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Increased numbers of $CD5^+$ B cells and T cell receptor (TCR) $\gamma\delta^+$ T cells are associated with younger age of onset in rheumatoid arthritis (RA)

J. HASSAN, G. YANNI*, V. HEGARTY*, C. FEIGHERY, B. BRESNIHAN* & A. WHELAN *Department of Immunology, St James's Hospital, and *Department of Rheumatology, St Vincent's Hospital, Dublin, Republic of Ireland*

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SUMMARY

Patients presenting with RA before the age of 45 years (younger onset) are known to have more aggressive disease compared with patients presenting after the age of 65 years (older onset). Coordinated expansion of circulating $CD5^+$ B cell and TCR $\gamma\delta^+$ T cell levels has been reported in patients with RA. This study assesses the peripheral blood levels of these two cell types in RA patients with younger and older onset of disease. $CD5^+$ B cell levels were significantly elevated in the younger onset RA group ($26{\cdot}6 \pm 4{\cdot}5\%$) compared with the older onset RA group ($14{\cdot}2 \pm 1{\cdot}2\%$; $P < 0{\cdot}01$). TCR $\gamma\delta^+$ T cell levels were also significantly raised in the young patients ($4{\cdot}0 \pm 0{\cdot}9\%$) compared with elderly patients ($1{\cdot}6 \pm 0{\cdot}2\%$; $P < 0{\cdot}01$). T cell levels ($CD3^+$) were similar in both groups (young $66{\cdot}4 \pm 3{\cdot}3\%$; old $74{\cdot}3 \pm 3{\cdot}4\%$ (mean $\pm$ s.e.m.); NS). Total B cell levels ($CD19^+$) were also similar in these groups ($7{\cdot}7 \pm 0{\cdot}7\%$ *versus* $8{\cdot}9 \pm 1{\cdot}8\%$; NS). A significant positive correlation was observed between the $CD5^-$ B and TCR $\gamma\delta^+$ T cell types in the patients ($r = 0{\cdot}72$, $P < 0{\cdot}05$). Compared with age-matched normal controls, the younger onset patients had similar $CD5^+$ B cell and TCR $\gamma\delta^+$ T cell levels to the elderly controls ($CD5^+$ B cells $30{\cdot}2 \pm 3{\cdot}0\%$; TCR $\gamma\delta^+$ T cells $3{\cdot}0 \pm 0{\cdot}8\%$). Conversely, older onset RA patients had $CD5^+$ B cell levels similar to the young controls ($12{\cdot}3 \pm 1{\cdot}9\%$). Spontaneous *in vitro* synthesis of immunoglobulins (IgM, IgA and IgG) and rheumatoid factors (IgM and IgA isotypes) were not significantly different in both patient groups. The coordinate expansion of circulating $CD5^+$ B cells and $\gamma\delta^+$ T cells seen in patients with RA presenting before 45 years of age and not after 65 years of age may suggest a potential role for these cells in more aggressive disease states.

Keywords rheumatoid arthritis younger onset $CD5^+$ B cells TCR $\gamma\delta^+$ T cells

INTRODUCTION

$CD5^+$ B cells are raised in a normal individual at birth [1], after tetanus toxoid immunization [2] and when elderly [3]. In these normal states, $CD5^+$ B cells are committed to the production of polyreactive natural antibodies where they might function as a rapid first line of defence against bacteria and viruses and/or they might function in clearing autoantigens from the circulation [3]. Because of the 'anti-self' reactivity of the antibodies they produce, $CD5^+$ B cells have been suggested to play a role in autoimmune conditions such as RA where patients show a striking increase in the number of $CD5^+$ B lymphocytes [4].

TCR $\gamma\delta^+$ T lymphocytes have also been implicated in various inflammatory and autoimmune conditions. Increased numbers of $\gamma\delta^+$ T cells in the synovia of patients with active RA [5], increased reactivity to mycobacterial antigens [6] and to heat shock protein [7], which is highly expressed in the inflamed joint, suggest a role for $\gamma\delta$ T cells in RA.

Similar to the observation in lupus erythematosus, there are reasons to suspect that RA in older patients differs from the disease seen in younger adults [8]. There is evidence that immunological reactivity changes with age, for example the presence of low titres of rheumatoid factor (RF) in healthy older people suggests that when exposed to similar antigenic stimuli, older patients may respond with different clinical symptoms from younger adults [8]. Coordinate expansion of $CD5^+$ B cells and TCR $\gamma\delta^+$ T cells has been reported in patients with RA [9]. However, the age of the patients studied has not been adequately addressed. The present study examines the circulating levels of $CD5^+$ B cells and TCR $\gamma\delta^+$ T cells in active RA patients with onset of disease before 45 years of age (younger onset) and patients with onset of RA after 65 years of age (older onset). Expansion of both cell types was observed in younger onset patients, but not in older onset patients,

Correspondence: Dr Jaythoon Hassan, Children's Research Centre, Our Lady's Hospital for Sick Children, Crumlin, Dublin 12, Republic of Ireland.

supporting the notion that the disease process differs depending on the age of onset of RA.

