Limited VH gene usage in B-cell clones established with nurse-like cells from patients with rheumatoid arthritis

S. Nakamura-Kikuoka¹, K. Takahi², H. Tsuboi², T. Toyosaki-Maeda¹, M. Maeda-Tanimura¹, C. Wakasa¹, N. Kikuchi¹, S. Norioka³, M. Iwasaki¹, T. Matsutani⁴, T. Itoh⁴, S. Yamane¹, H. Takemoto¹, Y. Tsuruta¹, Y. Shimaoka², M. Yukioka⁵, R. Suzuki¹,⁶ and T. Ochi⁶

Objectives. Nurse-like stromal cells (NLC) in synovia and bone marrow of patients with rheumatoid arthritis (RA) can support pseudoemperipolesis, protect from apoptosis and enhance immunoglobulin production of peripheral blood B cells isolated from healthy individuals, suggesting the profound contribution of hyperactivation of B cells in RA. In the course of establishing RA-NLC from RA patients, we observed the growth of B cells in the presence of RA-NLC.

Methods. We cloned B cells from the synovium or bone marrow of RA patients using the limiting dilution technique. For established clones, nucleotide sequences of immunoglobulin and surface antigens were investigated. To investigate the dependence of these clones on NLC, differences in the proliferation and the amount of immunoglobulin produced in the presence or absence of NLC were compared. Immunocytochemical staining of various cells was performed using the antibody these clones produced.

Results. Nine B-cell clones established from RA patients showed RA-NLC-dependent growth. These B-cell clones expressed CD19, CD20, CD38, CD39 and CD40, suggesting that the cloned cells were mature and activated. All clones secreted immunoglobulins in culture media, which were specific for intracellular components of various cell lines, including RA-NLC. Interestingly, we found limited usage of immunoglobulin heavy-chain variable regions (VH) among B-cell clones from RA patients. These repertoires were reported to be detected preferentially in fetal livers.

Conclusion. The present study provides a novel insight into the involvement of RA-NLC in the immunopathogenesis of RA via an autoreactive B cell development and/or activation mechanism.

Key Words: Rheumatoid arthritis, B cell, Immunoglobulin VH gene, Autoantibody.
dependent on direct cell-cell contact with RA-NLC. These B cells secreted a large amount of immunoglobulin, and were CD5+, CD19+, CD20+, CD38+ and CD39+. Biased usage of VH repertoires was observed in B-cell lines established from each individual. Similar limited VH repertoires were previously reported in the fetal liver B cells [19–21] and autoreactive B cells [22]. The evidence for selective B cell-activation in the presence of RA-NLC could provide new insights into the immunopathogenesis of RA.

Materials and methods

Patients and specimens

All specimens were obtained with consent and used in accordance with the policies and procedures of the research institutional review board for human subjects at each laboratory and hospital. Synovial tissues were obtained from five RA patients and a non-RA patient. All patients in this study fulfilled the American College of Rheumatology Revised Criteria [23] for the diagnosis of RA at the time of joint reconstructive surgery in Osaka University Hospital.

Cell lines

HEp-2 (human epidermoid carcinoma), SiHa (human squamous carcinoma), Hs729 (human rhabdomyosarcoma) and ACHN (human adenocarcinoma) cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (Gibco BRL) and antibiotics (complete DMEM) in 7.5% CO2 at 37°C. RA-NLC were established from primary cultures of synovial tissues and bone marrow of RA patients as described previously [16, 18]. Briefly, synovial tissues were teased apart with scissors and digested into single cells using collagenase and hyaluronidase. The cells were washed and cultured in complete DMEM in 7.5% CO2 at 37°C. Heparinized bone marrow was obtained from the iliac crest. Mononuclear cells were separated from those with allogenic RA-NLC (data not shown), clones were cocultured with RA-NLC (5 × 104 cells/well) which had been previously cultured for 2 days in a 24-well flat-bottomed culture plate and treated with mitomycin C (MMC; Kyowa Hakko Kogyo, Tokyo, Japan) at 25 μg/ml at 37°C for 1 h. Cells were washed and seeded onto a 24-well flat-bottomed culture plate (Costar). The next day, 100 000 B-cell clones were cocultured with MMC-treated RA-NLC for 9 days. Cells were then pulsed with 0.5 μCi of tritiated thymidine (Radiochemical Center, Amersham, UK) for 18 h and were harvested onto glass filters. Radioactivity was measured on a β-scintillation counter. Experiments were performed with no additional cytokine or growth factor.

