Low molecular weight IgM and CD5 B lymphocytes in rheumatoid arthritis

Hui Xu, Robin Geddes, Peter J Roberts-Thomson

Abstract

**Objectives**—To evaluate the role of low molecular weight (LMW) IgM and CD5 B cells in rheumatoid arthritis (RA) and to explore the possibility that LMW IgM is derived selectively from this subset of B cells.

**Methods**—LMW IgM in sera and culture supernatants was detected by a sensitive immunoblot technique with an enhanced chemiluminescence detection system. CD5 B cells were determined by FACScan cytometry. In vitro studies were established in culture plates containing pokeweed mitogen with or without 2-mercaptoethanol (2-ME). Supernatants were obtained from CD5 positive hybridomas and CD5 negative hybridomas. Other immunological indices were measured by laser nephelometry.

**Results**—Circulating LMW IgM was detected in all rheumatoid patients with significantly higher levels being observed in sero-positive patients. LMW IgM correlated significantly with total IgM and RF. Peripheral blood mononuclear cells (PBMC) from the majority of the patients with RA secreted LMW IgM in vitro as did mononuclear cells from a synovial fluid sample. The addition of low concentrations of 2-ME to the culture medium enhanced the proportions of secreted monomeric IgM. In contrast, PBMC from healthy subjects secreted only trace quantities of LMW IgM. In RA no significant correlations were observed between CD5 B cells and LMW IgM and RF. LMW IgM could be detected in the supernatants from both CD5+ and CD5− B cell lines. Finally, CD5 B cells were not significantly elevated in RA and levels remained constant over time.

**Conclusion**—LMW IgM exists in high concentrations in RA sera and synovial fluid. Serum level correlates with RF and IgM. In vitro studies have suggested that the occurrence of LMW IgM may be due to an intrinsic defect(s) in the assembly of the IgM pentameric molecule. LMW IgM is unlikely to be derived solely from CD5 B cells.

cells from patients with RA.\textsuperscript{15} These studies therefore suggest CD5 B cells are involved in autoimmune responses. Whether they contribute to the pathogenesis of autoimmune disease is still uncertain.

In this current study we have evaluated the role of LMW IgM and CD5 B cell in RA. We are particularly interested to observe any correlation between these two variables as it is possible that CD5 B cells may be the cellular origin of LMW IgM in RA.

Materials and methods

PATIENTS AND CONTROLS

Thirty eight patients with RA participated in the study. All fulfilled the American Rheumatism Association revised criteria for RA, and 31 of the patients were seropositive. There were 27 women and 11 men with a mean (SD) age of 68.3 (11.7) years and a mean (SD) duration of disease of 13.2 (6.3) years. Eight patients with other inflammatory arthritides and three patients with osteoarthritis were also studied. Control subjects consisted of 17 healthy adults from the clinical and laboratory staff. Sera and PB from patients and controls were collected at the same time. Synovial fluid (SF) was also collected from one patient with RA.

CELL PREPARATION

Peripheral blood mononuclear cells (PBMC) and mononuclear cell from SF were separated by Ficoll-Hypaque sedimentation using standard techniques. Ten mL sample was mixed with 10 mL phosphate-buffered saline (PBS) and layered slowly onto 10 mL Lymphoprep (Nyegaard, Oslo). The tubes were then centrifuged at 750 g for 15 minutes. The mononuclear layer was removed into PBS and washed once. Any remaining red blood cells were lysed by incubating at room temperature for 5 minutes with 5 mL ammonium chloride lysing solution. The cells were then washed twice with PBS, and resuspended at 10\textsuperscript{7}mL in RPMI 1640 medium with 10% fetal calf serum for immunofluorescence staining and in-vitro study.

