Systemic and Local Expression of Perforin in Lymphocyte Subsets in Acute and Chronic Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To investigate the role of the cytolytic action mediated by perforin in the course of rheumatoid arthritis (RA), we studied the immunophenotypic characteristics of lymphocytes containing perforin in peripheral blood (systemic level), in synovial fluid (SF), and in the synovial membrane (local level) in patients during the acute or chronic phase of RA. Cells from patients with osteoarthritis were used as controls.

Methods. Flow cytometry was used for simultaneous detection of intracellular (perforin) and cell surface antigens. Mean fluorescence intensity (MFI) was a measure of the mean perforin content per cell. Immunocytochemical staining was used to visualize perforin in the cytoplasmic compartment of cells.

Results. In acute RA highly significant changes in perforin expression were found in all compartments (peripheral blood, SF, and synovial membrane): (1) increase of percentage of total perforin positive cells; (2) increase of both subsets of cytolytic cells, T (CD8+P+) and NK (CD56+P+) cells; (3) increase in the frequency of perforin positive cells in CD8+ and CD56+ cell populations; and (4) the highest content of perforin/cell (MFI values) in all compartments, except in the synovial membrane.

Conclusion. Perforin positive cells may participate in the acute phase of RA by maintaining and perpetuating inflammation and contributing to tissue destruction. (J Rheumatol 2003;30:660–70)

Key Indexing Terms:
RHEUMATOID ARTHRITIS LYMPHOCYTE MEDIATED CYTOTOXICITY
BLOOD SYNOVIAL TISSUE PERFORIN

Rheumatoid arthritis (RA) is a human autoimmune disease characterized by chronic inflammation of synovial joints and infiltration of bone marrow derived mononuclear cells, predominantly T cells, macrophages, plasma cells, and natural killer (NK) cells, all of which show signs of activation and eventually lead to destruction of cartilage and bone in affected joints[1]. The pathogenetic role of different lymphocyte sets and subsets has been investigated broadly in both peripheral blood (PB) and synovial fluid (SF) as well as in the synovial membrane of patients with RA, and significant oscillations in their concentrations have been reported[2-5]. However, it is uncertain which T cells contribute to pathogenetic changes in the early phase of disease and which ones contribute to maintaining synovial inflammation. There are differences in T cell and NK cell subsets depending on the phase of the disease and compartment analyzed (systemic vs local)[3,6,7]. Progressive destruction of cartilage and bone is believed to be mainly mediated by cytokine induction of destructive enzymes, mainly matrix proteinases[8].

The role of lymphocyte mediated cytotoxicity in the pathogenesis of RA is not clear, particularly at the molecular level. Cytotoxic lymphocytes belong to the subpopulations of NK cells or cytotoxic T lymphocytes. Granules in the cytoplasm of cytotoxic cells contain the cytolytic pore-forming protein perforin and granzymes (serine esterases)[9-12]. Perforin is a cytolytic mediator in cell mediated cytotoxic reactions[13-16]. Perforin is constitutively present in NK cells and in a population of cytotoxic T lymphocytes and its expression in these cells can be upregulated[17].

The role of perforin mediated cytotoxicity has been investigated in various human autoimmune diseases; some

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interesting changes have been reported in lymphocytes infiltrating the thyroid gland in Hashimoto's thyroiditis[18] and in PB lymphocytes in multiple sclerosis, particularly in the active phase of disease[19], suggesting the possibility that cytotoxic lymphocytes can cause tissue damage directly. There are very few data in patients with RA showing a role of cytolytic and apoptotic molecules located in granules of cytolytic cells including perforin and granzymes[20]. Using *in situ* hybridization and immunohistochemistry for perforin and granzymes, Griffiths, *et al*[21] found both these molecules in SF lymphocytes from patients with RA, but not in SF lymphocytes from patients with osteoarthrosis. They concluded that cytolytic lymphocytes may be involved in the pathogenesis of RA.

We analyzed the expression of the cytolytic molecule perforin in RA in patients in the acute and chronic phase of disease. Systemic expression in PB lymphocytes was compared to the local expression in SF and synovial membrane lymphocytes. We examined perforin expression in all subpopulations of cytolytic cells to determine whether a specific cell population containing perforin (P) in their granules (CD4+P+, CD8+P+, CD16+P+ and CD56+P+) is associated with the clinical course of RA. We found in the acute disease phase a significant increase in the number of perforin positive cells in all compartments as well as an increase of the number of perforin molecules per cell (mean fluorescence intensity values). These results are consistent with a pathogenetic relevance of perforin mediated cytotoxicity, at least in the acute and initial phase of this autoimmune disease.

MATERIALS AND METHODS

Patients. The study was approved by Ethics Committee of the Medical Faculty, University of Rijeka. PB and SF samples were obtained from patients with definite or classical RA and osteoarthritis (OA). All patients were hospitalized in the Clinic of Orthopaedic Surgery Lovran, Croatia. The diagnosis of RA was based on the 1987 criteria of the American College of Rheumatology[22]. Patients who fulfilled a minimum of 4 out of the 7 possible criteria were enrolled. Selection of patients was solely based on availability of paired blood and synovial tissue samples (synovial membrane and SF) from patients undergoing synovectomy or knee joint replacement surgery. Pregnant women and patients with other immunological diseases, other chronic diseases (chronic pancreatitis, diabetes mellitus, hematopoietic organ diseases, etc.), and with malignant diseases were excluded.

Patients with RA were divided into 2 groups: acute and chronic stage. Acute disease activity was defined by a combination of clinical signs (swollen and tender joints, skin hyperthermia) and results of laboratory testing (erythrocyte sedimentation rate > 30 mm/h and high serum C-reactive protein). This group included patients in whom diagnosis of RA was determined for the first time and those already treated for RA, but hospitalized in the acute stage of the disease. Patients were not divided based on rheumatoid factor seropositivity. PB, synovial membrane, and SF samples from 30 patients with acute RA (21 women, 9 men, age range 37–60 yrs) were analyzed.

Patients with chronic RA were considered those in whom signs of acute inflammation were not visible at the time of examination, but who were hospitalized because of the knee joint replacement surgery (total knee endoprosthesis). Blood and synovial samples from 32 patients with chronic RA (21 women, 11 men, age range 50–74 yrs) were analyzed.

The third group comprised 20 patients with RA (14 women, 6 men, age range 35–67 yrs) who were treated with immunosupressive therapy (prednisone < 15 mg/day), and since they were not undergoing surgery only their blood samples were analyzed.

