Table 4. Levels of interleukin (IL)-6 and interleukin (IL)-8 mRNAs in nurse-like cells derived from rheumatoid arthritis synovium (RA-NLCs) and monocytes that were fractionated after 24-h coculture

Cytokines	Cells	Ratio of mRNA	
		mRNA/GAPDH mRNA	Ratio
IL-6	RA-NLCs cultured alone	0.016	1
	RA-NLCs cultured with monocytes	1.189	76.5
	Monocytes cultured alone	0.025	1
	Monocytes cultured with RA-NLCs	0.131	5.2
IL-8	RA-NLCs cultured alone	0.025	1
	RA-NLCs cultured with monocytes	15.627	616
	Monocytes cultured alone	0.021	1
	Monocytes cultured with RA-NLCs	0.076	3.7

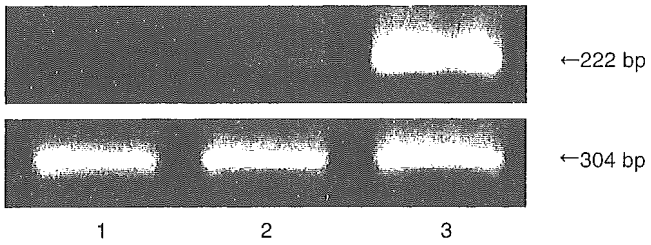

Fig. 4. Electrophoresis of polymerase chain reaction products of interleukin-6 (IL-6) (*top lane*) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*bottom lane*). The conventional revere transcription–polymerase chain reaction procedure was performed using Premix Tag (TaKaRa, Shiga, Japan) and LightCycler Primer Set (search-LC, Heidelberg, Germany) of human IL-6 and human GAPDH with annealing temperature at 60°C and amplification by 25 cycles for IL-6 and 20 cycles for GAPDH. Polymerase chain reaction products from monocytes cultured alone (*1*), nurse-like cells derived from rheumatoid arthritis synovium (RA-NLCs) cultured alone (*2*), and cocultured RA-NLCs and monocytes (*3*) are shown

coculture without direct contact was 347 ± 36 pg/ml, while that in the supernatant from RA-NLCs alone was 320 ± 25 pg/ml ($P = 1.000$) (Table 3). These results indicate that direct contact between RA-NLCs and monocytes is required for induction of cytokines.

Levels of mRNA were examined after RA-NLCs and monocytes were cocultured for 24 h by conventional RT-PCR (Fig. 4) and by quantitative RT-PCR (Table 4). For quantitative RT-PCR, cytokine mRNA levels were normalized using GAPDH mRNA as an internal control (Table 4). Relative levels of IL-6 and IL-8 mRNA in RA-NLCs cocultured with monocytes were approximately 80 and 620 times higher than those in RA-NLCs cultured alone, respectively (Table 4). Levels of IL-6 and IL-8 mRNA in monocytes cocultured with RA-NLCs were approximately 5 and 4 times higher, respectively, than those in monocytes cultured alone (Table 4).

Discussion

Coculture of RA-NLCs established from the synovial tissues of RA patients and monocytes freshly isolated from PBMCs of healthy donors resulted in the induction of high levels of IL-6 and IL-8. The levels of IL-6 and IL-8 were much higher when RA-NLCs were cocultured with CD14-positive cells (i.e., monocytes)[17] than when cocultured with CD14-negative cells, CD3-positive cells (i.e., T lymphocytes),[18] or CD19-positive cells (i.e., B lymphocytes).[19] The levels of IL-6 and IL-8 mRNA in RA-NLCs were also increased when cocultured with monocytes. When cultured together, RA-NLCs were more activated than monocytes, determined by the levels of IL-6 and IL-8 mRNAs in respective fractions. These results suggest that monocytes are more potent stimulators to RA-NLCs, than they are to monocytes.

Multiple inflammatory cytokines are known to be produced by various types of cells such as infiltrating T and B lymphocytes and monocytes/macrophages in the inflammatory synovial tissues.[8] Several studies have reported that interaction between synoviocytes and T and B lymphocytes promoted cytokine production.

RA-NLCs also interact with monocytes/macrophages. Our group[14] reported that monocytes differentiated into osteoclasts in two steps: cultured in the presence of RA-NLCs and then supplemented with IL-3 and distorted bones. Recently we also induced osteoclasts from CD14-positive cells in synovial fluids (SFs) from RA patients and OA patients by culturing whole cells in each SF and then with supplement of IL-3, and found that osteoclasts derived from RA-SF were larger, had more nuclei, and had more capacity of resorption pit formation on dentine slice and of resorption area formation on osteologic discs than those induced from OA-SF.[20] Chomarat et al.[21] reported that interaction of monocytes and synoviocytes from RA patients induced the expression of adhesion molecules, VCAM-1 and ICAM-1. There were reports of IL-6 production in the coculture of synoviocytes from RA patients and monocytes.[21,22] One study demonstrated that coculture of U937, monocytic cell line, and FLSs leads to enhanced production of IL-6.[23] The levels of IL-6 were, however, only three times higher in the supernatant fluids from coculture of RA synoviocytes and U937 cells than in those from cultures of RA synoviocytes alone. As U937 is an established cell line, use of monocytes freshly isolated from PBMCs is more appropriate and will provide more physiological information. Chomarat et al.[24] reported that coculture of monocytes from healthy donors and synoviocytes from RA patients resulted in IL-6 production; the levels of produced IL-6 were, however, only 15–25 times higher than the sum of those produced by monocytes and synoviocytes cultured alone. Moreover, they compared the effect of coculture of monocytes from healthy donors and synoviocytes from RA patients and that of coculture of monocytes and synoviocytes obtained from patients with knee ligament symptoms. There was no difference in the amount of IL-6 production.

The present study demonstrated that coculture of NLCs from RA patients (RA-NLCs), not FLSs from OA patients (OA-FLSs), and monocytes resulted in production of high levels of IL-6 and IL-8. The results suggest that NLCs from RA patients may have a unique property to be activated more easily than OA-FLSs and that, for RA-NLCs, monocytes are more potent stimulators than T or B lymphocytes.

