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Synovial fluid cells in juvenile arthritis: evidence of selective T cell migration to inflamed tissue

E. D. SILVERMAN*†‡, B. ISACOVICS‡, D. PETSCHE‡ & R. M. LAXER*‡ *Division of Rheumatology, Departments of *Pediatrics and †Immunology, and the ‡Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada*

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SUMMARY

The perpetuation of chronic synovitis in juvenile arthritis (JA) is a complex interaction of local and systemic regulatory mechanism. We examined the cell surface phenotype of synovial fluid cells and peripheral blood lymphocytes from 15 patients with JA to better understand the mechanism of local inflammation. Synovial fluid and peripheral blood mononuclear cells were analysed for cell surface expression of CD2, CD3, CD4, CD8, CD19, CD25, CD29, CD45R and Ia using flow cytometry. We found a very low percentage of B cells with a concomitant increase of T cells in synovial fluid as compared with peripheral blood. A large percentage of the synovial fluid T cells were HLA-DR$^+$, or activated T cells, and there was a relative decrease in CD4$^+$ cells in synovial fluid as compared with peripheral blood. There was only a minimal increase in CD25$^+$ synovial fluid cells. The synovial fluid CD4$^+$ cells were mainly of the CD2^{high}, CD29$^+$, CD45RO phenotype. This CD4 phenotype found on synovial fluid cells from patients with JA and in particular the CD29 cell surface marker, which recognizes a common β-chain of adhesion molecules, is associated with binding to extracellular matrix proteins and is also associated with 'primed' T cells. Our results demonstrated the presence of T cells which either selectively migrate to synovium and synovial fluid or are activated *in situ* in the joint.

Keywords juvenile/paediatrics synovial fluid cells lymphocyte markers

INTRODUCTION

Juvenile arthritis (JA) is characterized by systemic as well as local joint inflammation. Immunoregulatory abnormalities have been demonstrated in the peripheral blood of these patients but few studies have examined synovial fluid (SF), synovium or synovial fluid cells (SFC). In the peripheral blood there has been evidence of activated T cells as demonstrated by the presence of VLA-1 antigen on T cells, abnormal T cell subset ratios and T cell function with abnormal cytokine production [1–7]. Abnormal natural kill cell function, abnormal CD4:CD8 ratios and HLA-DR$^+$ (activated) T cells have been demonstrated in the SF [8–10].

The control of the immune system is a complex interaction of multiple factors including cellular interactions, secretion of cytokines and migration or homing of effector cells to target tissues. Even within the T cell compartment there appear to be multiple cellular interactions necessary for normal regulation. It has been shown that the CD4$^+$ (helper) subset can be divided into subsets by the presence of the CD29 (4B4) or CD45R (2H4) molecules on the cell surface. Examination of these subsets has shown that the CD4$^+$45R$^+$ (CD45RA) subset contains 'naive' cells which respond to mitogen but not to recall antigen and includes the suppressor-inducer population of cells [11,12]. Conversely, the CD29$^+$ (CD45RO) cells represent the CD4$^+$ cells which respond well to recall antigen, 'memory' cells, and include the cells with helper-inducer function [11–13]. The CD29 molecule recognizes the common β-chain of the VLA family of molecules, a family of molecules which may be important as homing receptors and as receptors for extracellular matrix proteins including fibronectin and collagen [14–18].

This study was undertaken to examine the cell surface phenotype of SFC and circulating peripheral blood lymphocytes (PBL) in JA patients to better understand the mechanism of local inflammation in JA. Specifically we compared SFC and PBL from JA patients for evidence of differences in the T cell subpopulations. We found that the greatest differences between SFC and PBL were increased numbers of CD4$^+$CD29$^+$ cells, increased numbers of CD2$^+$ cells with an increased number of CD2 molecules per cell and decreased numbers of CD4$^+$CD45R$^+$ cells in SF as compared with PBL. These results suggest that there is either local activation and clonal expansion of T cells within the joint or homing of preactivated T cells to the joint in JA.

Correspondence: Dr E. Silverman, Division of Rheumatology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8.