PATIENTS AND METHODS

Patients

Two groups of patients were recruited for the study [10]. Group 1 ($n = 11$) consisted of patients with onset of disease before the age of 45 years, and group 2 ($n = 10$) after the age of 65 years. All patients had active disease and were not receiving disease remittive agents at the time of study. All patients were seropositive on routine RF latex investigation. Healthy individuals made up the normal control groups (young, four males, six females, mean age 32·3 years; old, three males, seven females, mean age 71·7 years).

Enumeration of $CD5^+$ B cells and TCR $\gamma\delta^+$ T cells

Analysis of $CD5^+$ B cells and T cells was performed by two-colour fluorescence using a fluorescence-activated cell scan (FACScan) flow cytometer. The MoAbs used included: PE-Leu-1 (CD5), FITC-Leu-12 (CD19), PE-Leu-4 (CD3) and FITC-TCR delta 1 (T Cell Sciences, Cambridge, MA). All Leu antibodies were purchased from Becton Dickinson (Sunnyvale, CA). Briefly, 50 μl of EDTA whole blood were incubated with optimal amounts of MoAbs, mixed and left at room temperature in the dark for 10 min. Erythrocytes were lysed by adding 2 ml of a FACS lysing solution (Becton Dickinson) per tube, mixed and incubated for a further 10 min at room temperature. Cells were spun and then washed once with PBS pH 7·2 containing 0·1% bovine serum albumin (BSA) and 0·1% sodium azide. Cells were acquired on the flow cytometer and the results expressed as $CD5^+$ B cells (as per cent of $CD19^+$ cells) and $\gamma\delta^+$ T cells (as per cent of $CD3^+$ cells). Relevant isotype-matched control antibodies were included in the staining procedure in order to exclude non-specific binding.

Cell culture

Peripheral blood mononuclear cells (PBMC) were isolated on Ficoll–Hypaque density gradient by the method of Boyum [11]. The cells were washed three times in Hanks' balanced salt solution (HBSS) and resuspended in RPMI 1640 supplemented with 50 μg/ml gentamycin, 1% L-glutamine, 10% fetal calf serum (FCS) and 2 μg/ml fungizone. PBMC were resuspended at a concentration of 1×10^6/ml and incubated at 37°C in 200 μl volumes in flat-bottomed microtitre plates and a fully humidified 5% CO_2 atmosphere. Supernatants of these cultures were removed after 7 days and stored at −70°C. IgG, IgA and IgM levels were quantified by a modified ELISA.

ELISA assays

IgG, IgA and IgM levels in culture supernatants were quantified by the ELISA technique as previously described [12]. Briefly, microtitre plates (Nunc, Roskilde, Denmark) were coated with 100 μl goat anti-human IgG, IgA or IgM (Cappel Labs, Cochranville, PA) at a concentration of 1 μg/ml in a coating buffer (carbonate-bicarbonate pH 9·6) and incubated overnight at 4°C. After washing with PBS containing 0·05% Tween-20, 100 μl of neat supernatant were added. Standard dilutions of IgG, IgA or IgM (Behring) ranging from 0·01 to 5 μg/ml were included on every plate. After incubation at 37°C for 30 min, the plates were washed. Peroxidase-conjugated goat anti-human IgG, IgA or IgM (Cappel) at 1 : 500 dilution was then added. After incubation and washing as before, freshly prepared substrate (orthophenylenediamine in phosphate-citrate buffer pH 5·0 containing 0·012% H_2O_2) was added and the reaction stopped with 2·5 M H_2SO_4. The concentration of IgG, IgA and IgM in the supernatants was calculated from the standard curves.

IgM-RF and IgA-RF assays were performed similarly, except human IgG (1 mg/ml) was used for coating and the peroxidase-conjugated goat anti-human IgM $F(ab)_2$ or IgA $F(ab)_2$ was used at a 1 : 10 000 dilution. The reaction time was 20 min and results were expressed as ELISA Index (mean of the sample/mean of the background).

Statistical analysis

Data were analysed using the Wilcoxon signed-rank test (paired data), Wilcoxon two-sample test (unpaired data) and correlation coefficient tests.