The fourth group included patients with OA in whom diagnosis of degenerative processes were determined by clinical signs and radiologic methods. All patients with laboratory signs of inflammation and with inflammatory or neoplastic disease were excluded. PB and synovial tissue samples from 30 patients with OA (15 women, 15 men, age range 65–76 yrs) were analyzed.

Control blood samples were obtained from 27 healthy donors matched with patients for age and sex.

Synovial membrane and SF samples were routinely obtained in the clinic from patients undergoing diagnostic or therapeutic joint tapping, arthroscopic and open synovectomy of the knee joint (acute RA patients), or implantation of total knee prosthesis (chronic RA and OA patients).

Preparation of synovial membrane lymphocytes. Synovial membrane tissue free of blood coagulum was washed in cold RPMI 1640 medium (Institute of Immunology, Zagreb, Croatia) several times, cut into small pieces, and exposed to trypsin digestion [0.125% trypsin (Difco, Detroit, MI, USA) with 0.2% EDTA] at 37°C for 90 min with constant stirring. Trypsinization was stopped by adding human AB serum to a concentration of 10%. The isolated cells were separated from debris by passing twice through nylon mesh and sedimented by centrifugation (600 g/10 min). Supernatant was aspirated and 10 ml of RPMI 1640 was added to the pellet. The cell suspension was overlaid on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and centrifuged at 800 g for 20 min. The cells accumulating at the interface were collected, washed twice in RPMI 1640, and cultured overnight in tissue culture grade Petri dishes in a humidified CO_2 incubator. After adherence of the macrophages, the remaining mononuclear cell suspension was aspirated and was used for immunofluorescence phenotyping. All samples were > 95% viable as assessed by trypan blue dye exclusion.

Preparation of SF lymphocytes. SF was obtained from knee joints and chosen on the basis of SF volume obtained because of mononuclear cell (MNC) requirements for flow cytometry (a minimum of 10 ml). Grossly hemorrhagic SF was not used. MNC were collected after Ficoll-Hypaque density gradient centrifugation and washed 3 times in RPMI 1640 supplemented with 10% fetal calf serum (FCS). Further steps were the same as described for synovial membrane lymphocytes.

Preparation of PB lymphocytes. Roughly 20 ml heparinized PB was taken by cubital vein phlebotomy from patients undergoing diagnostic or therapeutic procedures before initiation of anesthesia. Blood was overlaid onto Ficoll-Hypaque, centrifuged for 20 min at 800 g, and PB monocytes from the interface were collected, washed, and cultured as described for synovial membrane lymphocytes.

Simultaneous detection of cell surface and intracellular antigens by flow cytometry. Perforin (intracellular antigen) and cell surface membrane antigens were detected simultaneously by indirect immunofluorescence and analyzed by flow cytometry immediately after cell separation as described[23]. Briefly, cells were aliquoted (10^6 per aliquot) and washed in FACS buffer [2% FCS, 1 mmol/l EDTA, 0.1% NaN_3 in phosphate buffered saline (PBS), pH 7.4], then fixed in 100 µl PBS containing 4% paraformaldehyde, pH 7.4, for 10 min at room temperature. After 2 washings in FACS buffer, the cells from each cell sample were permeabilized with 100 µl saponin buffer [0.1% saponin (Sigma, Poole, UK), 2% goat serum, 1 mmol/l EDTA in PBS] for 20 min at room temperature. Mouse anti-human perforin monoclonal antibody (Mab, δG9, IgG2b, purified from Balb/c ascites; produced by E.R. Podack) diluted in saponin buffer (3 µg/200 ml) was added to samples (100 µl) and incubated 30 min at 4°C. Cells were washed twice in saponin buffer, and secondary antibody (fluorescein conjugated goat anti-mouse IgG; Becton Dickinson, Mountain View, CA, USA) was added for a further 30 min at 4°C. After 2 washings

in saponin buffer, cells were resuspended in 1 ml FACS buffer to restore the integrity of the membranes. After 10 min incubation, cell surface antigens were labeled with avidin-phycoerythrin conjugated Mab. The following antibodies were used: anti-CD3, CD4, CD8, CD16, CD56 and CD25 (all Becton Dickinson). In all experiments irrelevant isotype matched murine Mab were used as a negative control. A minimum of 10^4 cells were analyzed on a FACScan (Becton Dickinson). Results are presented as a mean fluorescence intensity (MFI) of gated populations of cells.

Immunocytochemistry. Perforin was detected in cytospins of PB lymphocytes, synovial membrane lymphocytes, and SF lymphocytes isolated from the acute and chronic RA and OA groups of patients. Lymphocytes were washed twice in PBS and 100 µl of cell suspension (8×10^5/ml) was centrifuged onto glass microscope slides (500 RPM for 10 min). After drying at room temperature, the cells were fixed in cold acetone for 10 min and washed once in Tris buffered saline (TBS), then stored at –20°C until labeled. Before further procedure, cytospin cells were surrounded by PAP-PEN (Dako) and rehydrated twice in PBS for 5 min. All incubations were performed at room temperature in a humidified atmosphere. Endogenous peroxidase activity was blocked by H_2O_2, and nonspecific binding was stopped by preincubation with 1% bovine serum albumin. The primary antibody (mouse anti-human perforin) was applied at a concentration of 30 µg/ml for 1 h. Peroxidase labeled anti-mouse IgG (Boehringer, Mannheim, Germany) was used at a dilution of 1:100 for 45 min. After 2 washing steps in TBS, the reaction was developed by 50 µl of diaminobenzidine (DAB) solution [25 ml TBS, pH 7.5, 2.5 mg DAB, 7 mg $NiSO_4 6H_2O$, 1 µl 30% H_2O_2]. The red-brown reaction was stopped by distilled water. Nuclei were counterstained with hematoxylin for 2 min, then washed in distilled water. The slides were mounted in glycerol-gelatine.

Statistical analysis. Results were analyzed using Sigma Plot for Windows, Version 1.02 (Jandel Scientific, Chicago, IL, USA). Statistical analyses were performed using a standard Student t test with one-way analysis for comparison of means.