Establishment of RA-NLC-dependent B-cell lines and clones

To establish RA-NLC, primary cultures of the synovial tissue or the bone marrow were maintained over 6 months allowing the proliferation of lymphoblastic cells. The cells were adjusted to 2–3 × 105 in 75 cm2 culture flasks (Costar, Corning, NY, USA) every week. After lymphoblastic cells were sufficiently grown, these cells were stained for B-cell surface antigens as described below. B-cell clones were established from these B-cell lines by the limiting dilution method. Briefly, ten thousand autologous RA-NLC were cultured in 96-well plate (Costar) for 3 days, then B-cell lines were cocultured on RA-NLC at 0.5 or 1 cell per well in complete DMEM. Cells, when proliferated, were transferred into a 48-well culture plate (Becton Dickinson Labware, Bedford, MA, USA) and further expanded with autologous RA-NLC. Since no significant difference was seen in the phenotypes and immunoglobulin production of B cells cocultured with autologous RA-NLC from those with allogenic RA-NLC (data not shown), clones were maintained either with autologous or allogenic RA-NLC every month.

Molecular analysis of immunoglobulin genes

Immunoglobulin heavy chain genes of each B cell were amplified by adapter-ligation mediated PCR as described previously [24] with minor modification. Briefly, total RNA from each of the B cells isolated using Trizol reagent (Gibco BRL) were submitted for synthesis of first-strand cDNA using BSL-18 primer, which contains oligo-dT and the NotI cutting site. After second-strand synthesis, P20EA/P10EA adapter was ligated to double-stranded cDNA, then digested with NotI. Adapter-ligated cDNAs were submitted for nested PCR using P20EA and primers specific for C-regions. The sequences of PCR primers were as follows: IgG, first PCR (CG1: CAC CTT GGT GTT GGT GGG CTT), second PCR (CG2: TCC TGA GGA CTG TAG GAC AGC); IgA, first PCR (CA1: GGT TGC TCG TGG TGT AC), second PCR (CA2: GGA AAG TTT CTG GGG GTC AC); IgM, 1st PCR (CM1: TCC TGT GCG AGG CAG CCA A), second PCR (CM2: GTC TCC GAC GGG GAA TTC TC).

Nested PCR products were cloned into pGEM-T vector (Promega, Madison, WI, USA). Dye terminator cycle sequencing was carried out using T7 primer according to the manufacturer’s instruction (Beckman Coulter, Fullerton, CA, USA). The cDNA sequences were compared with the corresponding human germ-line VH gene segments in GenBank using the BLAST program.

Cell proliferation assay

RA-NLC (5 × 104 cells/well) were cultured for 2 days in a 24-well flat-bottomed culture plate and treated with mitomycin C (MMC; Kyowa Hakko Kogyo, Tokyo, Japan) at 25 μg/ml at 37°C for 1 h. Cells were washed and seeded onto a 24-well flat-bottomed culture plate (Costar). The next day, 100 000 B-cell clones were cocultured with MMC-treated RA-NLC for 9 days. Cells were then pulsed with 0.5 μCi of tritiated thymidine (Radiochemical Center, Amersham, UK) for 18 h and were harvested onto glass filters. Radioactivity was measured on a β-scintillation counter. Experiments were performed with no additional cytokine or growth factor.

Measurement of the amount of immunoglobulin production

To determine the immunoglobulin isotype, culture supernatants of each B-cell clone were tested with a human immunoglobulin isotyping kit (The Binding Site, Birmingham, UK) based on the ouchterlony immunodiffusion technique. B cells (1 × 105 cells) were cocultured with RA-NLC (5 × 104 cells/well) which had been previously cultured for 2 days in a 24-well flat-bottomed culture plate. In some experiments, B cells were cultured on Millicell culture plate inserts (Nihon Millipore Kogyo, Tokyo, Japan) at 25 μg/ml at 37°C for 1 h.