Our results also indicate that direct cell–cell contact is required for the interaction between RA-NLCs and monocytes. Cytokine induction through coculture of RA-NLCs and monocytes was inhibited by anti-human TNF-α mAb. No supernatant sample contained detectable levels of TNF-α by ELISA. Monocytes/macrophages are known to be a major producer of TNF-α.[25] Tumor necrosis factor α is produced as a membrane-bound, 26-kDa proform,[26] and the mature, 17-kDa TNF subunit is released from the proform by proteolytic cleavage.[27–31] The membrane-bound TNF-α has biological activities as soluble TNF-α: inducing apoptosis, proliferation, or cytokine induction.[32] Together, it is likely that interaction between RA-NLCs and monocytes is mediated by the membrane-bound TNF-α.

The present study also demonstrated that monocytes are more potent stimulators for RA-NLCs than T or B lymphocytes. The results suggest that production of a large amount of cytokines through the interaction between RA-NLCs and monocytes may be one mechanism in the pathogenesis and maintenance of arthritis in RA. Recently, infliximab,[33] a chimeric anti-TNF-α mAb, and etanercept,[34] a soluble TNF-α receptor conjugated to Fc fragment of IgG, have been clinically applied as therapeutic reagents to RA. It is expected that these will effectively inhibit the interaction between RA-NLCs and monocytes/macrophages in inflammatory synovium in RA.

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

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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.

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by progressive joint destruction resulting from chronic inflammation [1]. Various inflammatory cells, including T cells, B cells and monocytes, infiltrate the synovium, and the formation of lymphonodular infiltrates containing lymphoid follicles is frequently seen [2]. Infiltrating B cells show several characteristics suggestive of an activated state: they proliferate and produce large amounts of immunoglobulin, such as rheumatoid factor (RF) [3]. RF is a group of autoantibodies that recognizes the constant region of IgG, and is frequently detected in patients with systemic autoimmune diseases [4]. On the other hand, various autoantibodies, including anti-nucleoprotein [5], anti-collagen [6, 7] and others [8–10] have been reported in the patients with RA. These findings suggest relationships between autoantibodies and autoimmune disease; however, B-cell involvement in these diseases is still to be elucidated.

Immunoglobulin heavy chain variable region (VH) gene of B cells in synovium of RA patients have been shown to be highly mutated, indicating an antigen-driven process of affinity maturation [11]. Although several studies showed that B-cell maturation occurred in affected joints [12–14], little is known about the precise mechanism. Reparon-Schuijt and colleagues [3] reported that fibroblast-like RA synoviocytes isolated from synovial fluid of RA patients stimulate IgM-RF production of B cells, suggesting the contribution of non-lymphoid cells in B-cell activation in RA joints.

We previously reported the establishment of nurse-like stromal cell lines (NLC) from bone marrow and synovium of RA patients [15, 16]. The RA-NLC as well as bone marrow stromal cells [17] supported the survival of B cells and enhanced their function [18]. NLC were not detected in the synovium of osteoarthritis patients or of normal subjects. These results suggested that RA-NLC may be a specific component of RA synovia and play an important role in RA pathogenesis.

In the present study, we characterized NLC-dependent B cells established from RA synovial tissue. The growth of B cells was

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dependent on direct cell–cell contact with RA-NLC. These B cells secreted a large amount of immunoglobulin and were $CD5^-$, $CD19^+$, $CD20^+$, $CD38^+$, $CD39^+$. 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 heparinized bone marrow aspirate was obtained from one 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% CO_2 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% CO_2 at 37°C. Heparinized bone marrow was obtained from the iliac crest. Mononuclear cells were separated by Ficoll–Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation and the cell suspensions were cultured in complete DMEM for 2 weeks. After non-adherent cells had been removed, adherent cells were maintained in complete DMEM in 7.5% CO_2 at 37°C.

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 $2–3 \times 10^5$ in 75-cm^2 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 *Not*I cutting site. After second-strand synthesis, P20EA/P10EA adapter was ligated to double-stranded cDNA, then digested with *Not*I. 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 GCT GGG CTT), second PCR (CG2: TCC TGA GGA CTG TAG GAC AGC); IgA, first PCR (CA1: GCT GGC TGC TCG TGG TGT AC), second PCR (CA2: GGG AAG TTT CTG GCG GTC ACG); IgM, 1st PCR (CM1: TCC TGT GCG AGG CAG CCA A), second PCR (CM2: GTA 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 (5×10^4cells/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 (1×10^5 cells) were cocultured with RA-NLC (5×10^4cells/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, Yonezawa, Japan) to prevent direct contact with RA-NLC. On day 3, the culture supernatants were collected and the concentration of immunoglobulins was measured with the Human Immunoglobulin ELISA Quantitation Kit (Bethyl, Montgomery, TX, USA).

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)

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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) 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 FACScan™ (BD Pharmingen) flow cytometer and CellQuest software (BD Pharmingen). Dead cells were excluded by propidium iodide staining. Forward and side scatter gates were determined for lymphoblastic cells to exclude possible contamination by RA-NLC.

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 *Bam*HI 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% skim milk/1% BSA/PBS at 4°C for 18 h and 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 semiconfluent conditions. Cells were collected with a cell scraper (BD Labware) and lysed with RIPA buffer (1% NP-40, 0.5% sodium deoxycolate, 0.1% SDS, 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 μg/ml aprotinin, 1 mM PMSF) or hexadecyltrimethyl-ammonium 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 number of mutations of R and S was the same in RA79. Moreover,

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

Patient	Origin	VH gene[a]	Homology[b] (%)	Frequency[c] (N/T)
RA3	Synovial tissue	3–48	95.9	9/9
RA32	Bone marrow	**3–13**	96.6	6/10
		3–21	95.6	2/10
		4–4	94.9	1/10
		4–4	93.2	1/10
RA45	Synovial tissue	3–9	94.2	6/6
RA79	Synovial tissue	3–30	93.8	13/36
		3–7	93.9	9/36
		2–5	87.9	8/36
		3–9	91.5	3/36
		3–7	89.1	1/36
		3–33	85.7	1/36
		4–18	86.8	1/36

[a]Bold 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]. [b]Homology was calculated by similarity at the nucleic acid level. [c]Number of immunoglobulin genes detected (N) out of total number (T) of sequences analysed.