Table 1. Clinical and laboratory features of RA patients

	Young	Old	*P*
Total number	11	10	
M : F ratio	5 : 6	4 : 6	
Age (years)	40·9 ± 7·5	76·1 ± 4·9	0·001
Age of onset (years)	38·9 ± 6·4	73·5 ± 4·4	0·001
Disease duration (years)	2·2 ± 2·3	2·8 ± 3·9	0·687
Haemoglobin (g/dl)	12·8 ± 1·5	10·8 ± 2·3	0·036
ESR (mm/h)	32·7 ± 27·7	55·6 ± 37·3	0·230

ESR, Erythrocyte sedimentation rate.

RESULTS

Table 1 shows the clinical and laboratory parameters assessed in the two groups of RA patients. Disease duration was similar in both groups. The erythrocyte sedimentation rate (ESR) was higher in the older patients, but the difference did not reach statistical significance. PBMC were isolated from the patients

Table 2. Spontaneous *in vitro* synthesis of rheumatoid factors (RF) and immunoglobulins by peripheral blood mononuclear cells

	Group 1 ($n = 11$)	Group 2 ($n = 10$)	*P*
IgM-RF	3·6 ± 5·4	2·1 ± 2·0	0·990
IgA-RF	1·0 ± 0·2	0·9 ± 0·1	0·089
IgM	0·6 ± 0·9	0·4 ± 0·3	0·460
IgA	0·4 ± 0·2	0·3 ± 0·2	0·714
IgG	2·7 ± 1·9	2·5 ± 1·5	0·850

Results for RF are expressed as the ELISA index. An ELISA index of 1·0 represents background values. IgM, IgA and IgG are given as μg/ml. All data are expressed as mean ± s.e.m.

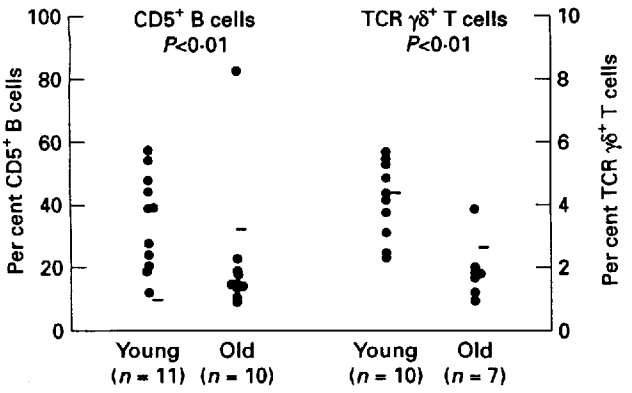

Fig. 1. Circulated CD5$^+$ B cell levels and TCR γδ$^+$ T cell levels in RA patients presenting before the age of 45 years (young) and after the age of 65 years (old). Horizontal bars represent the means of age-matched controls.

and cultured for 7 days. The spontaneous *in vitro* production of rheumatoid factors and immunoglobulins was measured and is shown in Table 2. Production of IgM, IgG, IgA, IgM RF and IgA RF were similar in both groups. The levels of CD5$^+$ B cells and TCR γδ$^+$ T cells in peripheral blood were assessed by two-colour FACS analysis, and results are shown in Fig. 1. CD5$^+$ B cells were significantly elevated in the young onset group (26·6 ± 4·5; mean ± s.c.m.) compared with the older onset group (14·2 ± 1·2%; $P < 0·01$) or with young normal individuals studies (12·3 ± 1·9%, $P < 0·01$). In contrast, CD5$^+$ B cell levels in elderly onset patients were significantly reduced compared with elderly controls (30·2 ± 3·0%, $P < 0·01$) but were similar to young controls (NS). With regard to the controls, the elderly group had significantly raised CD5$^+$ B cell levels compared with the younger controls studied ($P < 0·01$).

TCR γδ$^+$ T cell levels were also significantly raised in young patients (4·0 ± 0·9%) compared with elderly patients (1·6 ± 0·2%; $P < 0·01$), but were similar to young controls (4·46 ± 1·36%). Older onset RA patients did not have statistically different TCR γδ$^+$ T cell levels from age-matched controls (1·6 ± 0·2% *versus* 3·0 ± 0·8%; NS). Both young and old control groups had similar γδ$^+$ T cell levels (4·46 ± 1·36% *versus* 3·0 ± 0·8%, NS). In RA patients, a significant positive correlation was observed between the CD5$^+$ B cell and TCR γδ$^+$ T cell subsets ($r = 0·72$; $P < 0·05$). Total T cell levels (CD3$^+$) and B cell levels (CD19$^+$) were similar in patient groups and in age-matched controls (Fig. 2).

