Use of human chondrocyte cell cultures to identify and characterize reactive antibodies in rheumatoid arthritis sera

A. Sabbatini, C. Tacchetti[1], S. Tommasi, F. Bongiorni, P. Migliorini

Clinical Immunology Unit, Department of Internal Medicine, University of Pisa and the [1]Department of Experimental Medicine, Section of Anatomy, University of Genova, Italy.

Abstract

Objective

To study the reactivity of rheumatoid arthritis (RA) sera with human chondrocyte populations isolated from normal cartilage and expanded in vitro.

Methods

Human articular chondrocytes were cultured as adherent (non-differentiated) cells on plastic dishes or in suspension (differentiated) on dishes previously coated with a thin layer of 1% agarose. Sera from 28 RA patients and 5 paired synovial fluids were tested on lysates from chondrocytes and fibroblasts as control by immunoblot. Antigen expression on the cell membrane was evaluated by flow cytometry in a few sera.

Results

In 9/28 RA sera IgG antibodies specific for chondrocyte antigens (97kDa, 74kDa, 67kDa, 60kDa, 54kDa, 48kDa and 37kDa) were detected. Twelve sera reacted with proteins expressed both on chondrocytes and fibroblasts and 7 with fibroblasts only; two sera had no reactivity. When lysates from adherent or suspension chondrocytes were compared, RA sera reacted with higher intensity and detected more antigens on chondrocytes cultured in suspension. Flow cytometry assay demostrated that RA sera are able to recognize antigens expressed on the cell membrane of the human chondrocytes.

Conclusion

Our data indicate that: a) 32% of the RA sera contain antibodies reactive with antigens expressed exclusively by chondrocytes, but this value rises to 75% if antigens expressed both by chondrocytes and fibroblasts are considered; b) the reactivity of fully differentiated chondrocytes in suspension culture is higher than the reactivity of chondrocytes cultured in monolayer; and c) some of the chondrocyte-specific antigens identified are associated with the chondrocyte membrane. Thus, in vitro *cultured chondrocytes may be used to study both the specificity and the biological activity of autoantibodies in RA.*

Key words

Chondrocytes, rheumatoid arthritis, autoantibodies.

Alessandra Sabbatini, MD; Carlo Tacchetti, MD; Simona Tommasi, BS; Francesca Bongiorni, BS; Paola Migliorini, MD, Professor.

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Please address correspondence and reprint requests to: Paola Migliorini, MD, Clinical Immunology Unit, Department of Internal Medicine, Via Roma 67, 56126 Pisa, Italy.
E-mail: p.migliorini@int.med.unipi.it

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Introduction

Rheumatoid arthritis (RA) is a common human disease characterized by chronic inflammation of the joints followed by progressive destruction of the articular cartilage. Cellular and humoral immune responses to joint costituents play a central role in this destruction (1). Autoantibodies to various collagen types, nuclear proteins and DNA have been detected in RA sera. A tissue-specific immune response directed against articular cartilage and namely against chondrocytes has also been described (2, 3). Chondrocytes are actively involved in the synthesis of cartilage matrix, which isolates them and creates an immunologically privileged site. In the inflammatory process that characterizes RA, chondrocytes become targets of autoantibodies and T cells and play an active role in modulating this immune response. In fact, they express HLA class II antigens when stimulated with cytokines such as IFNγ and, according to some authors, act as antigen presenting cells (4). To characterize chondrocyte autoantigens, membrane preparations isolated from chicken cartilage (5, 6) or from a human chondrosarcoma cell line (7, 8) have been used.

However, in these studies heterologous antigens or tumor-associated antigens may be identified as targets of autoantibodies, while the use of human non-transformed chondrocytes allows the detection of bona fide autoantigens. These studies are difficult to perform because of the limited number of cells that can be obtained from the cartilage samples and the lack of standardized techniques for the *in vitro* culture of chondrocytes.

In this report we present a technique to culture human differentiated chondrocytes and we analyze the reactivity of RA sera with these cells by immunoblot and flow cytometry.