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

Patient	Origin[a]	Representative name of clone	V gene subgroup	VH gene[b]	Homology[c] %	Homology[c] D gene subgroup	Homology[c] J gene subgroup	Frequency[d] (N/T)
RA3	Sy	RA3a	VH3	3-48	95.9	DH5	JH4	9/9
RA32	BM	RA32a	VH3	**3-13**	96.6	DH6	JH4	6/6
RA45	Sy	RA45a	VH3	**3-9**	94.2	DH6	JH4	6/6
RA79	Sy	RA79a	VH3	**3-30**	93.8	DH3	JH4	7/7
RA133	Sy	RA133a	VH1	**1-69**	95.9	DH3	JH4	4/10
	Sy	RA133b	VH3	3-66	91.4	DH6	JH5	3/10
	Sy	RA133c	VH3	3-48	89.0	DH2	JH3	2/10
	Sy	RA133d	VH5	**5-51**	91.5	DH3	JH3	1/10
RA176	Sy	RA176	VH3	**3-30**	92.1	DH5	JH4	6/6

[a]BM, bone marrow; Sy, synovial tissue. [b]Bold 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]. [c]Homology was calculated by similarity at the nucleic acid level. [d]Number 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

Name of clone	FR[a] R[b]	FR[a] S[b]	FR[a] R/S	CDR[a] R[b]	CDR[a] S[b]	CDR[a] R/S
RA3a	4	1	4.0	4	1	4.0
RA32a	4	1	4.0	5	0	–
RA45a	5	3	1.7	5	2	2.5
RA79a	7	1	7.0	4	4	1.0

[a]FR, framework; CDR, complementarity-determining region; [b]replacement (R) and silent (S) mutations in the framework and CDR regions.

comparison of the R/S ratio in the framework region (FR) and the CDR showed that every clone was different (Table 3).

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.

Nucleotide and amino acid sequences of CDR3 region is are shown in Table 6 as supplementary data.

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 Ig 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.

immunoglobulin

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).

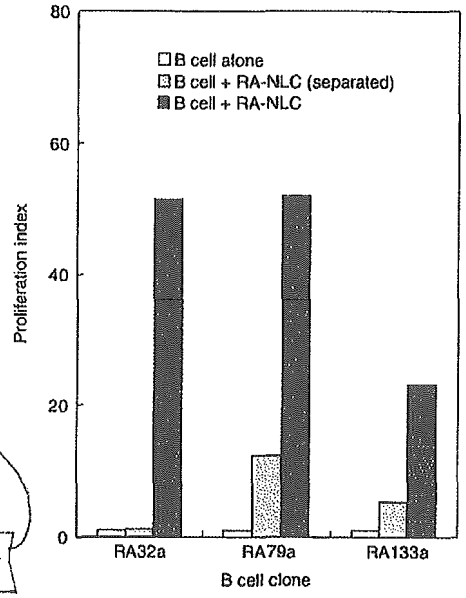

FIG. 1. Proliferation of established B-cell clones. B cells (1×10^5) were cultured alone or cocultured with or without direct contact with mitomycin C-treated RA-NLC (5×10^4) for 10 days. Proliferation index was calculated as experimental ^{3}H-TdR incorporation (mean c.p.m.)/^{3}H-TdR incorporation of B cells cultured alone (mean c.p.m.).

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

Patient	Representative name of clone	Isotype[a]	Secreted immunoglobulin[b]	Immunofluorescence staining pattern
RA3	RA3a	μ/m/κ	IgM	NT
RA32	RA32a	γ/g/κ	IgG	nucleus (speckled)
RA45	RA45a	α/a/λ	IgA	Cytoplasm
RA79	RA79a	γ/g/κ	IgG	Cytoplasm
RA133	RA133a	γ/g/κ	IgG	Cytoplasm
	RA133b	γ/g/κ	IgG	Cytoplasm
	RA133c	γ/g/κ	IgG	NT
	RA133d	γ/g/κ	IgG	NT
RA176	RA176	γ/g/κ	IgG	Cytoplasm

[a]Determined by FACS analysis; [b]determined by Ouchterlony immunodiffusion method with culture supernatants. NT, not tested.

in Greek alphabet, if you can

TABLE 5. Immunoglobulin production by the B-cell clones

Culture condition	Immunoglobulin production[a] (ng/ml)		
	RA32a	RA45a	RA79a
RA-NLC	<31.3[c]	<31.3	<31.3
B cells	49.1	33.8	<31.3
B cells + RA-NLC (separated)[b]	119.4	<31.3	<31.3
B cells + RA-NLC	150.2	>1000	>1000

B-cell clones (1×10^5) and NLC (5×10^4) were cultured for 3 days in 24-well plates. [a]The amount of immunoglobulin in the cell culture supernatants was measured with an ELISA kit as described in Materials and methods; [b]B-cell clones were cultured on a Millicell culture insert and RA-NLC were cultured on the bottom of the same well separately; [c]undetectable level.

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 (b) Clone RA79a. Original magnification, ×100.

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).

as supplementary data

TABLE 6. Nucleotide and amino acid sequences of CDR3 region of established B-cell clones

Clone	Sequence of CDR3 region																														
RA3a	GCG	AGA	GGG	TAT	AGT	GCG	ACC	CTC	GGC	GGA	AAC	TTT	GAC	TAC																	
	A	R	G	Y	S	A	T	L	G	G	N	F	D	Y																		
RA32a	GCA	CGA	GGA	TTG	GAT	AGC	AGC	AGC	TGG	AAC	GTT	TTT	GAC	TAC																	
	A	R	G	L	D	S	S	S	W	N	V	F	D	Y																		
RA45a	GCA	AAA	GAC	AGG	AAT	GCT	GGG	ACC	TTC	GAA	AGC	TTA	GAC	TCC																	
	A	K	D	R	N	A	G	T	F	E	S	L	D	S																		
RA79a	GCG	AGG	AGC	GGT	TTC	GGG	GAA	TTA	CCT	CTT	GAC	TAC																			
	A	R	S	G	F	G	E	L	P	L	D	Y																			
RA133a	GCT	CTG	GCC	CAC	TAC	GAC	CTT	GAT	AGT	AGT	GGT	TAT	GAC	TTG	TCG	GGT	CTT	AAT	TAC												
	A	L	A	H	Y	D	L	D	S	S	G	Y	D	L	S	G	L	N	Y												
RA133b	ACA	AGA	TGG	GAG	CAG	CTC	GGT	GGC	TGG	TTC	AAC	CTC																			
	T	R	W	E	Q	L	G	G	W	F	N	L																			
RA133c	GCG	AGA	GTT	TCG	ACC	GGA	GGG	GGA	TTT	TTT	ACT	GAA	GGT	GGT	GCC	TAT	AAT	GCT	TTT	GAT	TTG										
	A	R	V	S	T	G	G	G	F	F	T	E	C	G	A	Y	N	A	F	D	L										
RA133d	GCG	AGA	CCG	GGG	GGG	GGT	GCT	TTT	GAT	GTC																					
	A	R	P	F	F	F	A	F	D	V																					

