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Cartilage formed by syngeneic rat chondrocytes in joint surface defects is rejected in animals sensitized with allogeneic chondrocytes: involvement of the synovial lining

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A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Stanisław Moskalewski[ADE], **Anna Osiecka-Iwan**[BD], **Anna Hyc**[BD] **and Justyna Niderla**[B]

Department of Histology and Embryology, Medical University of Warsaw, Warsaw, Poland

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Summary

Introduction: The aim of the study was to discover the mechanism of rejection of chondrocyte transplants introduced into articular cartilage defects.

Materials and Methods: Chondrocytes from 3–5-day-old Lewis or WAG rats were liberated by enzymatic digestion from articular-epiphyseal cartilage complexes and implanted into defects made in the subpatellar region of the femur condyle of naive Lewis rats. Syngeneic transplants were also done after sensitization of the recipients with allogeneic chondrocytes injected intramuscularly. The transplants and synovial membrane were studied in periodate-lysine-paraformaldehyde-fixed material with antibodies against B lymphocytes, $CD4^+$ and $CD8^+$ cells, NK cells, and macrophages. For detection of humoral response, chondrocyte lysates were subjected to protein electrophoresis and Western blotting with sera from the transplant recipients.

Results: Cartilage produced in intracartilaginous transplants of syngeneic chondrocytes did not show any signs of rejection. $CD8^+$ lymphocytes and macrophages accumulated in the vicinity of cartilage produced by similar transplants in animals sensitized with intramuscular transplants of allogeneic WAG chondrocytes or bearing transplants of allogeneic WAG chondrocytes. $CD8^+$ cells penetrated into the peripheral part of the cartilage, while macrophages advanced much more deeply. No specific anti-chondrocyte antibody was detected. The synovium from rats bearing intracartilaginous transplants of allogeneic chondrocytes or syngeneic chondrocytes after sensitization contained macrophages and $CD8^+$ cells.

Conclusions: The rejection of cartilage formed by syngeneic chondrocyte transplants in sensitized animals argues in favor of a chondrocyte-specific antigen expression. The involvement of the synovial membrane during transplant rejection suggests that it should be included in observations of the behavior of chondrocyte transplants introduced into articular cartilage.

Key words: **chondrocyte transplants rejection • synovium • $CD8^+$ cells • macrophage**

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Author's address: Stanisław Moskalewski, Department of Histology and Embryology, Medical University of Warsaw, Chałubińskiego 5, 02-004 Warsaw, Poland, fax +48 22 629-52-82, e-mail: smosk@ib.amwaw.edu.pl

INTRODUCTION

Transplanted fragments of allogeneic cartilage are usually not rejected[9, 19, 31]. This can be explained by the protection of chondrocytes by the matrix[2, 25] and thus prevention of their contact with immunocompetent cells of the host. Cartilage produced by transplants of allogeneic chondrocytes is, however, rejected, presumably because their contact with immunocompetent cells occurs before the matrix accumulates in a sufficient amount to offer protection. On the other hand, transplants of syngeneic or autogeneic chondrocytes produce cartilage without signs of rejection[6, 25, 36, 37]. Nevertheless, the expression of tissue-specific antigen(s) by chondrocytes has been suggested, since syngeneic rat chondrocytes were found to develop autoreactivity as assayed by a leukocyte migration test[30] and to stimulate a proliferative response in rat lymphocytes in mixed lymphocyte-chondrocyte cultures[17, 18, 29]. A similar response has also been observed in the cases of rabbit, human, bovine, and canine chondrocytes[22, 54]. We have recently found that cartilage produced by intramuscular transplants of syngeneic chondrocytes is rejected in recipients that have been previously exposed to allogeneic chondrocytes[38]. It is conceivable that chondrocytes express tissue-specific antigen(s) at a level insufficient to cause sensitization after transplantation but sufficient to provoke rejection in a sensitized host.

Autogeneic chondrocyte transplants for the treatment of articular cartilage defects have gained widespread clinical acceptance[5, 7, 15, 41, 44, 45]. Therefore it seemed important to establish whether syngeneic chondrocytes introduced into articular cartilage defects may also evoke an immunological response in a sensitized host. Thus, in this study we have compared syngeneic and allogeneic chondrocyte transplants into articular cartilage defects in naive rats with similar syngeneic transplants in recipients sensitized with allogeneic chondrocytes. The synovial membrane lining the knee joint was also studied to establish whether it is affected by the rejection of the transplants introduced into the articular cartilage.

