Clin. exp. Immunol. (1991) **84**, 78–82 ADONIS 0009910491001071

Elevation of a γδ T cell subset in peripheral blood and synovial fluid of patients with rheumatoid arthritis

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(Accepted for publication 26 October 1990)

SUMMARY

We examined the levels of TcRδ1$^+$ T cells (total γδ T cell) and δTCS1$^+$ (γδ T cell subset) T cells in the peripheral blood (PB) and synovial fluid (SF) of 16 patients with rheumatoid arthritis (RA) and compared them to the levels in PB of patients with Felty's syndrome (FS) and 21 healthy control subjects (NML). Synovial fluid from eight patients with seronegative spondyloarthropathies (SSA) was also examined. The results demonstrated elevated levels of the δTCS1$^+$ subset in the PB of RA and FS patients relative to NML ($P < 0.05$). No such differences were observed in the levels of PB TcRδ1$^+$ T cells. The results did not appear to reflect a non-specific inflammatory response since δTCS1 T cells were elevated in the SF of RA patients relative to SSA SF and NML PB. δTCS1 T cells in SSA PB and SSA SF were comparable to NML PB. TcRδ1$^+$ T cell levels in RA SF were higher than SSA SF levels but were comparable to those of NML PB. Taken together, the results support a pathogenic role for δTCS1$^+$ T cells in RA.

Keywords T cells γδ T cells rheumatoid arthritis synovial fluid

INTRODUCTION

Two distinct types of T cell, bearing different antigen receptor molecules (TcR) on the cell surface, have been well established in man (Strominger, 1986; Brenner *et al.*, 1986). The bulk (about 95%) of peripheral blood T cells express αβ TcR as disulphide-linked heterodimeric polypeptides in association with the CD3 protein complex. The αβ T cells contain the classic helper/inducer (CD4$^+$) subset and the cytotoxic/suppressor T cell (CD8$^+$) subset.

The minority (about 5%) of peripheral blood T cells, however, express γδ TcR, which possess a similar protein structure to that of αβ TcR. The two molecules exhibit moderate homology in DNA sequence to each other (Brenner *et al.*, 1986; Moingeon *et al.*, 1986). The γδ T cells appear to be functionally competent; they manifest cell-mediated cytotoxicity restricted by both HLA (Moretta *et al.*, 1989) and non-HLA systems (Borst *et al.*, 1987) and mount proliferative responses to mitogenic stimuli, such as monoclonal antibodies against CD3 and γδ TcR (Wu *et al.*, 1988). Bucy, Chen & Cooper (1989) recently reported that γδ T cells exhibited preferential homing to the sinusoidal areas (red pulp of the spleen) and into the epithelial layer of the intestine in humans. In contrast to earlier reports (Lanier *et al.*, 1986) that γδ T cells are mostly CD4$^-$CD8$^-$, these investigators observed that 30% of γδ T cells in the blood and 50% γδ T cells in the spleen are CD8$^+$. The γ and δ polypeptides each contain a variable and constant domain encoded in different gene segments (V, variable; d, diversity; J, joining; and C, constant) that join through gene rearrangement during ontogeny (Brenner, Strominger & Kraugel, 1988). There are multiple and distinct V genes in the germ-line DNA for both the γ and the δ chains. Monoclonal antibodies produced against the Vγ and Vδ were reported (Faure *et al.*, 1988).

Several monoclonal antibodies (MoAbs) against the δTcR protein were found to be useful in studying the morphological and functional heterogeneity of γδ T cells. The MoAb TcRδ1, which is reactive with the constant domain of the δ chain, stains all γδ T cells (Band *et al.*, 1987) while MoAbs δTCS1 and BB3 reactive with Vδ, and Vδ2 gene products respectively, identify approximately 20% and 70% of peripheral blood γδ T cells in normal subjects respectively (Wu *et al.*, 1988; Ciccone *et al.*, 1988). Among the γδ cells, the δTCS1 T cell subset appears most interesting in that it is motile and exhibits a non-typical lymphocyte morphology (Grossi *et al.*, 1989).