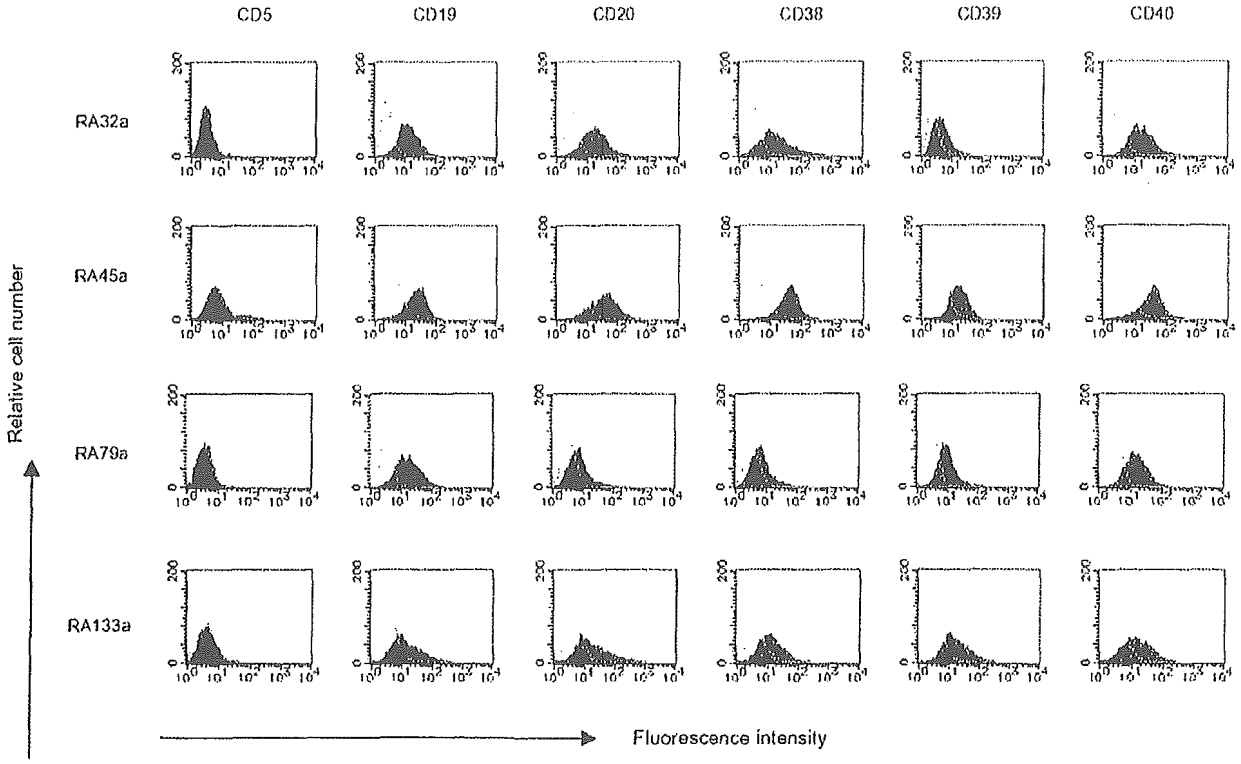

FIG. 3. Phenotypic analysis of B-cell clones for B-cell-specific markers. Horizontal and vertical axes illustrate log fluorescence and relative cell numbers, respectively. The histogram corresponding to each monoclonal antibody (shaded area) is superimposed on that of the negative control (open area) stained with an isotype-matched unrelated monoclonal antibody. Each row represents data from an individual RA patient.

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 *Bam*HI 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 immunocyto-chemical staining. Antibodies purified from culture supernatants 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

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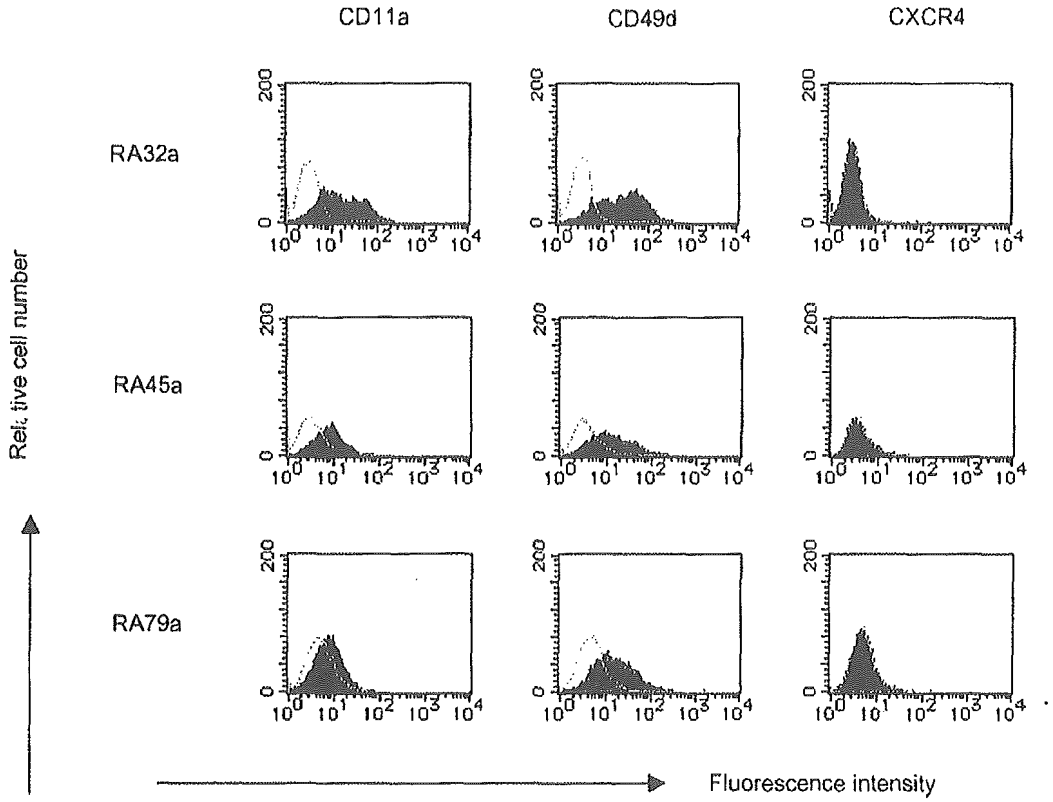

