Expression of CD69 antigen on synovial fluid T cells in patients with rheumatoid arthritis and other chronic synovitis

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Abstract

Objectives—The expression of the CD69 antigen on synovial fluid and peripheral blood lymphocytes was studied in 12 patients with rheumatoid arthritis (RA), five subjects with other forms of chronic synovitis, and on the peripheral blood lymphocytes of 15 patients with systemic lupus erythematosus (SLE) and immune vasculitis.

Methods—The CD69 antigen and other activation markers (HLA-DR, interleukin 2 receptor (IL-2R), transferrin receptor) were measured by cytometric analysis. In patients with RA soluble IL-2R was determined by enzyme linked immunosorbent assay (ELISA).

Results—The percentage of T cells bearing CD69 was significantly increased in synovial fluid from patients with RA (30.3 (13)%) and other chronic synovitis (18 (9)%). The expression of CD69 on peripheral blood lymphocytes of patients with RA, other chronic synovitis, and SLE and immune vasculitis was within the normal range 2.1(1.2)%. According to previously published work, a high proportion of synovial fluid T cells are HLA-DR positive (64.2 (12.4)% in synovial fluid from patients with RA and 61 (1.2)% in synovial fluid from patients with other chronic synovitis). Transferrin receptor expression on synovial fluid was upregulated compared with that on peripheral blood.

The increase of IL-2R expression on synovial fluid lymphocytes *v* peripheral blood was not significant; the quantitative determination of soluble IL-2R levels gave a mean value of 921 (351) U/ml in synovial fluid of patients with RA, 672 (229) U/ml in the serum of the same patients, and 273 (100) U/ml in serum from normal subjects.

Conclusions—Synovial fluid lymphocytes are in a different functional state than peripheral blood lymphocytes. CD69 antigen is an interesting cellular marker which should be studied in patients with chronic synovitis. The unusual expression of the activation antigens and the sequence of their appearance require further study.

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interleukin 2 (IL-2), insulin and transferrin receptors, 4F2 and EA-1 antigens appear early, before DNA synthesis, whereas HLA-DR, T10, Ta1, VLA1, and TLI SA1 appear later (after two to six days) on activated cells.

Some early activation antigens, also called activation inducer molecules, have been found to be associated with earlier stages of *in vitro* T cell activation. One of them, the CD69 antigen, is a phosphorylated 28-32 kilodalton disulphide linked homodimer. It is induced within two hours of the stimulation of TCR/CD3 or from the direct activation of protein kinase C by phorbol esters¹⁻³ and precedes the expression of CD25, HLA class II, and transferrin receptors during T cell activation. It is known that small resting peripheral blood lymphocytes are negative for CD69 and that 20-30% of resting human thymocytes are stained by monoclonal antibodies to CD69.^{4 5}

Much has been published on peripheral blood lymphocyte activation markers *in vitro*, but studies comparing the kinetics of expression of the various membrane molecules evaluated in parallel during the activation process have not been reported.

Biselli *et al*⁶ have shown that CD69 expression on human peripheral blood activated T lymphocytes peaks at 24 hours, and that an average 40% of cells are positive at 168 hours; IL-2 and transferrin receptor expression peak at 48-72 hours and remain at a plateau value for 72 hours.

Several activation antigens have been measured in synovial fluid T cells from patients with rheumatoid arthritis (RA) to clarify the role of activated T cells in the pathogenesis of the disease. Most of these investigations analyse the expression of IL-2 receptor (IL-2R), HLA-DR antigens and transferrin receptor, and few data are available on the behaviour of early activation antigens in autoimmune diseases.^{7 8} In this work we studied the expression of some activation markers and, in particular, the CD69 antigen in the synovial fluid and peripheral blood of patients with RA and other kinds of chronic synovitis.

Patients and methods

PATIENTS

Peripheral blood and synovial fluid samples were obtained at the same time from 12 patients (nine women, three men) with active RA according to the American Rheumatism Association 1987 revised criteria.⁹ Ten of them were positive for IgM-19S rheumatoid factor.

