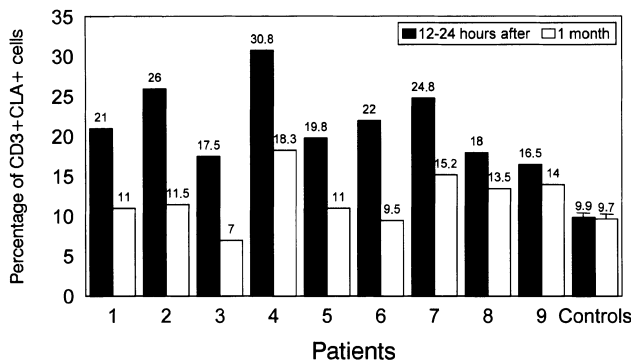


Figure 1. Expression of CLA in circulating CD3+ cells from nine subjects with delayed allergic drug reactions with skin involvement when first evaluated in emergency department (T1). Second value (T2) was determined 1 month after drug reaction. Ten healthy subjects with no history of allergy to drugs and normal skin were used as negative control group. Median CD3+CLA+ value for study group was 20.4 ($P_{25}=17.2$, $P_{75}=25.1$), and for healthy control group 9.4 ($P_{25}=8.4$, $P_{75}=10.1$) ($P<0.001$) at T1, and 11.2 ($P_{25}=9.6$, $P_{75}=14.3$) and 10.9 ($P_{25}=9.0$, $P_{75}=14.0$), respectively, at T2 ($P<0.1$).

expression of CLA in comparison with HLA-DR in CD3+ lymphocytes in patients 1, 4, and 8 can be seen in Fig. 3.

Re-exposure data

Patients 4, 5, 6, and 8 were re-exposed to the offending drug, and all gave a positive response. Patient 4 developed erythema on the abdomen and neck 20 h after the administration of 100 mg of cloxacillin, patient 5 developed generalized exanthema 30 h after the administration of 60 mg of metronidazole, patient 6 developed exanthema on the abdomen and legs 12 h after the administration of 25 mg of phenytoin, and patient 8 developed generalized erythema 8 h after the administration of 50 mg of amoxicillin. In these patients, there was an increase in CLA expression in CD3+ cells 24 h after the re-exposure (Fig. 4), with a progressive decrease and a tendency to reach those

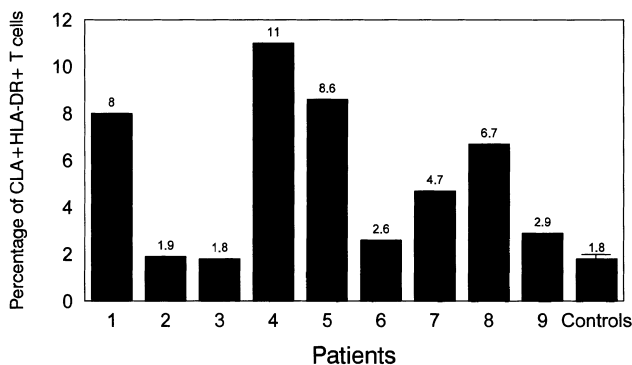


Figure 2. Activation marker (HLA-DR) expression in CD3+CLA+ subpopulations of patients who developed allergic drug reactions with skin symptoms. Median of patient group at moment of reaction was higher than control group, 3.8 ($P_{25}=2.1$, $P_{75}=8.1$) vs 1.4 ($P_{25}=0.6$, $P_{75}=1.9$) ($P<0.005$).

levels observed in the control group after 1 week (median=10.6, $P_{25}=9.3$, $P_{75}=11.7$ at time 0, median=16.5, $P_{25}=15.2$, $P_{75}=17.7$ at 24 h, and median=13.3, $P_{25}=11.7$, $P_{75}=15.4$ at 1 week). A similar increase in the DR expression in the CD3+CLA+ cells was also observed (median=1.9, $P_{25}=1.8$, $P_{75}=2.2$ at time 0, median=3.6, $P_{25}=2.3$, $P_{75}=5.2$ at 24 h, and median=2.8, $P_{25}=1.6$, $P_{75}=3.9$ at 1 week). The Friedman test showed statistical significance in these sequential determinations in both CLA ($P<0.05$) and HLA-DR expression ($P<0.05$). We found no increase in the CD3+CLA- subpopulation or in the DR expression in these cells.

