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Induction of multinucleated giant cells from rheumatoid arthritis (RA) synovial adherent cells by anti-DR antibody

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SUMMARY

To determine the effects of signalling through the DR molecule on synoviocytes from RA patients, the synovial adherent cells were incubated with anti-DR antibodies. After 24 h incubation, we found the formation of multinucleated giant cells in that culture. These multinucleated giant cells showed characteristics of monocyte-macrophage lineage cells and precursor of osteoclasts. Cyclohexamide inhibited the formation of multinucleated giant cells, but not the aggregation of synovial cells, suggesting that newly synthesized proteins are associated with the cell fusion. These results revealed a new mechanism in multinucleated giant cell formation.

Keywords multinucleated giant cells DR antigen rheumatoid arthritis

INTRODUCTION

A DR molecule has two separate MHC-encoded polypeptides (DRα and DRβ) which are essential for reactions of immune recognition [1]. Much effort has been devoted to defining the role of the DR molecule in antigen presentation. It has also been reported that the ligation of a DR molecule with anti-DR MoAbs or superantigens causes the activation of B cells, Ia-positive T cells and monocytes [2–5]. Elevation of PI turnover, increase in intracellular Ca^{2+} and tyrosine phosphorylation have been reported as some of the biochemical events associated with the DR molecule [3,4]. Furthermore, several reports indicated cytokine production [6,7] and the proliferation of leucocytes subsequent to those biochemical signals [3,7,8]. Recently, Mourad *et al.* reported that signalling through the DR molecule induced the expression of IL-6 and IL-8 mRNA on RA type B synoviocytes by utilizing the combination of anti-DR MoAb or superantigens and interferon-gamma (IFN-γ) [9]. These findings prompted us to determine the further effects of signalling through the DR molecule on RA synoviocytes.

In this study, we found that anti-DR MoAbs induced the formation of multinucleated giant cells (MGC) from RA synoviocytes. To date, most studies concerning MGC formation have focused on cytokines, including 1.25 dihydroxyvitamin D_3 and phorbol myristate acetate (PMA) and monocyte-macrophage lineage cells from different sources [10–17]. Also, using antibody which recognizes 120-kD surface protein, a similar study was presented in rat bronchoalveolar macrophage [18]. However, the mechanism leading to MGC formation is still unknown. Our study suggests that biochemical events following the ligation of the DR molecule are associated with MGC formation.

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PATIENTS AND METHODS

Patients

Twenty-four synovial tissues and 14 peripheral blood samples from 38 patients with RA, seven synovial tissues from patients with osteoarthritis (OA) and one synovial tissue from a patient with calcium pyrophosphate were included in this study. All RA patients met the revised criteria of the American College of Rheumatology [19], and OA was diagnosed based on clinical symptoms and typical changes seen on knee radiographs, i.e. cartilage loss, subchondral sclerosis, and formation of osteophytes. All patients had been taking non-steroidal anti-inflammatory drugs. Ten RA patients had been taking less than 15 mg/day of predonisolone. Two RA patients were not using DMARDs. The rest of the RA patients had been taking either gold salts, methotrexate, salazopyrazone or bucillamine (D-penicillamine analogue). Synovial tissues from total knee replacements were pathologically examined. The pathological activity was determined by the three components: synovial intimal lining thickness, subintimal mononuclear cell infiltration, and lymphoid aggregates. The synovial tissues with two or

more scores of inflammation score described by Firestein *et al.* [20] were used for MGC formation.

Twenty-four blood samples from 14 RA patients and 10 healthy controls were used in the experiments. Activity of RA was defined as the presence of six or more swollen joints and at least two of the following features: (i) an erythrocyte sedimentation rate >30 mm/h; (ii) morning stiffness > 45 min duration; and (iii) nine or more joints painful to palpation.

