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Expression of Cytolytic Mediators by Synovial Fluid Lymphocytes in Rheumatoid Arthritis

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To understand the role of cytolytic lymphocytes in the pathogenesis of rheumatoid arthritis, we investigated the expression of lymphocyte cytotoxicity mediators, perforin, and serine esterases, in lymphocytes derived from the synovial fluid of 15 patients with rheumatoid arthritis. Previous work has shown that $CD8^+$ lymphocytes that possess markers of activation appear to be present in rheumatoid arthritis (RA). By means of* in situ *hybridization techniques and immunohistochemical analysis, the authors show that perforin and two serine esterases (serine esterase 1/Hanukah factor/granzyme A, and serine esterase 2/granzyme B) are expressed by subpopulations of $CD8^+$ and $CD56^+$ lymphocytes obtained from synovial fluid. The presence of these cytotoxic mediators suggests a possible mechanism for tissue damage, and provides evidence implicating cytolytic lymphocytes in the pathogenesis of RA. (Am J Pathol 1992, 140:1261–1268)

Rheumatoid arthritis (RA) is considered to be immunologically mediated, with both humoral and cellular immunity participating in pathogenesis. The role of humoral immunity has been investigated in some detail.[1–4] However, a majority of lymphocytes in synovial fluid (SF) from affected joints are T cells. The phenotypes, the dynamics of migration, and the functional abnormalities of infiltrating SF lymphocytes have been the objects of intense investigation.[4–7] The T cells found in the synovial tissue and fluid are highly activated, and differ in many respects from circulating T cells in patients with RA and in normal subjects. The CD4/CD8 ratio of SF lymphocytes, for instance, is lower than that seen in peripheral blood.[5] Also, SF $CD8^+$ lymphocytes appear to be enriched in the T cytotoxic ($Leu15^-$) rather than the T suppressor ($Leu15^+$) cells.[8]

The presence of cytotoxic lymphocytes that are activated and selectively enriched in synovial fluid indicates a role for them in RA. However, demonstration that these cytolytic lymphocytes possess the wherewithal to inflict tissue damage is crucial for assigning them a substantiative role in the pathogenesis of RA.

Cytotoxic T lymphocytes (CTL) and natural killer (NK) cells are important components of the immune response to tumors and to viral and other intracellular infections, and have also been implicated in the pathogenesis of autoimmune diseases.[9] Several mediators produced by these effector lymphocytes have been described, including a potent, cytolytic pore-forming protein (PFP, perforin, or cytolysin). In the presence of calcium, perforin assembles tubular lesions in the target cell membranes.[10–13] A family of serine esterases (SE) has also been found in lymphocyte granules[14,15]; however, the function of these proteases is still unclear. Both PFP/perforin and SE are known to be associated almost exclusively with lymphocytes that have been activated to become competent killer cells by various stimuli, including interleukin-2 (IL-2), lectins and phorbol esters, and antibodies against CD3.[16]

Materials and Methods

Patients

Synovial fluid (SF) and synovial biopsy samples were obtained from 15 adult patients (5 men, 10 women, age 54

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± 6 yr) with classical or definite RA according to the criteria of the American Rheumatism Association.[17]

Isolation of Lymphocytes

Lymphocytes from synovial fluid were isolated by Ficoll-Hypaque (Pharmacia) density gradient centrifugation.[18] $CD8^+$ cells were separated using a cell sorter (FACSTAR).

Antibodies and RNA Probes

Rabbit polyclonal antisera,[19,20] prepared against purified murine perforin (antiserum #1), against an N terminal peptide of human perforin (#2) and against a human perforin cDNA expressed in *E. coli* as a fusion protein (#3) were used. We have previously shown[19,20] that these antisera specifically recognize human perforin in both immunoblot and immunofluorescence analyses, and do not react with human complement proteins. A rabbit antiserum to human SE-2 fusion protein[21] was provided by Dr. J. Trapani (Memorial Sloan Kettering Cancer Center). The following monoclonal antibodies (MAbs) were used for subset analysis: Leu11 (Becton-Dickinson), directed against the immunoglobulin Fc receptor CD16, which is expressed on natural killer cells and neutrophils;[22] NKH-1 (Coulter Immunology), the NK cell-specific reagent directed against CD56 of NK cells and large granular lymphocytes (LGL);[23] OKT3, OKT4, and OKT8 (Ortho Diagnostic Systems), directed against CD3, CD4, and CD8, expressed on T cells. Biotin-conjugated, fluorescein-conjugated, and rhodamine-conjugated, goat anti-mouse IgG and goat anti-rabbit IgG antisera were purchased from Boehringer-Mannheim.