Materials and methods

Patient sera

Sera were obtained from 28 RA patients followed at the Rheumatology and Clinical Immunology Units of the University of Pisa. The diagnosis of RA was made based on the criteria of the American College of Rheumatology. Clinical and serological data were available for 26 of the 28 patients. Eleven patients had active disease and rheumatoid factor was detected in 19. Normal sera were obtained from healthy laboratory personnel.

Chondrocyte isolation and culture

Cartilage tissue was obtained from the non-affected areas of surgical specimens removed for knee arthroplasty and used after informed consent was obtained.

Cartilage specimens were processed as described (9). Briefly, cartilage slices free of synovial and subchondral tissue were finely minced. The extracellular matrix was digested for 1 h at 37°C with 0.2% collagenase II and 0.25% trypsin in PBS. After centrifugation the pellet was digested for 12 h with 0.2% collagenase II, 0.25% trypsin and 0.05 mg/ml DNase I in Coon's modified Ham F12 medium (10) without calf serum at 37°C in a humidified atmosphere of 5% CO_2 in air. The resulting cell suspension was resuspended in 10 ml of medium with 15% fetal calf serum and seeded in 10 cm^2 plastic culture dishes.

After 2-3 weeks of anchorage-dependent growth, cells were harvested by trypsinization and transferred to tissue culture dishes previously coated with a thin layer of 1% agarose; the culture medium was changed every third day.

Collagen production by chondrocytes

The precipitation of collagen present in the supernatant of chondrocyte culture is accomplished by the slow addition of appropriate quantities of crystalline NaCl (up to the concentration of 4M) with costant stirring. The precipitate is allowed to form over night at 4°C, then recovered by centrifugation at 35,000 g for 1 h. The pellet was resuspended in 0.1 ml of 0,5 M acetic acid (11) and separated on 7.5% acrylamide gel, blotted to nitrocellulosa and probed with rabbit serum anti-collagen II.

Fibroblasts

Human foreskin fibroblasts (a kind gift from Dr. M. De Luca, Istituto Dermopatico dell'Immacolata, Pomezia, Rome) were cultured with RPMI medium supplemented with 10% FCS.

Sera reactivity with condrocytes

Immunoblot. The total cell lysate was loaded onto a 10% acrylamide gel (12) and transferred to nitrocellulose (13). The nitrocellulose filters were cut into 0.5 mm strips and saturated for a one-hour incubation period in 0.05M TRIS, 0.15M NaCl and 5% dry non-fat milk. Sera, diluted 1: 250 in the same buffer, were incubated on the filters for 4 hours at room temperature. The bound antibodies were detected by goat anti-human IgG $(Fab)_2$ conjugated with alkaline phosphatase (Sigma Chemical Co., St. Louis, MO). The immunoactive bands were visualized using BCIP and NBT as substrates (14) .

Flow cytometry. Antigen expression on the cell membrane was evaluated by flow cytometry on human articular chondrocyte or foreskin fibroblasts. The fibroblasts were harvested by trypsinization, washed with PBS and counted. The suspension chondrocytes were dissociated from matrix treating the aggregates with a solution containing 1000 U/ml of collagenase II, 1% trypsin and 1 mg/ml hyaluronidase at 37°C for 30 min. The cells were then washed with PBS and counted; 10^5 cells were incubated with RA sera, synovial fluids or normal sera (1:10 in PBS, 1% BSA, 10% normal goat serum) for 90 min. in an ice bath. After washing with PBS and 1% BSA, the cells were incubated with FITC-labeled goat anti-human IgG (Southern Biotechnology Associates, Birmingham, AL) for 30 min. in ice. After washing twice with PBS and 1% BSA, the cells were fixed with 0.500 ml of 1% paraformaldehyde.