Purification of antibody from culture media

The antibodies were purified using protein A and/or protein G Sepharose 4B (Amersham Pharmacia Biotech) chromatography according to the manufacturer’s instructions. For the detection of RF, purified antibodies were incubated with denatured human IgG-coated latex particles using total RA Test-N (Nissui Pharmaceutical, Tokyo, Japan).

Immunocytochemical staining

RA-NLC, HEP-2, SiHa, Hs729 and ACHN cells were cultured in a chamber slide (Nalge-Nunc International, Roskilde, Denmark)
at 37°C. After 16 h, culture medium was removed and cells were fixed on the slides with cold acetone at −20°C. These slides were air-dried and stored at −80°C until use. For immunocytochemical staining, slides were incubated with purified antibodies at 100 μg/ml with 0.1% bovine serum albumin (BSA)/phosphate-buffered saline (PBS) at 4°C for 18 h. Slides were washed with PBS, then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-human immunoglobulin antibody (Dako, Carpinteria, CA, USA) diluted 1:200 with 0.1% BSA/PBS for 2 h at 37°C. After washing with PBS, the slide was covered with a coverglass in 50% glycerol/PBS and examined under a fluorescence microscope.

**Phenotype of B-cell clones**

The clones were stained with FITC-conjugated anti-human monoclonal antibodies specific for CD5, CD11a, CD20, CD38 and CD40 (BD Pharmingen, San Diego, CA, USA) and for human IgA, IgG, IgM, IgD, kappa and lambda (Dako). They were also stained with phycoerythrin-conjugated anti-human monoclonal antibodies specific for CD19 and CD39 (BD Pharmingen). Anti-human monoclonal antibodies specific for CD49d (Upstate Biotechnology, Lake Placid, NY, USA) was non-labelled and FITC-conjugated goat anti-mouse IgG (BD Pharmingen) was used as the secondary antibody. Anti-human CXCR4 (R&D Systems, Minneapolis, MN, USA) was detected by staining with Avidin-R-Phycoerythrin (Serotec, Raleigh, NC, USA). The stained cells were analysed using a FACSscan 

**Detection of Epstein–Barr virus (EBV) genome**

Genomic DNA samples were isolated from each B-cell clone using the DNeasy™ Tissue Kit (Qiagen, Valencia, CA, USA). To remove RA-NLC, B-cell clones were collected, washed and stained with CD19 antibody conjugated with magnetic microbeads (Miltenyi Biotec, Germany) and passed through the magnetic column. CD19+ purity of the B-cell clones after the procedure was >98%, using FACS analysis. A PCR specific for the BamHI W repeat region of the EBV genome was performed. Primers for the above region were synthesized and the PCR reaction was conducted as described previously [25]. Amplified products were electrophoresed in 1% agarose gel and visualized with ethidium bromide staining.

**Western blot analysis of B-cell clones**

The cell lysates were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reduced conditions, and the electrophoresed protein was transferred to PVDF membrane (Millipore, Bedford, MA, USA) in a semi-dry blotting system. The membrane was blocked with 3% skimmed milk/1% BSA/PBS at 4°C for 18 h, then cut into strips and incubated with antibodies diluted with 1% BSA/PBS at room temperature for 3 h. Strips were washed with 0.5% Tween 20/PBS and reacted with horseradish peroxidase-labelled anti-human immunoglobulin antibodies diluted 1:2000 with 0.1% BSA/PBS for 1 h. After washing, bound antibodies were detected with an electrochemiluminescence system (ECL; Amersham Pharmacia Biotech).

**Immunoprecipitation**

RA-NLC, HEP-2, SiHa, HS729 and ACHN were cultured in semi-confluent conditions. Cells were collected with a cell scraper (BD Labware) and lysed with RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 μg/ml aprotinin, 1 mM PMSF) or hexadecyltrimethylammonium bromide (CTAB) buffer [1% CTAB, 5 mM EDTA, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 μg/ml aprotinin, 1 mM PMSF]. Cell lysates were mixed with purified antibodies at 4°C for 18 h. The immune complexes were recovered by protein G Sepharose 4B and the eluates were subjected to SDS-PAGE. The reacted proteins were visualized by silver staining (Wako Pure Chemical, Osaka, Japan).