MATERIALS AND METHODS

Animals

Inbred Lewis (Lew, Rt-1^l) and Wistar Albino Glaxo (WAG, Rt-1^u) rats were obtained from the Animal Unit of the Medical University of Warsaw and were housed under standard conditions. Four-day-old and 5–6-month-old animals served as chondrocyte donors and recipients, respectively. The syngeneic chondrocytes originated from Lewis and the allogeneic from WAG rats. Each type of transplant (syngeneic, allogeneic, or syngeneic in animals sensitized with allogeneic chondrocytes) was done on 12 animals. Additionally, 4 rats were sensitized with allogeneic chondrocytes and killed after 2 weeks for examination of the synovial membranes.

The study was approved by the Animal Ethics Committee of the Medical University of Warsaw.

Isolation and transplantation of chondrocytes

The chondrocytes were isolated from articular-epiphyseal cartilage complexes by constant stirring in an enzymatic solution for about 3 h at 37°C. The enzymatic solution contained 0.25% collagenase (type I, Sigma-Aldrich, MO, USA), 0.05% DNase (Sigma-Aldrich), and 17.5 mM N-α-tosyl-L-lysine chloromethyl ketone (TLCK, Sigma-Aldrich) in F-12 medium (Gibco BRL, Paisley, Scotland, UK)[26]. The addition of TLCK, a potent inhibitor of clostripain activity, which is present in commercial collagenase preparations, significantly increases the yield of viable chondrocytes[20]. After isolation, the chondrocytes were rinsed 3 times with F-12 medium with antibiotics. Chondrocytes for intraarticular cartilage transplants were suspended in a 2% solution of high-molecular-weight hyaluronic acid (ZND-88, Biotechnology General, Kiriat-Weizmann, Rehovoth, Israel, kindly supplied by Dr. Z. Nevo)[46] in MEM (Gibco) at a density of 2×10^7 cells/ml.

Rats were anesthetized by ketamine and xylazine. In the syngeneic or allogeneic intracartilaginous transplants in animals not previously subjected to experiments (naive animals), approximately 4×10^4 chondrocytes suspended in hyaluronic acid were introduced with a syringe into defects made in the subpatellar region of the femur condyle by a 1.3-mm-diameter bore, as described[26]. The hole reached into the bone and bone marrow beneath the cartilage. Lewis rats were sensitized by injection of WAG chondrocytes into the muscle of both forelimbs and into the posterior tibial muscle of the right leg (3.0×10^6 cells/0.1 ml of MEM). After 2 weeks, syngeneic chondrocytes were transplanted into the intraarticular cartilage of the left leg. Two weeks after the intracartilaginous transplantation of chondrocytes, the animals were bled from the heart and killed by cervical dislocation.

Histological evaluation of the grafts

The joint cavity was opened and the synovial membrane together with the patella, patellar ligament, and joint capsule as well as a fragment of bone with the

articular cartilage bearing the chondrocyte transplant were excised. Six transplants from each group were fixed in 4% formaldehyde and the rest in periodate--lysine-paraformaldehyde (PLP)[56]. To study the synovial membrane, this was excised together with the patella, infrapatellar fat pad, and joint capsule after detachment of the patellar ligament from the upper part of the tuberosity of the tibia. For gross observations, the whole preparation was fixed in 4% formaldehyde, rinsed in water, stained in a saturated solution of Sudan IV in 70% ethanol, again rinsed in water, and photographed. For microscopic study the synovial membrane and adhering tissues were fixed in PLP at 4°C for 24 h. The synovial membrane, together with the infrapatellar fat pad and joint capsule, was excised perpendicular to the long axis of the patellar ligament during dehydration in 70% ethanol, since immediately after fixation the tissue was soft and difficult to handle. Dehydration in 70, 80, and 96% ethanol followed by clearing in butanol lasted 24, 2, 6, and 18 h, respectively, at room temperature. The synovial membrane together with the infrapatellar fat pad was separated in butanol from the patellar ligament and joint capsule to facilitate sectioning. After final dissection it had a rectangular shape, with the longer margin perpendicular to the leg's long axis. Excised tissue was embedded in MEDIM-Plast (MEDIM, Giessen, Germany), with a melting point 52–53°C, for 6 h. Bone was trimmed to facilitate access of reagents, decalcified in 14% EDTA solution at 37°C for 4 days, dehydrated, and embedded similarly as the synovial membrane. The synovial membrane was cut parallel to its long margin on a rotary microtome at 6 μm and mounted on glass microscope slides precoated with silane. The transplants were similarly cut perpendicular to the articular surface. To check whether the embedding procedure did not destroy antigenicity, fragments of the spleens of the Lewis rat were fixed in PLP. Some of them were processed as suggested by Whiteland et al.[56], others in exactly the same manner as the bone-bearing transplants, including exposure to the decalcifying solution. Furthermore, synovial membranes from Lewis and WAG rats were fixed in PLP and simultaneously processed for comparison of the reaction with macrophage-detecting antibody. Sections of transplants or synovial membrane were stained with H&E. Cells infiltrating the transplants were identified with monoclonal antibody MCA1427 against rat CD161 (NK cells), MCA 1432 against a 160/180 kDa surface antigen expressed by rat B lymphocytes, and MCA 341R, clone ED1 against rat macrophages CD68 (Serotec, Ltd., Oxford, England). Rat $CD4^+$ cells were identified with MCA against rat CD4, and for the identification of rat $CD8^+$ cells MCA against CD8 was used (Cymbus Biotechnology Ltd., Chandlers Ford, Hants, England). Primary antibodies were used at 1:25 dilution except MCA 341R, which was diluted 1:50. The secondary antibody was biotinylated rabbit $F(ab')_2$ fragments of anti-mouse IgG diluted 1:400 (Serotec). Incubation with primary antibodies lasted 30 min. Incubation of synovial membrane of naive Lewis rats with anti-ED1 antibody was prolonged to 1 or 18 h. Incubation with the second antibody lasted 30 min. Reaction was completed with extravidin-peroxidase conjugate and diaminobenzidine tablets (Sigma--Aldrich). Sections intended for macrophage detection were treated with trypsin before application of the first antibody. Control sections were incubated with diluted mouse serum instead of primary antibody. Unspecific reaction with the second antibody was blocked by preincubation of sections with 3% rabbit serum before application of the first antibody. Some sections were counterstained with 0.1% Alcian blue GX8.