Antibodies

Anti-CD1 (IgG1), anti-CD3 (IgG2a), anti-CD11a (IgG1), anti-CD11b (IgM), anti-CD29 (IgG1), anti-CD45 (IgG2a) and anti-class I (IgG2a) MoAbs were gifts of S. F. Schlossman (Dana-Farber Cancer Institute, Boston, MA). Anti-β_2-microglobulin (IgG1) and anti-DR (L243, IgG2a) MoAbs were generated from hybridoma cells (American Type Culture Collection, Bethesda, MD). Two other anti-DR MoAbs (IgG1) were a gift of F. Obata (Department of Immunology, School of Medicine, Kitazato University, Japan) and M. Yokoyama (Department of Immunology, School of Medicine, Kurume University, Japan). The characterization of anti-DQ MoAb (IgG2a) used here was previously reported [21]. Anti-CD14 (IgG1) was made in our laboratory. These MoAbs were incubated with RA synovial adherent cells as described below. In some experiments, anti-DR MoAb was used in $F(ab')_2$ form. The purification of $F(ab')_2$ was done as described previously [22]. Anti-human IFN-γ, anti-human IL-4 and anti-human tumour necrosis factor-alpha (TNF-α) polyclonal antibodies were obtained from Genzyme Corporation (Cambridge, MA) and used as neutralizing units.

Reagents

Human recombinant IL-1β, IL-3, IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF; Genzyme Corporation) were used at 12·5–100, 10–1000, 100–2500 and 50–500 U/ml, respectively. TNF-α (Endogen, Boston, MA) was used at 20–250 U/ml. IFN-γ (Shionogi Research Lab., Tokyo, Japan) was used at 50–500 U/ml. 1.25 dihydroxyvitamin D_3 (Japan Roche, Tokyo, Japan) was used at 10^{-9}–10^{-7} M. Phorbol myristate ester (Sigma, St Louis, MO) was used at 25–200 ng/ml. Staphylococcal enterotoxin B (Sigma) was used at 1–40 μg/ml. Cyclohexamide (Sigma) was used at 1 μg/ml.

Cell culture

Immediately upon surgical removal, synovial tissues from knee joints were placed in Iscove's modified Dulbecco's medium and 10% fetal calf serum (FCS) supplemented with 100 μg/ml Kanamycin monosulphate. After a few hours, the tissues were removed from the medium, minced with scissors, and then incubated with 1 mg/ml of collagenase type V (Sigma) and 1 mg/ml of hyaluronidase type V (Sigma). The materials were filtered through nylon mesh, and pelleted through centrifugation. The pellet was resuspended in the medium and layered on Ficoll–Hypaque (specific gravity 1·077) and centrifuged at 800 *g* for 15 min. The upper layer was collected and centrifuged with the medium. The synovial cells were resuspended at 1×10^6 cells/ml in the medium. The peripheral blood mononuclear cells (PBMC) were prepared as described previously, and adjusted to a concentration of 5×10^6 cells/ml in the medium [23]. The synovial cells or PBMC were incubated in 0·2 ml of the medium on eight-well Lab Tek chamber slides (Nunc, Naperville, IL) overnight. The wells were then washed with the medium three times to remove non-adherent cells and the adherent cells were cultured with MoAbs or reagents for the indicated number of days. After incubation, adherent cells were fixed in cold acetone until further examination.

Estimation of multinucleated giant cells

The adherent cells were stained in Mayer's haematoxyline solution and the cells with more than three nuclei were counted as MGC. The specimen were analysed by one of us and the blinded observer. In general, no major differences were found between the analyses by two different observers.

The fusion rate of more than 300 cells from each treatment was calculated as follows:

$$\text{fusion rate (\%)} = \frac{\text{total number of nuclei within MGC}}{\text{total number of nuclei counted}} \times 100.$$

The Δ fusion rates presented in Table 1 and in the figures were calculated as follows:

Δ fusion rates (%) = fusion rate with anti-DR – fusion rate with anti-DQ.

Cytochemistry

Using diagnostic kits (Sigma), the detection of non-specific esterase and tartrate-resistant acid phosphatase was performed according to the manufacturer's instruction (Sigma).