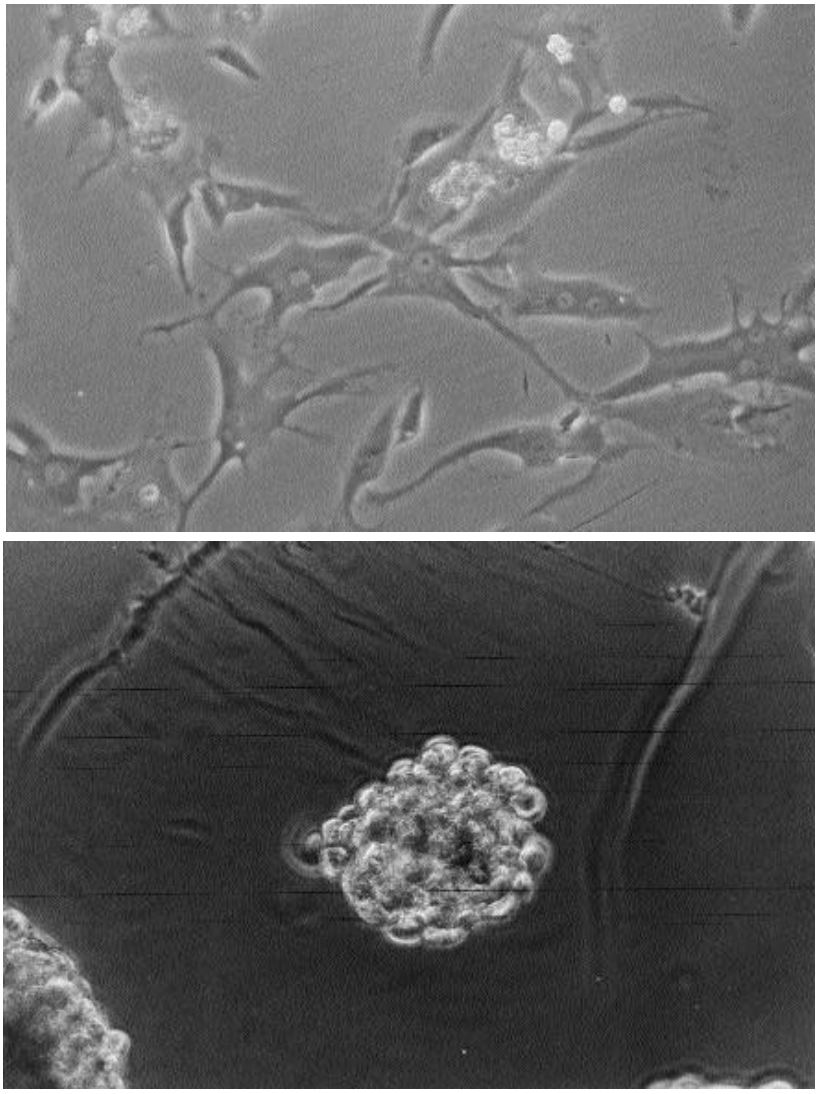

Fig. 1. (**a**) Culture of human chondrocytes as dedifferentiated cells:cells adhere to plastic and show a fibroblast-like phenotype. (**b**) Culture of human chondrocytes on agar-coated dishes:cells grow in suspension and form aggregates.

Results

Isolation and culture of human chondrocyte

Human chondrocytes were isolated and cultured according to the methods previously described for chicken chondrocytes (isolated from tibial cartilage of early chicken embryos) (15). Human chondrocytes cultured on plastic dishes stay in suspension without growth for approximately 6-7 days. After this lag period they become adherent and, as observed for the chicken chondrocytes, assume a fibroblast-like morphology (Fig. 1a). When transferred to suspension culture they re-assume the chondrocyte phenotype, form aggregates and secrete type II collagen (15-18) (Fig. 1b).

However, it has been reported that adherent chondrocytes in monolayer culture may secrete high molecular weight (4) proteoglycans and collagen type II during the first weeks of culture (2). We therefore evaluated the levels of type II collagen secreted in the supernatant of chondrocytes, at different times of monolayer culture (2, 3, 6 and 14 days), and after 3 and 7 days of suspension culture. Our results showed that type II collagen was secreted in monolayer cultures, but the levels increased when chondrocytes were transferred to suspension culture (data not shown).

Sera from 28 RA patients and 10 normal subjects were tested for the presence of antibodies binding to chondrocyte antigens; in 5 cases paired synovial fluid (SF) samples were also tested.