**Results**

**Establishment of B-cell clones and molecular analysis of immunoglobulin repertoire**

We found that B cells proliferated in a long-term culture with NLC from RA bone marrow and synovial tissue. We analysed the immunoglobulin VH gene repertoire of these B-cell lines (Table 1). Since only one VH gene was detected in RA3 and RA45 cell lines, these lines were considered to be monoclonal. The B-cell lines generated from RA32 and RA79 had broader repertoires. Highly limited VH repertoires have been reported in B-cells of the human fetal liver [19–21]. Interestingly, the majority of the detected VH gene usage of our B-cell lines was contained within the limited repertoire found in the human fetal liver.

Using the limiting dilution method, we established B-cell clones and analysed their immunoglobulin repertoire (Table 2). For clones established from RA3, RA32, RA45, RA79 and RA176, nucleotide sequences of the V-D-J region were all identical in each patient. In RA32 and RA79, broader repertoires were detected before cloning and only the most frequently used repertoire in each B-cell line was cloned. From RA133, four different B-cell clones were detected, with the VH1-69 gene subgroup used most frequently. Nucleotide sequences of clones from RA79 and RA176 belong to the same putative germ-line gene, 3-30, and RA3 and RA133 belong to the same germ-line gene, 3-48.

The replacement/silent (R/S) ratios in mutations of complementarity-determining region (CDR) of VH gene differed for every clone. All mutations were R in RA32; on the other hand, the replacement/silent (R/S) ratio of mutations in RA79 was S. These lines were considered to be monoclonal. The B-cell lines generated from RA32 and RA79 had broader repertoires. Highly limited VH repertoires have been reported in B-cells of the human fetal liver [19–21]. Interestingly, the majority of the detected VH gene usage of our B-cell lines was contained within the limited repertoire found in the human fetal liver.

**Table 1. Repertoire of immunoglobulin heavy-chain variable regions of B-cell lines established from RA patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Origin</th>
<th>VH gene</th>
<th>Homology (%)</th>
<th>Frequency (N/T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA3</td>
<td>Synovial tissue</td>
<td>3–48</td>
<td>95.9</td>
<td>9/9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3–13</td>
<td>96.6</td>
<td>6/10</td>
</tr>
<tr>
<td>RA32</td>
<td>Bone marrow</td>
<td>3–21</td>
<td>95.6</td>
<td>2/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4–4</td>
<td>94.9</td>
<td>1/10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4–4</td>
<td>93.2</td>
<td>1/10</td>
</tr>
<tr>
<td>RA45</td>
<td>Synovial tissue</td>
<td>3–9</td>
<td>94.2</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3–10</td>
<td>93.8</td>
<td>13/36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3–3</td>
<td>93.9</td>
<td>9/36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2–5</td>
<td>87.9</td>
<td>8/36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3–9</td>
<td>91.5</td>
<td>3/36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3–7</td>
<td>89.1</td>
<td>1/36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3–33</td>
<td>85.7</td>
<td>1/36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4–18</td>
<td>86.8</td>
<td>1/36</td>
</tr>
</tbody>
</table>

aBold face signifies VH usage corresponding to that previously reported in B cells in fetal liver [19–21]. Underlined values correspond to VH usage previously reported in autoantibodies [22].

Homology was calculated by similarity at the nucleic acid level.

Number of immunoglobulin genes detected (N) out of total number (T) of sequences analysed.
CDR showed that every clone was different (Table 3). Nucleotide and amino acid sequences of CDR5 region are shown in Table 6 as supplementary data.

In total, nine B-cell clones were established from six patients. Clones predominantly expressed members of VH3 family with no representation of the VH2 or VH4 family. The homology of VH regions of B-cell clones ranged from 89.0 to 96.6%. For JH regions, dominant usage of the JH4 family was observed, but there was no correlation between VH, JH and DH usage.

Immunoglobulin production of B-cell clones

The growth of B-cell clones was found to be RA-NLC-dependent, similar to previous findings in B-cell lines established from RA patients [16]. Direct contact with RA-NLC appeared necessary for the proliferation of B-cell clones. B cells showed no growth when cultured in medium alone, while proliferation was remarkably high in the presence of RA-NLC (Fig. 1).