FIG. 4. Phenotypic analysis of B-cell clones for adhesion molecules and/or chemokine receptors. Horizontal and vertical axes illustrate log fluorescence intensity and relative cell numbers, respectively. The histogram corresponding to each monoclonal antibody (shaded area) is superimposed on that of the negative control (open area) stained with an isotype-matched unrelated monoclonal antibody. Each row represents data from one patient.

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 (Figs 3 and 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 pseudoemperipolesis 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 pseudoempelipolesis, 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 pseudoemperipolesis observed in this study ~~(Figs 3 and 4)~~. (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 possibly 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 hypersomatic mutation and extension of the N 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 novel insight into the involvement of RA-NLC in the immunopathogenesis of RA.

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[Ver: A3B2 8.07r/W] [29.10.2005–4:23pm] [FIRST PROOFS] [1–9] [Page No. 9] {Oup}rheumatology/kei170.3d (RHE) Paper: kei170

リウマチの破骨因子

■新たなリウマチの破骨細胞分化誘導系

関節リウマチ(RA)の罹患者は日本国内で約80万人ともいわれ，これらの患者の機能障害をいかに軽減するかは医療福祉上・医療経済上重要な問題である．RAは四肢の関節破壊を主徴とするが，これに伴って加齢によるものよりはるかに高度な骨粗鬆症が生じ，これも患者の機能を大きく損ねる要因である．しかし高度な骨粗鬆の病態は解明されていない部分が多く，その予防・治療の方法はまったく確立されていないといっても過言ではない．

RAでは関節腔内からも骨破壊が進行するのが特徴的である．関節滑膜組織内に破骨細胞様の形態をとる多核巨細胞が存在することは以前より知られており，これが関節内からの骨吸収に関与する可能性も示唆されていた．われわれはRA関節腔内に前駆破骨細胞が存在することを予測し，RA患者関節液中に大量のCD14陽性単球様細胞の存在と，これらがサイトカインの刺激により骨吸収能を持つ成熟破骨細胞に分化することを見出し，前駆破骨細胞であるCD14陽性単球様細胞は，健常人末梢血単球とは異なる表面抗原プロファイルを有しており，分化段階の異なる細胞であることが示唆された[5]．

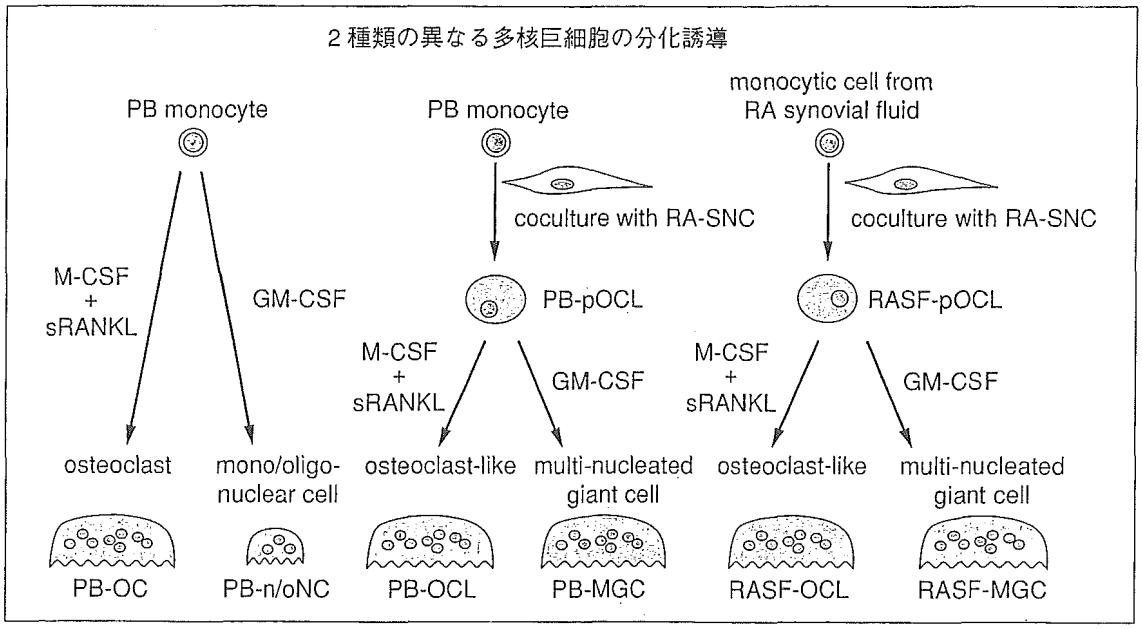
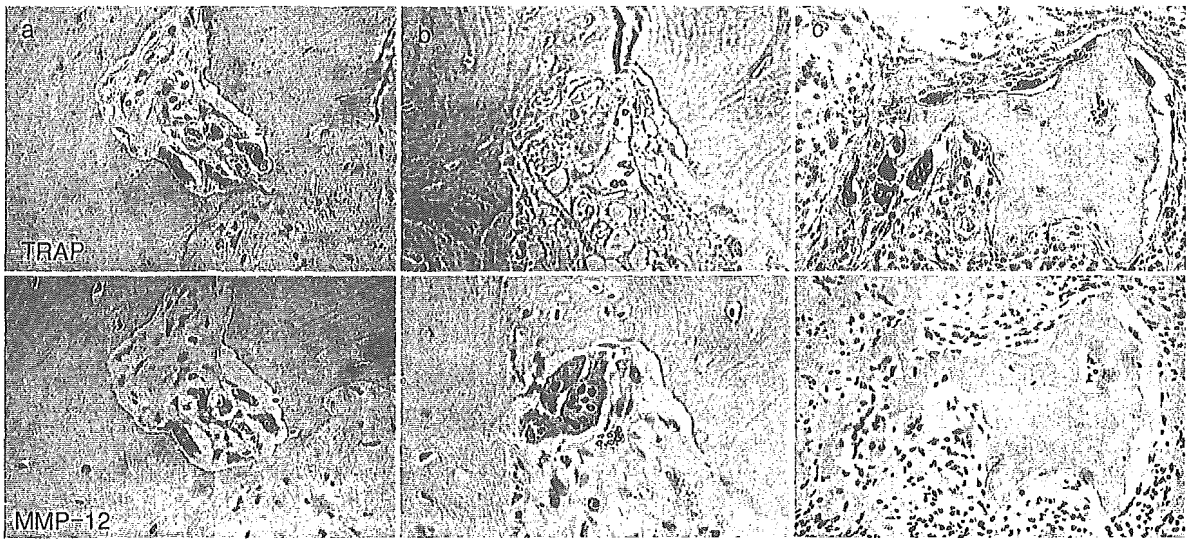