Isolation of skin fibroblasts

Five-day-old rats were killed by cervical dislocation and put into 10% antibiotic-antimycotic solution at 37°C for 1 h. Skin was cut into small fragments and stirred in the enzymatic solution used for the isolation of chondrocytes for 1 h. The cells were then filtered through a 40 μm mesh nylon filter and used for the preparation of cell lysates.

Preparation of cell lysates

Freshly isolated chondrocytes or fibroblasts were rinsed 3 times in F-12 medium at 4°C and seeded into 35 mm Petri dishes at a density of 3×10^6 cells per dish and cultured for 24 h. RIPA buffer (200 μl) enriched with protease inhibitors was then added to each dish. The cells were passed 5–6 times through a 26-gauge needle and sonicated in a sonificator (BANDELIN electronic, GmbH&Co KG, Berlin, Germany). All lysates were stored at –25°C.

Protein determination

One μl of lysate or RIPA buffer alone (blank test) and 9 μl of deionized water were placed in a flat-bottomed 96-well plate and 0.2 ml of BCA protein assay reagent (Pierce, Rockford, Illinois, USA) was added to each well. The plate was incubated at 37°C for 30 min. Protein concentration was determined spectrophotometrically at 550 nm (SLT Spectra Labinstruments, Crailsheim, Germany).

Protein electrophoresis and Western blotting

Cell lysates (10 μg of protein) mixed in sample buffer were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 12% acrylamide)[28]. Sepa-

rated proteins were transferred onto a PVDF membrane by semi-dry blotting at 25 V for 30 min, using a Trans-Blot SD apparatus (Biorad Laboratories, Hercules, CA, USA). Sera from naive rats and rats bearing allogeneic intracartilaginous transplants or syngeneic transplants after sensitization were used at a 1:25 dilution. Biotinylated rabbit anti-rat immunoglobulins served as the second antibody. For comparison of antigen(s) detected by rat sera and by sera produced in rabbits sensitized with rat chondrocytes[39], the latter serum was used at a dilution of 1:120. Incubation lasted 1 h. Biotinylated $F(ab')_2$ fragments of swine anti-rabbit immunoglobulins (Dako A/S DK-2600 Glostrup, Denmark) served as the second antibody. Actin, used as an internal control, was detected with mouse monoclonal anti-β-actin antibody (Sigma-Aldrich) followed by biotinylated goat anti-mouse immunoglobulins (Dako). In all experiments, antibody binding was demonstrated by an amplified alkaline phosphatase detection system (Sigma-Aldrich).

RESULTS

Syngeneic transplants into articular cartilage defects in naive rats

Cartilage produced by transplanted chondrocytes filled the defect within the articular cartilage. Chondrocytes in the layer of cartilage directed into the joint cavity were flattened. In the deeper layers of cartilage, the chondrocytes were rounded or oval and enlarged, but limited hypertrophy of the chondrocytes was observed only in 3 out of 12 transplants. The cartilage matrix was weakly stained with Alcian blue after formaldehyde fixation and remained unstained after PLP fixation, but the chondrocyte nuclei stained well. In some places, cartilage adhered to the subchondral bone, in others it was separated from the bone and bone marrow cells by a layer of loose connective tissue, often with numerous blood vessels similar to bone marrow sinusoids (Fig. 1a). A number of macrophages ($CD68^+$ cells) was present within this area (Fig. 1b), but lymphocytes could not be observed. They were, however, encountered within the bone marrow of metaphyseal bone.