RESULTS

Lymphocyte subpopulations. Single staining of surface molecules (Figure 1) showed no significant difference in the percentages of total T lymphocytes (CD3+ cells) at either systemic or local level among the groups of patients investigated. CD4+ T lymphocytes were at the lowest concentration in SF in patients in acute phase RA, but at the highest level in synovial membrane in this phase of disease. By contrast, CD8+ T lymphocytes were at the highest values in SF in the acute disease phase. These changes were accompanied by changes of the CD4/CD8 ratio, which was decreased in SF but increased in synovial membrane compared to PB values. The frequency of CD16+ cells was the lowest in SF in acute RA, but significantly higher values were found in synovial membrane. The highest levels of CD56+ cells were found in SF in acute disease phase, and this was significantly higher than in chronic disease phase of RA.

Total perforin positive cells in PB, SF, and synovial membrane lymphocytes. PB lymphocytes. The percentage of perforin positive PB lymphocytes from acute and chronic RA was compared with results obtained in healthy controls and OA and RA patients undergoing immunosuppressive therapy (Figure 2). A highly significant increase (47.6% positive cells) of the percentage of perforin positive cells was found in patients in the acute disease phase compared to all other groups ($p < 0.001$). The frequency of perforin positive cells in PB lymphocytes of patients in the chronic disease phase and in OA patients was at the level of healthy controls. Immunosuppression by corticosteroids efficiently suppressed the percentage of total perforin positive cells compared to all other groups and controls.

SF and synovial membrane lymphocytes. The percentage of total perforin positive cells was determined in suspensions of lymphocytes from SF and synovial membranes (Figures 3A, 3B). The highest percentages of perforin positive cells were found in SF (22.9%) and synovial membrane (19.5%) of patients with acute RA, and they were significantly higher ($p < 0.001$) compared to both chronic phase RA and OA, where the lowest levels were found. In the chronic phase of RA, both SF and synovial membrane lymphocytes contained significantly higher concentrations of perforin than patients with OA.

Comparative analyses of perforin expression at the systemic and local level in RA and OA. Comparative analyses of perforin expression in different body compartments (Figures 2 and 3) showed in all groups (acute and chronic RA and OA) the highest frequency of perforin positive cells in the PB lymphocytes, and this difference was highly significant ($p < 0.001$ or $p < 0.01$). Interestingly, there was no difference in the frequencies of perforin positive cells between SF and synovial membrane in any of the groups investigated. The results of comparative immunocytochemical staining analyses are shown in Figure 4.

Cell surface antigen expression by perforin positive cells (double positive cells). As explained in Materials and Methods, cell suspensions were double labeled simultaneously for perforin (intracellular antigen) and for a panel of cell surface antigens characteristic for cells known to express this cytolytic molecule. For T lymphocytes we labeled CD4 and CD8 antigens and for NK cells CD56 and CD16 antigens. CD25 molecule was labeled as well. Double positive cells are represented as a fraction (%) of total lymphocytes counted (10,000 cells), set at 100% (Figure 5 shows percentages of double positive cells for suspensions of PB lymphocytes, SF lymphocytes, and synovial membrane lymphocytes). In the suspension of PB lymphocytes (Figure 5A), a significant increase of cytotoxic T lymphocytes (CD8+P+) and NK cells (CD16+P+ and CD56+P+) was found in acute phase RA, as well as a significant increase of perforin positive cells expressing high affinity interleukin 2 receptor (CD25 molecule). There were no differences in percentages of CD4+P+ cells among PB lymphocytes from all examined groups. However, a marked increase of this cell population was found in SF of patients with acute RA, compared to the 2 other groups (Figure 5B). In acute RA SF, similarly to PB, increased values of CD8+P+ and CD56+P+ cells were found. However, the percentage of CD16+P+ cells in acute RA was significantly higher only when compared to the OA group. CD25+P+