In a first set of experiments we tested the reactivity of the RA sera with chondrocytes from a 7-day suspension culture, and with human foreskin fibroblasts as control. We grouped the sera according to their reactivity. Normal sera showed only a weak reactivity with chondrocytes or fibroblasts. We identified 9 sera (group A) reacting exclusively with chondrocyte cell lysates (97kDa, 74kDa, 67 kDa, 60 kDa, 55kDa, 47kDa and 36kDa). Of the remaining sera, 12 (group B) reacted

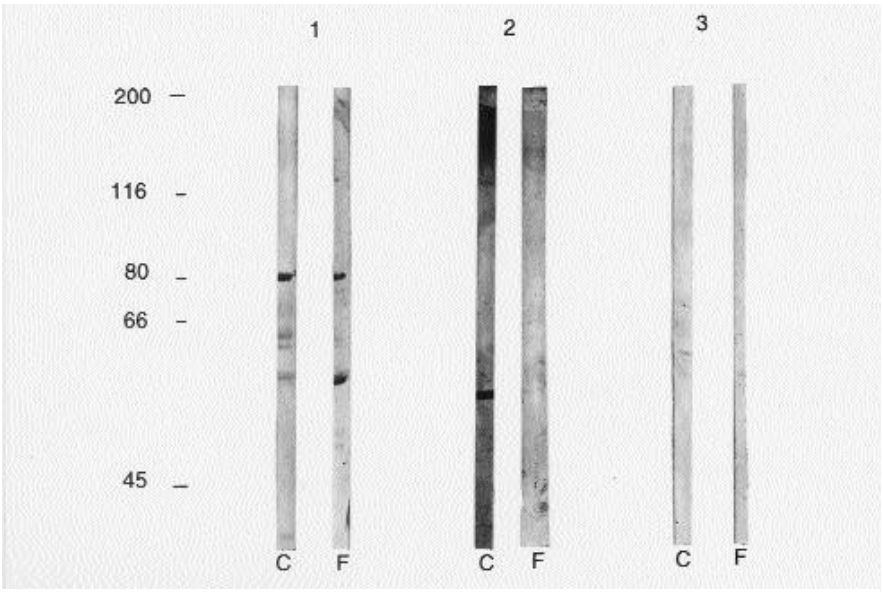

Fig. 2. Reactivity of sera or SF from RA patients on chondrocyte (**C**) or fibroblast (**F**) lysate.
Cell lysate was fractionated on acrylamide gels, transferred to nitrocellulose and probed with RA serum (**1**); RA SF (**2**); or normal serum (**3**). Molecular weight markers in kDa are given on the left.
The RA serum detected antigens present both in chondrocyte and fibroblast lysate, while its paired synovial fluid reacts with chondrocyte-specific antigens.