Allogeneic transplants of chondrocytes into articular cartilage defects

The general morphology of the cartilage in transplants fixed in formaldehyde was similar to that produced by syngeneic chondrocytes. Alcian blue staining of the cartilage matrix was weak after formaldehyde fixation and absent after PLP. In the region adjacent to cartilage, large vascular spaces filled with erythrocytes and distinct infiltrations were visible. The infiltrations contained numerous macrophages and some $CD8^+$ cells. Both types of cells penetrated into the cartilage matrix, and the peripheral lacunae were often devoid of chondrocytes, but the range of $CD8^+$ cell penetration was much shorter than that of macrophages (Figs. 1c, e). CD161 (NK cells), $CD4^+$ lymphocytes, and cells expressing 160/180 kDa surface antigen (B lymphocytes) were encountered only occasionally. Normal articular cartilage around defects bearing transplants was devoid of infiltrating cells and did not differ from that in naive rats.

Syngeneic transplants of chondrocytes into articular cartilage defects in animals sensitized with allogeneic chondrocytes

In these transplants, areas of cartilage with easily recognizable matrix and cartilage lacunae were usually separated from the bone by a layer of loose connective tissue with delicate network of fibers. Within this area, numerous large blood vessels, filled with erythrocytes and similar to bone-marrow sinusoids, were present. Macrophages invaded the tissue adjacent to cartilage and penetrated into the cartilage matrix, reaching the central area (Fig. 1d). In some transplants, only remnants of cartilage filled with macrophages could be observed. Some $CD8^+$ cells were observed in the vicinity of transplants or penetrated into the peripheral part of the cartilage (Fig. 1f). Other types of lymphocytes were only occasionally observed.

Detection of antibodies in rat sera

Antigen with a M_r of ~19 kDa was detected by sera from naive and experimental rats in immunoblots of syngeneic chondrocyte or fibroblast lysates. The intensity of immunoblot staining varied considerably from serum to serum and no clear difference between the sera of particular groups of rats could be observed. The M_r of the antigen remained unchanged if reduction in 2-mercaptoethanol was omitted. The sera did not react with the ~23 kDa antigen detected in our previous work by antibody from rabbits sensitized with rat chondrocytes (Fig. 2).

Synovial membrane

Staining of fat cells with Sudan IV enabled observation of the topology of the synovial membrane. On its surface, two grooves, usually assuming a U shape, were present (Fig. 3a). On the microscopic level, the synovial membrane consisted of synoviocytes covering a basal layer of the membrane with fibroblasts and collagen fibers resting on the infrapatellar pad of