Discussion

T cells have been shown to be involved in delayed-type skin reactions induced by drugs (7, 23), and, although their role in the pathophysiologic mechanism is relevant, the precise process by which they participate needs to be clarified. In recent years, there has been an increased interest in the evaluation of immunologic reactions to drugs, including allergy, but most of the data come from *in vitro* studies, and extrapolation to *in vivo* models still needs to be confirmed. Generation of T-cell lines and clones *in vitro* from skin-infiltrating lymphocytes has enabled the study of their antigen specificity, cytokine production, and antigen-presentation requirements with different drugs (6, 24). The *in vivo* monitoring of the acute response in peripheral blood from allergic patients has been carried out by several groups (25–27), but few data have been provided in studies of the allergic response to drugs (6). Therefore, for a better understanding of the immuno-

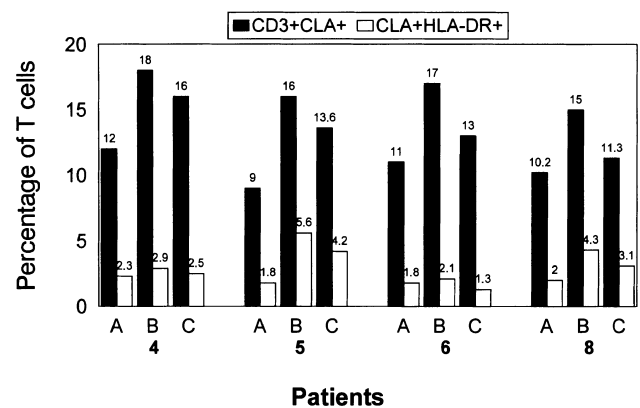


Figure 3. CLA and HLA-DR expression in T cells in four different patients who were re-exposed. Determinations were made before (A), 24 h after re-exposure (B), and 7 days afterward (C). Median of CLA expression in CD3+ cells was 10.6, $P_{25}=9.3$, $P_{75}=11.7$ at time 0, median=16.5, $P_{25}=15.2$, $P_{75}=17.7$ at 24 h, and median=13.3, $P_{25}=11.7$, $P_{75}=15.4$ at 1 week. Median of DR expression in CD3+CLA+ cells was 1.9, $P_{25}=1.8$, $P_{75}=2.2$ at time 0, median=3.6, $P_{25}=2.3$, $P_{75}=5.2$ at 24 h, and median=2.8, $P_{25}=1.6$, $P_{75}=3.9$ at 1 week.