PATIENTS AND METHODS

Patient population

We studied 10 boys and five girls who met the American College of Rheumatology (ACR) criteria for the diagnosis of juvenile rheumatoid arthritis [19]. The mean age at the time of study was 12·7 ± 3·7 years (mean ± s.d.). There were five patients with antinuclear antibody-positive (ANA^+) pauciarticular JA, three with ANA^- pauciarticular JA, five patients with $HLA\text{-}B27^+$ JA and two patients with rheumatoid factor (RF)-negative, ANA^- polyarticular JA. None of the patients had RF^+ JA or systemic JA. Three patients had their SF studied on two different occasions. Ten patients were on non-steroidal anti-inflammatory drugs while none was on corticosteroids at the time of study. None of the patients was on slow acting anti-rheumatic drugs. Five patients were not on any medication at the time of the study.

Preparation and staining of lymphocytes

Synovial fluid cells. When clinically indicated, arthrocentesis was performed on inflamed knee joints. Mononuclear cells were then isolated from the patients' heparinized SF by HISTOPAQUE-1077 (Sigma Chemical Co., St Louis, MO) density gradient centrifugation. Fluorescein- and PE-conjugated MoAbs (Coulter, Hialeah, FL) were incubated with 5×10^5–1×10^6 isolated mononuclear cells. The majority of cells were analysed on the day of sample collection and processed, while in a minority of samples the cells were fixed with 1% formaldehyde/PBS before analysis.

Peripheral blood. After consent, whole blood from each patient was lysed using the leucocyte preparation system and Q-prep (Coulter) with staining by two-colour MoAbs (Coulter). In a minority of patients' blood, samples were separated by HISTOPAQUE-1077 density gradient centrifugation (Sigma), before staining peripheral blood lymphocytes. When directly tested there was no difference in the two methods of preparation of cells.

Monoclonal antibodies. Specific antibodies used were anti-CD2, anti-CD3, 4B4 (anti-CD29), 2H4 (anti-CD45R), anti-HLA-DR(Ia), anti-CD4, anti-CD8 and anti-CD19 (Coulter), and anti-IL-2R (CD25) (Becton Dickinson, Mississauga, Ontario). Control antibodies were mouse isotype-specific (IgG1 or IgG2a) control MoAbs (Coulter).

Cell surface phenotype analysis. Two-colour fluorescence flow cytometric analysis was performed on an EPICS Profile Analyser (Coulter). Lymphocytes were enumerated by gating as defined by forward scatter and side scatter. Single histogram plots were used for a single surface antigen while contour graphs were used for dual antigen expression. The percentage of single antigen-positive and double antigen-positive subpopulations were determined: In all experiments, a minimum of 5000 cells were counted.

In the Coulter Profile® Flow Cytometer, the FITC and PE fluorescence signals are logarithmically amplified over four decades on a 256-channel abscissa. The relative fluorescence index (RFI) is used to convert the logarithmically amplified scale to a linear scale in order to directly compare the mean fluorescence intensities (antigen density) of different cell populations. The statistics necessary for the RFI calculations are generated directly by computer software for every sample including isotype background controls. The RFI of isotypic control is assigned a value of 1·0.

Statistical analysis

All results were analysed using Student's *t*-test for unpaired values and paired Student's *t*-test or Mann–Whitney *U*-test for paired samples. Data were analysed using STATVIEW software on a Macintosh computer.

RESULTS

Circulating PBL and SF lymphocytes were examined for cell surface phenotype. There was a decreased percentage of B cells ($CD19^+$ cells) in the SF (mean ± s.d. 3·7 ± 2·5) as compared with the peripheral blood (17·5 ± 7·4; $P \leqslant 0{\cdot}0001$) (Table 1). Similar results were seen when only the 14 paired PBL-SF samples were examined (17·5 ± 7·4 *versus* 4·3 ± 2·3; $P \geqslant 0{\cdot}0001$). Within the pauciarticular population of patients there was no significant difference between percentage of SF B cells of $B27^+$ patients *versus* ANA^+ patients (3·7 *versus* 4·13; $P \leqslant 0{\cdot}1$).