図1　M-CSF+RANKL依存性破骨細胞とM-CSF+RANKL非依存性破骨細胞

PB monocyte：末梢血単球　　M-CSF：マクロファージコロニー刺激因子
OCL　　：破骨細胞　　RASF：リウマチ滑膜線維芽細胞
RANKL　　：NF-κB活性化受容体リガンド

リウマチ骨吸収部位における MMP-12 の発現



健常な成人の骨・軟骨においては MMP-12 の発現は認められない.

図 2　RA 罹患部に存在する MMP-12 陽性の破骨細胞(文献 1)
a：$TRAP^{+}$ $MMP12^{+}$の破骨細胞
b：$TRAP^{-}$ $MMP12^{+}$の破骨細胞
c：$TRAP^{+}$ $MMP12^{-}$の破骨細胞

従来，破骨細胞はその分化および成熟のすべての過程において骨髄の間質細胞や骨芽細胞などの支持細胞が必要であるとされてきた．また近年，支持細胞の代わりに支持細胞の発現する分子(M-CSF，RANKL)を使用する破骨細胞誘導系も報告されている．一方，筆者らが RA 関節滑液から発見した RA 患者由来 CD14 陽性単球様細胞は，前駆破骨細胞としての性状を有しながらも，このような支持細胞や M-CSF＋RANKL を必要とせず，サイトカイン(IL-3, IL-5, IL-7 または GM-CSF)の刺激のみによって成熟破骨細胞へと分化する(図 1)．この分化に関わるこれらサイトカインは，RA 患者の関節液中に高頻度・高濃度に存在するものであることから，われわれが発見した CD14 陽性単球様細胞は関節腔内で直接的に破骨細胞に分化するものと推測される．

この仮説を実証することは，RA における骨吸収の新たなメカニズムを示すことになるが，いままでこれに類した報告は見当たらない．この方法は RA 患者から前駆破骨細胞を得る方法であり，培養前駆細胞は 98%以上の純度で繰り返し大量に実験に使用することが可能である．また，この方法では，末梢血単球から直接に破骨細胞を誘導してしまう M-CSF＋RANKL の実験系では得られない，前駆破骨細胞の性状解析と，前駆細胞から成熟破骨細胞への分化機序の解明が可能になると考えられる．

このような方向からの破骨細胞形成過程の解明は，われわれが RA 患者滑液から見出した方法以外のアプローチでは困難と考えられる．従来の実験系では解明できなかった，末梢血単球—前駆破骨細胞—成熟破骨細胞への分化過程と，各ステージに特異的な遺伝子産物の発現を明らかにすることができる．さらに得られた結果をもとに，骨代謝を各ステージ特異的に制御できる治療法を開発することが可能と考えている．

■RA 関節組織の免疫化学的検討

RA 患者の関節組織を免疫化学的に解析した結果，酒石酸耐性酸フォスファターゼ(tartrateresistant acid phosphatase；TRAP)陽性の単核球と多核巨細胞が滑膜組織と軟骨下骨付近に見出され，これらの関節滑膜組織中に存在していた TRAP 陽性単核球と多核巨細胞からは MMP(macrophage metalloelastase)-2 と MMP-9 が検出された．さらに TRAP 陽性多核巨細胞から MMP-12 と MMP-14 も検出された．RA 関節由来の TRAP 陽性単核球は，*in vitro* で軟骨組織中のプロテオグリカンを切断することも確認された．RA と変形性関節症(OA)の関節液中に見出される CD14 陽性前駆破骨細胞の性状と機能を比較するために，各疾患の患者の関節液の初代培養から得た CD14 陽性前駆破骨細胞を IL-3，IL-5，IL-7 または GM-CSF により刺激し，破骨細胞への分化誘導を行った．その結果，RA，OA それぞれの関節液由来前駆破骨細胞が破骨細胞へと分化したが，破骨細胞ごとの核数，単核細胞から多核細胞への融合率，骨吸収能のいずれにおいても RA 由来前駆破骨細胞によるもののほうが大きかった．このことより，RA 関節腔においては OA のそれより強く活性化された前駆破骨細胞が存在し，関節破壊に関与している可能性が示唆された[2]．

RA 患者において，組織マクロファージが関節滑膜組織中に集簇する機序を明らかにするため，われわれが以前からその存在を報告してきた RA 滑膜組織に特異的に存在する間質細胞(RA ナース細胞)[3,4]の培養上清による末梢血単球の遊走を測定したところ，RA ナース細胞培養上清は対照群である OA 由来間質細胞や皮膚由来線維芽細胞の培養上清と比較し，有意に単球の遊走を刺激する結果を得ている．さらに MCP-1(monocyte chemotactic protein-1)と IL-8 に対する中和抗体により単球遊走が阻害されたため，MCP-1 および IL-8 が RA ナース細胞における単球遊走に関与することが示唆された．また，TNFα 刺激により RA ナース細胞によるこれらの遊走因子産生が増強されることを見出している．