^{35}S UTP-labeled sense and antisense RNA probes for human perforin were prepared from a 1.4 kb *Bam*HI—*Eco*RI fragment of the perforin cDNA clone HP10,[16] subcloned into the transcription vector pGEM-1 (Promega). Similarly, RNA probes were prepared from a 0.8 kb *Eco*RI-*Bam*HI restriction fragment of SE-1 cDNA[24] and a 0.9 kb *Eco*RI fragment of SE-2 cDNA,[21] both subcloned into pGEM-1.

Immunocytochemical Analysis

SF cells sedimented onto slides in the cytocentrifuge were fixed with 2% paraformaldehyde (RT, 20′) and permeabilized with acetone (−20 C, 5′). Immunofluorescence and immunoperoxidase staining were performed as described.[25] Briefly, slides were preincubated with 15% normal goat serum in phosphate-buffered saline (PBS) for 1 hour at 4 C, and incubated with indicated primary antibody for 1 hour at 4 C. Fluorochrome-conjugated secondary antibodies detected by fluorescence optics, or biotin-conjugated secondary antibodies detected with an ABC-immunoperoxidase kit (Vector Laboratories), were used to identify cells reacting with primary antibodies.

In Situ *Hybridization*

Mononuclear cells were collected by cytocentrifugation onto slides treated with Denhardt's solution (0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), fixed with 4% paraformaldehyde in PBS (RT, 20′), and stored at −20 C in a desiccator until use. Hybridization and washing were performed as described.[26] Briefly, slides were hybridized overnight, at 42 C in a humidified chamber, with 1 X 10^6 cpm ^{35}S-labeled RNA probe in 10 μl of hybridization solution (50% formamide, 1 X Denhardt's, 10% dextran sulfate, 0.3 mol/l NaCl, 80 μg/ml of salmon sperm DNA, 0.5 μg/ml of yeast tRNA in 10 mmol/l TRIS-HCl, pH 7.5). After hybridization, slides were washed twice in 2 X SSC/50% formamide and then twice in 2 X SSC (1 × SSC = 0.015 mol/l Na citrate, 0.15 mol/l NaCl, pH 7.0), treated with RNAse A and RNase T1 (Boehringer-Mannheim), dehydrated, dipped in emulsion (Kodak NTB2), and exposed at 4 C for 2 weeks. After development, slides were stained with Giemsa stain. Cells that had three times more grains than background were considered positive. Sense RNA probes were used as controls.

Electron Microscopic Examination

Synovial biopsy tissues were fixed for 2 hours at 4 C in 0.1 mol/l phosphate buffer (PB) (pH 7.0) with 2% glutaraldehyde and 1% paraformaldehyde. After rinsing twice in 0.2 mol/l PB, the tissues were postfixed in 0.1 mol/l PBS containing 2% OsO_4 for 2 hours at 4 C and rinsed again in 0.2 mol/l PB. The sections were then dehydrated by immersing successively in 50%, 70%, 95%, and absolute alcohol and embedded in Epon 812. Semithin sections (0.2 μm thick) were stained with Richardson's stain (Methyl Blue-AzurII). Ultrathin sections were contrasted with lead citrate and uranyl acetate.