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The mean (SD) age of the patients was 53.9 (9.6) years and the mean disease duration was 10 (6) years.

All patients were receiving non-steroidal anti-inflammatory drugs (NSAIDs). In addition, two patients were receiving treatment with gold salts, four with low doses of corticosteroids (prednisone, 5 mg daily), and one with methotrexate.

CONTROLS

Blood samples from 15 healthy donors matched for age and sex were studied as normal controls. Blood and synovial fluid samples were obtained from patients with other chronic synovitis (one seronegative polyarthritis, four juvenile chronic arthritis). The expression of the CD69 marker on peripheral blood lymphocytes was studied in

15 patients with systemic lupus erythematosus (SLE) and immune vasculitis; disease activity was assessed by a scoring system of clinical and laboratory features as reported previously.¹⁰

CELL PREPARATION, MONOCLONAL ANTIBODIES AND FLOW CYTOMETRIC ANALYSIS

Synovial fluid and peripheral blood samples were drawn into heparinised tubes. Synovial fluid was treated with hyaluronidase (Sigma) for 20 minutes at 37°C to reduce viscosity, then mixed with an equal volume of RPMI 1640 and centrifuged at 400 *g* for 20 minutes. Resuspended cells of synovial fluid and peripheral blood samples were separated by Lymphoprep (Nyegaard) density gradient centrifugation. Synovial fluid and peripheral blood mononuclear cells were analysed by a panel of monoclonal antibodies (antibodies to CD3, HLA-DR, CD25 (IL-2R), CD71 (transferrin receptor), and CD69 (Becton-Dickinson)) tagged with either fluorescein isothiocyanate (FITC) or phycoerythrin.

The optimum dilution of monoclonal antibodies was established in preliminary experiments and the cytometric analysis was performed on a Becton Dickinson FACScan. The tubes were immediately applied to the flow without paraformaldehyde fixation. A total of 10 000 cells was counted in each analysis; they were gated for lymphocyte characteristics to minimise interference from other cell types such as monocytes or dead cells and they were scored as positive or negative according to their fluorescence (red or green). The specific percentages of positive cells were obtained by subtracting the background fluorescence determined by FITC or phycoerythrin irrelevant mouse IgG (Becton-Dickinson), applied instead of the specific antibody.

Figure 1 Expression of activation markers on synovial fluid (SF) and peripheral blood (PB) lymphocytes of patients with rheumatoid arthritis (RA) and other chronic synovitis (OCS). CD69: the difference between CD69 expression on synovial fluid and peripheral blood T lymphocytes from patients with RA was statistically significant ($p < 0.02$). Interleukin 2 receptor (IL-2R): the comparison between IL-2R expression on synovial fluid and peripheral blood lymphocytes in the two groups of patients and with normal peripheral blood was not significant. Transferrin receptor (Tr-R): the difference between transferrin receptor expression on synovial fluid and peripheral blood cells was statistically significant ($p < 0.01$). HLA-DR: the difference between the expression of HLA-DR on synovial fluid and peripheral blood of the same patients with RA and between peripheral blood of patients with RA and normal peripheral blood values was statistically significant ($p < 0.002$ and $p < 0.02$ respectively). The horizontal dotted area represents the normal range.

EVALUATION OF SOLUBLE IL-2R

The quantitative determination (enzyme linked immunosorbent assay (ELISA)) was performed for the peripheral blood and synovial fluid of patients with RA using a commercial kit (Cellfree; T Cell Science). A standard curve was prepared from four IL-2R standards and the concentrations in the samples determined from the standard curve.

STATISTICAL ANALYSIS

All results were expressed as mean (SD) values. Statistical significance was determined using the Wilcoxon signed rank test for comparison of peripheral blood and synovial fluid values of patients with RA and the Mann-Whitney *U* test to compare the RA group with the control or other chronic synovitis groups.

Results

The percentage of CD3+ cells was in the normal range in the synovial fluid and peripheral blood of all patients (73.3 (10)% in synovial fluid from patients with RA, 78.7

Figure 2 CD69 expression on synovial fluid and peripheral blood lymphocytes from a patient with rheumatoid arthritis. (A, C) Control antibody staining on synovial fluid and peripheral blood lymphocytes respectively. The right hand panels compare CD69 expression on synovial fluid (B) (32.9%) and peripheral blood (D) (2.3%) lymphocytes.