The involvement of T cells in the pathogenesis of rheumatoid arthritis (RA) is well documented (Decker *et al.*, 1984). Previously, it was reported that T cells infiltrating the synovial membrane of rheumatoid patients are predominantly CD3$^+$-CD4$^+$ and presumably are αβ$^+$ T cells (Cush & Lipsky, 1988). The possibility that γδ T cells might be of pathogenic significance in RA was supported by a recent preliminary report which

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demonstrated elevated levels of $CD4^-CD8^-$ T cells in the synovial fluid and synovial membrane of some patients with RA (Brennan *et al.*, 1988) and in the blood of some patients with Felty's syndrome (FS) (Brennan *et al.*, 1989).

In this report, we used two direct enumerating MoAb reagents for the study of $\gamma\delta$ T cells in RA. Our results suggest that TCS$\delta 1^+$ (V$\delta 1^+$) T cell subset, not the total $\gamma\delta$ T cells, is significantly elevated in the peripheral blood and synovial fluid of patients with RA and FS.

MATERIALS AND METHODS

Patient selection

Sixteen patients with definite or classical RA (Ropes *et al.*, 1958) and 10 additional patients with FS seen in the Rheumatic Disease Unit of the Wellesley Hospital were studied. Controls consisted of 21 healthy volunteers from the Wellesley Hospital and T Cell Sciences, USA. Twelve of the controls were age-matched for the RA group. Since the data obtained from the age-matched control subjects from the two groups were comparable to those not matched for age, all control data were pooled. In addition, eight patients with seronegative spondyloarthropathies (SSA) (five with psoriasis, one with Reiter's syndrome and two with ankylosing spondylitis) were examined. All RA patients were receiving non-steroidal anti-inflammatory drugs (NSAIDs). One patient was receiving NSAIDs only. In addition to NSAIDs, the RA patients were receiving the following: remittive agents (11): gold (five), chloroquine (two) and penicillamine (four); cytotoxic agents (two): methotrexate (two): prednisone alone (one); and prednisone in combination with remittive/cytotoxic agents (two): prednisone in combination with penicillamine (one) and prednisone in combination with methotrexate (one). Patients with FS were treated as follows: no medications (two); remittive agents: gold (four); cytotoxic agents: Imuran (one), prednisone alone (two), and prednisone in combination with Imuran (one). All SSA patients were receiving NSAIDs while four patients were receiving remittive drugs (gold) in addition. None was receiving steroids or cytotoxic agents. Patients receiving steroids were taking $\leqslant 10$ mg per day.

Clinical evaluation

A number of clinical variables were examined in each patient, including disease duration, number of actively inflamed joints (defined as those with tenderness or effusions), ESR (Westergren), and medications.

Preparation of mononuclear cells from peripheral blood and synovial fluid

Equal volumes of anticoagulated whole blood and Sepracell-MN (Sepratech Corp.) were added to a centrifuge tube. After gentle mixing, the tube was centrifuged at 1500 ***g*** at room temperature for 20 min. After density separation, mononuclear cells found in the opalescent compact band just below meniscus were collected. They were washed twice by mixing the cells with four volumes of phosphate-buffered saline/bovine serum albumin (PBS–BSA) (0·1% w/v), and the mixture subjected to centrifugation at 300 ***g*** for 10 min per wash.

Synovial fluid cells were first pelleted and resuspended in PBS. Mononuclear cells were then isolated by density gradient centrifugation.