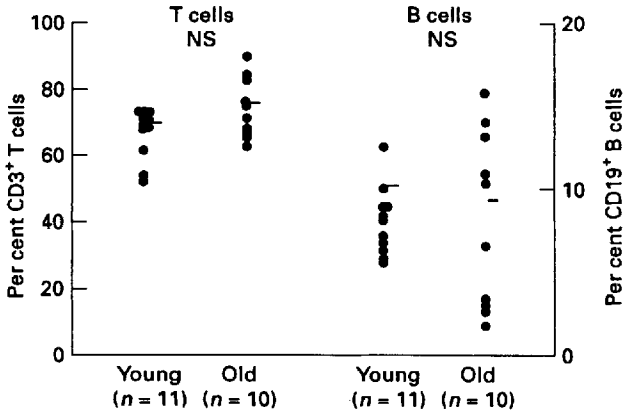

Fig. 2. Total T and B cell levels in the peripheral blood of RA patients as defined in Fig. 1. Horizontal bars represent the means of age-matched controls.

DISCUSSION

The principal observation of this study is the expansion of CD5$^+$ B cells and TCR γδ$^+$ T cells in the peripheral blood of RA patients with onset of disease before 45 years of age, but not in patients with disease onset after the age of 65 years. These findings support the concepts that these cell types play a role in the more aggressive disease process seen in young RA patients, and that the immunological reactivity and manifestation of the disease differs depending on the age of onset in patients.

Although CD5$^+$ B cells have been generally defined as polyclonal and secreting multispecific antibodies, clonal expansion and monoreactivity have been observed in RA [13]. Such clonal dominance may be due to mechanisms such as antigenic stimulation or an intrinsic proliferative advantage which expand the CD5$^+$ B cell subset. Greater rates of immunoglobulin synthesis have been reported in the CD5$^+$ B cell population compared with the conventional CD5$^-$ B cell [14,15]. The inheritance of greater concentrations of CD5$^+$ B cells may therefore represent part of the genetic susceptibility to produce autoantibodies [16] and perhaps in predisposed young adults it may contribute to the development of autoimmune disease such as RA. In the elderly, however, RA may develop as a consequence of perturbation of the immune response and as such, expansion of the CD5$^+$ B cells which is seen in healthy elderly individuals does not occur. This may explain the less aggressive disease intensity seen in elderly onset RA patients, where CD5$^+$ B cells may not have as important a role in the pathogenesis of the disease.

Other studies examining CD5$^+$ B cell levels have not adequately addressed the contribution of therapy of patients. We have previously reported that patients receiving gold therapy had significantly lower CD5$^+$ B cells compared with patients on first line drugs [17]. In the present study, both groups of patients were carefully selected so that they were not receiving remittive therapy at the time of study, so the influence of treatment is not a contributing factor to the expansion of CD5$^+$ B cells in younger onset RA patients only.

In agreement with other reports, we showed that patients with onset of RA after the age of 65 years had a high ESR (Table 1). Furthermore, weak RF positivity as detected by latex agglutination has been reported in older onset patients. Similarly, we have shown lower spontaneous production of IgM-RF *in vitro*, although statistical significance was not reached (Table 2). This is probably due to the small numbers of patients studied in each group. No correlation was observed between ESR or IgM-RF levels and CD5$^+$ B cell levels.

A recent report has shown that there is a progressive decrease in the complexity of the γδ T cells in blood with age [18]. Giachino and colleagues [18] demonstrate that the elderly display oligoclonal expansions and tend to have a reduction of the polyclonal component. This was observed in elderly healthy individuals and patients with RA, suggesting that reports of

oligoclonal $\gamma\delta$ cells in the peripheral blood of RA patients may simply reflect the age of the patient and not the disease state [18].

Our data suggest the following scenario. In the younger onset RA patients, the $CD5^+$ B cell and $\gamma\delta^+$ T cell repertoire are unrestricted, and continuous antigenic stimulation may result in clonal expansions which reflect the selective stimulation as seen by $\gamma\delta$ T cell clones responding to *Mycobacterium tuberculosis* or hsp60. On the other hand, elderly onset patients have restricted repertoire of their $CD5^+$ B cells and $\gamma\delta^+$ T cells, because by their age a few particular clonotypes have already been expanded and these may not be due to the 'suspected antigens' causitive of RA. However, further studies are required to compare the specificities and repertoires of $CD5^+$ B cells and $\gamma\delta^+$ T cells in younger and older onset RA patients.

The findings in the present study suggest that $CD5^+$ B cells and $\gamma\delta^+$ T cells may play a role in the pathogenesis of RA in younger onset patients, but not older onset patients. Because the disease process appears to be different in these patient groups, the $CD5^+$ B cell and $\gamma\delta^+$ T cell status and age of patient need to be considered before implementation of any treatment protocol. Finally, since paediatricians have reclassified juvenile RA into five subsets, one wonders whether adult RA being a heterogeneous condition needs to be subdivided into subsets based on age, clinical course, RF positivity and HLA association.

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