Results

Synovial Biopsies

Semithin sections of synovial biopsies showed stromal edema accompanied by infiltration of lymphocytes containing numerous granules (Figure 1). Plasma cells were

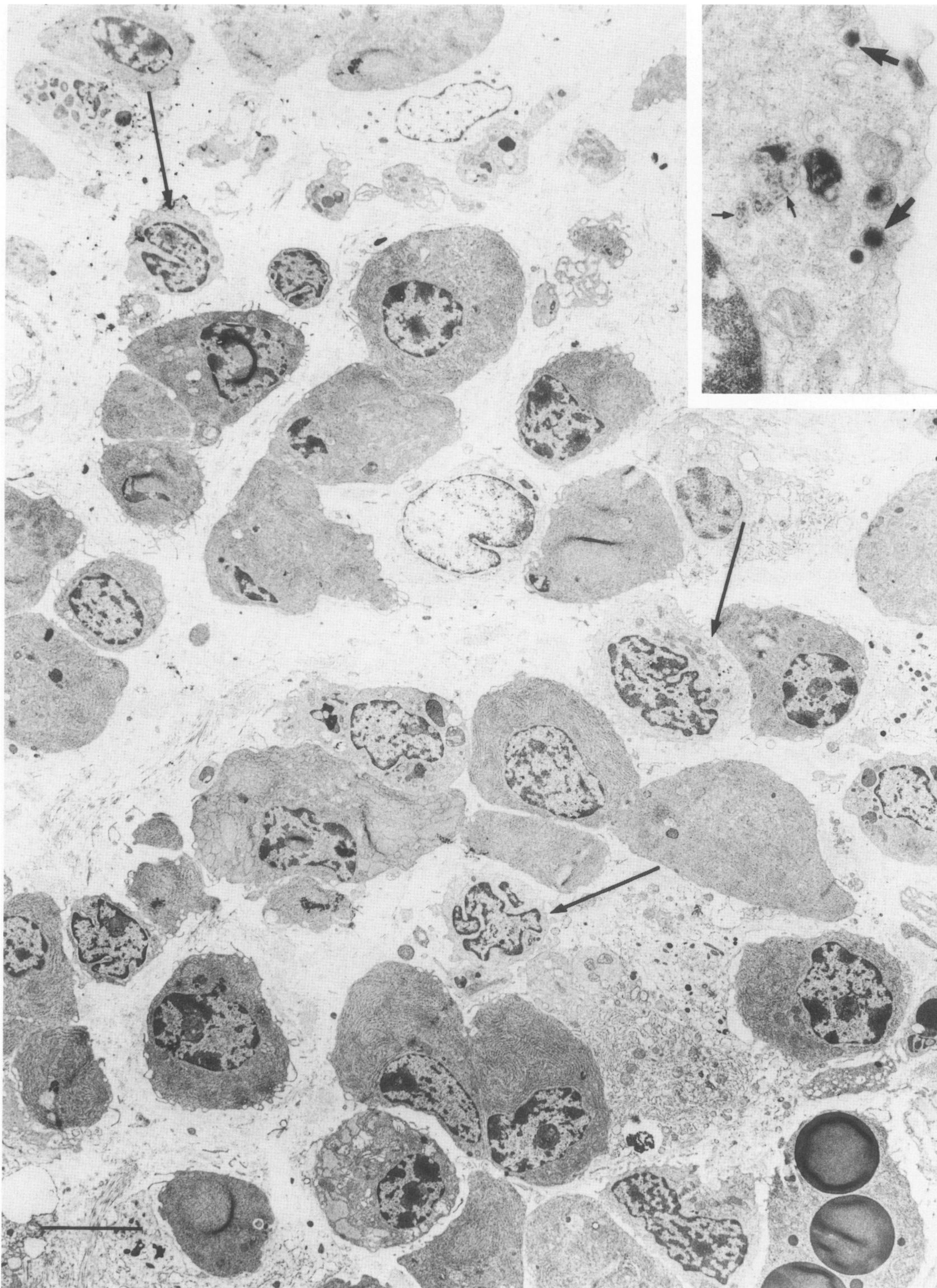

Figure 1. *Synovial biopsy from a patient with rheumatoid arthritis: ultrathin section stained with uranyl acetate and lead citrate. Notice numerous infiltrating T cells containing granules (long arrows) and plasma cells. Inset: Higher magnification view of the granules showing the dense core (large arrows) and small vesicles (small arrows). Bar = 8 μm.*

also present in these sections. At higher magnification (Figure 1, inset), the mononuclear cells resembled activated cytolytic lymphocytes, containing the characteristic granules. The granules consisted of a dense core with a relatively electron-lucent cap that contains several small vesicles.