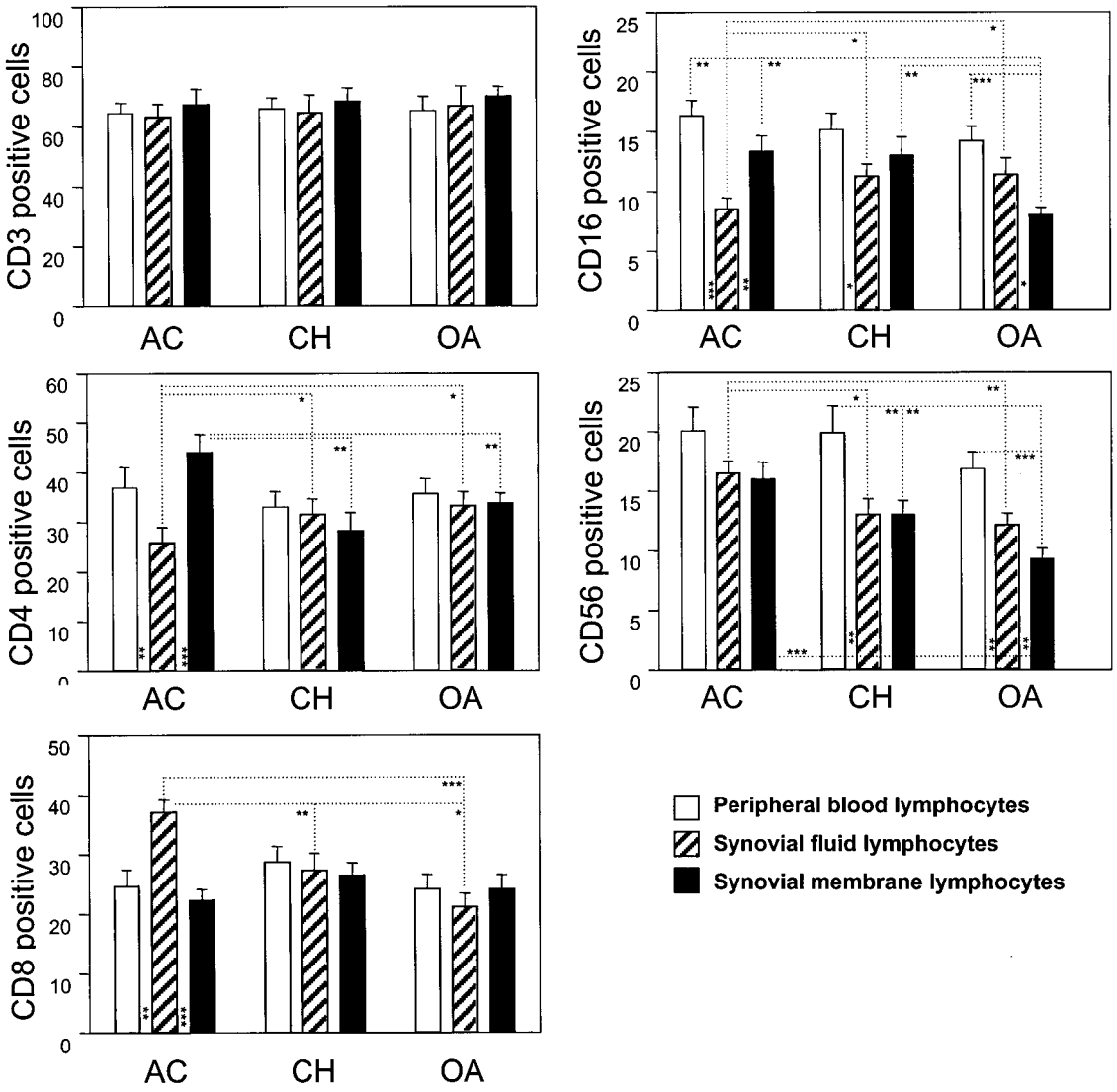

Figure 1. The phenotype of PB, SF, and synovial membrane lymphocytes in patients with acute RA (AC), chronic RA (CH), and osteoarthritis (OA). Bars represent the percentage (mean ± SE) of surface marker positive cells. *p < 0.05, **p < 0.01, ***p < 0.001.

cells were significantly higher in acute phase compared to chronic phase RA. In contrast to the results obtained for PB, in SF we found a significantly higher value for all variables analyzed compared to patients with OA. Analyzing double positive cells in the synovial membrane lymphocyte suspensions (Figure 5C), we found similar results for CD8+P+, CD56+P+, CD16+P+, and CD25+P+ cells as in SF. However, in synovial membrane samples the prevalence of CD4+P+ cells was higher in patients with chronic RA compared to other groups.

Increase of frequency of perforin-expressing cells in cytolytic cells in acute phase RA. Figure 6 illustrates the findings for perforin positive cells in acute and chronic RA as the fraction of cells with a certain phenotype; this relates to the percentage of cytolytic cells (either cytotoxic T lymphocytes or NK cells) that have the potential for perforin mediated cytotoxicity. A highly significant increase of perforin expression in PB lymphocytes was found in acute phase RA samples for cytotoxic T lymphocytes (CD8+) and NK cells (CD16+ and CD56+). A dramatic increase in the frequency of perforin positive cells among the population of CD8+ cytotoxic T lymphocytes is illustrated by the data for acute phase RA: 78% of CD8+ cells are simultaneously perforin positive; and in chronic phase RA the finding is only 35% (Figure 6A). In the acute phase almost all PB NK cells, both CD16+ and CD56+, are also perforin positive (89.02% and 89.36% of cells, respectively). A similar, less dramatic increase was found in the frequency of perforin positive cells for SF and synovial membrane lymphocytes (Figures 6B, 6C). The only difference was for CD4+ cells —

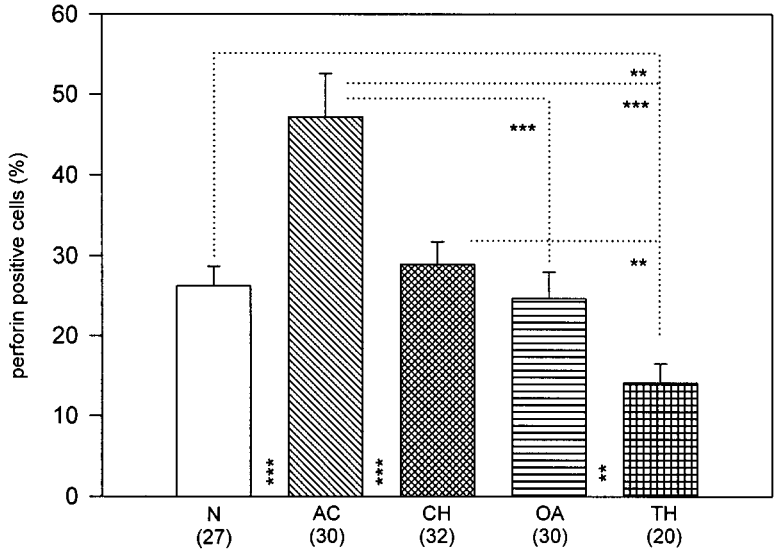

Figure 2. Percentage of perforin positive cells (mean ± SE) in PB lymphocytes of healthy controls (N), patients with acute RA (AC), chronic RA (CH), osteoarthritis (OA), or RA patients subjected to immunosuppressive therapy (TH). **p < 0.01, ***p < 0.001.