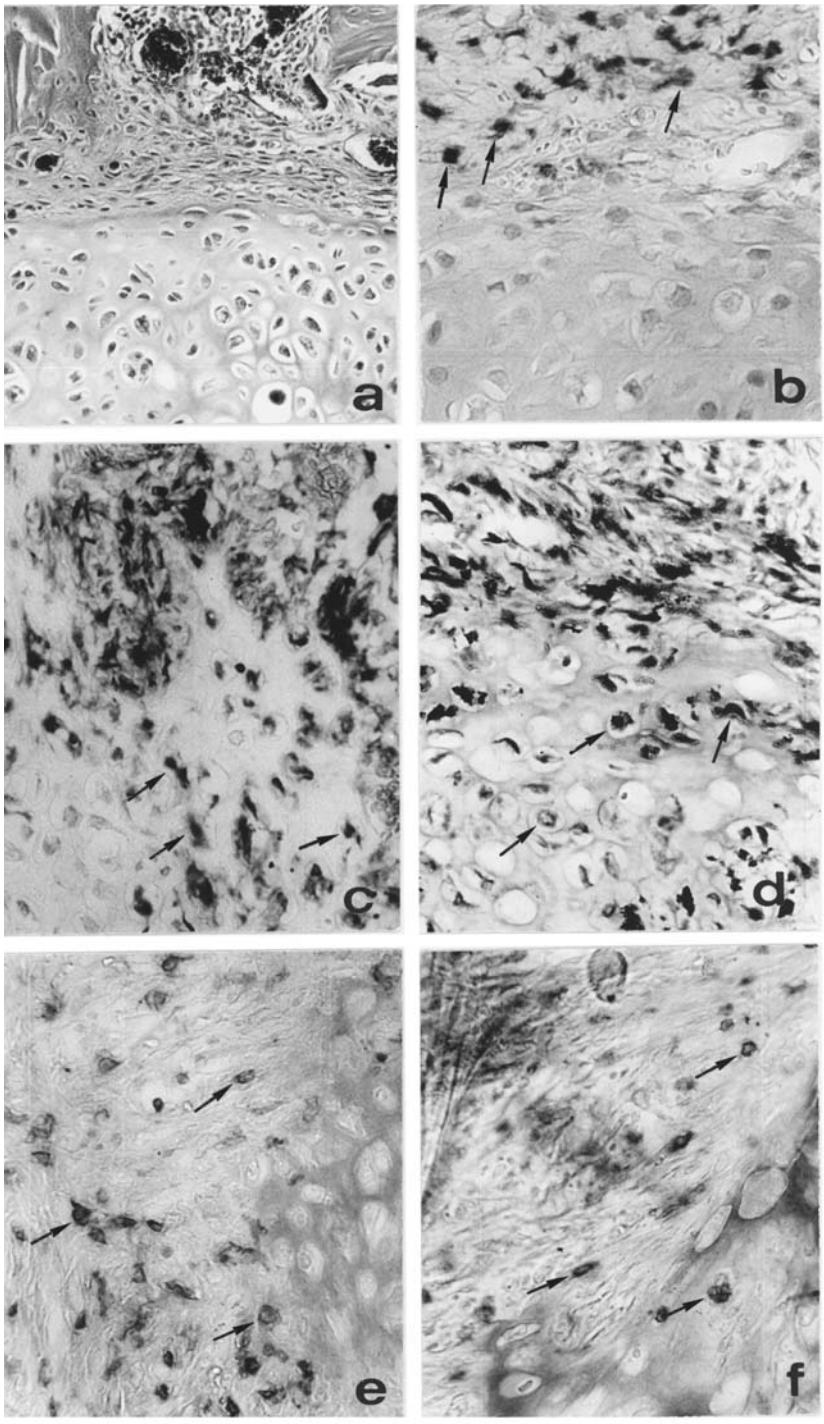

Figure 1. Intracartilaginous, 2-week-old transplant of chondrocytes. Syngeneic transplants general appearance (**a**) and macrophages are present in the tissue separating the cartilage from the bone, but they do not penetrate into the cartilage matrix (**b**). Macrophages invading the cartilage matrix in the transplant of allogeneic chondrocytes (**c**) and in the transplant of syngeneic chondrocytes in a sensitized rat (**d**). In large vessels similar to bone-marrow sinusoids are visible (**d**; arrows indicate macrophages) $CD8^+$ cells in allogeneic (**e**) and syngeneic transplants of chondrocytes in a sensitized rat (**f**). The $CD8^+$ cells (indicated by arrows) are present at the periphery of the transplants or penetrate into the peripheral matrix H&E, ×240 (**a**), MCA 341R (**b**–**d**), clone ED1 detecting macrophages followed by biotinylated rabbit $F(ab')_2$ fragments of anti-mouse IgG and the extravidin-peroxidase detection system. Sections counterstained with Alcian blue (×440), MCA against T cells $CD8^+$ detected as above; ×440 (**e, f**).

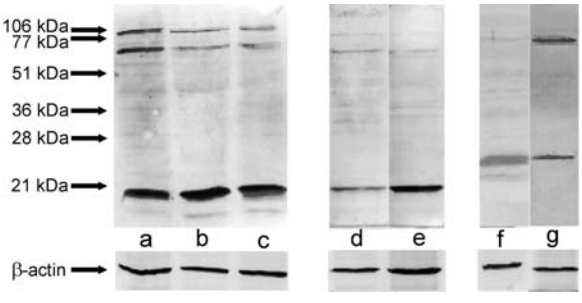

Figure 2. SDS-PAGE and immunoblot of lysates of chondrocytes (lines a, b, d–g) and skin fibroblasts (line c). Lysate in line b and g was run without reduction in mercaptoethanol, the remaining lysates were run under reducing conditions. Lanes a, b, c were incubated with serum from rats bearing syngeneic transplants of chondrocytes after sensitization with allogeneic chondrocytes. Lanes d, e were incubated with serum from naive rats. Lanes f, g was incubated with serum from rabbit sensitized with rat chondrocytes.