Table 2. Analysis of immunoglobulin heavy-chain regions of the B-cell clones

<table>
<thead>
<tr>
<th>Patient</th>
<th>Origina</th>
<th>Representative name of clone</th>
<th>V gene subgroup</th>
<th>VH gene b</th>
<th>%</th>
<th>D gene subgroup</th>
<th>J gene subgroup</th>
<th>Frequencyd (N/T)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA3</td>
<td>Sy</td>
<td>RA3a</td>
<td>VH3</td>
<td>3–48</td>
<td>95.9</td>
<td>DH5</td>
<td>JH4</td>
<td>9/9</td>
</tr>
<tr>
<td>RA32</td>
<td>BM</td>
<td>RA32a</td>
<td>VH3</td>
<td>3–13</td>
<td>96.6</td>
<td>DH6</td>
<td>JH4</td>
<td>6/6</td>
</tr>
<tr>
<td>RA45</td>
<td>Sy</td>
<td>RA45a</td>
<td>VH3</td>
<td>3–9</td>
<td>94.2</td>
<td>DH6</td>
<td>JH4</td>
<td>6/6</td>
</tr>
<tr>
<td>RA79</td>
<td>Sy</td>
<td>RA79a</td>
<td>VH3</td>
<td>3–30</td>
<td>93.8</td>
<td>DH3</td>
<td>JH4</td>
<td>7/7</td>
</tr>
<tr>
<td>RA133</td>
<td>Sy</td>
<td>RA133a</td>
<td>VH1</td>
<td>1–69</td>
<td>95.9</td>
<td>DH3</td>
<td>JH4</td>
<td>4/10</td>
</tr>
<tr>
<td>RA176</td>
<td>Sy</td>
<td>RA176</td>
<td>VH3</td>
<td>3–30</td>
<td>92.1</td>
<td>DH5</td>
<td>JH4</td>
<td>6/6</td>
</tr>
</tbody>
</table>

aBM, bone marrow; Sy, synovial tissue.
bBold face signifies VH usage corresponding to that previously reported in B cells in fetal liver [19–21]. Underlined values correspond to VH usage previously reported in autoantibodies [22].
cHomology was calculated by similarity at the nucleic acid level.
dNumber of immunoglobulin genes detected (N) out of total number (T) of analysed sequences.

Table 3. Analysis of immunoglobulin heavy-chain regions of the B-cell clones

<table>
<thead>
<tr>
<th>Name of clone</th>
<th>FRb</th>
<th>CDRb</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA3a</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>RA32a</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>RA45a</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>RA79a</td>
<td>7</td>
<td>1</td>
</tr>
</tbody>
</table>

bFR, framework; CDR, complementarity-determining region.
CR, replacement (R) and silent (S) mutations in the framework and CDR regions.

CDR showed that every clone was different (Table 3). Nucleotide and amino acid sequences of CDR5 region are shown in Table 6 as supplementary data.

In total, nine B-cell clones were established from six patients. Clones predominantly expressed members of VH3 family with no representation of the VH2 or VH4 family. The homology of VH regions of B-cell clones ranged from 89.0 to 96.6%. For JH regions, dominant usage of the JH4 family was observed, but there was no correlation between VH, JH and DH usage.

Immunoglobulin production of B-cell clones

The growth of B-cell clones was found to be RA-NLC-dependent, similar to previous findings in B-cell lines established from RA patients [16]. Direct contact with RA-NLC appeared necessary for the proliferation of B-cell clones. B cells showed no growth when cultured in medium alone, while proliferation was remarkably high in the presence of RA-NLC (Fig. 1).

To determine the isotype of secreted antibodies produced by these B-cell clones, culture supernatants were examined. B-cell clones predominantly produced IgG (7/9) while a minority produced either IgA (1/9) or IgM (1/9) (Table 4). The production of immunoglobulin was also observed only when cocultured with RA-NLC. If B-cell clones were cocultured with RA-NLC but separated by a cell culture insert, proliferation and immunoglobulin production were markedly down-regulated (Fig. 1, Table 5). In summary, the proliferation of B-cell clones and their production of immunoglobulin are dependent upon direct contact with RA-NLC.

