Assessment of Perforin Expression in Peripheral Blood Lymphocytes in Psoriatic Patients During Exacerbation of Disease

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INTRODUCTION
Psoriasis is an inflammatory papulosquamous skin disease which is immunologically characterized by increased keratinocyte proliferation, infiltration of inflammatory cells, especially T lymphocytes, and production of inflammatory cytokines, mostly of Th1 phenotype (1).

Immunological changes characteristic of psoriasis occur at the systemic and local levels, in the region of the psoriatic lesion in the skin (2). It is generally considered that T lymphocytes play an important role in the pathogenesis of psoriasis. Skin lesions in psoriatic patients are strongly infiltrated with T lymphocytes and, depending on the stage of disease, CD4+ or CD8+ cells predominate. Variations in the levels of these cells are observed in the peripheral blood and in lesions.

There are very few data concerning the role played by cell-mediated cytotoxicity, particularly at the molecular level, in the course of psoriasis. Both cytotoxic T lymphocytes (CTL) and natural killer cells contain in their granules the cytolytic protein perforin, a mediator in cell-mediated cytotoxicity reactions. The aim of this study was to analyze perforin expression in various sets and subsets of perforin-positive peripheral blood lymphocytes in patients with chronic psoriasis vulgaris in the exacerbation phase. The results were compared with those of an age- and sex-matched healthy control group (n= 21). Perforin (intracellular antigen) and cell surface antigens were detected using the simultaneous double-staining method. We found a significant increase in perforin (P) expression in the patient group for CTL (CD3+ P+ cells), which are located mostly in the CD8+ population of T lymphocytes (CD8+ P+). Key words: psoriasis; perforin; cytotoxic T lymphocytes; NK cells.

MATERIALS AND METHODS

Patients
This study was approved by the Ethics Committee of the Medical Faculty, University of Rijeka. For flow cytometric analyses 2 groups of patients were recruited: (i) 17 patients (8 males, 9 females; 23–60 years) who were in the early exacerbation phase of chronic psoriasis; and (ii) 21 healthy persons (laboratory personnel and volunteer blood donors; 9 males, 12 females; 20–57 years) who served as controls.

Lymphocyte preparation
Heparinized venous peripheral blood (10 ml) was layered onto Ficoll/Hypaque density gradient and centrifuged for 20 min at 800 g. Cells accumulating at the interface were washed twice in RPMI 1640 and resuspended at a final concentration of 1x10^6 peripheral blood lymphocytes per sample in fluorescent-activated cell sorting (FACS) buffer. Cell viability was checked using trypan blue.

Monoclonal antibodies
A murine antihuman perforin monoclonal antibody (MoAb) 6G9 (IgG2b) was purified from Balb/c ascites (produced by E. R. P.). The following MoAbs were obtained conjugated to phycoerythrin from Becton Dickinson (Mountain View, CA): Leu-4 (anti-CD3), Leu-3a (anti-CD4), Leu-2a (anti-CD8), Leu-11b (anti-CD16) and Leu-19 (anti-CD56).

Simultaneous detection of cell surface and intracellular antigens by flow cytometry
Our procedure for the simultaneous detection of intracellular (perforin) and cell surface membrane antigens has been described elsewhere (8). Briefly, peripheral blood lymphocytes were cultured overnight at 37°C in complete RPMI. Non-adherent cells were aliquoted (10^6 per aliquot), washed in FACS buffer and then fixed in 100 µL PBS containing 4% paraformaldehyde, pH 7.4, for 10 min at room temperature. After 2 washes in FACS buffer, the cells were permeabilized with saponin buffer (0.1% saponin) (Sigma, Poole, UK) for 20 min at room temperature. Anti-perforin MoAb in saponin
buffer (3 μg/100 ml) was added to the cell suspension at a final concentration of 8–10 μg per sample and incubated for 30 min at room temperature. A second antibody (fluorescein-conjugated goat anti-mouse IgG; Becton Dickinson) was added for a further 30 min at room temperature. Cell surface antigens were then labeled by incubating for 30 min at room temperature with phycoerythrin-conjugated MoAbs after the integrity of the membranes was restored by a 10-min incubation in FACS buffer. In some experiments, only cell surface labeling was performed. An irrelevant isotype-matched murine MoAb was used as a negative control.

Lymphocytes were gated on the basis of forward and side scatter. A minimum of 10⁶ cells were analyzed on a FACSscan (Becton Dickinson) and the results are shown as contour graphs. Thresholds for positive staining were set at <2% using the negative control and percentages of positive cells were obtained by subtracting the value of the control.

Statistical analysis
Results were analyzed using Sigma Plot for Windows, version 1.02 (Jandel Scientific). Statistical analyses were performed using a Student’s t-test 1-way analysis for comparison of means.

RESULTS
Lymphocyte subpopulations (T and NK cells) and total perforin-positive cells

Significant increases in CD3+ and CD8+ cells in the peripheral blood lymphocytes of psoriatic patients in the acute phase of disease were found compared to healthy controls (Fig. 1). The incidence of CD3+ cells increased from 62% to 74.4% and the incidence of CD8+ cells increased from 23.2% to 36.5% (p < 0.001). There was no difference between the healthy control group and the patient group in terms of either CD4+ T lymphocytes or both markers of NK cells (CD16+ and CD56+). The percentage of lymphocytes containing cytolytic molecule perforin (P+ cells) was slightly higher in the patient group than in the healthy control group, but this difference was not significant (Fig. 1). The incidence of total P+ lymphocytes was 51.4% for the patient group and 27% for the healthy controls.

Double positive peripheral blood lymphocytes

Double positive cells (simultaneously perforin-positive and surface marker-positive) are represented as a fraction of the total peripheral blood lymphocytes counted (10,000 cells), which is set at 100% (Fig. 2). A significant increase in perforin expression was found in the patient group for the population of T lymphocytes, i.e. CD3+ P+ cells (19.2% vs. 10%; p < 0.02). This increase occurred mostly in the CD8+ population of T lymphocytes (CD8+ P+), which was significantly higher in the patient group (p < 0.01). A slight but non-significant increase was also found for CD4+ P+ cells. There was no significant difference for both populations of NK cells (CD16+ P+ and CD56+ P+), in spite of the fact that slightly lower levels were found in the psoriasis group.

DISCUSSION
It has been shown that the perforin-mediated cytolytic pathway plays a role in the rejection of foreign tissue, autoimmunity, control of tumor growth and viral infections and infections by intracellular pathogens (9–11), and is greatly weakened in elderly subjects (8). In the cytotoxic pathway mediated by regulated secretion of granules the formation of membrane pores by perforin is a prerequisite for apoptosis of the target cell (12, 13). In addition to perforin the main protein component of granules is a group of serine proteases known as granzymes (14). Their main function is to mediate target DNA degradation upon gaining entry to the target cell by means of perforin pores (7).

In the present investigation we noticed significant differences in some lymphocyte subpopulations between healthy controls and psoriatic patients. The cloning of skin-infiltrating lymphocytes from lesional skin showed that some clones are cytotoxic against autologous lymphoblastic cell lines. Cytolytic clones were mostly CD4+ and some exhibited characteristics of NK cells (15). Eruption of acute psoriatic lesions coincides with the epidermal influx and activation of CD4+ T cells, whereas spontaneous disease resolution is associated with recruitment of CD8+ cells (16). Accordingly, the absence of CD8+ cytotoxic clones in the experiments...
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REFERENCES