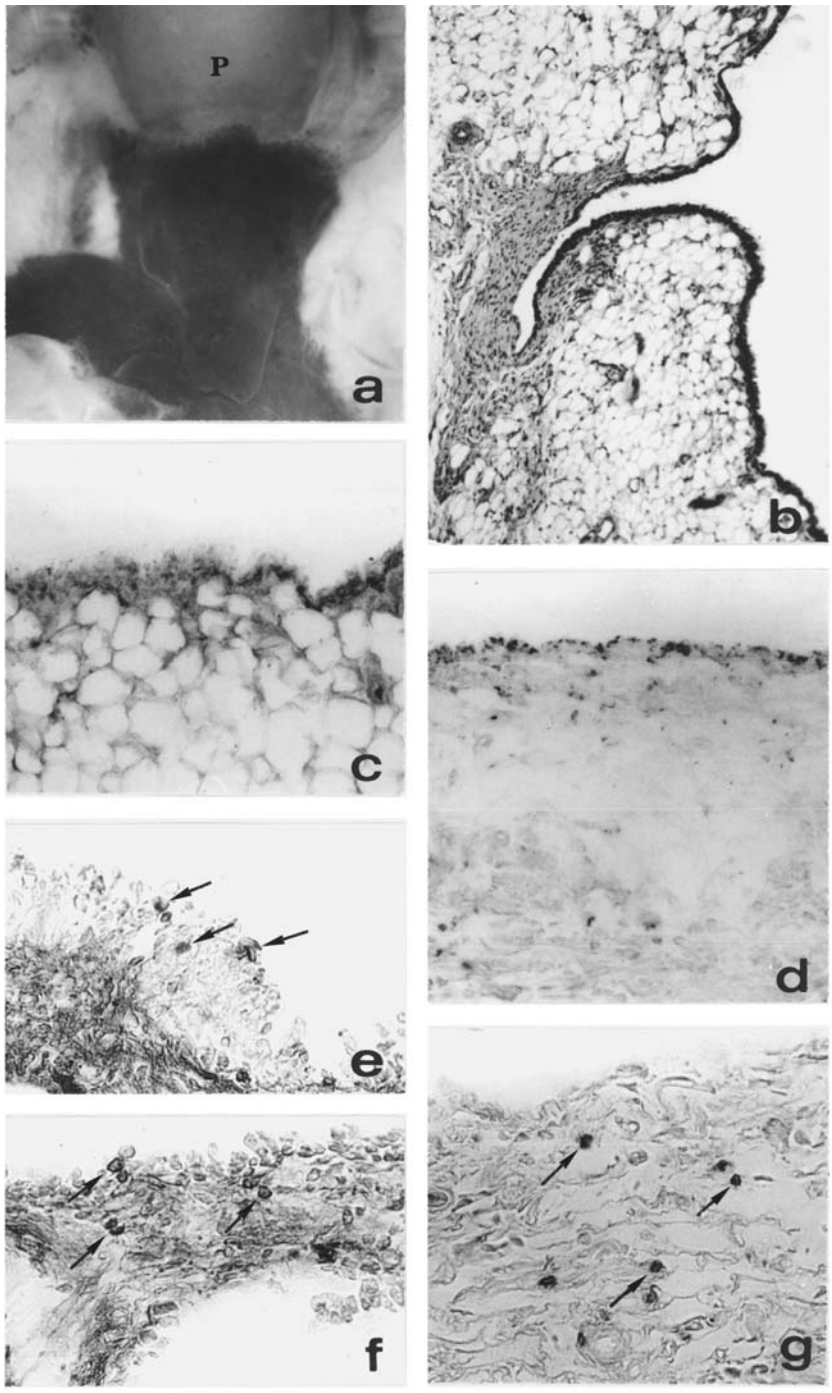

Figure 3. Joint capsule from the left knee joint. A whole mount of the synovial membrane and infrapatellar pad showing distinct grooves within the synovium (**a**). Cross-sections of the synovial membrane and infrapatellar pad from a naive rat (**b**). The surface of the synovial membrane and a deep groove with the bottom formed by dense fibrous tissue and slopes resting on fat cells are visible. Macrophages in the synovial membrane (**c, d**) from rats with allogeneic (**c**) and syngeneic (**d**) transplants of chondrocytes after sensitization.T $CD8^+$ cells in the synovial membrane (**e**–**g**) from rats with allogeneic (**e, f**) and syngeneic (**g**) transplants of chondrocytes after sensitization. Show cells in the synovial lining covering the surface of the synovial membrane (**e, f**) and in the pseudovillous extension of the membrane (**f**). T $CD8^+$ cells in the deeper part of the synovial membrane (**g**). Arrows (in **e**–**g**) indicate T $CD8^+$ cells. Sudan IV staining (**a**), photographed in water; P – patella. ×10. H&E (**b**) ×100. Anti-ED1 serum followed by biotinylated rabbit $F(ab')_2$ fragments of anti-mouse IgG and extravidin-peroxidase detection system (**c, d**) ×440. MCA against T $CD8^+$ cells detected (**e, f, g**) as above, ×440.