Phenotypic Analysis of SF Mononuclear Cells

Phenotypic analyses performed on samples of SF from all 15 RA patients showed that 75% (range 69–80%) of the isolated mononuclear cells reacted with OKT3, a pan T anti-CD3 MAb. Double-labeling studies showed that these consisted of equal proportions of $CD4^+$ and $CD8^+$ cells (CD4/CD8 = 0.9 ± 0.3). In all patients 20% (range 15–26%) of the cells were $CD16^+$, whereas 10% (range 7–12%) were $CD56^+$. These results are in agreement with previous studies.[27,28]

Expression of Perforin and Serine Esterase Genes in SF Lymphocytes

Mononuclear lymphocytes that had been isolated from SF by Ficoll-Hypaque density gradient centrifugation were examined for the expression of perforin, SE-1, and SE-2 mRNA by *in situ* hybridization using ^{35}S-labeled sense and antisense RNA probes. The result shown in Figure 2 represents the typical hybridization pattern seen. The percentage and intensity of perforin-positive cells were lower than those seen for SE-1 (Table 1). Perforin-positive cells did not exceed 10% of the total lymphocyte population.

Production of Perforin and Serine Esterase 2 by SF Lymphocytes

We attempted to detect perforin and serine esterase proteins in SF lymphocytes by immunocytochemical analysis. Results obtained using anti-perforin antiserum 1 are shown in Figures 3 and 4; similar results were obtained with anti-perforin antisera 2 and 3.

Synovial fluid lymphocytes from arthritic joints were stained with antisera to perforin (Figure 3a, b) and SE-2 (Figure 3c, d), detected by immunofluorescence techniques (3a, c, e) and by immunoperoxidase procedures