T cells were enumerated using CD2 and CD3 markers. There was no significant difference between the percentage of $CD3^+$ PBL compared with SF (67·4 ± 8·3 *versus* 74·2 ± 10; $0{\cdot}1 \leqslant P \geqslant 0{\cdot}05$). This was also true when only paired PBL-SF were examined (66·5 ± 8·2 *versus* 75·9 ± 5·2; $0{\cdot}25 \leqslant P \geqslant 0{\cdot}1$). However, the percentage of $CD2^+$ cells was significantly increased in SF compared with PBL. The percentage of $CD2^+$ cells in SF was 92·4 ± 5·0 compared with PBL values of 73·4 ± 8·3 ($P \leqslant 0{\cdot}0001$). This was also true in the 14 paired samples (93·6 ± 3·6 *versus* 72·8 ± 8·4; $P \leqslant 0{\cdot}0001$). More importantly, the RFI (a measure of the number of antigen molecules per cell) was increased in SF compared with peripheral blood (representative experiment shown in Fig. 1a and Table 2). This demonstrates increased density of CD2 on SF cells. In contrast, the values for $CD3^+$ were similar in PBL and SF (Fig. 1b, Table 2). These findings were true in all patients regardless of disease subtype.

T cell subsets were analysed using CD4 and CD8 cell surface antigens. There was a significantly increased percentage of $CD8^+$ cells (38·9 ± 12·7% *versus* 22·5 ± 5·3%; $P \leqslant 0{\cdot}0001$) with a concomitant decreased proportion of $CD4^+$ cells (32 ± 12% *versus* 41 ± 7·3%; $P \leqslant 0{\cdot}01$) in SF compared with PBL. As a result, the $CD4^+ : CD8^+$ ratio was significantly decreased in SF compared with PBL (1 ± 0·2 *versus* 1·9 ± 0·6; $P \leqslant 0{\cdot}0001$). In fact, only 6/18 SF samples had CD4:CD8 $\geqslant 1{\cdot}4$ while all peripheral blood samples had CD4:CD8 $\geqslant 1{\cdot}4$ (Table 1). $CD4^+$ SF and PBL were subdivided into mutually exclusive $CD45R^+$ ($2H4^+$) and $CD29^+$ ($4B4^+$) subsets. There was a significantly increased percentage of $CD4^+$ $CD29^+$ cells in SF compared with PBL (27·4 ± 11·4 *versus* 8·4 ± 3·5; $P \leqslant 0{\cdot}0001$). There was a concomitant decrease in the percentage of $CD4^+$ $CD45R^+$ cells in SF compared with PBL (2·3 ± 3·1 *versus* 32·1 ± 13·2; $P \leqslant 0{\cdot}0001$). This resulted in a significantly increased $CD4^+$ $CD29^+ : CD4^+$ $CD45R^+$ cells in SF compared with PBL (26·0 ± 18·3 *versus* 0·28 ± 0·14; $P \leqslant 0{\cdot}0005$) (Figs 2 and 3). The percentages of $CD4^+$ subsets were within the expected percentages for age in peripheral blood.

T cells were measured for evidence of 'activation' (i) using the cell surface expression of CD25 (IL-2 receptor) as a measure of early activation; and (ii) using DR surface acquisition on T cells as a measure of late activation. All samples contained less

Table 1. Cell surface phenotype of peripheral blood lymphocytes (PBL) and synovial fluid (SF) cells