Immunofluorescence staining of cell surface markers

All patient cells were phenotyped by flow cytometry using the Ortho Diagnostics Cytofluorograph II (Ortho Diagnostics, Raritan, NJ). Fluorescein-conjugated MoAbs specific for various cell surface determinants were used for a direct immunofluorescence staining procedure. Briefly, $2–5 \times 10^5$ mononuclear cells were suspended in 100 μl PBS with 0·2% BSA and 0·05% sodium azide (flow buffer) at 4°C. Conjugated MoAbs (100 ng) were added to the cell suspension, mixed well, and incubated for 30 min at 4°C. The stained cells were washed with flow buffer twice and finally resuspended in the same buffer for cytometric analysis. The MoAbs utilized for immunofluorescence include the following from Ortho Diagnostics Systems: OKT3, OKT4, OKT8, which recognize the CD3, CD4 and CD8 determinants, respectively; and from T cell Sciences, Inc. (Cambridge, MA) TcR$\delta 1$ and δTCS1, which recognize all human $\gamma\delta$T cells and a subset of them, respectively. The $\gamma\delta$T cells were expressed as per cent of total $CD3^+$ T cells.

Statistical analysis

The arithmetic mean values for each of the patient sample groups was calculated and significant differences analysed with the Wilcoxon rank sum test. Spearman's correlation coefficients were obtained between levels of TcR$\delta 1^+$ and δTCS1^+ T cells and between these T cell subsets and variables of disease activity. Where indicated, χ^2 analysis was employed.

RESULTS

$\gamma\delta$ T cells in peripheral blood

We first examined the levels of TcR$\delta 1^+$ T cells and δTCS1^+ T cells (as percentage of $CD3^+$) in the peripheral blood of patients with RA and compared them to the levels in peripheral blood of patients with FS and healthy control subjects (NML). The results revealed that the mean ($\pm$s.e.) levels of peripheral blood δTCS1^+ T cells in RA ($1·8 \pm 0·4$) and FS ($4·5 \pm 1·6$) were significantly elevated relative to NML ($0·8 \pm 0·2$) ($P = 0·007$ and $P = 0·006$, respectively) (Fig. 1b). No such differences were observed in TcR$\delta 1^+$ T cell levels (Fig. 1a). The proportion of patients with elevated δTCS1 cells (>2 s.d above the normal mean) in the FS group (5/10) and RA population (4/16) was statistically greater than that in the group of healthy subjects (1/20) with $\chi^2 = 8·9$ ($P = 0·003$) and 2·9 ($P = 0·08$) respectively. Moreover, in patients with FS who demonstrated elevated levels of TcR$\delta 1^+$ T cells, δTCS1^+ T cells accounted for the majority of TcR$\delta 1^+$ T cells, while in normals δTCS1^+ cells accounted for $< 20\%$ of the TcR$\delta 1^+$ T cell population (data not shown). These results suggest a possible correlation between the level of δTCS1^+ T cells and TcR$\delta 1^+$ T cells in FS. Indeed, the level of δTCS1^+ T cells correlated strongly with the level of TcR$\delta 1^+$ T cells in FS ($r = 0·98$, $P = 0·001$) while no such correlation was observed in RA and NML ($P < 0·05$ for both) (data not shown).

The level of peripheral blood δTCS1^+ T cells was next examined as a proportion of TcR$\delta 1$ cells. The results revealed an elevation in the mean ($\pm$s.e.) ratio of δTCS1/δTcR$\delta 1$ in both RA ($0·4 \pm 0·08$) and FS ($0·6 \pm 0·07$) relative to NML ($0·2 \pm 0·05$) ($P = 0·02$ and $P = 0·005$, respectively). We also evaluated the relationship between the peripheral blood levels of δTCS1^+ and TcR$\delta 1^+$ T cells in RA and FS and the clinical variables outlined above (See Materials and methods). While patients with FS and RA could be differentiated from normals by measuring of