Table 1. Multinucleated giant cell formation in RA synovial cell cultures with various reagents

	Δ fusion rate (%)				
Experiments	IgG anti-DR	$F(ab')_2$ anti-DR	SEB	PMA	IgG anti-DR + CH
I	36	22	0	8	10
II	37	12	8	15	6
III	51	23	2	16	6
IV	70	31	2	5	5

IgG anti-DR, $F(ab')_2$ anti-DR and cyclohexamide (CH) were used at 1 μg/ml in RA synovial adherent cell culture. Staphylococcal enterotoxin B (SEB) and phorbol myristate acetate (PMA) were used at 5, 10, 20 and 40 μg/ml and 50, 100, 200 and 400 μg/ml, respectively. Maximal values are presented. The fusion rate shows multinucleated giant cell (MGC) formation after 3 days incubation with various reagents. Each experiment was done in different specimens.

Detection of surface antigen

For the detection of surface antigen of multinucleated giant cells, adherent cells on chamber plate were incubated with PE-conjugated mouse immunoglobulin, anti-CD1, anti-CD3, anti-CD14, anti-CD20, anti-CD25, and PE-conjugated anti-DR MoAb (which reacts with the epitope differently from one recognized by L243) (Coulter Immunology, Miami, FL) for 15 min at room temperature. For fluorescence measurements, the chambers were mounted in the ACAS 570 interactive laser cytometer (Meridian, Okemos, MI). Dual monitors simultaneously provided fluorescence and video image displays. This allowed us to compare light optical and fluorescence images. The multinucleated giant cells were illuminated by a 488-nm line of the argon ion laser, and PE fluorescence was recorded at a 575-nm wavelength with a photomultiplier. Fluorescence intensity was converted to digitized images graduated by 16 colours.

For the detection of surface antigen of adherent cells, acetone-fixed adherent cells were reacted with biotinylated mouse immunoglobulin, anti-CD14 MoAb or anti-DR MoAb for 15 min, and after washing with PBS, the activity of peroxidase in these cells was blocked with 0·6% H_2O_2 in methanol, and the cells were subsequently incubated with rabbit serum to prevent non-specific binding. After washing with PBS, adherent cells were reacted with peroxidase-conjugated streptoavidin for 15 min. After washing with PBS, 3.3′ diaminobenzidine was used as a substrate, and the nuclei were stained in Mayer's haematoxylin solution. All reagents for peroxidase staining were obtained from Nichiray Co. (Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using Student's *t*-test and correlation analysis.

RESULTS

Anti-DR MoAbs induce multinucleated giant cells in synovial adherent cell culture

To examine the effect of anti-DR MoAbs on synovial adherent cells from RA patients, we added anti-DR MoAbs in RA synovial adherent cell culture for the indicated number of days. Three different anti-DR MoAbs (two IgG1 and one IgG2a subclass) induced MGC in culture, while anti-DQ and the other MoAbs described in Patients and Methods did not (Fig. 1). On day 1 there was no significant difference in the numbers of synovial adherent cells, but there was significant difference in the numbers of MGC cultured with or without anti-DR MoAb (data not shown). This finding suggests that MGC induced by anti-DR MoAbs are derived from the fusions of synovial adherent cells and not from cell division. Next, the