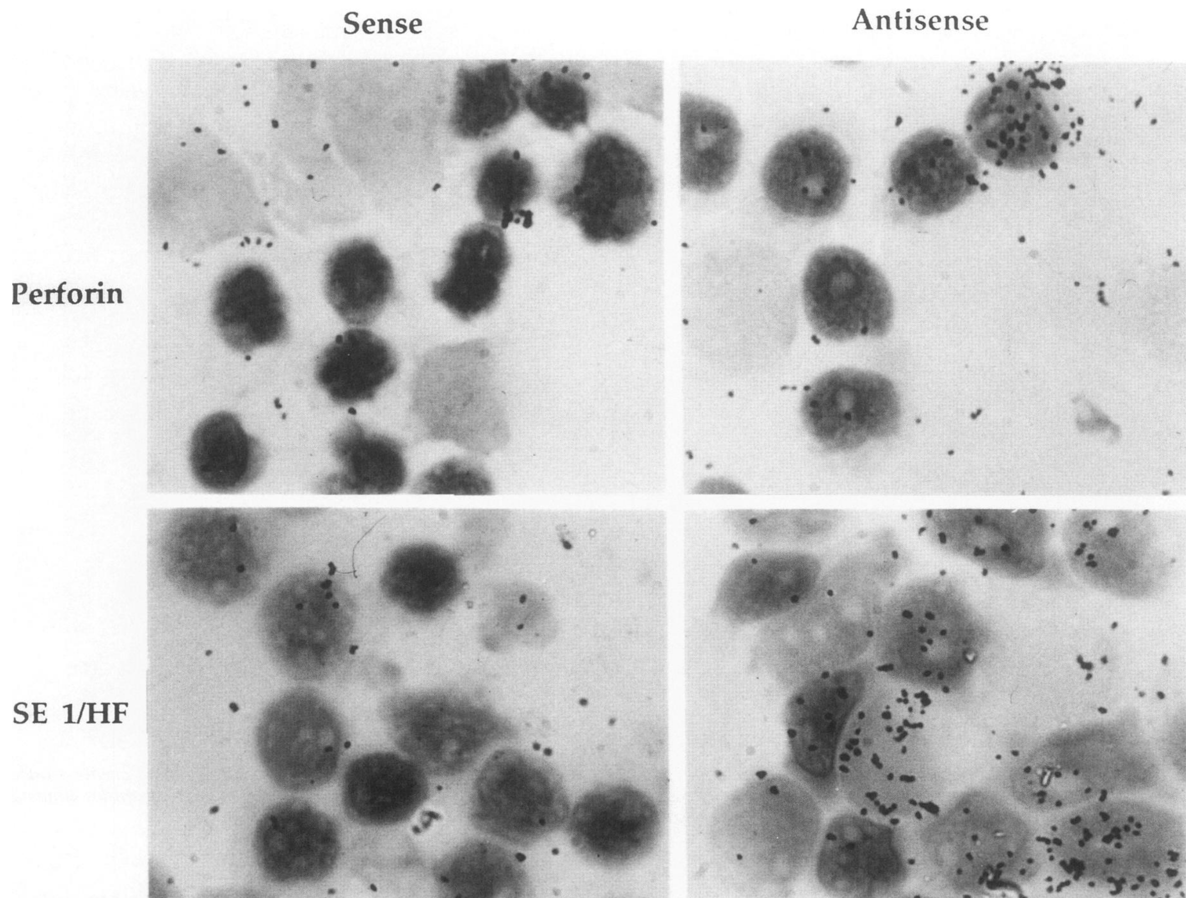

Figure 2. *Detection of perforin and SE-1 mRNA in synovial fluid lymphocytes by* in situ *hybridization. SF lymphocytes were hybridized with either sense probes (left) or antisense probes (right), specific for either perforin or SE-1/HF/GA. The results obtained with SE-2/GB probe were similar to those obtained with SE-1.*

Table 1. *Frequency and Intensity of Expression of Perforin and SE-1 by Synovial Fluid Lymphocytes*

Probe*	Percentage†	Number of grains per cell (mean ± SD)‡
Perforin	7.7 ± 1.53	23.0 ± 6.69
SE-1	14.7 ± 3.06	34.6 ± 13.22

* Antisense RNA probes were prepared and used as described in Materials and Methods.

† The percentage of cells positive for the given marker was determined by counting at least 300 cells from each of three sampled patients.

‡ The mean number of grains per cell was determined from at least 100 positively labeled cells collected from three patients.

(3b, d, f). Normal rabbit serum (Figure 3e, f), used as a control, did not stain SF lymphocytes. Although in the majority of the patients examined the number of perforin-positive cells did not exceed 10% of the total lymphocyte population, in three patients the number of perforin-staining cells ranged between 35–45%.

Perforin and serine esterases have been identified in lymphocytes with the killer phenotype (CTL or NK cells).[29,30] The phenotypes of SF lymphocytes expressing perforin and serine esterases were investigated with double-labeling immunofluorescence, using antisera to perforin, to SE-2, or specific cell surface markers. Only a minority (range 15–25%) of cells positive for perforin (Figure 4a) and SE-2 (Figure 4c) were also positive for CD56 (Figure 4b, d), a marker for NK cells. However, the majority (60%) of perforin$^+$ cells colabeled with OKT8 (CD8). Double-labeling immunofluorescence studies using anti-perforin antiserum #1 and a mixture of NKH-1, OKT-8, and LeuII mAb showed that a subpopulation (range 10–20%) of the perforin$^+$ cells was not recognized by NKH-1 (CD56), or OKT8 (CD8), or LeuII (CD16). In all patients examined, perforin$^+$ cells did not colabel with OKT4 (CD4).

Perforin Expression in SF Lymphocytes is enhanced by IL-2

Since we had shown that IL-2 and various mitogens are capable of inducing perforin and serine esterase expression in peripheral blood lymphocytes,[16] we asked whether the expression of perforin in SF lymphocytes could also be enhanced by IL-2. SF lymphocytes enriched for CD8$^+$ cells by cell sorting were cultured *in vitro* for short periods in medium containing 100 U/ml rIL-2 and then examined by immunofluorescence staining with anti-perforin antisera. Cells that had been stimulated with rIL-2 showed more frequent (Table 2) and stronger (not shown) expression for perforin than unstimulated cells, indicating that the expression of perforin was upregulated by rIL-2.