IMMUNOFLUORESCENCE STAINING CD5 B CELLS

Prepared PBMCs were mixed with both 20 µl of FITC-anti-CD19 and the 20 µl of PE-labelled anti-Leu-1 antibody (anti-CD5, Beckton Dickinson) for 30 minutes. In some cases, the cells were first incubated with 50 µl of optimal diluted monoclonal antibody FMC63 (anti-CD19, Flinders Medical Centre) for 30 minutes. After washing twice, the cells were then incubated with 50 µl of FITC-conjugated sheep antibody to mouse Ig F(ab) fragment (Silenus Laboratory, Victoria) for 30 minutes. Following washing and blocking using normal mouse serum, the cells were finally incubated with the 20 µl of PE-labelled anti-Leu-1 antibody (anti-CD5, Beckton Dickinson) for 30 minutes. The cells were then washed three times and fixed.

Fluorescence analysis was carried out using FACScan flow cytometer (Beckton Dickinson, Mountain View, California) with standard settings. The efficacy of gating of the lymphocyte population was checked by the monocye marker CD14 (FMC33, Flinders Medical Centre). The monoclonal antibody X-63 (negative control for lymphocytes, Flinders Medical Centre), FMC16 (positive control for lymphocytes, Flinders Medical Centre), OKT3 (Pan-T-cell marker, American Type Culture Collection) were also used for control. For staining of X63, CD14, FMC16, and OKT3, the cells were first incubated with 50 µl of the optimal diluted monoclonal antibodies, and then incubated with 50 µl of FITC-conjugated sheep antibody to mouse Ig F(ab) fragment (Silenus Laboratory, Victoria) as described above. Ten thousand cells were analysed from each sample. The results were stored and analysed in list mode of lysis II program. To further analyse if the fluorescence distribution was unimodal or bimodal, 10 000 B cells were selected by live gating.

SECRETION OF LMW IgM IN VITRO

 Cultures were established in 24 well flat-bottomed 2 mL culture plate containing 1 \times 10\textsuperscript{5} PBMCs or mononuclear cells from SF/mL and pokeweed mitogen (PMW, Gibco, Ohio USA) at a final dilution of 1:800 with or without 2-mercaptoethanol (2-ME) at 10\textsuperscript{4}. Cultures were carried out in RPMI 1640 medium supplemented with penicillin G (200 U/mL), gentamicin (10 mg/mL), L-glutamine (0.3 mg/mL), and 10% fetal calf serum. The cells were incubated at 37°C in a humidified atmosphere of 5% CO2 for three days, the supernatants collected, and then with fresh culture medium for further four days culture (two stages) or continuing seven days culture (without collecting the supernatants at day 3). The collected culture supernatants (total volume 20–30 mL) were concentrated in an Amicon ultrafiltration unit to a final volume of 300–400 µL for LMW IgM quantitation. At day 7 the cell viability was determined.

CD5 HYBRIDOMAS CULTURE Supernatants

Culture supernatants from three CD5 positive hybridomas and two CD5 negative hybridomas were kindly provided by Dr C Brown (Division of Clinical Immunology, Kennedy Institute of Rheumatology, London). The hybridomas were established using in vivo activated B cells present in RA synovium immortalised by cell fusion using the heteromyeloma fusion partner, SPAZ-4. They all produced IgM. The supernatants (total volume 40–50 mL each) were concentrated in an Amicon ultrafiltration unit to a final volume 1–3 mL for further IgM size examination.

DETECTION OF LMW IgM

LMW IgM was detected by an enhanced chemiluminescence detection system combined with a modified immunoblot technique.\textsuperscript{31} In brief, serum and culture super-
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Results
LMW IgM IN THE SERA OF PATIENTS AND NORMAL CONTROLS
Sera from all patients and normal controls were examined for LMW IgM by the sensitive immunoblot method. In all patients' sera, distinct monomeric IgM bands were observed and in the majority of these dimeric, trimeric and other oligomeric IgM bands were also visible in addition to the pentameric band (fig 1). Among the patient groups, LMW IgM levels were significantly higher in sero-positive RA patients compared with sero-negative RA and other patients, (p<0.01) (table 1). In contrast, LMW IgM was detected only in low level or trace quantities in the sera from normal controls as compared with patient group (table 1, the immunoblot for the normal controls not shown).