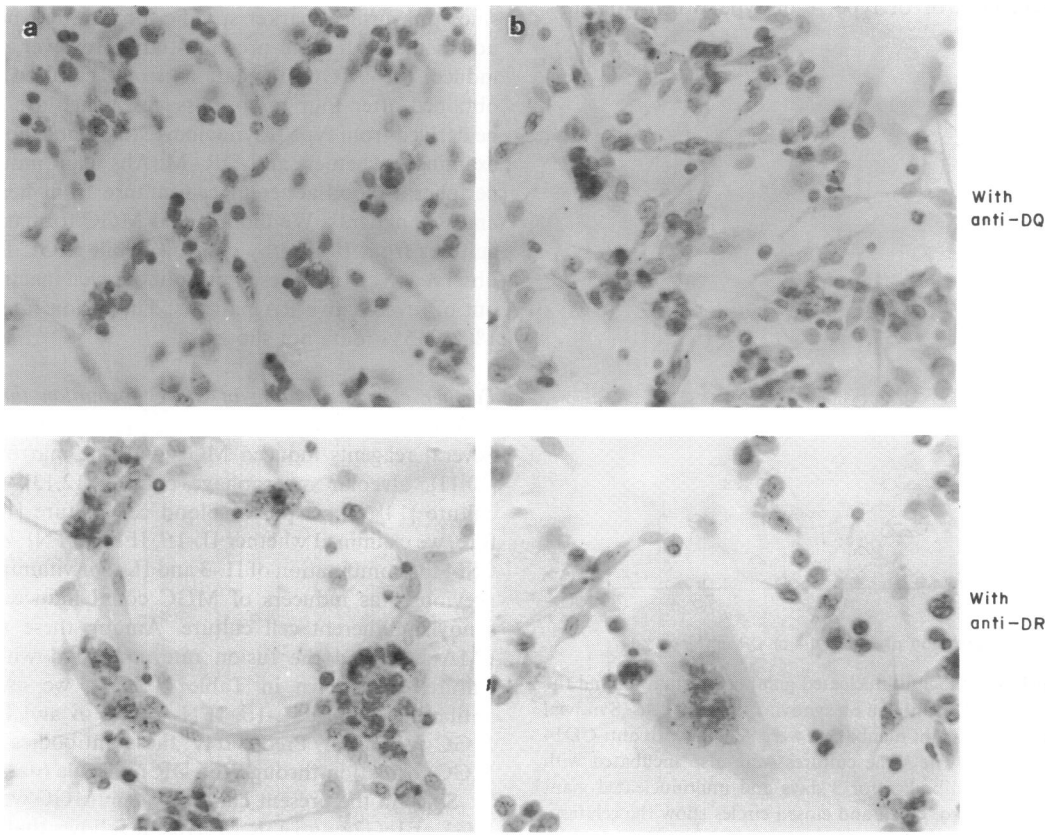

Fig. 1. Multinucleated giant cells induced by the anti-DR antibody in RA synovial adherent cell culture. (a) RA synovial cells were incubated with anti-DQ (IgG2a) or anti-DR (IgG2a) antibodies for 1 day. The nuclei are randomly placed in each cell. (b) RA synovial adherent cells were incubated with anti-DQ or anti-DR antibodies for 6 days. The nuclei in multinucleated giant cells aggregated in the centre of each cell.

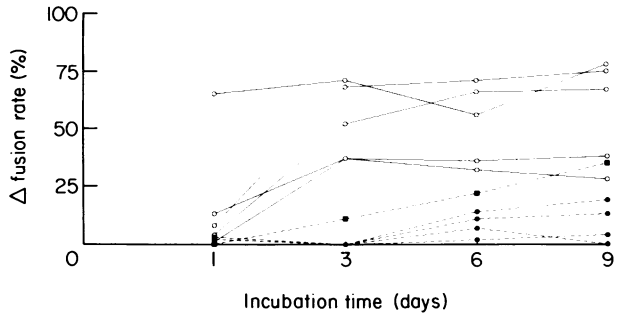

Fig. 2. Time course of multinucleated giant cell formation. Synovial adherent cells were incubated with anti-DQ or anti-DR antibodies. Multinucleated giant cell formation was expressed as the fusion rate in synovial adherent cell cultures from RA (○), osteoarthritis (OA) (●) and arthritis with calcium pyrophosphate (■), respectively.

time course of MGC formation was examined in synovial adherent cell culture from five RA, four OA and one arthritis with calcium pyrophosphate (Fig. 2). For convenience, we represented the frequency of MGC formation in terms of fusion rate. While there were variations in MGC formation from RA samples, their fusion rates were consistently higher than those of OA and one arthritis with calcium pyrosphosphate. Variations in MGC formation among samples might be attributed to differences in the proportion of DR antigen-positive synoviocytes, or of type A or type B cells, or subintimal tissue macrophages which express the DR antigen. Therefore, we tried to determine the relationship between the fusion rate and the expression of DR antigen or CD14 antigen as a marker of type A cells and tissue macrophages on synovial adherent cells. As shown in Fig. 3, significant correlation was found only in the case of the fusion rate and DR antigen expression on synovial adherent cells, suggesting the participation of DR antigen-positive and CD14 antigen-negative cells in MGC formation.