Discussion

Previous studies have shown that T lymphocytes from SF of affected joints are activated, as shown by increased expression of Ia, IL-2 receptor, and transferrin receptor, and their response to mitogens.[31–33] The CD4/CD8 ratio of SF lymphocytes is lower than in peripheral blood.[8,27,34] Although a majority of SF T lymphocytes are either CD4$^+$ or CD8$^+$, a small subset lack both these markers, and may represent γ/δ T cells.[18,35,36] These cells may account for some of the MHC-unrestricted (or NK-like) cytotoxicity observed in SF lymphocytes. γ/δ-T cells are known to respond to stress proteins, including mycobacterial heat shock protein.[37] Recently, SF lymphocytes have been shown to respond to stimulation with mycobacterial antigens; the responding cells appear to be γ/δ T cells.[38,39] Preliminary colabeling experiments indicate that a fraction of the perforin$^+$ SF lymphocytes are γ/δ-T

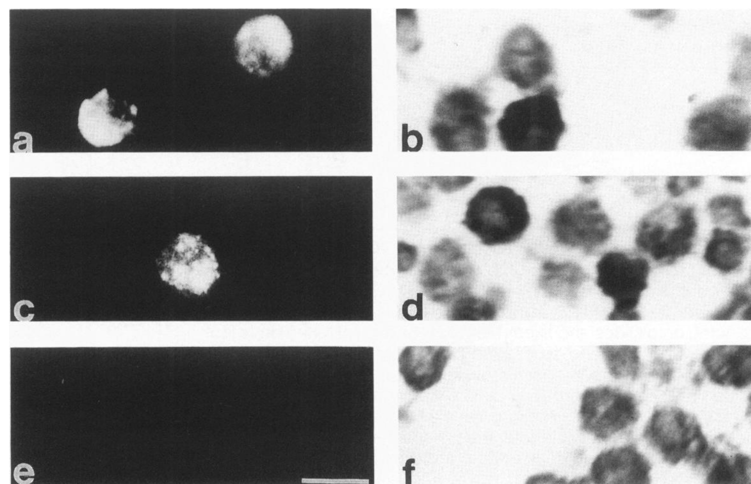

Figure 3. *Immunohistochemical detection of perforin and SE-2 in synovial fluid lymphocytes. SF lymphocytes were incubated with either anti-perforin antiserum 1* (a, b) *or anti-SE-2 antibody* (c, d) *and then visualized by either immunofluorescence* (a, c, e) *or immunoperoxidase* (b, d, f) *methods;* (e, f) *show control staining with preimmune serum. Similar results were obtained with anti-perforin antisera 2 and 3. Bar = 9 μm.*