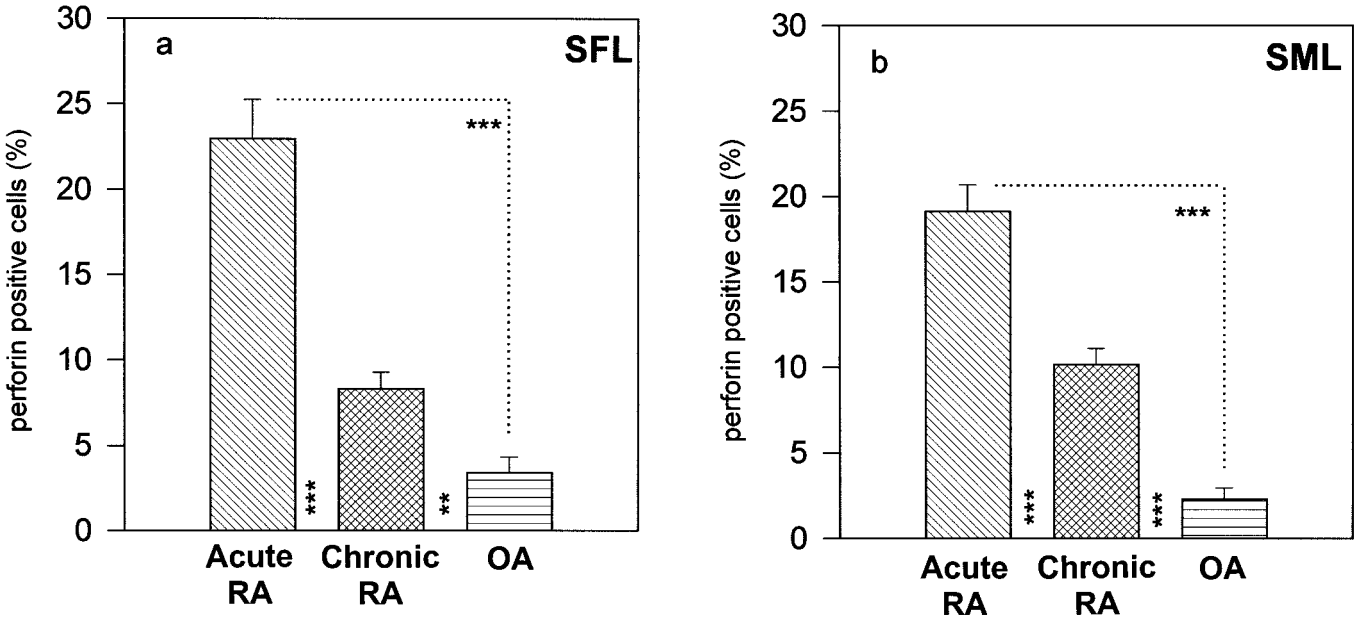

Figure 3. Percentage of perforin positive cells (mean ± SE) in SF lymphocytes (SFL) (a) and synovial membrane lymphocytes (SML) (b) of patients with acute RA, chronic RA, and osteoarthritis (OA). **p < 0.01, ***p < 0.001.

in the SF lymphocytes the percentage of perforin positive cells was increased in the acute phase RA samples and in the synovial membrane lymphocytes from chronic phase RA; in PB lymphocytes it was unchanged.

Perforin protein expression in T and NK subsets in PB, SF, and synovial membrane lymphocytes. Simultaneously with the determination of perforin positive cells by flow cytometry in various lymphocyte subsets, we measured the level of fluorescence for perforin protein expression, which is proportional to the number of perforin molecules present in the cells. The mean fluorescence intensity (MFI) was determined by applying standard variables for forward light scatter, side scatter, fluorescence intensity 1 (FITC as FL1 fluorochrome), and fluorescence intensity 2 (phycoerythrin as FL2 fluorochrome). MFI for perforin was analyzed in subsets of T lymphocytes (CD4+P+ and CD8+P+) and NK cells (CD56+P+ and CD16+P+). The results are presented in Figure 7. The highest content of perforin per cell (the

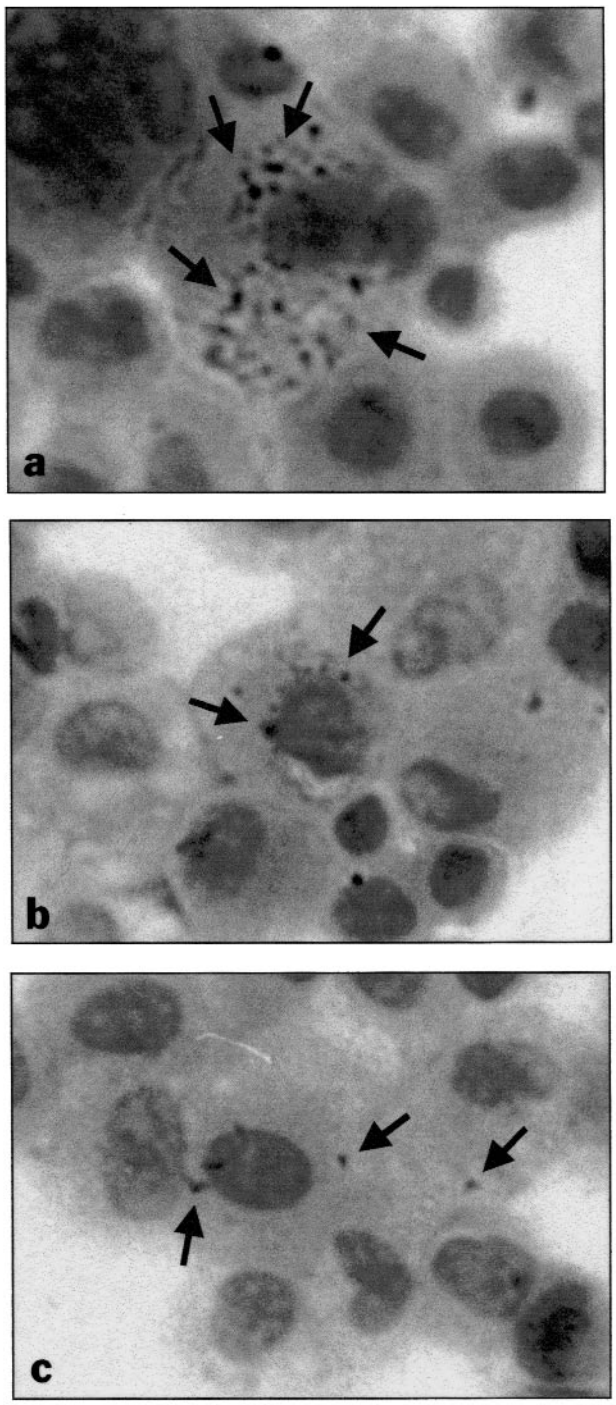

Figure 4. Immunocytochemistry of perforin expresion in (A) PB lymphocytes, (B) synovial membrane lymphocytes, and (C) SF lymphocytes from patients with acute RA. Arrows indicate perforin granules in the lymphocyte cytoplasm.

highest levels of MFI) was found in all subsets of cytotoxic T lymphocytes and NK cells in the PB of patients with acute phase RA (Figure 7A): MFI values were significantly higher ($p < 0.001$) compared to SF and synovial membrane lymphocyte subsets. Further, the content of perforin/cell (MFI values) was significantly higher in all subsets of acute phase RA PB lymphocytes compared to all other groups (Figure 7A). In chronic phase RA the content of perforin/cell was higher than in OA samples, and all values were lowest in patients subjected to immunosuppressive therapy. In SF lymphocytes similar results (highest level of perforin/cell) were found in acute phase samples compared to all other groups (Figure 7B). The highest levels of perforin/cell were obtained only in synovial membrane lymphocytes in chronic phase disease for CD4+P+ and in the OA group for CD8+P+ and CD16+P+ cells (Figure 7C).