STATISTICAL ANALYSIS
The liner correlation method was used for the analysis of any correlations between two variables. Comparison between patients and controls for LMW IgM and CD5 B cells were performed using the Student's t test. Significance was accepted if p<0.05.

IN VITRO STUDIES
PBMC were prepared from 25 patients with RA, two with osteoarthritis and 12 normal controls. In addition, mononuclear cells from the SF from a patient with active RA was also used for in vitro studies. Using the immunoblot method, distinct LMW IgM bands were clearly revealed in the culture supernatants of 20 patients with RA and two patients with osteoarthritis whilst only four normal controls secreted trace amounts of monomeric and oligomeric IgM in addition to pentameric IgM (fig 2). Remarkably, LMW IgM accounted for 40-1% of the total secreted IgM from mononuclear cells from SF. In these cell culture experiments cell viability at day 7 was 96% with no differences being observed between patients and controls. There was no significant correlation between amount of LMW IgM in the sera and supernatant (r = 0.2, p>0.05). Further studies found the addition of 2-ME to the cultures in final concentrations of 10^{-4} M resulted in the occurrence of increased quantities of monomeric and oligomeric IgM in the culture supernatant (fig 3). We have previously shown that this low concentration of 2-ME does not directly reduce secreted pentameric IgM to monomeric state in-vitro using the same experimental conditions as described above.22

Table 1 Serological and cellular indices in patients

<table>
<thead>
<tr>
<th>Patients and controls</th>
<th>Serological and cellular mean (SD) indices</th>
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<tbody>
<tr>
<td></td>
<td>LMW IgM (mg weight)</td>
</tr>
<tr>
<td>Sero-positive RA (n = 31)</td>
<td>279.3 (417.6)</td>
</tr>
<tr>
<td>Sero-negative RA (n = 7)</td>
<td>85.3 (47.5)</td>
</tr>
<tr>
<td>Other inflammatory arthritides (n = 8)</td>
<td>144.4 (131.1)</td>
</tr>
<tr>
<td>Osteoarthritis (n = 3)</td>
<td>110.9 (46.7)</td>
</tr>
<tr>
<td>Normal controls (n = 17)</td>
<td>52.4 (28.9)</td>
</tr>
</tbody>
</table>

*RF = Rheumatoid Factor, IU/mL (N<30)
*CRP = C Reactive Protein, mg/L (N<6)
Figure 2  Immunoblot appearance of culture supernatants from PBMCs (lane 1–7 and 9–15). One distinct monomeric IgM band (2 × 10⁶ D) can be seen in lane 2–7, 9–10 and 12 in addition to the pentameric band (1 × 10⁶ D). Lane 8 is a purified IgM containing both HMW IgM and LMW IgM as standard control. Note: the chemiluminescence reaction was visualised by exposure of the NC to radiographic film for 30 minutes.

Figure 3  (A) Immunoblot appearance of the supernatants from an in vitro study: 1) without 2-ME in culture medium; 2) with 2-ME in culture medium. An arrow indicates the band of LMW IgM. (B) Denstometric profiles of PBMC culture supernatants with corresponding to A. Enhanced secretion of LMW IgM and other oligomeric IgM from PBMC with 2-ME in culture medium was noted.

CD5 B cells in RA and normal controls
Using FACScan flow cytometry and two-colour fluorescence, CD5 B cells were enumerated in the PB of patients and controls. A comparison of the percentage of CD5 B cell in the different study groups is shown in fig 4. An increased mean (SD) percentage of CD5 B cells in sero-positive RA [35.0 (20.3)] and other inflammatory arthritides [41.6 (3.2)], and a decreased percentage in sero-negative RA [32.2 (10.7)] and osteoarthritis [30.9 (22.3)] was observed, when compared with normal controls [33.4 (9.3)]. These differences however were not significant (table 1).

CD5 B cells were measured in eight patients with RA and two with other inflammatory arthritides for more than two occasions over a one year period. CD5 B cells remained constant in these patients despite different disease activity or treatment regime (table 2).