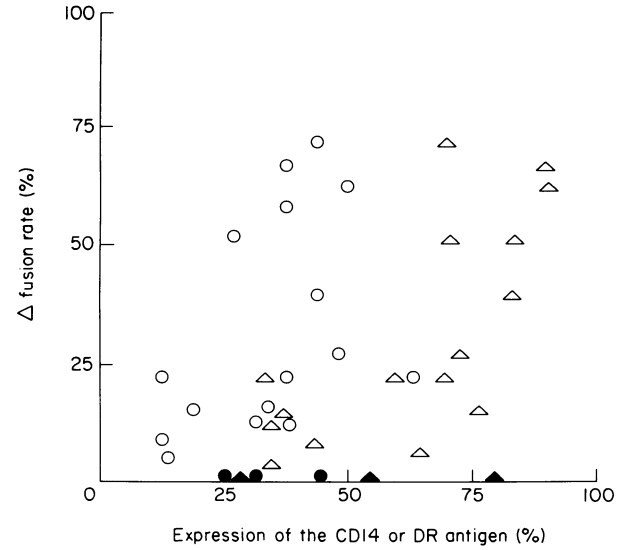

Fig. 3. Relationship of the multinucleated giant cell formation and the expression of CD14 or DR antigen on synovial adherent cells. Synovial adherent cells after overnight incubation were stained with anti-CD14 or anti-DR antibodies. The same cultures were also incubated with anti-DQ or anti-DR antibodies for 3 days and multinucleated giant cells formed were counted. Open and closed circles show the relationship between the fusion rate and the expression of the CD14 antigen from RA and osteoarthritis (OA) patients, respectively. The correlation coefficient was 0·38. Open and closed triangles show the relationship between the fusion rate and the expression of the DR antigen from RA and OA, respectively. The correlation coefficient was 0·65 ($P = 0·01$).

The origin of MGC induced by anti-DR MoAbs in RA synovial adherent cell culture

DR antigen-positive cells in the synovial adherent cells obtained from our method are considered to be mainly composed of type A and type B and subintimal tissue macrophages. We examined whether MGC induced by anti-DR MoAbs present the surface markers of macrophages in immunofluorescent and cytochemical studies. When MGC were stained with FITC-conjugated antibodies, they were DR and CD14 antigen-positive and CD1, CD3, CD20 and CD25 antigen-negative. Interestingly, spotty staining was observed in each cell stained with anti-CD14 antibody (Fig. 4). In addition, MGC demonstrated non-specific esterase and tartrate-resistant acid phosphatase activity (data not shown). These results support the notion that MGC induced by anti-DR MoAbs in RA synovial adherent cell culture may partially consist of monocyte-macrophage lineage cells. Of course, we cannot exclude the contamination of type B synoviocytes in MGC, since the surface marker specific for type B synoviocytes was not available for the present study. However, we could not induce the MGC formation from cultured synoviocytes obtained after four to six passages, which are considered to be derived from type B synoviocyte (data not shown). Next, we examined whether anti-DR MoAbs can induce MGC in peripheral blood-adherent cell culture from healthy controls and RA patients. We did not find MGC formation in the cell cultures from 10 healthy controls, while MGC formation was observed in cell cultures of one out of seven inactive RA and six out of seven active RA patients. The fusion rates varied from 1% to 15% (data not shown).