■今後の課題

RA 患者関節液の初代培養から CD14 陽性かつ破骨細胞特異的酵素である TRAP 陽性の単核球を見出し，その表面抗原プロファイルが健常人 CD14 陽性末梢血単球と異なることを発見した．また，これら CD14 陽性 TRAP 陽性単核球が IL-3，IL-5，IL-7 あるいは GM-CSF の存在下，支持細胞を必要とせずに強い骨吸収能を有する成熟破骨細胞へと分化することを確認している．前駆破骨細胞は高純度で長期にわたり維持され，最長 8 カ月まで繰り返し本誘導実験系に使用できることも明らかにした．さらに，健常人からの CD14 陽性単球を RA 滑膜ナース細胞と共培養することにより，正常末梢血由来単球は TRAP 陽性の前駆破骨細胞に分化し，IL-3，IL-5，IL-7 あるいは GM-CSF により関節液由来の前駆細胞同様，成熟破骨細胞へ分化することが判明した．また，RA に特異的に存在する RA ナース細胞が RA における破骨細胞の誘導に重要な関与をすることが示唆されてきた．

現在，RA 罹患部の免疫組織学検討から，M-CSF＋RANKL で誘導される破骨細胞が保有しない MMP-12 陽性の破骨細胞が RA 患者にのみ特徴的に存在することから(図 2)，RA における破骨細胞は，われわれが新たに見出した M-CSF＋RANKL 非依存性の分化経路による破骨細胞が，RA の骨破壊に関与している可能性を示唆したと考えている．

われわれの研究ではまず，RA 患者の関節における RA ナース細胞による前駆破骨細胞ならびに，これから分化誘導される破骨細胞に特異的に発現する遺伝子を現在探索中である．既に，この探索から見出された未解明の遺伝子に関しては，現在，これらがコードするタンパクの特異性の検討ならびに機能を解析中である．RA の骨破壊に

関与すると予想される同定されたタンパクのうち，破骨細胞の分化あるいは機能(骨吸収能)発現に関与するものをターゲットとして，その抑制による骨粗鬆の抑制を試みることが可能となってきている．

以上はRAの骨破壊に関わるわれわれ独自の発見であるが[5,6]，最近，米国・韓国・日本で複数のグループによっても，本実験系がRA病態を反映し，骨・関節破壊治療法開発に有用であると報告されたが，本実験系による破骨細胞分化解析はわれわれが先行している．先進各国における社会の高齢化を鑑みても，骨代謝の中心的役割を担う破骨細胞の分化・機能制御が医療福祉上極めて重要な課題であることは間違いないと考えている．

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(12) **United States Patent**
Maeda et al.

(10) **Patent No.: US 6,861,257 B1**
(45) **Date of Patent: Mar. 1, 2005**

(54) **METHODS FOR ISOLATION OF OSTEOCLAST PRECURSOR CELLS AND INDUCING THEIR DIFFERENTIATION INTO OSTEOCLASTS**

(75) Inventors: **Tomoko Maeda**, Osaka (JP); **Ryuji Suzuki**, Osaka (JP); **Takahiro Ochi**, Hyogo (JP)

(73) Assignee: **Shionogi & Co., Ltd.**, Osaka (JP)

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§ 371 (c)(1),
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PCT Pub. Date: **Oct. 21, 1999**

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(51) **Int. Cl.**[7] **C12N 5/06**; C12N 5/00; C12N 5/08

(52) **U.S. Cl.** **435/377**; 435/375; 435/372

(58) **Field of Search** 435/377, 375, 435/372

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Primary Examiner—Vera Afremova
(74) *Attorney, Agent, or Firm*—Oblon, Spivak, McClelland, Maier & Neustadt, P.C.

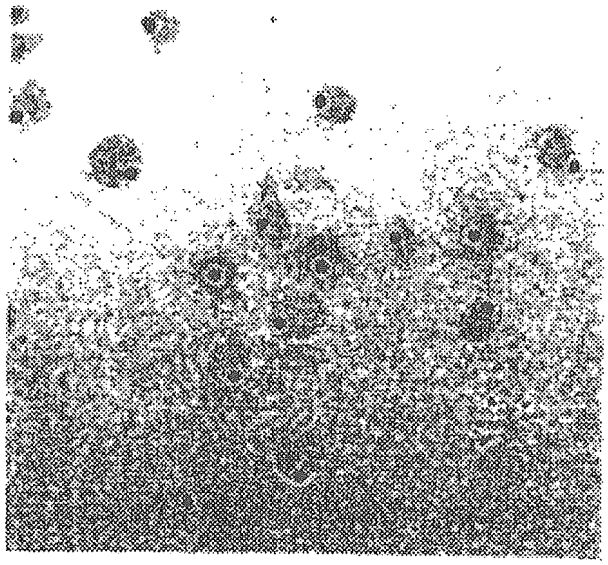
(57) **ABSTRACT**

This invention relates to: a method for differentiating osteoclast precursor cells (preosteoclasts) into osteoclasts, which comprises culturing the preosteoclasts in the absence of accessory cells; a method for isolating preosteoclasts, which comprises culturing peripheral blood or joint fluid in the absence of cytokine for 1 to 3 weeks; an preosteoclasts, which is obtainable by the above method; a method for differentiating the preosteoclasts obtained by the above method into osteoclasts, which comprises culturing the preosteoclasts in the absence of accessory cell; an osteoclast, which is obtainable by the above method; a method for screening agents for treating metabolic bone diseases, which comprises using the preosteoclasts or the osteoclasts as described above; and agents for treating metabolic bone diseases, which is obtainable by the above screening method.