Immunocytochemical staining with antibodies produced by B-cell clones

B-cell clones from our RA patients produced 10–50 mg/l of immunoglobulins in their culture supernatants (data not shown). We purified immunoglobulins from culture supernatants by column chromatography and conducted immunocytochemical staining using antibodies secreted by these B-cell clones to examine their reactivity to human cell lines.

A monoclonal antibody purified from the culture supernatant of the RA32 B-cell clone showed speckled nuclear staining, while
eight purified antibodies from the other RA patients showed a diffuse cytoplasmic pattern of RA-NLC (Table 4, Fig. 2). All antibodies from culture supernatants stained human stromal cell lines established from other tissues, i.e. HEp-2, SiHa, Hs729 and ACHN. Our data suggest that the antibodies produced by the B-cell clones recognized antigens that were ubiquitously expressed in various tissues (data not shown).

**Surface phenotype of B-cell clones**

Individual B-cell clones showed a similar phenotype of cell-surface antigens (Figs 3 and 4). Although fluorescent intensities of antigens were slightly different from clone to clone, all clones expressed B-cell surface markers CD19, CD20 and CD40, which were not expressed on plasma cells. These B-cell clones showed a unique profile, staining double-positive for CD38 and CD39. Antigens CD38 and CD39 are known as markers of germinal centre (GC) B cells, though GC B cells usually expressed either CD38 or CD39, but not both. These clones did not express CD5, a marker of autoreactive B cells in humans [26]. We also analysed a panel of adhesion molecules, including CD11a [lymphocyte function-associated antigen 1 (LFA-1)], CD49d [very late antigen 4 (VLA-4)] and CXCR4, which are believed to be important for B-cell adhesion and pseudoemperipolesis activity involving RA-NLC [17] or fibroblast-like synoviocytes [27]. All clones expressed CD11a and CD49d but not CXCR4, suggesting CXCR4 is not essential for survival of these B-cell clones. In summary, surface marker analysis showed these cloned cells to be activated and mature B cells. Our data also suggest a less important role for the adhesion molecule CXCR4 in the survival of these clones.

**EBV transformation of B-cell clones**

B-cell clones were examined for EBV transformation by PCR. All samples showed amplification of the BamHI W repeat region of the EBV genome (data not shown).

**RA45 antibody reacts with 48-kDa protein expressed in HEp-2 cells**

In preliminary experiments, we could not detect any specific reactivity of these antibodies by western blot analysis (data not shown). This suggested that autoantibodies produced by the clones recognized the tertiary structure of the antigen(s). Further examination of the antigen-specificity of autoantibodies produced by B-cell clones was done in immunoprecipitation studies. Using purified RA45 autoantibody and RA-NLC lysate in CTAB buffer, a 48-kDa precipitate was detected (data not shown). This precipitate was also detected in lysate derived from HEp-2 cell, consistent with the antigen specificity observed in immunocytochemical staining. Antibodies purified from culture supernatants

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**Table 4. Recognition of autoantigen by immunoglobulins produced by B-cell clones**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Representative name of clone</th>
<th>Secreted immunoglobulin</th>
<th>Immunofluoresence staining pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA3</td>
<td>RA3a</td>
<td>IgM</td>
<td>NT</td>
</tr>
<tr>
<td>RA32</td>
<td>RA32a</td>
<td>IgG</td>
<td>Nucleus (speckled)</td>
</tr>
<tr>
<td>RA45</td>
<td>RA45a</td>
<td>IgA</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>RA79</td>
<td>RA79a</td>
<td>IgG</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>RA133</td>
<td>RA133a</td>
<td>IgG</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>RA133b</td>
<td>IgG</td>
<td>Cytoplasm</td>
<td></td>
</tr>
<tr>
<td>RA133c</td>
<td>IgG</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>RA133d</td>
<td>IgG</td>
<td>NT</td>
<td></td>
</tr>
<tr>
<td>RA176</td>
<td>RA176</td>
<td>IgG</td>
<td>Cytoplasm</td>
</tr>
</tbody>
</table>

* Determined by FACS analysis.
* Determined by Ouchterlony immunodiffusion method with culture supernatants. NT, not tested.