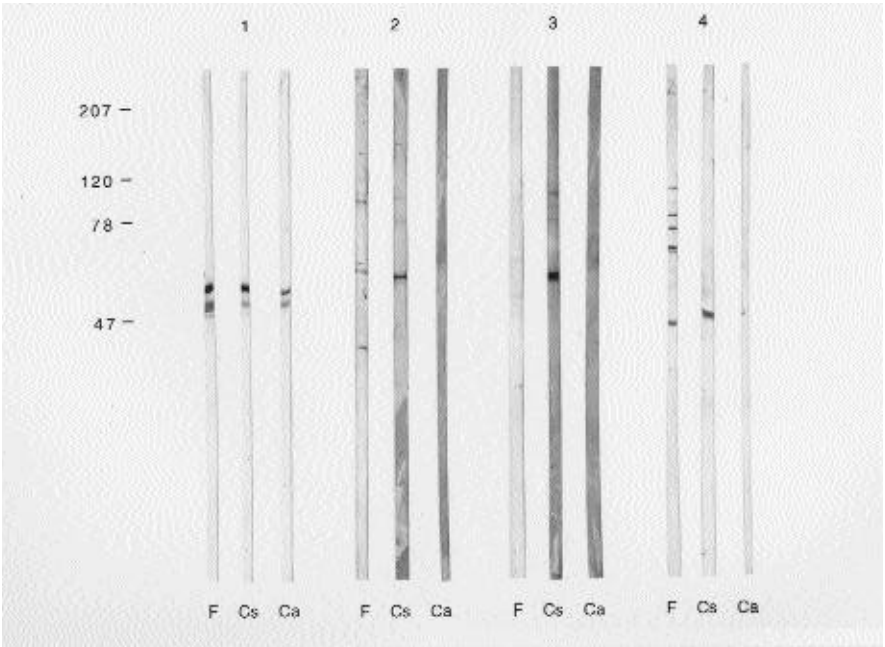

Fig. 3. Reactivity of sera from RA patients on lysates of suspension chondrocytes (**Cs**),adherent chondrocytes (**Ca**) or fibroblasts (**F**).
Cell lysates were fractionated on acrylamide gels, transferred to nitrocellulose and probed with RA serum group B (**1**); RA sera group A (**2, 3**); normal serum (**4**). Molecular weight markers in kDa are given on the left.

with both chondrocytes and fibroblasts, recognizing proteins of identical molecular weight (89kDa, 77kDa, 71kDa, 59kDa, 56kDa, 53kDa, 52kDa, 50kDa, 48kDa, 45kDa, 42kDa, 40kDa), 7 sera (group C) reacted only with fibroblasts, and 2 sera (group D) with neither of the cell lysates. Out of the 9 group A sera, 4 were from patients with active disease and 8 were rheumatoid factor-positive.

When paired synovial fluids (2 from group A patients, 3 from group B patients) were tested, 3 (1 group A, 2 group B) out of 5 dectected antigens in chondrocyte lysates (67 kDa, 60 kDa, 36 kDa). A representative example of group B sera with its paired synovial fluid recognizing chondrocyte-specific antigens is given in Figure 2.

Because of the increase in collagen expression upon transferring chondrocytes to suspension culture, we evaluated whether the culture conditions were influencing the expression of the detected antigens. The reactivity of a small number of RA sera (4 from group A; 2 from group B; 1 from group C; 1 from group D), was tested on extracts of chondrocytes in monolayer cultures or fully differentiated chondrocytes in suspension cultures. The group A sera reacted with extracts from 7-day suspension cultures and weakly with chondrocytes in monolayer culture, the group B sera reacted with antigens present both on chondrocytes and fibroblasts, the group C sera reacted only with fibroblasts and the group D sera did not display any reactivity (Fig. 3).

These results indicated that the two types of cells differed in their expression of antigens recognized by RA sera, with chondrocytes in suspension displaying a wider number of antigens and in higher amounts (Fig. 3).

Antigen expression on the cell membrane.

We tested whether the antigens were expressed on the cell membrane of human chondrocytes and fibroblasts by flow cytometry. The cells were incubated with 4 RA sera (2 group A, 2 group B), one SF serum, and one normal serum and stained with FITC-labeled goat anti-human immunoglobulins. Chondrocytes incubated with 2 RA sera and with the SF were intensely fluorescent as compared to cells incubated with normal serum (Fig. 4). In immunoblot, one of the 2 positive sera reacted with chondrocyte antigens only, whereas the other reacted with antigens shared by chondrocytes and fibroblasts. All the sera reacted very weakly with the fibroblasts membrane (Fig.4).

Discussion

The results of this study can be summarized as follows: a) 32% of the RA sera contain antibodies reactive with antigens expressed exclusively by chon-