Further characterization of MGC formation through the DR molecule

Several reagents induced MGC in bone marrow cell culture [10,11], alveolar macrophage culture [12,13], umbilical cell culture [14] or peripheral blood cell culture [15–17]. Therefore, we examined whether IL-1β, IFN-γ, TNF-α, PMA, GM-CSF, the combination of IL-3 and IL-4 or vitamin D_3 described previously as inducers of MGC could induce MGC in RA synovial adherent cell culture. Among these reagents, only PMA increased the fusion rate compared with the control cultures, as shown in Table 1. Next, we used polyclonal antibodies against IL-1β, IFN-γ, TNF-α and IL-4 to inhibit MGC formation. Predictably, these antibodies did not affect MGC formation through the DR molecule (data not shown).

Since in the present culture system MGC were induced by IgG1 or IgG2a anti-DR MoAbs, it follows that MGC formation is not related to IgG subclass of anti-DR MoAbs. Whether or not the Fc portion of IgG is required for the induction of MGC remains questionable. When $F(ab')_2$ anti-DR MoAb instead of IgG anti-DR MoAb was used for the induction of

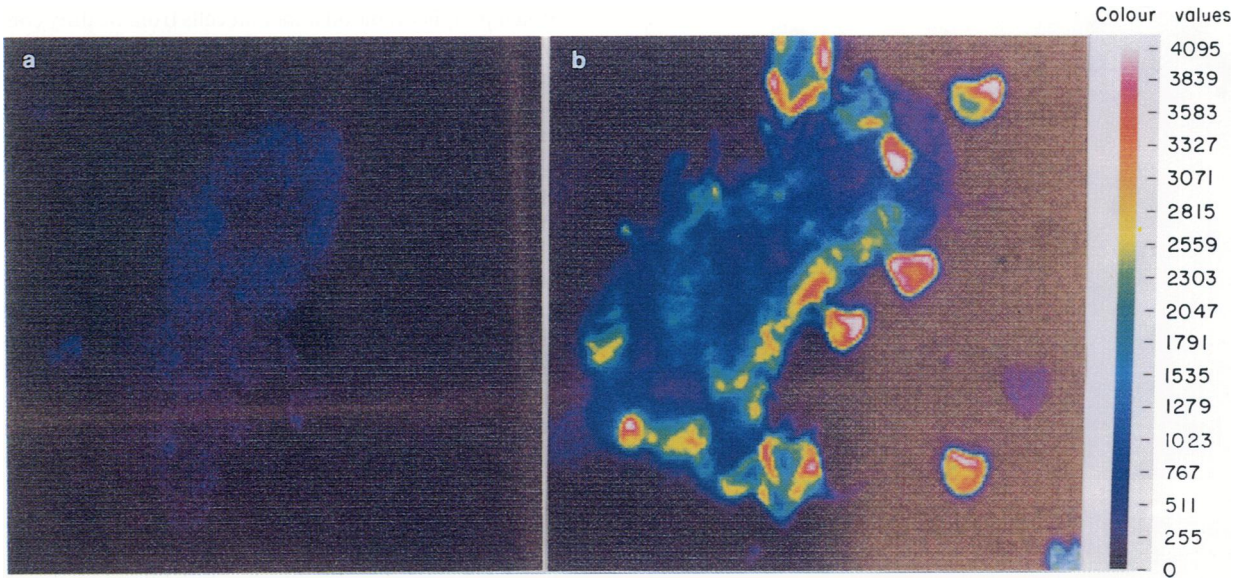

Fig. 4. The staining pattern of the CD14 antigen on a multinucleated giant cell. RA synovial cells were incubated with anti-DR antibody for 3 days. Multinucleated giant cells induced in that culture were stained with PE-conjugated anti-CD3 antibody (a) as a negative control, or PE-conjugated anti-DR antibody (b), and the fluorescent intensity was analysed by adherent cell sorting and analysis 570. Depending on the intensity of fluorescence, the colour in the digitized image varies as indicated by the scale on the right. In b, spotty staining of the CD14 antigen was on a multinucleated giant cell. As shown on the right in b, regular size macrophages were also CD14 antigen-positive.

MGC, MGC formation still occurred, although the fusion rate was reduced compared with that induced by IgG anti-DR MoAb (Table 1).