**Table 5. Immunoglobulin production by the B-cell clones**

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>RA32a</th>
<th>RA45a</th>
<th>RA79a</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA-NLC</td>
<td>&lt;31.3*</td>
<td>&lt;31.3</td>
<td>&gt;31.3</td>
</tr>
<tr>
<td>B cells</td>
<td>49.1</td>
<td>33.8</td>
<td>&lt;31.3</td>
</tr>
<tr>
<td>B cells + RA-NLC</td>
<td>119.4</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

* B-cell clones (1 × 10⁵) and NLC (5 × 10⁴) were cultured for 3 days in 24-well plates.
* The amount of immunoglobulin in the cell culture supernatants was measured with an ELISA kit as described in Materials and methods.
* B-cell clones were cultured on a Millicell culture insert and RA-NLC were cultured on the bottom of the same well separately.
* Undetectable level.

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**Fig. 2. Immunostaining pattern of RA-NLC with a monoclonal antibody purified from culture supernatant of B-cell clone.** Positive reaction was seen using FITC-conjugated anti-human immunoglobulin antibody, using supernatant from (a) clone RA32a and (b) clone RA79a. Original magnification, ×100.
of other B-cell clones did not show significant antigen specificity. Identification of the 48-kDa precipitate is now under way.

Discussion

In this study, we reported the establishment of B-cell clones from synovium and bone marrow of patients with RA. Previous studies have looked at the clonal analysis of B cells in the synovium of RA patients, but this is the first study to do this analysis on B-cell clones. These B-cell lines showed interesting characteristics, such as CD38 and CD39 double-positive phenotypes, autoantibody production, a restricted VH repertoire, and dependence on direct contact with RA-NLC for proliferation, differentiation and activation.

A large diversity of immunoglobulin is generated by recombination between V, D and J segments. However, immunoglobulin repertoire usage is not a random process. The VH3 family is used most frequently, followed by VH4 and VH1 in adult peripheral blood [28, 29]. Also, in each VH family, biased usage of particular VH gene segments was seen; for example, in the VH3 family, the 3-23 gene was most frequently used by healthy adults in peripheral blood [28]. While the 3-23 gene was found most frequently in the peripheral blood [30] and in the synovial tissue of patients with RA [31], we did not find the same germ-line gene in established B-cell lines and clones (Tables 1 and 2). Huang et al. [30] showed a lower frequency of the 3-30 gene in RA patients than in healthy donors. However, we detected 3-30 gene usage in two out of nine clones. Our data suggest that the process by which B-cell clones survive is not a random one.

B cells in the human fetal liver are believed to have highly restricted sets of VH gene segments [19–21], and these limited VH segments were widely used for various autoantibodies, including RF, anti-DNA and anti-thyrotropin receptor antibodies [22]. Our established B-cell clones recognized ubiquitous antigens. Consistent with these findings, autoreactive B-cell clones of this study used VH genes which were also frequently found in the fetal liver.

Autoreactive B cells are not always CD5+ [32], though CD5+ B cells have been reported to secrete autoantibodies in several autoimmune diseases [26]. The percentage of CD5+ cells increased in the peripheral blood of RA patients with CD5+ B cells detected in the synovium [33]. Natural antibodies are also produced by CD5+ B cells [34]. In this study, B-cell clones from RA patients did not express CD5 (Fig. 4).

Kim and colleagues reported three different subsets of infiltrating B cells in inflamed synovium [31]: (i) terminally differentiated plasma cells (CD20−, CD38+); (ii) mature CD20+, CD38− B cells; and (iii) activated B cells with GC phenotypes (CD20+ and CD38+ or CD39+). Our B-cell clones expressed CD19, CD20, CD38, CD39 and CD40, which suggests mature, activated cells. Instead of the similarities between RA synovia and lymph nodes, the surface antigens of our B-cell clones differed from those of these activated B cells. This unique expression pattern of surface antigens might be due to EBV, since EBV has been shown to induce B-cell activation [35]. Although our established clones were all transformed by EBV, they rapidly died when cultured in medium alone. Further study of this unique B cell phenotype in the peripheral blood and joints of RA patients is under way.
Rheumatoid synovia can support differentiation of activated B cells into plasma cells [12, 36–38]. RA-NLC and fibroblast-like cells found in RA synovia rescue B cells from apoptosis and have similar phenotypes to follicular dendritic cells in GC [17, 39]. For proliferation, activation and differentiation of these cloned B cells, direct contact and pseudoperoxisis between B cells and RA-NLC were necessary. In RA-NLC and B-cell interactions, LFA-1-ICAM-1 (intercellular adhesion molecule-1 protein) and VLA-4–VCAM-1 (vascular adhesion molecule 1) adhesion pathways appear to be involved [17, 27, 40]. Recently, Burger et al. [27] reported that, for pseudoperoxisis, stromal cell-derived factor-1 (SDF-1) and CXCR4 were involved. However, CXCR4 was not expressed on our B-cell clones, suggesting that another regulation system might be contributing to the pseudoperoxisis observed in this study (Fig. 4).