fat. In some areas, synovial membrane formed villous extensions. On the slopes of the grooves, the fat pad was covered by a 2–3 cell-thick layer of the synoviocytes. The bottom of the groove slope consisted of a band of fibrous tissue covered by 1–2 cell layer of cells (Fig. 3b). In the synovium from the naive Lewis rats, reaction with ED1 antibody failed to visualize macrophages. Only occasionally could a few positive granules in the synovial lining be observed. Increasing the concentration of ED1 from 1:50 to 1:25 and prolonging the incubation to 1 or 18 h had no influence. In contrast, numerous macrophages were detected in the synovial lining from WAG rats used as a technical control. In the synovium of Lewis rats bearing syngeneic transplants, macrophages were detected only in limited areas among superficial cells and sometimes in groups in deeper parts of the membranes. In rats bearing allogeneic transplants or syngeneic transplants after sensitization, the whole superficial layer of cells located between the synovial grooves or on their slopes contained ED1-positive cells (Fig. 3c, d). They were, however, practically

absent at the bottoms of the grooves. Furthermore, in the synovium of rats sensitized with allogeneic intramuscular transplants of chondrocytes, reaction with ED1 antibody was similar to that in rats which additionally received intracartilaginous transplants of syngeneic chondrocytes. In the synovium of rats with intraarticular transplants of allogeneic chondrocytes or syngeneic chondrocytes after sensitization, a number of $CD8^+$ cells were present. In the former transplants they predominated in the superficial layer of the synovial membrane and were frequently present in its small pseudovillous extensions (Fig. 3e, f). In the latter they were more often observed in the deeper part of the membrane or in the underlying fat tissue (Fig. 3g). In naïve rats, rats bearing syngeneic transplants, or rats sensitized with intramuscular transplants of allogeneic chondrocytes, lymphocytes belonging to all the studied subsets ($CD4^+$, $CD8^+$, B, and NK) were absent or occurred only sporadically, either between synoviocytes or in the underlying fat tissue.

Control staining of spleen

Macrophages and all subsets of lymphocytes were stained in the spleen sections, although specimens embedded after exposure to decalcifying solution required a higher concentration of primary antibody to obtain an intensity of staining comparable to that in specimens processed in accord with the original description[56].

DISCUSSION

We have previously reported the rejection of intramuscular transplants of allogeneic chondrocytes in naive rats or similar transplants of syngeneic chondrocytes done in rats sensitized with allogeneic chondrocytes[38]. In this study, the rejection of similar types of transplants, but introduced into articular cartilage defects, was observed. Previous studies indicated that cartilage produced by allogeneic chondrocytes in intramuscular transplants was infiltrated in the course of rejection by $CD8^+$ or $CD4^+$ cells and cells of macrophage lineage. Since $CD4^+$ cells were demonstrated with a monoclonal antibody which also reacts with macrophages, their identification was not certain[47]. Among the cells infiltrating intracartilaginous transplants, $CD8^+$ cells, which accumulated close to the transplants and invaded only their peripheral part, and macrophages, penetrating into the peripheral and also central zone of the transplants, predominated. $CD4^+$ cells, B lymphocytes, and NK cells occurred only occasionally. Antigenic markers of these lymphocytes are susceptible to fixation and embedding procedures[56], but all of them could be demonstrated in control sections of spleen and also in the bone marrow present in the bone bearing the transplant. Thus the absence of these cells in infiltrations connected with transplants does not seem to depend on technical error. The absence of NK cells in infiltrates suggests that they are not involved in transplant rejection although they cause lysis of isolated chondrocytes in cytotoxic tests[43].

The composition of the infiltrates was similar in the cases of allogeneic and syngeneic transplants, suggesting a similar mechanism of rejection. The existence of chondrocyte-specific antigen is still uncertain. Sera from patients with osteoarthitis and rheumatoid arthritis contained antibodies against several peptides from the chondrocyte surface with molecular weights ranging 28–155 kDa[34]. Moreover, chondrocyte autoantigen (CH65) reacting with sera from patients with rheumatoid arthritis and possibly involved in the pathogenesis of adjuvant arthritis in Lewis rats was identified[3, 16]. Recently, Sabbatini et al.[48] found that some rheumatoid arthritis sera reacted with 7 human chondrocyte antigens in the 37–97 kDa range which were absent on human foreskin fibroblasts. Moreover, synovial fluid immunoglobulins from patients with rheumatoid arthritis exhibited complement-dependent cytotoxicity against isolated bovine articular chondrocytes[54]. On the other hand, 5 monoclonal antibodies raised against human articular chondrocytes reacted not only with chondrocytes, but also with different epitopes of non-cartilaginous tissues, suggesting that no antibody was cartilage specific[58].

Besides chondrocytes, all major cartilage matrix components, such as collagen types II, IX and XI[8, 40, 53], core protein of proteoglycans and, particularly, the G1 domain of aggrecan[11, 21, 23, 60], or link proteins[13], may stimulate antibody production or cause lymphocyte sensitization[57] in various pathological or experimental conditions. Thus a possibility remains that during rejection of allogeneic chondrocyte transplants, sensitization occurs against cartilage matrix components and not chondrocyte-specific antigen.