DISCUSSION

In the pathogenesis of RA T cells are involved both in disease induction and in disease maintainance[24,25]. However, the question of which T cells contribute to inducing and maintaining the synovial inflammation in RA remains unanswered. The importance of analysis of T cell subpopulations was reinforced in our investigation. Although we found no differences in the concentrations of CD3+ cells, significant changes were found for CD4+ cells, which were lowest in acute phase RA in SF but highest in the synovial membrane, where the pathological process occurs. The opposite was found for CD8+ cells. The association of RA with a class II MHC epitope implies that antigen-specific responses of CD4+ T lymphocytes are required for development of the disease[26]. The whole CD4+ T cell subset, in both PB and SF, shows increased activation with the expression of activating markers (interleukin 2 receptor and HLA-DR molecule) and may be involved in the inflammatory process[24]. The investigation of Th1/Th2 profiles of T cell cytokines did not fit into any of the classical divisions of cytokine production, but suggested a sequence of pathologic cell-cell interaction in RA that eventually leads to the transformation of synovial tissue to a component of the systemic lymphoid system[26]. Recent information about the local production of the Th1-type cytokine interleukin 15, which has some similarities to but also significant differences with interleukin 2, whose production is suppressed, points to the importance of local cellular interactions in joints[27].

Only a few reports elucidate the role of perforin mediated cytotoxicity in RA, and they are focused primarily on the detection of mRNA for perforin[20]. By strictly defining the acute and chronic phases of RA disease we investigated the potential role of perforin mediated cytotoxicity in the clinical course of RA. Significant differences were found in perforin expression in various lymphocyte subpopulations depending on the compartment investigated (PB, SF, synovial membrane), clinical entity (RA vs OA), and the phase of the disease (acute vs chronic RA). The most interesting result related to the potential role perforin could play in the pathogenesis of RA, particularly during the acute phase. Perforin is expressed only in activated killer cells, and the level of perforin expression in the cell (expressed as

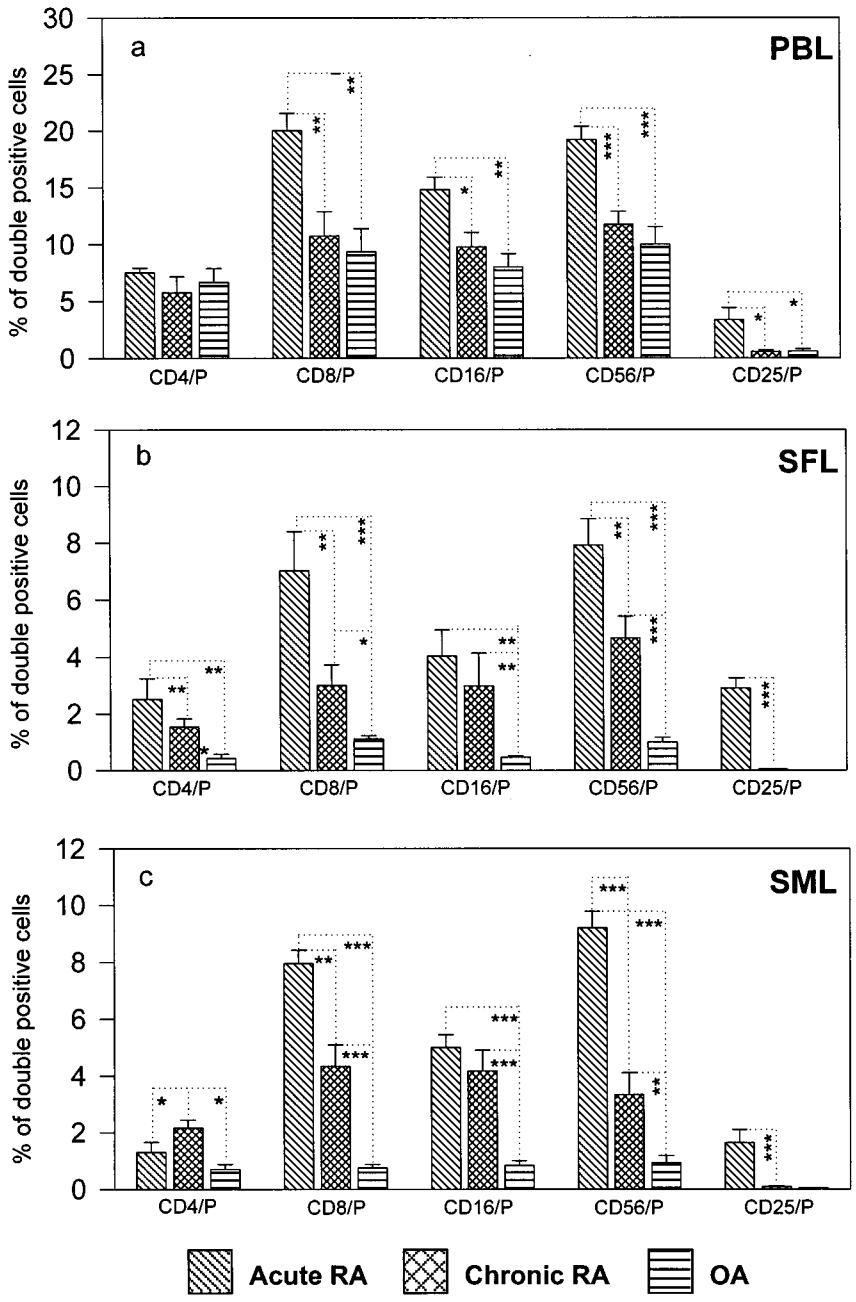

Figure 5. The percentage of double positive (perforin+ and surface marker+) lymphocytes (CD4+P+, CD8+P+, CD16+P+, CD56+P+, and CD25+P+) in peripheral blood (A), synovial fluid (B), and synovial membrane (C) from patients with acute RA, chronic RA, and OA. Results are mean ± SE. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

MFI values) generally correlates with the cytolytic potential of these cells[28]. We observed that during the course of the disease, significant oscillations of perforin expression are present — both the percentages of perforin positive cytotoxic T lymphocytes and the expression of perforin protein in the cells (MFI values). In our opinion these oscillations are not merely an epiphenomenon, they are the confirmation for the role of cytolytic lymphocytes in the pathogenesis of RA. High levels of expression of perforin in cytolytic cells (cytotoxic T lymphocytes and NK cells) in the SF and synovial membranes in acute phase RA suggest that these cells are probably functionally cytolytic cells and consequently can destroy the target cells that they recognize. Accordingly, the perforin mediated cytolytic pathway could be one of the major mechanisms of cell mediated cytotoxicity operating in RA joints.