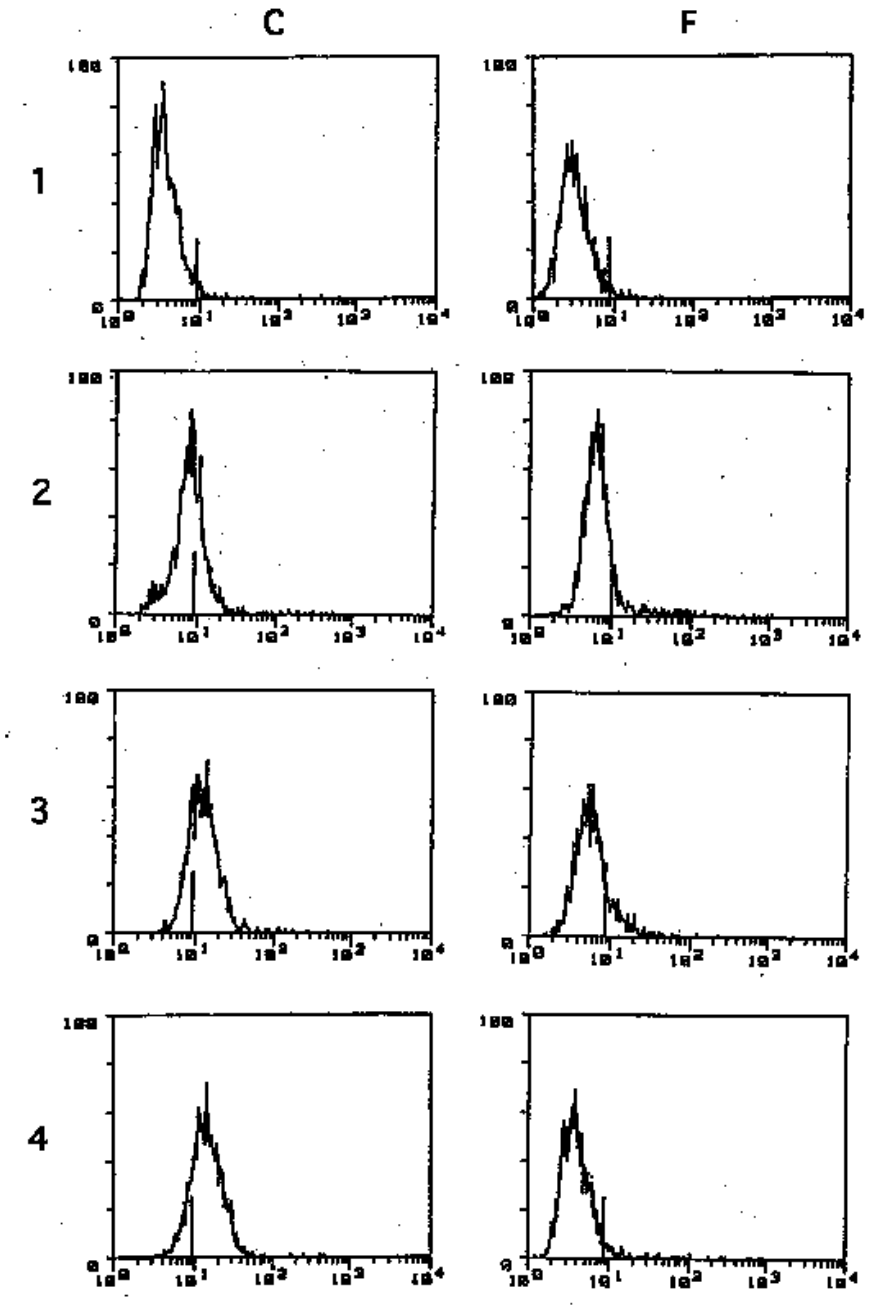

Fig. 4. Flow cytometric analysis of human chondrocytes (**C**) and fibroblasts (**F**) stained with RA sera, SF and normal serum and developed with fluorescein-labeled anti-human IgG. Single-colour fluorescence analysis was performed using a FACScan (Becton Dickinson) flow cytometer with logarithmic intensity scales using a FACScan research software program (FRSP).
1: buffer; **2**: normal serum; **3**: RA serum; **4**: synovial fluid.

drocytes; however this value rises to 75% if antigens expressed both by chondrocytes and fibroblasts are considered; b) the reactivity of fully differentiated chondrocytes in suspension culture is higher than the reactivity of chondrocytes cultured in monolayer; and c) some of the chondrocyte-specific antigens identified are associated with the chondrocyte membrane.

A number of studies have addressed the reactivity of RA sera with chondrocyte antigens. A crucial issue in comparing the results of these studies is the source of chondrocytes and the technique used for the *in vitro* culture of these cells.