Since staphylococcal enterotoxin B (SEB) is known to bind to DRβ chain [24], we tried to determine whether, like anti-DR MoAbs, SEB induces MGC. Unexpectedly, SEB induced lower frequencies of MGC in three of four experiments compared with those induced by anti-DR MoAbs (Table 1). Next, the effect of cyclohexamide was examined on the formation of MGC induced by anti-DR MoAb. As shown in Table 1 and Fig. 5, cyclohexamide clearly inhibited MGC formation, but it did not inhibit cell aggregation induced by anti-DR MoAb, suggesting that the newly synthesized protein is required for cell fusion.

DISCUSSION

We used IL-1β, IFN-γ, TNF-α, the combination of IL-3 and IL-4, 1.25 dihydroxyvitamin D_3 and PMA to induce MGC from RA synovial adherent cells, and neutralizing antibodies against IL-1β, IFN-γ, TNF-α and IL-4 to inhibit MGC formation by anti-DR MoAb. Of these, only PMA was effective. Thus, it is clear that MGC formation by anti-DR MoAbs is not due to the secretion of cytokines known previously as inducers of MGC formation. The MGC induced by anti-DR MoAb showed characteristics of monocyte-macrophage lineage cells. However, the CD14 antigen expression on RA synovial adherent cells is not correlated to MGC formation, as shown in Fig. 3. Spotty staining was observed in the expression of CD14 antigen on single MGC. The expression of CD25 antigen on MGC was negative. Since the CD14 antigen is expressed on monocyte-macrophage lineage cells but not on activated macrophages [25], and the CD25 antigen is mainly expressed on subintimal tissue macrophages but not on type A synoviocytes [26], these results suggest that type A synoviocytes primarily participate in MGC formation. This interpretation is consistent with the *in vivo* finding that MGC from macrophage lineage cells and MGC from type B synoviocytes are dominantly present in RA and OA synovial tissues, respectively [27].

MGC formation was clearly inhibited by cyclohexamide, although the aggregation of RA synovial cells induced by anti-DR antibody was not affected by cyclohexamide. These results suggest that the DR molecule could act as a signal transduction molecule also on RA macrophage-like cells as described on the RA fibroblast-like cells pretreated with IFN-γ [9], and that the newly synthesized protein subsequent to cell activation is required for MGC formation. Several reports have used superantigens such as staphylococcal enterotoxins as ligands of the DR molecule instead of anti-DR MoAbs. In the present study, SEB was not as effective on MGC formation as anti-DR MoAbs. This discrepancy may be because SEB could not induce the cell aggregation of RA synovial cells, which is considered to be necessary for MGC formation [28]. It is conceivable that anti-DR MoAbs induce cell aggregation of RA synovial cells by antibody-mediated bridge of the DR molecule to Fc receptors, or cross-linking of adjacent DR antigens on the surface of the same cell to induce the secondary expression of adhesion molecules. Both mechanisms seem active in the present experiment using anti-DR MoAb, because even when we used $F(ab')_2$ fragment of anti-DR MoAb, we still observed MGC formation, but to a lesser extent. In the case of