To analyse if the fluorescence distribution of the CD5 antigen was unimodal or bimodal, 10 000 B cells were selected by live gating. It was shown that there were three populations of B cells, with the majority of B cells unstained, a small number of cells briefly stained for CD5 and a few intermediate staining cells. There was a significant low level of highly stained CD5 B cells in RA compared with controls (1.7% v 4.0%) (fig 5).

Correlation between LMW IgM, CD5 B cells and other immunological indices
Significant correlations were noted between LMW IgM and total IgM in RA (both sero-positive and negative. r = 0.76, r = 0.94, p<0.05, respectively), but not in patients with other inflammatory arthritides (r = 0.41, p>0.05) and osteoarthritis patients (r = 0.46, p>0.05). Significant correlations were also observed in LMW IgM and RF in sero-positive RA (r = 0.49, p<0.05) (fig 6). However, there were no significant correlations between total IgM and CD5 B cells (r = 0.018, p>0.05), LMW IgM and CD5 B cells (r = 0, p>0.05), RF and CD5 B cells (r = 0, p>0.05), and CRP and CD5 B cells (r = 0.016, p>0.05) in sero-positive RA and all other patient groups (fig 6).

LMW IgM in supernatants from IgM secreting B cell lines
To explore the cellular origins of LMW IgM in RA, CD5 B cell lines were cultured and the
supernatants examined for IgM size. It was shown that LMW IgM could be detected in the supernatants from both CD5+ and CD5− B cell lines (fig 7).

Discussion
Using our sensitive chemiluminescence detection system we have shown that LMW IgM existed in all our patients with RA and significant higher levels was found in the sero-positive patients (fig 1, table 1). Furthermore, a significant correlation was noted between LMW IgM and RF in these patients (fig 6).

The significance of LMW IgM in these sera is unknown. LMW IgM has been detected in both sera and SF of the patients with RA but not SF from other inflammatory arthritides.2,23,24 Anderson-Imbert23 reported that the concentrations of LMW IgM in SF was always exceeded that of the corresponding sera, but Roberts-Thomson et al,2 found that only two of 10 RA SF had higher LMW IgM levels than the corresponding sera, implying the local synovial membrane synthesis of LMW IgM. This is confirmed in our in vitro study which showed that secreted LMW IgM was found from mononuclear cells from the SF of a patient with RA and the amount of LMW IgM accounted for 40–1% of the total secreted IgM. This finding provides direct evidence that LMW IgM can be synthesised in pathogenic joints.

The mechanism(s) for the occurrence of LMW IgM in human disease are still uncertain. Previous reports clearly showed that LMW IgM was not the product of pentameric IgM metabolism in vivo and not derived from membrane-bound IgM as well.4 In this study we found a significant correlation between the levels of circulating LMW IgM and total IgM (fig 6); the presence of LMW IgM with other LMW oligomers of IgM (fig 1); and the secretion of LMW IgM from PBMC in vitro (fig 2). All these findings suggest that LMW IgM may be released into circulation due to a defect of assembly of the IgM pentameric molecule. This could be a deficiency in any of the multiple steps involved in IgM polymerisation. However, in these steps J chain and glycosylation of µ heavy chain is not required for IgM polymerisation, per se.25 In this study we showed the addition of low quantities of 2-ME to the culture medium could enhance

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**Table 2** CDS B cells in patients over time