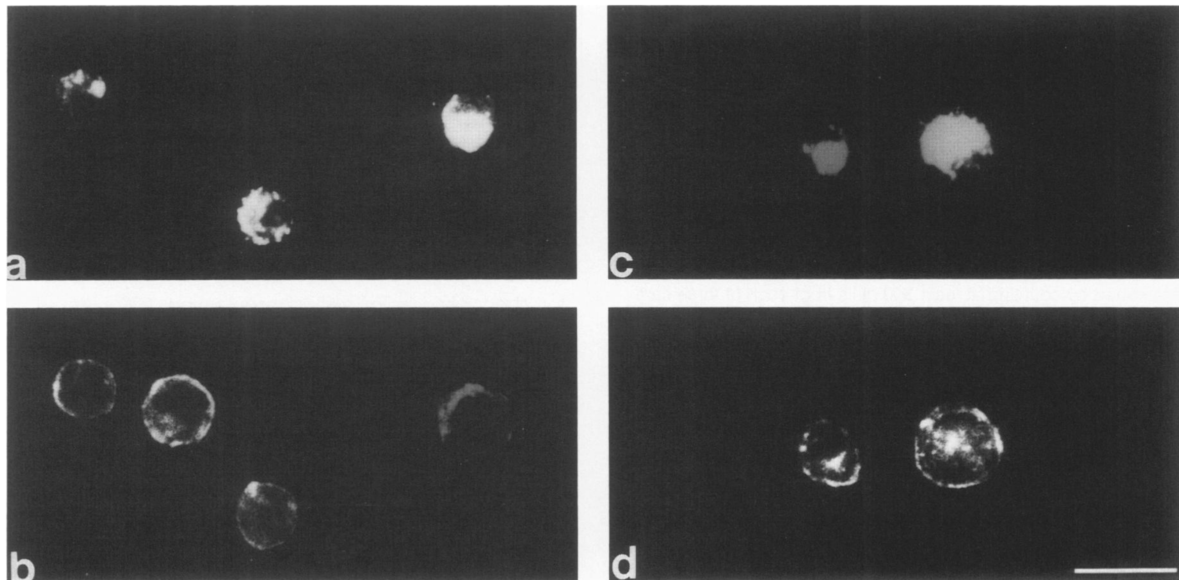

Figure 4. *Expression of perforin and SE by a subgroup of synovial fluid lymphocytes. SF lymphocytes were colabeled with either anti-perforin antiserum 1* **(a)** *or anti-SE-2* **(c)** *antiserum, and anti-CD56 mAb* **(b,d)** *and visualized by double immunofluorescence. Similar results were obtained with anti-perforin antisera 2 and 3. Bar = 16 μm.*

cells, an observation strengthened by recent reports[40,41] that both primary and cloned γ/δ-T cells can be induced to express perforin. NK cells have also been reported in SF of patients with RA.[18] The phenotypic characteristics of SF lymphocytes observed in our studies are in concordance with previous studies.

Perforin and serine esterases appear to be expressed only in NK cells and CTL activated by lymphokines or mitogens, and may therefore serve to distinguish activated cytolytic effector lymphocytes.[16,42] Based on the evidence of three specific polyclonal antiperforin antisera and on the basis of *in situ* hybridization with specific riboprobes, we show here that a well-characterized mediator of cytotoxicity, perforin, is present in SF lymphocytes. The presence of two serine esterases in SF lymphocytes is also demonstrated both by immunocytochemical analysis and *in situ* hybridization. Our observations close an important gap in the argument for a role for cytolytic lymphocytes in the pathogenesis of rheumatoid arthritis, as the SF lymphocytes were not shown in earlier studies to be cytotoxic or possess cytotoxic mediators. The expression of perforin and serine esterase genes in activated lymphocytes is downregulated by cyclosporin A and by glucocorticoids, both known to be immunosuppressive agents. Rheumatoid arthritis patients with severe inflammatory reactions refractory to conventional therapy are sometimes treated with these drugs. In a preliminary study of a few RA patients treated with glucocorticoids and cyclosporin A, we have observed decreased levels of perforin and serine esterase expression concomitant with clinical improvement. However, the number of patients studied so far is inadequate to permit statistical analysis, and the correlation between reduced expression of perforin/SE and clinical improvement is as yet only tentative.

Table 2. *Frequency of Cells Labeled with Perforin After Stimulation with IL-2*

Experiment number	+/− IL-2	% Perforin⁺ cells on day			
		0	2	4	6
1	+	5	15	16	21
	−	5	2	2	BD
2	+	3	18	21	30
	−	3	1	BD	BD
3	+	14	25	33	41
	−	14	7	4	2

Lymphocytes were obtained from three different patients, enriched for $CD8^+$ cells (over 96% purity) by cell sorting (Methods), cultured in RPMI-1640 supplemented with (+) or without (−) 100 U/ml of rIL-2 (Genzyme). Samples from the same experiment were collected onto slides and fixed (Methods) on the indicated days, but were stored frozen and processed for immunohistochemistry all at the same time. Perforin-specific antisera were used as the primary antibodies (Methods). Positive cells were scored by visual inspection and compared with background staining using preimmune antisera; 100 cells from each patient were counted. Samples for day 0 represent unstimulated cells prior to addition of rIL-2. BD = below detection levels.

Perforin has been found in CTL and NK cells infiltrating affected tissue in mice with viral infections and autoimmune diseases,[25,43,44] whereas SE-1 expressing lymphocytes have been reported in human dermatoses.[45] Our present study, however, shows for the first time that perforin and SE are coexpressed in human disease.

The pathogenesis of rheumatoid arthritis appears to be multifactorial, and cytolytic lymphocytes are not the only effector mechanism involved. However, several lines

of evidence suggest that cytolytic lymphocytes play an important role. The altered CD4/CD8 lymphocyte ratio, compared with peripheral blood, suggests that a subset selectively migrates into affected joints. The cells possess markers of activation, suggesting that they are unlikely to be passive participants in the inflammatory process. Moreover, the presence of cytotoxic mediators provides the lymphocytes with the means to cause tissue damage. Our data thus suggests that cytolytic lymphocytes are an effector mechanism in the pathogenesis of rheumatoid arthritis.

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