Patient	Cell type	CD19$^+$	CD2$^+$	CD3$^+$	CD3$^+$DR$^+$	CD4$^+$	CD8$^+$	CD4:CD8 ratio
1	PBL	27·3	72·9	69·8	1·4	40·8	20·9	1·9
	SF	3·0	95·5	80·2	46·1	17·7	63·7	0·3
2	PBL	13·9	79·6	69·8	1·3	43·3	28·0	1·5
	SF	4·7	96·5	75·2	25·5	18·4	49·3	0·4
2a	SF	0·9	ND	55·3	ND	30·1	38·2	0·8
3	PBL	16·5	77·7	70·5	1·3	43·8	25·2	1·7
	SF	4·5	96·4	75·6	26·6	30·6	51·2	0·6
3a	SF	2·1	95·0	76·3	24·6	26·4	49·8	0·5
4	PBL	10·1	72·6	ND	ND	37·4	27·1	1·4
	SF	2·5	95·0	76·0	ND	26·0	20·5	1·7
5	PBL	10·2	87·5	ND	ND	46·1	33·3	1·4
	SF	5·9	96·3	92·7	11·0	42·6	33·3	1·3
6	PBL	16·6	80·0	76·4	0·9	50·4	24·7	2·0
	SF	4·8	ND	ND	ND	47·1	25·7	1·8
7	PBL	13·6	81·4	77·7	2·7	32·4	19·7	1·6
	SF	2·0	95·8	84·4	25·3	29·3	44·0	0·7
8	PBL	26·8	62·6	54·8	0·9	40·0	21·6	1·8
	SF	3·8	94·8	81·6	31·5	55·1	30·4	1·8
9	PBL	27·3	59·8	54·3	1·0	24·0	16·9	1·4
	SF	7·2	92·0	78·1	24·0	43·5	27·2	1·6
10	PBL	10·4	64·8	55·3	0·3	41·7	12·5	3·3
	SF	9·4	94·9	71·7	45·0	18·9	62·1	0·3
10a	SF	1·0	ND	75·1	ND	39·0	25·0	1·6
11	PBL	16·6	78·6	75·3	3·4	56·0	17·7	3·2
	SF	5·7	96·2	67·3	43·3	41·7	51·1	0·8
12	PBL	18·0	69·6	69·3	1·7	38·8	25·1	1·5
	SF	1·3	79·4	52·2	6·8	27·7	32·8	0·8
13	PBL	7·6	77·1	66·5	2·1	45·0	21·6	2·1
	SF	4·6	89·6	69·6	11·3	36·6	39·9	0·9
14	PBL	29·4	63·3	68·7	4·7	36·9	20·1	1·8
	SF-left	0·8	85·4	ND	ND	40·3	30·1	1·3
	SF-right	0·5	88·6	ND	ND	30·8	37·2	0·8
15	SF	6·5	87·6	76·6	24·7	43·3	26·9	1·6

ND, Not done.

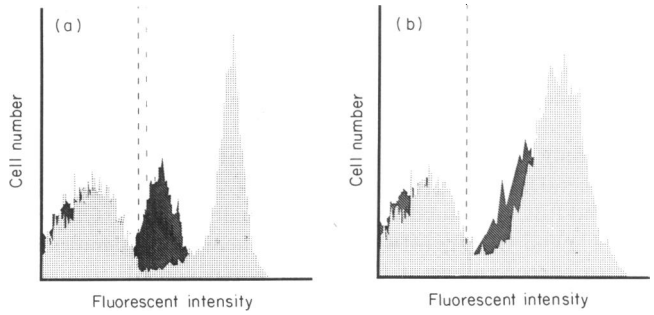

Fig. 1. (a) Comparison of the presence of CD2 molecules on paired synovial fluid and peripheral blood mononuclear cells. This figure demonstrates both the fluorescent intensity (abscissa) and number of CD2$^+$ cells present in paired synovial fluid (▧) and peripheral blood mononuclear cells (▦) (ordinate). This is a representative patient of the 13 patients. All patients were similar. (b) Comparison of the presence of CD3 molecules on paired synovial fluid and peripheral blood mononuclear cells. This is a representative patient comparing both the fluorescent intensity (abscissa) and number of CD3$^+$ cells present in paired synovial fluid (▧) and peripheral blood mononuclear cells (▦) (ordinate). This is a representative patient of all 13 patient experiments, which gave similar results.

Table 2. Comparison of relative fluorescent intensity (RFI) of CD2 and CD3 on peripheral blood and synovial fluid cells*

	RFI	
	CD2	CD3
Peripheral blood cells	10·2	11·6
Synovial fluid cells	58·3	13·9

* This is a representative experiment using flow cytometric analysis of fluorescent intensity of cell surface expression of CD2 and CD3 molecules.