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex/age</th>
<th>Diagnosis</th>
<th>Disease duration</th>
<th>Disease activity</th>
<th>Drug regime</th>
<th>Tested date</th>
<th>CDS B cells %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/71</td>
<td>SPRA</td>
<td>20 years</td>
<td>Active</td>
<td>Meth</td>
<td>01/07/91</td>
<td>31-8</td>
</tr>
<tr>
<td>2</td>
<td>F/60</td>
<td>SPRA</td>
<td>39 years</td>
<td>Active</td>
<td>Meth</td>
<td>07/10/91</td>
<td>32-2</td>
</tr>
<tr>
<td>3</td>
<td>F/71</td>
<td>SPRA</td>
<td>10 years</td>
<td>Active</td>
<td>Imuran/Pred</td>
<td>15/07/91</td>
<td>25-8</td>
</tr>
<tr>
<td>4</td>
<td>F/29</td>
<td>SPRA</td>
<td>4 years</td>
<td>Active</td>
<td>Pred</td>
<td>11/11/91</td>
<td>23-9</td>
</tr>
<tr>
<td>5</td>
<td>F/59</td>
<td>SPRA</td>
<td>18 years</td>
<td>Active</td>
<td>Pred/Plaq</td>
<td>16/03/92</td>
<td>56-1</td>
</tr>
<tr>
<td>6</td>
<td>F/61</td>
<td>SPRA</td>
<td>&gt;10 years</td>
<td>Inactive</td>
<td>None</td>
<td>04/05/92</td>
<td>10-9</td>
</tr>
<tr>
<td>7</td>
<td>M/62</td>
<td>SPRA</td>
<td>6 years</td>
<td>Active</td>
<td>Pred</td>
<td>06/07/92</td>
<td>9-8</td>
</tr>
<tr>
<td>8</td>
<td>M/69</td>
<td>SNRA</td>
<td>8 years</td>
<td>Active</td>
<td>Meth</td>
<td>06/07/92</td>
<td>25-5</td>
</tr>
<tr>
<td>9</td>
<td>F/88</td>
<td>OIA</td>
<td>1 year</td>
<td>Active</td>
<td>Sal/Pred</td>
<td>01/07/91</td>
<td>57-5</td>
</tr>
<tr>
<td>10</td>
<td>M/24</td>
<td>OIA</td>
<td>5 years</td>
<td>Active</td>
<td>Plaq</td>
<td>15/07/91</td>
<td>47-2</td>
</tr>
</tbody>
</table>

SPRA: Sero-positive RA; SNRA: Sero-negative RA; OIA: Other inflammatory arthritides; Meth: Methotrexate; Imuran: Azathioprine; Pred: Prednisone; Plaq: Plaquenil; Sala: Salazopyrin.
the secretion of LMW IgM from PBMC in vitro (fig 3). Our more recent experiment using a JVM3 cell line and others using a B lymphoma cell line have observed the same finding.22 26 This raises the possibility that alteration in the redox potential of the cellular microenvironment or the provision of free-SH radicals which might interrupt interchain disulphide bonds might be important. It is known that IgM polymerisation is catalysed by two protein enzymes-protein disulphide isomerase and protein disulphide oxidoreductase.27 28 Protein disulphide isomerase has multiple roles in the modification of nascent secretory proteins.27 Protein disulphide oxidoreductase, on the other hand, is identified to be a B lymphocyte specific enzyme that catalyses pentamer IgM assembly.28 The addition of 2-ME to the culture medium could interfere with the functional activity of this enzyme or the sulphydryl radicals of the cysteine involved in inter-chain disulphide links. It is found that cysteine in μ tailpiece plays a crucial role in the polymerisation of IgM.28 Thus molecular cloning and the characterisation of disulphide oxidoreductase should ultimately shed some insight into the gene regulation of polymerisation of IgM and provide basic insights into the mechanism underlying development and differentiation of B lymphocytes in human disease.

Whether LMW IgM is derived from a specific subset of B lymphocytes has not been previously explored. We therefore focused our investigation on CD5 B cells. Previous studies have found that LMW IgM commonly occurs in four disease groups.1 Coincidentally, in these diseases there invariably exist higher levels of circulating CD5 B cells or activated CD5 B cells.14 16 17 21 30 CD5 B cells are responsible for the production of high affinity RF in RA.15 Furthermore, LMW IgM and CD5 B cells from patients with RA shared the same major RF cross-reactive idiotype.31 These data indirectly suggest that LMW IgM may come from CD5 B cells. However, in this study no significant correlation was found between LMW IgM and CD5 B cells in patients with RA (fig 6). In vitro study showed that variable levels of LMW IgM could be detected in PBMC from the majority of patients with RA. Furthermore, LMW IgM could be detected in the supernatants from both CD5+ and CD5− B cell lines (fig 7). This study therefore suggests that LMW IgM is unlikely to be derived solely from CD5 B cells.
Low molecular weight IgM and CD5 B lymphocytes in rheumatoid arthritis