It is known that chicken embryo and mammalian (9, 15, 19) chondrocytes, when cultured adherent to a substrate, dedifferentiate and do not secrete type II collagen. The authors described these chondrocytes as dedifferentiated, to emphasize the reversible loss of differentiated functions, not implying regression to an earlier bipotent or multipotent embryonic state. When these chondrocytes were cultured on agarose coated dishes, they re-expressed the differentiated phenotype. Human chondrocytes cultured for the purpose of autologous transplantation are grown *in vitro* for 2-3 weeks as adherent cells and then injected back into the patient joint. In such cases, differentiation of chondrocytes takes place *in vivo*, as shown by the histological study of cartilage after transplantation (20).

Taken together, all these data suggest that the phenotype of chondrocytes in suspension resembles that of mature chondrocytes *in vivo*.

According to our results, adherent chondrocytes release type II collagen after 2 weeks in monolayer culture, whereas after 3 days in suspension culture they undergo a further differentiation, as type II collagen is dectected in higher amounts. Thus, the high reactivity of RA sera with chondrocytes in suspension, as compared with adherent chondrocytes, suggests that in this disorder the antibodies are mainly directed against a set of antigens expressed on highly differentiated cells.

The difficulty in obtaining human cartilage specimens and in culturing human differentiated chondrocytes has limited the number of studies in which these cells have been used to study the autoimmune response in RA. However, plasma membrane preparations from freshly isolated chondrocytes have been tested with peripheral or synovial T lymphocytes from RA patients (21). Both lymphocyte populations proliferated in response to chondrocyte membrane antigens presented by peripheral blood monocytes, while there was no significant response to membranes from fibroblasts or epithelial tumor cells.

Plasma membranes have also been isolated by Mollenhauer *et al*. (5) from human, chick and rat cartilage. However, only rat and chick membranes have been obtained in sufficient amounts to study RA reactivity by immunoblot.

The yield of human membranes allows the detection of antibodies specific for this antigen preparation only by ELISA.
Using chick membranes 10 proteins were identified as specific chondrocyte antigens recognized by autoantibodies. One of these, a protein of 65 kDa denominated RA 65, has been purified and analyzed (6); it displays structural and sequence similarities with heat shock proteins and cytokeratins.
Chondrocytes produce and secrete a glycoprotein (YKL-39) that is the target of autoantibodies in RA sera (22). Moreover, immunization with YKL-39 is able to induce arthritis in normal strains of mice (23). However, the frequency of autoantibodies to YKL-39 is low in RA sera and similar to what is observed in osteoarthritis patients (24).
Hattori *et al.* (8) analyzed the reactivity of sera with plasma membranes prepared from a stable human chondrosarcoma-derived cell line (7), synthesizing a matrix consisting of cartilage-specific proteoglycans and type II collagen. These authors described three proteins of 105 kDa, 76kDa and 47kDa as chondrocyte-specific autoantigens. The 47 kDa protein was sequenced and found to be identical to a hsp47-like protein, product of the colligin 2 gene. It is worth noting that hsp47 is a collagen-specific molecular chaperone.
Despite the different source of chondrocyte antigens used, our results show some overlap with these studies: the 67 and 47 kDa proteins we detected as chondrocyte-specific antigens might be identical to RA 65 and hsp47. As we used chondrocyte lysate in immunoblot, we do not know whether these antigens are expressed on the cell membrane.
However, a few sera containing chondrocyte-specific antigens have been shown to react with the cell membrane by flow cytometry. To our knowledge, the only study that analyzed RA sera or SF binding to chondrocytes by flow cytometry was performed by Takagi *et al.* (25). These authors detected IgG and IgM antibodies cytotoxic for bovine chondrocytes grown as adherent cells; membrane binding by flow cytometry, however, was analyzed only for IgM antibodies, while we detected IgG binding to human chondrocytes.
On the whole, these results show that *in vitro* cultured chondrocytes, grown in suspension culture, may be used to study both the specificity and the biological activity (such as cytotoxicity or induction of apoptosis) of autoantibodies in RA. The possibility of expanding chondrocytes *in vitro* overcomes the limits posed by the low amounts of human cartilage usually available and would allow the planning of more detailed biochemical and functional studies on human chondrocytes.

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