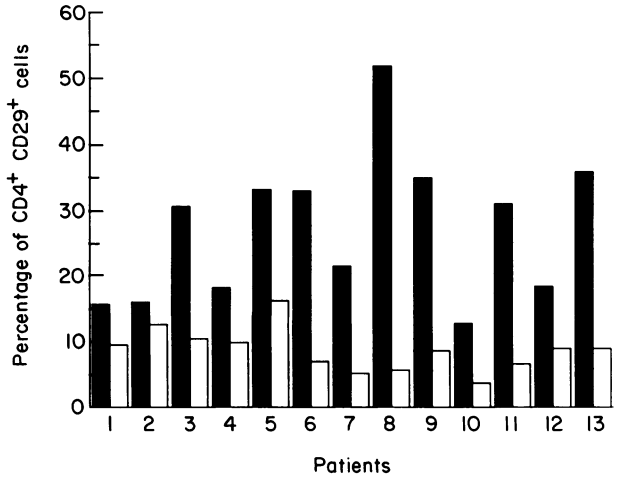

Fig. 2. Comparison of CD4$^+$ CD29$^+$ in paired peripheral blood and synovial fluid samples. This figure demonstrates the results of two-colour immunofluorescent analysis of the simultaneous expression of both CD4 and CD29 molecules on synovial fluid cells (■) and peripheral blood mononuclear cells (□) from 13 individual JA patients. All paired samples were obtained within 1 h of each other.

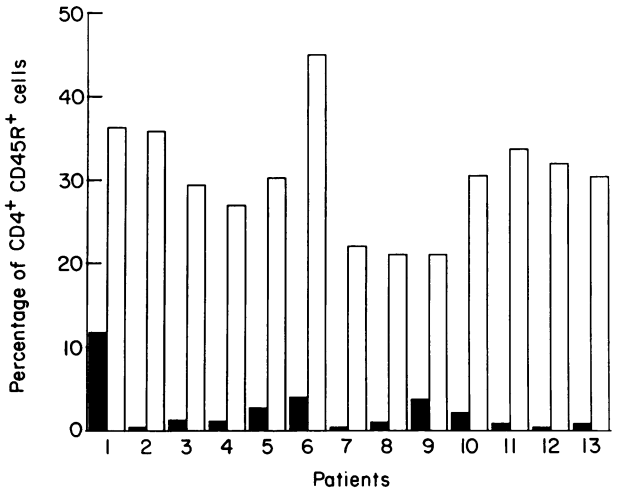

Fig. 3. Comparison of CD4$^+$ CD45R$^+$ in paired peripheral blood and synovial fluid samples. The results of two-colour immunofluorescent analysis of the simultaneous expression of both CD4 and CD45R molecules on synovial fluid (■) and peripheral blood mononuclear cells (□) from 13 different JA patients. All paired samples were obtained within 1 h of each other.

than 5% CD3$^+$ CD25$^+$ cells and there was no difference in the percentage CD25$^+$ T cells between SFC and PBL (data not shown). However, there was a significantly increased percentage of CD3$^+$ DR$^+$ cells in SF compared with PBL ($26{\cdot}6 \pm 12{\cdot}6$ *versus* $1{\cdot}8 \pm 1{\cdot}2$; $P \leqslant 0{\cdot}0001$) (Table 1). The percentage of CD3$^+$ DR$^+$ cells in PBL was not elevated when compared with laboratory controls. There was no difference in the results of 10 patients who were on medication compared with the five who were not on any treatment (data not shown).

DISCUSSION

In this study, we have demonstrated significant differences in both the percentage of T and B lymphocytes and the cell surface antigens present on SF T lymphocytes compared with the peripheral blood of patients with JA. The most important differences are: (i) the low percentage of B cells present in SF with a concomitant increase in T cells; and (ii) the distribution of cell surface molecules within the T cell population. This latter finding suggests that most of the SF T cells present are either activated and expanded in the joint or have specific receptors which has allowed them to localize (home) to the joint and then proliferate. Our findings in the peripheral blood did not differ from the expected for age-matched controls.