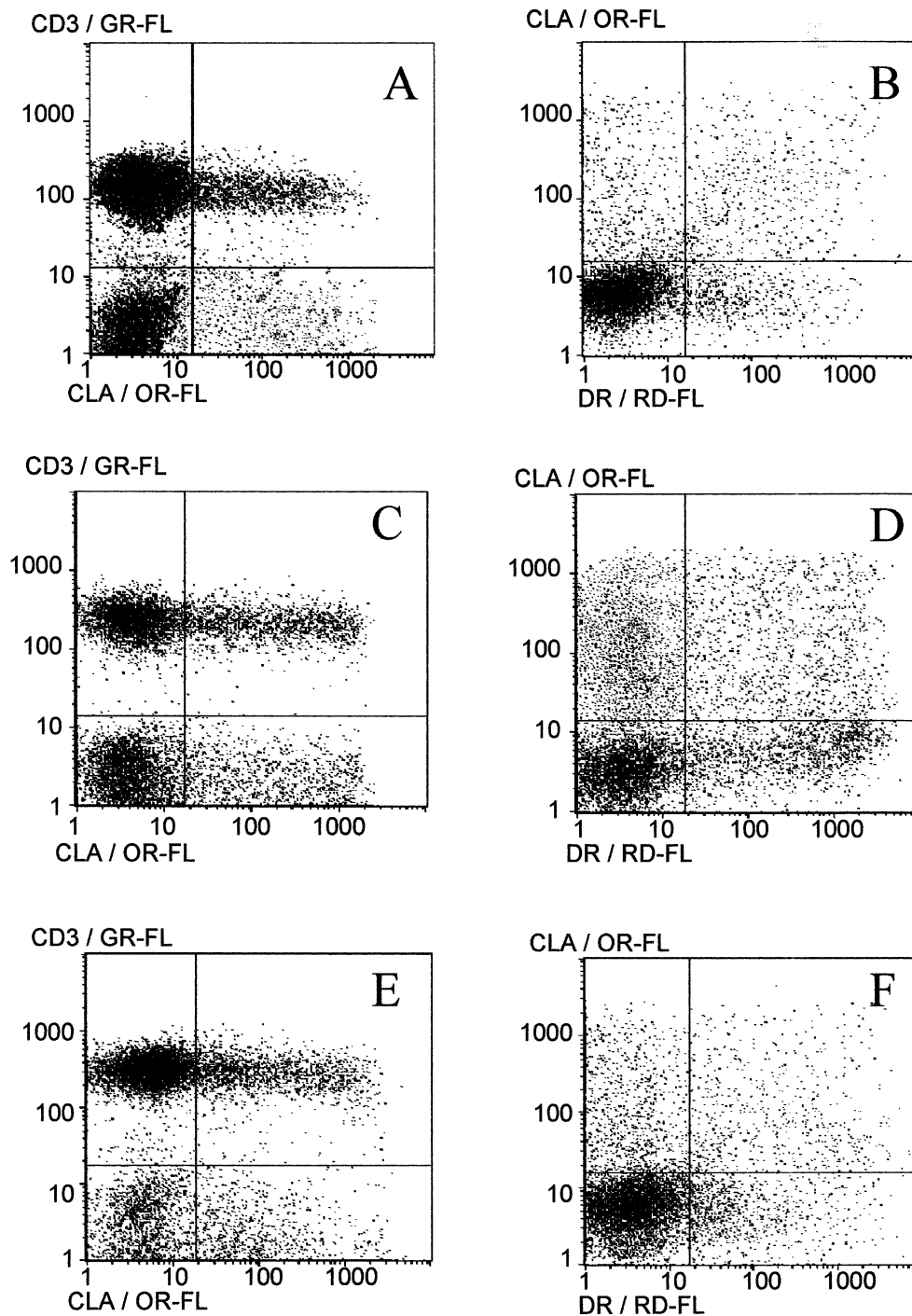


Figure 4. Evaluation of CLA vs HLA-DR expression in peripheral blood T cells from patients 1 (A and B), 4 (C and D), and 8 (E and F). PBMC were sequentially stained with HECA-452, goat anti-rat PE-conjugated, anti-CD3 FITC-conjugated, and anti-HLA-DR PE-Cy5-conjugated. The lymphocyte gate was established on basis of forward and side light scatter. A, C, and E are based on this gate. Events gated on CD3+ lymphocytes are shown in B, D, and F. Vertical and horizontal lines delineate extent of negative control staining (IgM PE-conjugated, IgG1 FITC-conjugated, and IgG1 PE-Cy5-conjugated) for each patient. Values on x- and y-axes indicate relative fluorescence intensity. Of CD3+ lymphocytes, 21% were CLA+ T cells in patient 1, 30.8% in patient 4, and 18% in patient 8. CD3+ cells expressing both CLA and HLA-DR were 8% in patient 1, 11% in patient 4, and 6.7% in patient 8.

logic mechanisms implicated in delayed-type, drug-induced cutaneous reactions, we studied *in vivo* the population of circulating skin-homing T cells, its activation state, and its association with the evolution of the clinical manifestations of this type of reaction.

Preliminary data indicated that subjects with allergic drug reactions expressed increased levels of CD3+ CLA+ lymphocytes, and in some their activated state paralleled the clinical symptoms (28–30). We have now compared the percentage of circulating CLA+ T cells in patients with delayed-type reactions induced by drugs, and our results indicate a clear increase in the percentage of circulating CLA+ T cells compared with healthy controls. In addition, the presence of the activation marker HLA-DR in the CLA+ population was also increased. Circulating CLA+ T cells decreased in parallel with the improvement of the clinical symptoms. These data indicate a close relationship between skin symptoms and the expansion and activation status of circulating CLA+ T cells.

In milk-induced eczema, it has been suggested that oral administration of antigen may lead to its deposition in the skin, where it can induce a T-cell-mediated immune response (31). It has been shown that casein can induce *in vitro* the expansion of specific CLA+ T cells from patients with milk-induced eczema (31). In our study, there was also an oral administration of antigen inducing cutaneous symptoms and CLA+ T-cell expansion *in vivo*. We think that these cells play an important role not only in those allergic reactions in which the antigen penetrates the skin (17) but also in those induced by the oral route.

To clarify the relationship between circulating CLA+ T cells and drug-induced cutaneous symptoms,

we studied four patients who developed the same reaction after a controlled re-exposure to the original drug involved. The symptoms appeared within an interval of 12–30 h after drug intake, and there was a significant increase in the percentage of circulating CLA+ T cells with an increase in DR expression also, as had occurred in the initial reaction reported in each patient. These data support the hypothesis of the immunologic nature of the adverse drug reaction, since an increase of activated CLA+ lymphocytes followed the administration of the drug.

It has been previously shown that in antigen-induced, delayed-type hypersensitivity reactions CLA+ T cells are localized in the skin blisters (19, 32), and our results concerning the CLA+ T-cell population may help to clarify the mechanism involved in this process. We hypothesize that an immunologic stimulus originating in the skin induces and amplifies an immunologic response with the increase in peripheral blood of lymphocytes expressing the CLA and homing to the skin, contributing in this way to the inflammatory response. Since the circulating CLA+ T-cell subset is easily accessible and can be recognized by a specific marker, this will enable further information to be obtained about antigen specificity and cytokine production in drug-allergic reactions.

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