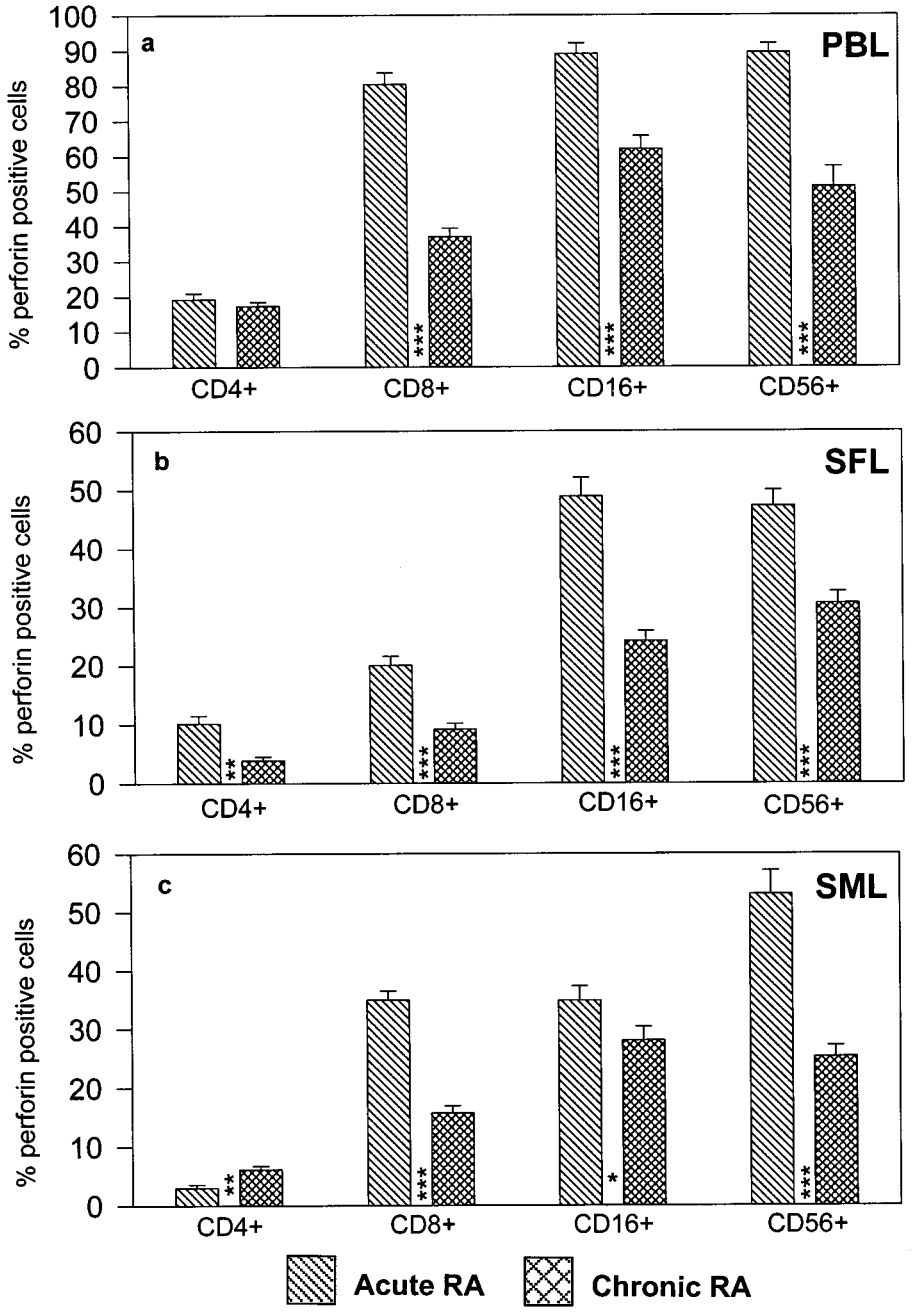

Figure 6. The frequency of perforin-expressing cells in T lymphocytes and NK cell populations (CD4+, CD8+, CD16+, and CD56+) in peripheral blood (A), synovial fluid (B), and synovial membrane (C) from patients with acute and chronic RA. Results are mean ± SE. *p < 0.05, **p < 0.01, ***p < 0.001.

Perforin expression in lymphocyte-infiltrating tissue *in vivo* correlates with the manifestation of T cell mediated cytotoxicity[18,19,29-32]. Concurrent opposite changes of perforin expression in MHC class I and class II dependent cytolytic cells (CD8+P+ and CD4+P+) shed new light on the potential pathogenetic role(s) of these subsets of cytolytic cells in the clinical course of RA. CD4+ cells are generally regarded as Th1 or Th2 cells producing either an inflammatory or noninflammatory network of cytokines. However, they can contain perforin and may also be cytotoxic[33]. This investigation provides the first direct evidence of the generation of cytolytic CD4+ cells containing perforin protein (CD4+P+) *in vivo* in the joints of patients with RA.

CD8+P+ cells are the predominant cytolytic population of cells, because most of the somatic cell targets (cells