(1.7)% in synovial fluid from patients with other chronic synovitis, 71 (8)% in peripheral blood from patients with RA, 76 (6.8)% in peripheral blood from patients with other chronic synovitis, and 71.3 (6)% in normal peripheral blood). The expression of activation markers on T cells was evaluated as a percentage of the total number of CD3+ cells.

Figure 1 gives the results of HLA-DR, IL-2R, and transferrin receptor expression; these do not differ significantly from those reported elsewhere.

The expression of CD69 was high on synovial fluid T cells from patients with RA (30.3 (13)% compared with peripheral blood from patients with RA and normal peripheral blood values (3.2 (2.9) and 2.1 (1.2)% respectively). The difference between mean values in synovial fluid and peripheral blood from patients with RA was statistically significant ($p < 0.02$) (figs 1 and 2). The expression on synovial fluid cells from patients with other chronic synovitis was 18 (9)% and it was 1.7 (1.6)% in the peripheral blood of the same patients (fig 1).

Expression of CD69 on peripheral blood T lymphocytes from 15 patients with SLE and immune vasculitis was 2 (0.9)%.

The quantitative determination of soluble IL-2R levels, performed only in patients with RA, gave a mean value of 921 (351) U/ml in

synovial fluid from patients with RA, 672 (229) U/ml in the serum of the same patients, and 273 (100) U/ml in normal serum samples.

Discussion

The role of activated T cells in the pathogenesis of RA has been studied by the evaluation of activation markers such as IL-2R, the HLA-DR antigen, and transferrin receptor on peripheral blood and synovial fluid lymphocytes.^{11 12} A high expression of HLA-DR and a relative paucity of IL-2R positive and transferrin receptor positive cells on synovial fluid T lymphocytes have been reported. Late activation antigens have been found on synovial fluid T cells¹³ and a dissociation between the expression of IL-2R and VLA-1 on synovial fluid lymphocytes has been reported.¹⁴ We have already shown the increased expression of CD69 on synovial fluid lymphocytes in patients with RA.⁷ These data have been confirmed on synovial fluid and synovial membrane T cells.⁸ The aim of this study was to verify the expression of CD69 with respect to other surface markers that appear later in the T cell activation pathway.

Normal values of this marker were found on peripheral blood lymphocytes from patients with RA, other chronic synovitis, and patients with systemic immune inflammation such as

clinically active immune vasculitis and SLE. In contrast, an increased expression was found on synovial fluid lymphocytes from patients with RA and those with other chronic synovitis, suggesting that CD69 may represent an important T cell activation marker in the synovial environment.

The increase of CD69 could be due either to a continuous antigen presentation mechanism at the site of the immune inflammation, or to a selective recruitment in the joints of systemically activated T cells. Indeed, such an event has been already shown in different forms of chronic inflammation using other T cell markers.^{15 16}

Of the markers considered in this study, CD69 and HLA-DR reached the highest state of positivity. In contrast, IL-2R expression was not significantly increased as determined by cytometric analysis. As CD69 is the first cell surface glycoprotein detected after lymphocyte activation, it is not clear why we did not find a parallel increase of IL-2R on the synovial fluid T lymphocytes of patients with RA. It is possible that IL-2R, which is expressed transiently, disappeared from the cell surface. After the activation process, part of the 55 kilodalton subunit is released from the membrane and becomes the soluble receptor for IL-2. The quantitative determination of soluble IL-2R showed increased values in synovial fluid compared with peripheral blood for patients with RA and normal subjects. Another explanation is that the expression of CD69 may trigger an alternative T cell activation pathway. Lastly, it is also possible that the CD69 antigen characterises a still unknown CD69+ IL-2R- HLA-DR+ population which might play an important part in the pathogenesis of immunophlogosis. Further studies are in progress in our laboratory to investigate these hypotheses.

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