CD4$^+$ cells can be divided, using cell surface molecules, into so-called memory cells and naive T cells [11–13]. The memory cells have the surface phenotype of: LFA-3$^+$, CD-2^{high}, CD29$^+$ and CD45RO [13]. The antibodies used in our study recognize this subset of cells [20,21]. The CD4$^+$ cells found in the SF were >90% CD2^{high}, CD29$^+$, CD45RO (CD45R$^-$). Furthermore, there was a large portion of T cells which were CD3$^+$ HLA-DR$^+$ while only a small number of these cells expressed surface IL-2 receptors. Although at first this latter finding may appear contradictory, the presence of IL-2R expression on T cells reflects only recent activation while an increase in surface CD2 molecules or the presence of CD29 are markers of prior activation. Similar results have been seen in JA and adult RA patients when IL-2R expression was compared with the presence of DR and/or VLA-1 (marker of prior activation) [9,22–24]. More recently, it has been shown that CD2^{high}, CD45RO memory T cells are cells which recirculate from blood to tissue [25]. This change in cell surface phenotype has been linked to enhanced migration of cells into areas of inflammation and it has been suggested that chronic stimulation leads to an increased number of primed T cells which can then seed to tissues. We found an increased percentage of CD2$^+$/CD3$^-$ cells in SF compared with peripheral blood. This finding was previously seen and the authors suggest these cells have both cytolytic and natural killer function [26]. We did not test the function of these cells nor did we further delineate their cell surface phenotype.

We found a very low percentage of B cells in the SF of JA patients compared with the peripheral blood. This was true in all subtypes of JA patients. Previous authors have demonstrated similar findings [9] and this may suggest a minor role for B cells in the pathogenesis of the chronic joint inflammation in all subtypes of JA. As opposed to adult RA, where RF is probably important in the pathogenesis and B cells have been shown to have abnormal regulation (reviewed in [27]), most patients (95%) with JA are rheumatoid factor-negative (RF$^-$). All patients in our study were RF$^-$. In fact, most previous authors have demonstrated abnormal T cell rather than B cell function in JA [2,4,6,7].

We have demonstrated increased numbers of CD4$^+$ CD29$^+$ cells which were also CD2^{high}. The CD29 marker recognizes the β chain of the VLA family of molecules. The common β chain of VLA family of molecules associates with different α chains to form different receptors for numerous extracellular matrix products including collagen and fibronectin [17,18]. The VLA molecules are felt to be part of a family of receptors which are important in migration (so-called homing receptors) and in adhesion [14,15,17,25,28]. The CD2 molecule is important in T cell interaction with cells which express the leucocyte-associated antigen 3 (LFA-3) (CD58) [29,30]. This includes the interaction of lymphocytes with endothelial cells [31]. It has

recently been shown that T cell activation via the physiologically important TCR/CD3 complex results in up-regulation of cell surface CD2 molecules [32]. When taken together, our results are suggestive that the cells present in SF of JA patients are probably cells which are activated in the circulation and then migrate to the specific target tissue via specific homing receptors. The putative receptor on these cells would consist of the VLA subfamily of integrin molecules which bind to extracellular matrix proteins [15,17,18]. This may be especially important as there are high levels of fibronectin in inflamed rheumatoid synovium and that cartilage contains readily detectable amounts of fibronectin as well as collagen [33,34]. In fact, high levels of VLA-1$^+$ and VLA-2$^+$ T cells have been isolated from sites of inflammation including inflamed rheumatoid joints [24,25]. In RA, it has recently been shown that SF T cells have increased numbers of VLA-4 receptors which were able to bind fibronectin [35,36]. Taken together, the evidence demonstrates a selective homing or persistence of primed T cells to articular joints in JA and RA.

We have demonstrated that the SF of JA patients consists mainly of T cells rather than B cells and that the CD4$^+$ T cells have the CD2^{high}, CD29, CD45RO phenotype. Whether these cells are activated in a joint by a yet unidentified antigen or are in fact selected to the sites of inflammation in this disease is yet to be determined. Although our results and the evidence in the literature would support the latter theory, the determination of which hypothesis is true may await the identification of putative antigens which lead to the development of JA. Whatever the reason for the presence of these T cells, it appears that JA is secondary to a T cell disorder rather than to abnormal B cells or antibody production and that these abnormal T cells are important in the persistent synovial inflammation characteristic of this disease.

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