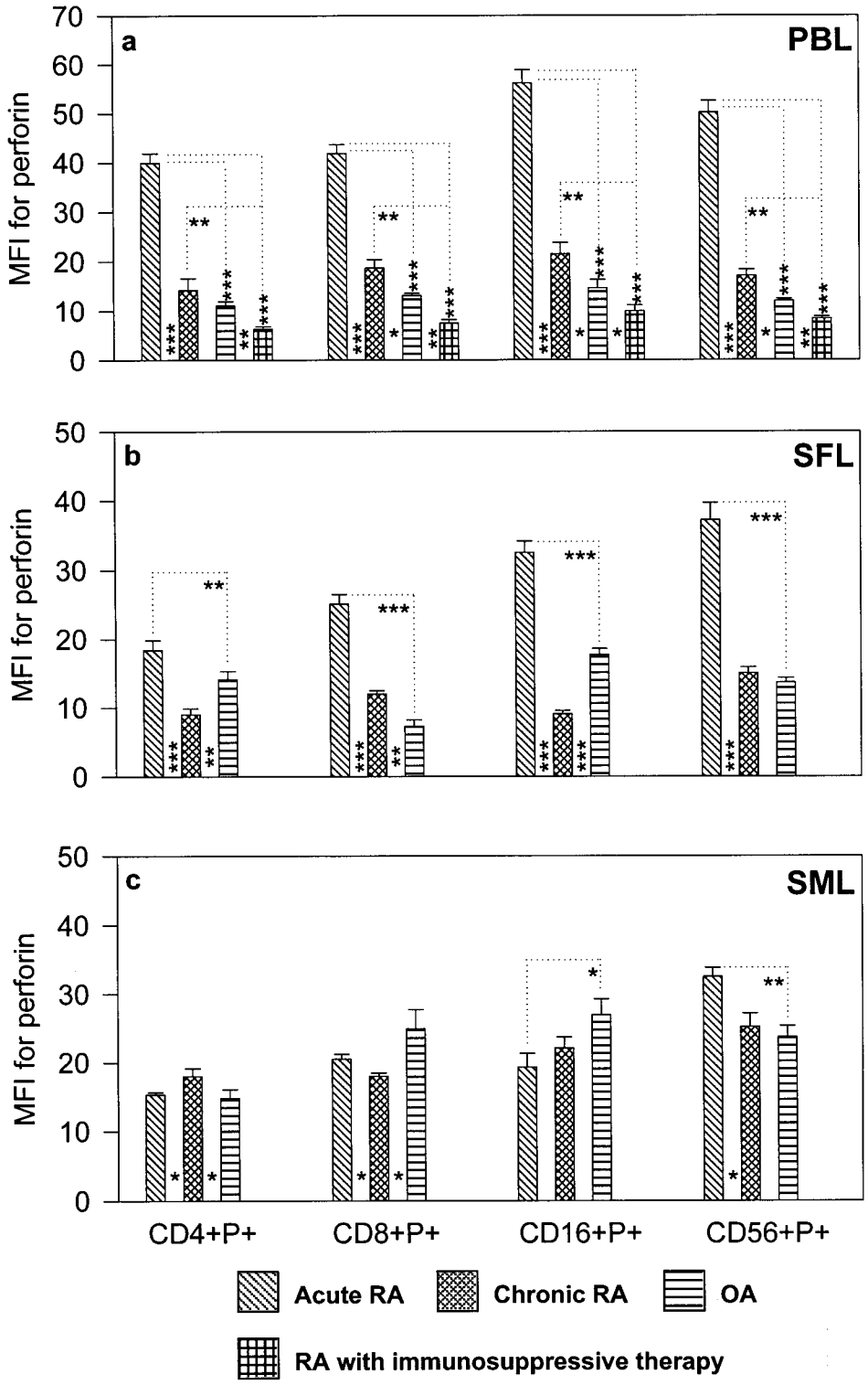

Figure 7. Level of perforin expression/cell (MFI values) for cytotoxic T lymphocytes (CD4+P+ and CD8+P+), NK cells (CD16+P+) in peripheral blood lymphocytes (A), synovial fluid lymphocytes (B), and synovial membrane lymphocytes (C) for patients with acute and chronic RA and with OA, and RA patients subjected to immunosuppressive therapy. Results are mean ± SE. *p < 0.05, **p < 0.01, ***p < 0.001.

infected by intracellular parasites, transformed and transplanted cells) express MHC class I antigens only. However, MHC class II antigens can be induced under the influence of inflammatory cytokines on a number of somatic cells

including inflamed synovium[34]. We have shown the presence of both CD8+ and CD4+ cytolytic cells expressing perforin. It has been shown that perforin and granzymes can degrade some components of basement membrane type IV collagen and open the way for migration of circulating activated T lymphocytes through the vascular endothelial basement membrane into inflammatory foci, consequently contributing to synovial hyperplasia[20]. Perforin and granzyme mRNA positive lymphocytes were found in SF of patients with RA[21]. Our observations of a highly significant increase of perforin positive cells simultaneously in the PB, SF, and synovial membrane of patients with acute phase RA confirm the possibility for these functions. Further, perforin and other cytolytic effectors, particulary FasL, may be important for the homeostasis of immune response at the local level, as in the joints. Recently, we described a new homeostatic role of perforin-expressing cytolytic cells in restraining the immune response through control of the afferent arm of the immune response by lysing antigen-presenting cells[35], which can be very useful at the local level in controlling and suppressing inflammatory processes. Oscillations in the level of perforin expression in various cell subsets, as we observed, should be investigated in light of these data.

The role of NK cells in the pathogenesis of RA is far from clear. In this investigation we found that the lowest frequency of CD16+ cells, but the highest frequency of CD56+ cells, was in SF in acute RA. The CD16 molecule is the Ig Fc-γ RIIIA receptor that is expressed on NK cells and that links cellular and humoral immunity. This molecule is exclusively responsible for antibody dependent, cell mediated cytotoxicity, and the CD16– NK subset lacks this function[36].

The lowest levels of percentages of perforin positive cells and contents of perforin per cell were found in the PB lymphocytes (all subpopulations) of patients subjected to immunosuppressive therapy with corticosteroids. We have reported that corticosteroid therapy for rejection crisis in kidney transplant recipients was followed by drastic decreases of these cells among PB lymphocytes and decreases of the content of perforin/cell in all subpopulations[32]. Corticosteroids are the treatment of choice in the therapy of acute RA because of broad antiinflammatory effects. In our opinion the antiperforin effects of corticosteroids additionally justify the use of these agents in therapy of the acute phase of the disease.

It is not known whether RA is initiated by an unrestrained inflammatory response to a microbial antigen, or is the result of an inappropriate autoimmune response to a joint-specific or ubiquitously expressed self-constituent[1,37]. Our results suggest direct involvement of functionally cytolytic cells in the pathogenesis of RA. There was no change in the percentage of CD8+ lymphocytes in the peripheral blood in the acute compared to the chronic phase of RA. However, we found a highly significant increase of CD8+P+ cells and of the frequency of perforin positive cells within the CD8+ population. Since the increase of the percentages of CD8+P+ cell populations in all compartments during the acute phase of RA was highly pronounced, it suggests that this may represent an increase in the frequency of cognate cytotoxic T lymphocytes that can recognize joint-specific targets. At least some of these cells could locally perform their cytolytic action by discharging their content of perforin, as shown by the lower MFI values for perforin in synovial membrane lymphocytes. It seems possible that a higher percentage of perforin positive cytolytic cells in peripheral blood compared to synovial membrane of patients with acute RA reflects the state of upstream initiation events in acute RA that remain to be elucidated.

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