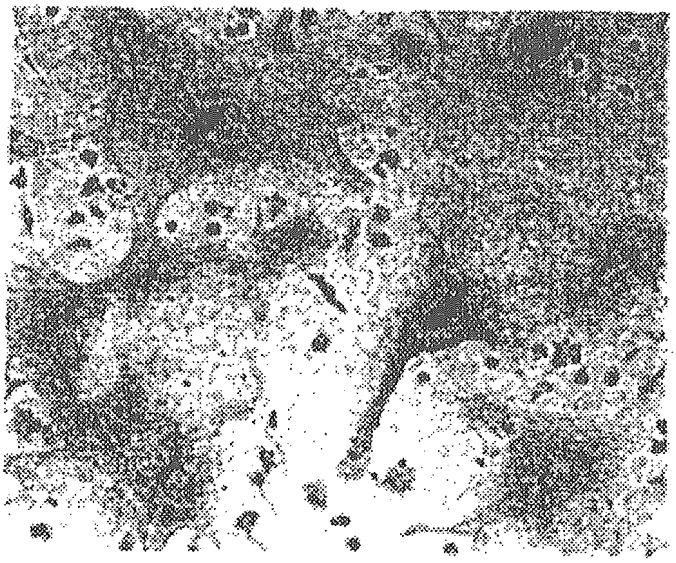
16 Claims, 5 Drawing Sheets

Figure 1

May-Giemsa stain

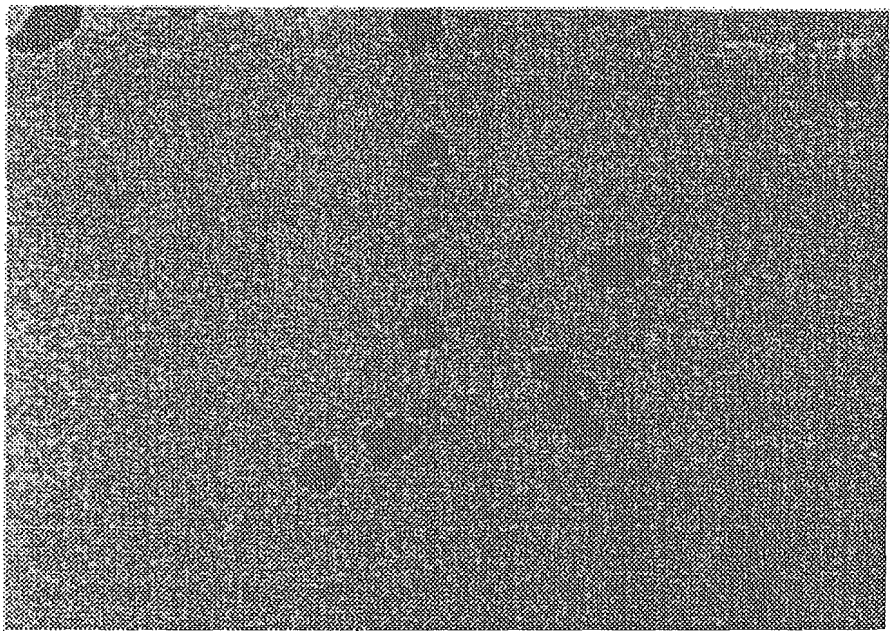


Preosteoclast (×40)



Osteoclast (×20)

Figure 2



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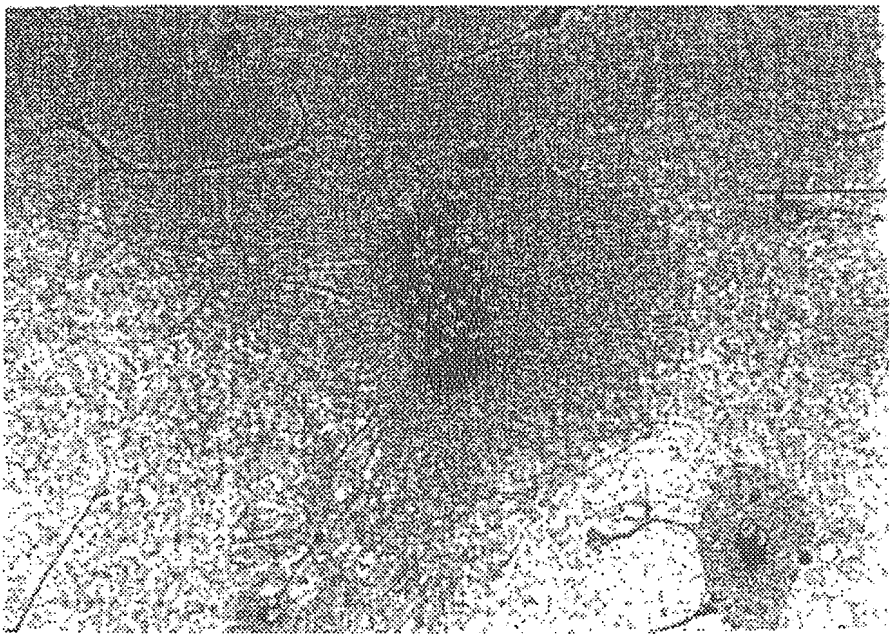
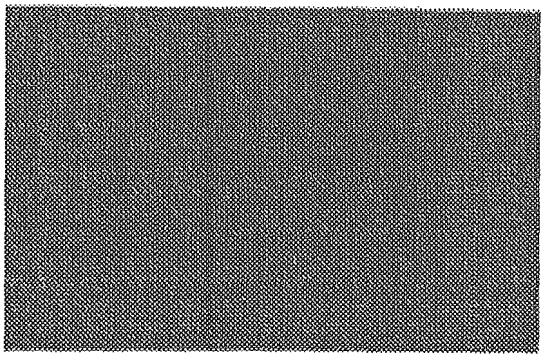


Osteoclast

Figure 3



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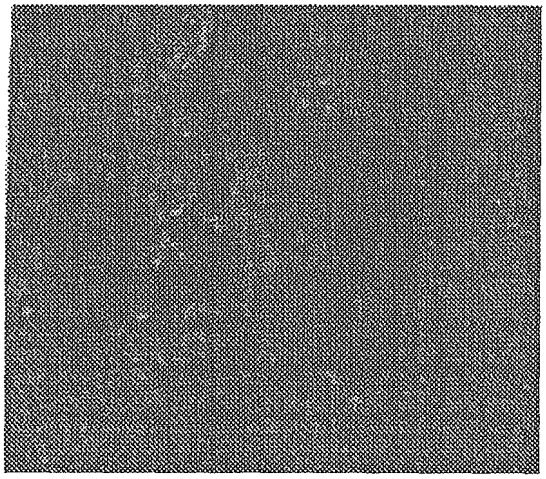

Osteoclast