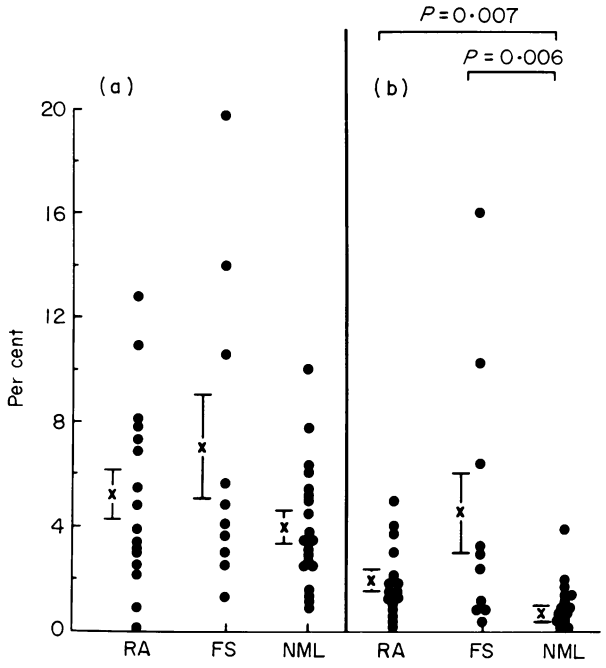

Fig. 1. Levels of TcRδ1-bearing T cells (a) and δTCS1-bearing T cells (b) in the peripheral blood of patients with rheumatoid arthritis (RA), Felty's syndrome (FS) and control subjects (NML). Arithmetic mean values are represented by the 'X' and the error bars represent ± 1 s.e.m.

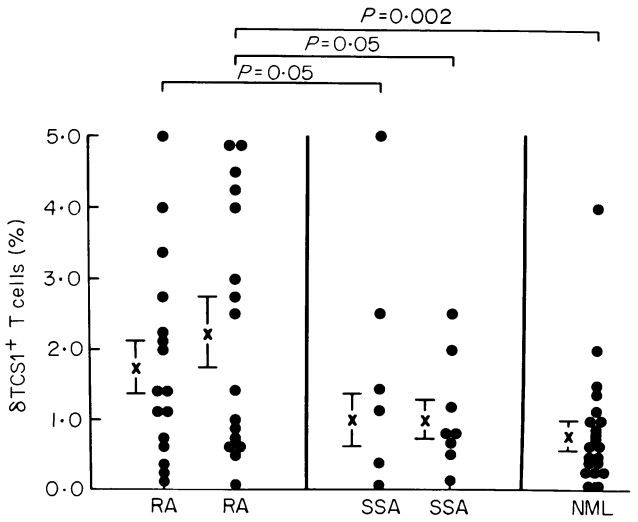

Fig. 2. Levels of δTCS1-bearing T cells in the peripheral blood (left) and synovial fluid (right) of patients with rheumatoid arthritis (RA) and seronegative spondyloarthropathies (SSA) in relation to peripheral blood levels in control subjects (NML). Arithmetic mean values are represented by the 'X' and the error bars represent ± 1 s.e.m.

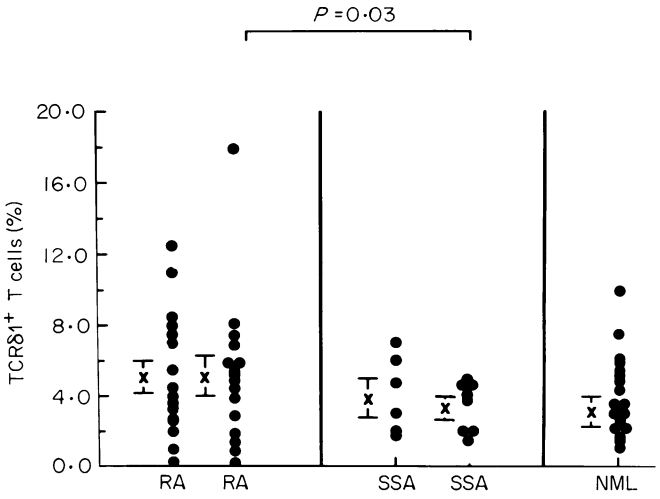

Fig. 3. Levels of TcRδ1-bearing T cells in the peripheral blood (left) and synovial fluid (right) of patients with rheumatoid arthritis (RA) and seronegative spondyloarthropathies (SSA) in relation to peripheral blood levels in control subjects (NML). Arithmetic mean values are reperesented by the 'X' and the error bars represent ± 1 s.e.m.