Determining the antigen that antibodies generated by B-cell clones recognize might be a clue to the immunopathogenesis of RA. Efforts were made to detect antigen(s) recognized by B cells by western blotting, but none showed specific signals. Since modification of Fc region might have weakened the affinity of autoantibodies produced by the B-cell clones, we did not use any labelled immunoglobulins which might have been useful in analysing antigen specificity. We did find, by the immunoprecipitation method, that the RA45 antibody recognized a 48-kDa molecule. Further work will be needed to define the antigens recognized and to delineate their role in the immunopathogenesis of RA.

Numerous studies have been done which have noted the accumulation of B cells in the inflamed synovium of RA. With the development and spread of molecular biology techniques, these B cells have been shown to be oligoclonal [41, 42], with hypervariation and extension of the D region in the VH gene [43–45]. It is known that GC B cells develop into memory cells which show increasing affinity to antigen through numerous somatic mutations of the VH gene of the CDR region. Most likely, the same mechanism applies to B-cell clones established from the synovium. However, our established B cells appear to be unique in terms of surface antigen pattern and the somewhat low R/S ratio of the CDR region. All antibodies secreted by these B cells recognize self-antigen. This suggests the existence of another mechanism by which self-reactive B cells are activated/maintained in the presence of RA-NLC.

Further questions to be studied include whether or not autoreactive B cells are the only cell subset that proliferates in the presence of RA-NLC. In order to answer this question, it is necessary to compare VH repertoires of peripheral B cells and B-cell clones obtained from identical RA patients. Although more detailed studies on the interaction between B cells and RA-NLC are required, we observed that RA-NLC support spontaneous growth of B cells with very limited VH repertoires. The antigen-specificity of these B cells is under study in our laboratory.

Conclusion

RA-NLC are indispensable for the generation and the activation of autoreactive B cells of patients with RA. Our results provide a
References


Clinical Vignette

Symmetrical muscle involvement in necrobiosis lipoidica

A 31-yr-old factory manager presented with a 6-month history of painless swellings over the extensor aspect of both forearms, associated with marked weakness, but no overlying skin changes. Plain X-rays were normal but MRI identified symmetrical signal change throughout the extensor muscle groups, with overlying subcutaneous oedema. Muscle enzymes, inflammatory markers and urinalysis were normal; muscle biopsy was inconclusive. The presentation was thought to be of a focal myositis.

The swellings improved over 12 months, leaving atrophied areas. Skin biopsy showed degenerate collagen, granulomatous inflammation and a vasculopathy affecting the small- and medium-sized vessels. These features were consistent with necrobiosis lipoidica.

Eighteen months later, the patient developed polyuria and polydipsia. His fasting plasma glucose was 15.8 mmol/l, and he was commenced on hypoglycaemic agents.

Necrobiosis lipoidica occurs in 0.3% of diabetic patients and approximately two-thirds of patients with necrobiosis lipoidica have diabetes at presentation. A further 15% will develop diabetes after the onset of the skin lesions.

This case is unusual in that the initial presentation was of swellings involving the extensor muscle compartment of both forearms. Only later did he develop the cutaneous features of necrobiosis lipoidica. No other cases of necrobiosis lipoidica presenting with clinical and radiological features of symmetrical muscle involvement have been reported.

The authors have declared no conflicts of interest.

D. SREERANGAIAH, C. PAGE, T. LAWSON, G. HERDMAN
Princess of Wales Hospital, Rheumatology, Bridgend, United Kingdom

Correspondence to: D. Sreerangaiah.
E-mail: dee.sreerangaiah@gmail.com