We also addressed the question of the role of CD5 B cells in RA. We found increased percentages of CD5 B cells in RA (fig 4), however, these differences were not significant compared with healthy controls (table 1). The increased frequency of CD5 B cells in RA blood was initially reported by Plater-Zyberk et al., in 1983 and observed by others, but not all reports have confirmed these findings. These controversial results may be due to a number of reasons, including the wide differences in the epitope density of CD5 on the B cell membrane, patient sampling, and the different sensitivities of the methods used. Our study found different epitope density of CD5 on the B cells with three populations of CD5 B cells being observed (fig 5). The significance of this bimodal fluorescence distribution in CD5 B cells is unknown but it appeared that the increased CD5 B cells in RA are the intermediate staining cells. Methods with different sensitivities for the detection of CD5 B cells give rise to the different levels of CD5 B cells in the circulation. For example, using fluorescence-microscopic techniques, CD5 B cells were found to comprise an average of 20% of the circulating B cells of patients with RA compared with less than 5% of the B cells of normal controls. However, Dauphine et al., using a sensitive flow-cytometric technique, reported that 46% of rheumatoid B cells were CD5 positive compared with 20% CD5 B cells in normal controls. Another report showed the control group of individuals in which the proportions of CD5 B cells rose from <5% when detected by microscopy to 19-5% when identified by the FACStar cytometry. In comparative evaluation CD5 B cells in RA patients using FACS analyser and FACSscan, different levels of CD5 B cells have been found in the same patient’s sample. As such, more sensitive techniques have resulted in smaller differences in the proportions of CD5 B cells in RA and normal controls.

Our data showed the levels of CD5 B cells in the PB of patients with RA and other inflammatory arthritis were stable over time (table 2). This finding has been supported by other investigations. The exception was patients treated with high-dose corticosteroids or combined immunosuppressive chemotherapy and irradiation. However, there appears to be no clear correlation between the numbers of CD5 B cells and the therapy. The increase of CD5 B cells also appears to be independent of disease activity in juvenile arthritis. The proportion of circulating B cells that express CD5 appears to be relatively constant in any given individual. A recent study found that elevated levels of CD5 B cells were a distinctive feature of selected families with RA, rather than a peculiar feature of patients with RA compared with their healthy relatives. Moreover, monozygotic twins discordant for RA have identical levels of CD5 B cells. Thus, these data suggest that the expression of CD5 on B cells, within the framework of RA is, at least partly, genetically determined. This conclusion is further supported by our finding that CD5 B cells are higher in certain ethnic groups such as Chinese. This finding is consistent with another report that Japanese have elevated levels of CD5 B cells.

In the present study no significant correlations were observed between CD5 B cells and other immunological indices including RF (fig 6). Several studies have also shown no apparent relationship between the relative CD5 B cell levels and the titre of RF. Moreover, not all patients with RA have high levels of CD5 B cells, and normal unaffected individuals without RF may have high levels of CD5 B cells.

What is the role of CD5 B cells in the pathophysiology of RA? The answer is still uncertain. Perhaps these uncertainties are secondary to the fact that the population of circulating lymphocytes in the PB is not representative of the cells found at sites of primary disease activity such as the peripheral joints. A study has already demonstrated that percentages of CD5 B cells in patients with RA were significantly higher in SF compared with that in PB. A closer examination of the cells and autoantibodies secreted within these sites may therefore reveal a more definitive relationship between the CD5 B cells and autoimmune diseases.

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35 Dauphinee M, Kincaid W, Talal N. Increased incidence of B cells coexpressing a T cell marker in rheumatoid arthritis. *Sixth International Congress of Immunology (abstr)* 1991; 3, 12, 9.
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