TCRδ1+ and δTCC1+ T cells, they could not be rank ordered with respect to their disease activity with these T cell counts in this study (data not shown). There were no obvious effects of remittive or cytotoxic agents on the γδ T cell levels. The sample size examined with each drug category, however, prevented statistical comparisons.

γδ cells in synovial fluid

Since elevated levels of peripheral blood δTCS1+ T cells were observed in RA, we next asked whether the increased peripheral blood levels could be a reflection of a local increase in δTCS1+ T cells within the affected joint. Similar to RA peripheral blood levels, RA synovial fluid δTCS1+ T cell levels were increased relative to NML peripheral blood levels ($P=0{\cdot}002$) (Fig. 2). In order to assess the specificity of these observations for RA, we examined δTCS1+ T cells and TcRδ1+ T cells in synovial fluid samples from eight patients with seronegative spondyloarthropathies (SSA), six of whom had concurrent peripheral blood levels determined. In contrast to RA, δTCS1+ T cell levels in SSA synovial fluid ($1{\cdot}1 \pm 0{\cdot}3$) and SSA peripheral blood ($0{\cdot}9 \pm 0{\cdot}4$) were not differentiated and were comparable to NML peripheral blood. Of significance, RA peripheral blood and synovial fluid levels of δTCS1+ T cells were elevated relative to SSA peripheral blood and synovial fluid levels ($P=0{\cdot}05$ for both comparisons) (Fig. 2).

The levels of TcRδ1+ T cells (Fig. 3) in paired RA peripheral blood and RA synovial fluid samples were not significantly different from each other or NML peripheral blood levels (Fig. 3). The levels of TcRδ1+ T cells in SSA peripheral blood and SSA synovial fluid were similar to each other and those of NML peripheral blood ($P>0{\cdot}05$). Of note, RA synovial fluid levels were higher than SSA synovial fluid levels ($P=0{\cdot}03$).

When we examined δTCS1/TcRδ1 ratios in paired peripheral blood and synovial fluid samples, we observed that the ratios in RA synovial fluid were significantly elevated relative to that in NML peripheral blood ($0{\cdot}2 \pm 0{\cdot}05$) ($P=0{\cdot}005$). The δTCS1/TcRδ1 ratio in SSA synovial fluid was comparable to that in RA synovial fluid ($P>0{\cdot}05$).

DISCUSSION

The results of the present study demonstrate elevated levels of a γδ T cell subset (δTCS1+) in the peripheral blood and synovial

fluid of RA patients and in the peripheral blood of patients with FS. Contrary to the data of Brennan *et al.*, 1988, 1989), we did not observe an elevation in total $\gamma\delta$ T cells in the peripheral blood or SF of RA patients relative to normal peripheral blood levels. The reasons for such divergent findings are unclear but may reflect differences in the patient population in terms of seropositivity, disease activity, stage of disease and therapy. We determined, in a preliminary way, the specificity of our findings for RA by examining $\gamma\delta$ T cell levels in patients with SSA. Our data support the possibility that elevated δTCS1$^+$ cell levels are more specific for RA since normal levels of peripheral blood and synovial fluid δTCS1$^+$ T cells were observed in SSA. Moreover, the mean levels of δTCS1$^+$ T cells and TcRδ1$^+$ T cells in RA synovial fluid were elevated relative to those in SSA synovial fluid. Further studies with larger numbers of patients are required to address this issue.