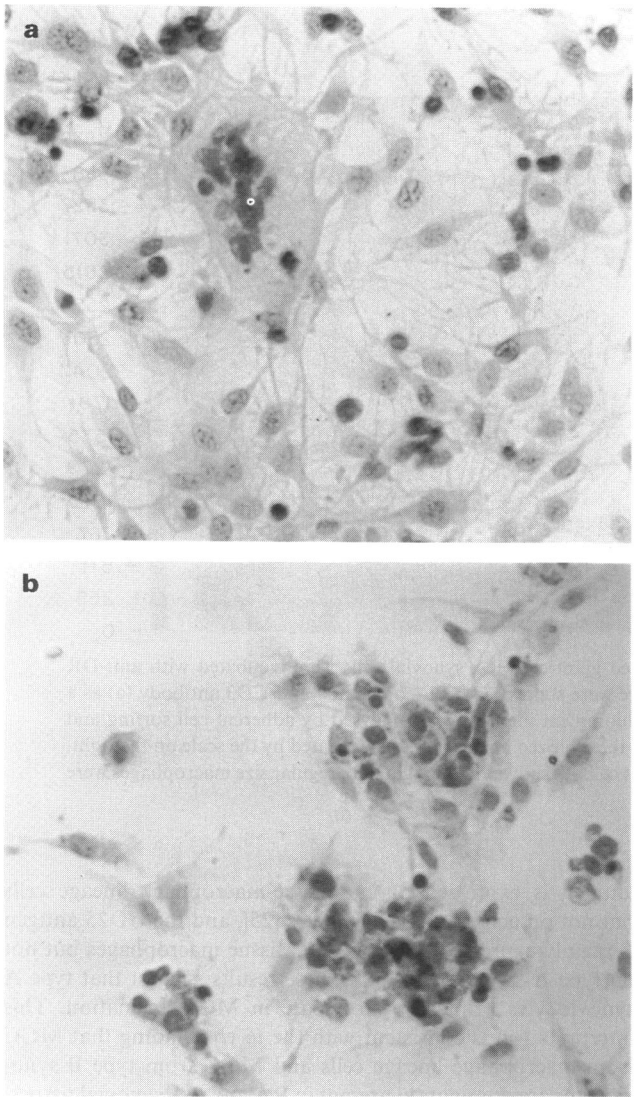

Fig. 5. The effect of cyclohexamide on the multinucleated giant cell formation. RA synovial adherent cells were incubated with anti-DR antibody in the absence or presence of cyclohexamide (a or b) for 3 days. Cyclohexamide clearly inhibited multinucleated giant cell formation, but not cell aggregation of RA synovial adherent cells.

SEB as a ligand of the DR molecule, Fc-mediated mechanism for cell aggregation would be unlikely, and the mechanism through adhesion molecules might be less involved compared with anti-DR MoAb, since it was reported that SEB could not induce B cell aggregation through adhesion molecules [29].

Considering the present findings along with the previous reports, it is conceivable that various stimuli for the induction of MGC would be active, depending on the state of differentiation or activation of monocyte-macrophage lineage cells. In the case of RA tissue macrophage or type A synoviocytes which are not precisely defined in their relation to normal monocyte-macrophage, the signal transduction pathway through the DR molecule would be different from that of the normal monocyte-macrophage, probably in the process of leading proteins for cell fusion. A similar situation of signal transduction through the DR molecule was reported in B cell proliferation, where anti-DR MoAb activity depended on the state of differentiation or activation of B cells [7,8].

When peripheral blood adherent cells from healthy controls or synovial adherent cells from OA or arthritis with calcium pyrophosphate patients were used as the cell source, MGC formation by anti-DR MoAbs was not observed in the peripheral blood adherent cells, and lower percentages of MGC formation were found in OA and arthritis with calcium pyrophosphate patients compared with those in RA patients. Interestingly, we found MGC formation in peripheral adherent cells from active RA. It is intriguing to ask whether these cells originated from bone marrow of active RA where myeloid cells with abnormal phenotypic marker were found [30], or whether the character was acquired, subsequent to the activation of cells by inflammatory cytokines in the circulation of RA synovial vessel.

In the RA synovium, it is known that MGC are present on the lining layer and pannus lesion [27,31]. Although it remains unclear how these MGC are formed in RA synovitis, the present data suggest that several ligands to bind to the DR molecule, such as superantigens from bacteria or viruses, soluble CD4 molecule, or anti-Ia autoantibodies, would be involved in MGC formation in RA synovium. What is the role of MGC in the pathogenesis of RA synovitis? Recently, macrophage polykaryon was reported as the precursor of osteoclast [32,33]. The character of MGC formed *in vitro* is consistent with the precursor of osteoclasts in macrophage-lineage cells, and the presence of tartrate-resistant acid phosphatase. Further examination will verify whether such induced MGC have osteoclastic activity.

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