In this study we used two MoAbs, δTCS1 against Vδ1 (a subset of δ T cells) and TcRδ1 against the common epitope of human TcRδ chain. The TcRδ1$^+$ and δTCS1$^+$ T cells account for 4% and 1% of peripheral blood T cells in normal subjects. As noted, the proportion of RA and FS patients with elevated levels of δTCS1$^+$ T cells above normal was significantly larger than the control subjects. In some patients the blood δTCS1$^+$ level reached 25% of CD3$^+$ T cells or higher. The pathogenetic significance of this elevation in RA patients remains unclear. Since the δTCS1$^+$ T cells represent the motile T cells, it is possible that the blood level reflects a traffic pattern of these cells moving between localized tissues. To date, $\gamma\delta$ T cells were found in the sinusoidal areas of spleen, synovium membrane, and other lymphoid tissues. It would be interesting to study the $\gamma\delta$ T cells in the spleen of Felty's patients and correlate the cell level in this organ with bone marrow and blood of these patients.

Elevation of the δTCS1$^+$ subset of $\gamma\delta$ T cells may reflect a differential propensity to differentiation within the synovium since known compartmentalization of $\gamma\delta$ T cells has been demonstrated in various tissues. Thus BB3$^+$ and δTCS1$^+$ subsets of $\gamma\delta$-bearing cells show distinctive tissue distribution patterns within the human lymphohaemopoietic system (Falini *et al.*, 1989). However, it is unlikely that compartmentalization alone accounts for our results since δTCS1 T cells were elevated in the synovial fluid of RA patients but not in the synovial fluid of SSA patients.

The elevation of δTCS1$^+$ T cells may reflect disease progression or a subtype of RA that tends to exhibit vasculitis, because of the preferential homing of $\gamma\delta^+$ T cells to vascular space in spleen (Bucy *et al.*, 1989). Longitudinal studies are needed to ascertain its implication in RA pathogenesis or patient management. Some groups suggest that $\gamma\delta$ T cells may regulate the functions and development of $\alpha\beta$ T cells (Ferrick *et al.*, 1989) and monocytes. To this end, the elevation of δTCS1$^+$ T cells may indicate certain immunodysfunction in RA patients.

$\gamma\delta$ T cells may be autoreactive in immune disorders. Thus, most studies in mice (O'Brien *et al.*, 1989; Janis *et al.*, 1989) and humans (Holoschitz *et al.*, 1989), have demonstrated reactivity of $\gamma\delta$ lymphocytes to mycobacterial antigens in a non-MHC restricted manner. A recent study by Modlin *et al.* (1989) claimed that the level of $\gamma\delta$ T cells increased five- to eightfold in particular granulomatous reactions of leprosy. These cells from the lesion reacted to mycobacterial antigens in the context of self molecules and produced factors inducing adhesion aggregation of monocytes. Moreover, Holoshitz *et al.* (1989) isolated mycobacterial-reactive $\gamma\delta$ T lymphocyte clones from rheumatoid arthritis synovial fluid. These findings suggest autoreactivity of $\gamma\delta$ T cells in RA since an acetone precipitable fraction of mycobacterium tuberculosis (MT) has been shown to be cross-reactive with proteoglycans, a major constituent of cartilage (Holoschitz *et al.*, 1986). Moreover, MT reactivity is restricted by HLA DR4, an MHC antigen strongly associated with RA (Palacios-Boix *et al.*, 1988). Taken together, the data suggest the possibility that $\gamma\delta$ T cells in RA synovium may contribute significantly to the perpetuation of rheumatoid disease by reacting against elements of cartilage within the joint. Studies in RA of $\gamma\delta$ T cell reactivity to cartilage constituents are currently underway to address this issue.

ACKNOWLEDGMENTS

The authors wish to acknowledge the excellent secretarial assistance of Ms N. Jellison. We also acknowledge Dr R. I. Fox, Scripps Clinic, La Jolla, CA, for helpful discussion. This work was partially supported by a grant from Pfizer Corporation.

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