We have previously found in cytotoxic testing that sera from naive rats contain autoantibodies directed against syngeneic chondrocytes. The titer of these antibodies increased in the sera of rats sensitized with chondrocytes. They could be at least partially absorbed by fibroblasts and thymocytes, and thus their specificity to chondrocytes remained doubtful[38]. In this study, sera from naive or sensitized rats were tested in immunoblots, but no specific reaction could be observed. Presumably, both tests detected the same type of antibodies. Specific antibodies against

chondrocytes could also not be produced in rats by intravenous injection of syngeneic chondrocytes[29]. Furthermore, sera from sensitized rats failed to identify 23 kDa antigen in reduced and the 74 kDa in unreduced state, detected previously in sera from rabbits sensitized with rat chondrocytes[39]. Since this antigen was not expressed by rat fibroblasts, endotheliocytes, and thymocytes, it appeared to be a good candidate for a chondrocyte-specific molecule. The possibility remains, however, that in rats it is involved in cellular rather than humoral reaction against chondrocytes. On the other hand, we cannot exclude the possibility that some chondrocyte-specific antibody occurred in the transplant area and participated in the transplant rejection though its concentration in serum was too low to be detected.

Immunocytochemical observations suggest that the destruction of transplanted chondrocytes was accomplished by $CD8^+$ cells which invaded the peripheral zone of the cartilage. Removal of the cartilage matrix was presumably left to macrophages, with the possible involvement of metalloproteinases released from chondrocytes[32]. Macrophages, however, similarly as in the previously reported experiments[47], penetrated into the central zone of the transplants, beyond the territory of $CD8^+$ cell operation. Similarly, in small bowel allografts macrophages infiltrated preferentially the deep layers of the small bowel wall, whereas only a slight T cell infiltration was observed[24]. A large number of host macrophages also migrated into the hepatic allograft[33].

Macrophages secrete a variety of proteinases with the capacity to degrade connective tissue matrix[52]. The long-range penetration of macrophages was probably facilitated since the matrix in transplants of only 2--week duration was immature, but this does not explain why it took place at all. One possibility is that the cells accumulating at the periphery of the transplant release a signal triggering secretion of macrophage-attracting chemokine(s) by chondrocytes. For example, the chemokine RANTES produced by activated T cells[50] could attract macrophages to the periphery of the transplant since it is chemotactic for monocytes[49]. Interleukin (IL)-1β produced by macrophages would stimulate the expression of MCG-1, IL-8, or RANTES by chondrocytes[1, 42], resulting in a further progression of macrophages into the cartilage matrix.

Rejection of syngeneic transplants in animals sensitized with allogeneic chondrocytes reported previously[38] and confirmed in this study, together with observations of the proliferative response of rat lymphocytes in mixed lymphocyte-chondrocyte cultures[17, 18, 22, 29, 54] support the notion that chondrocytes may express tissue-specific antigen.

Examination of the synovial membranes of joints with intracartilaginous transplants indicates that they are affected by the rejection process. The lining of the synovial membrane consists of macrophage-like type A cells and fibroblast-like B cells[4]. Macrophages in the synovial lining of naive Wistar rats are easily demonstrated by monoclonal antibody directed against ED1 antigen[12, 55]. This is a single-chain glycoprotein of 110 kDa expressed predominantly on the lysosomal membranes of myeloid cells, and also present on plasma membrane[10]. Surprisingly, anti-ED1 antibody did not detect macrophages in the synovial lining of naive Lewis rats, although under identical conditions of fixation, embedding, and staining, macrophages in WAG rat synovial membrane were distinctly marked. This particular difference between WAG and Lewis rats allowed observation of the influence of various experimental procedures on synovial membrane macrophages with a simple immunocytochemical reaction. Some macrophages expressing ED1 antigen appeared in joints with intracartilaginous transplants of syngeneic chondrocytes and were prominent after allogeneic or syngeneic transplants in sensitized rats. Furthermore, ED1 antigen could also be detected in macrophages present in the synovial lining of intact joints of rats sensitized with allogeneic chondrocytes. This suggests that the response of synovial macrophages was not caused, or was only partially caused, by the inflammation inherent to the surgical procedure.

The appearance of macrophages with ED1 antigen could result from the increased expression of antigen in macrophages already present in the synovial lining[4] or from the recruitment of bone marrow-derived mononuclear phagocytes[14]. The first possibility seems more probable, since sensitization by allogeneic chondrocytes could stimulate the expression of ED1 antigen as a result of the general response caused by the inflammatory process involved in allogeneic transplant rejection.

The synovium of rats bearing allogeneic or syngeneic chondrocyte transplants following sensitization contained cells reacting with antibody directed against $CD8^+$. This antibody, according to the manufacturer, reacts with cytotoxic lymphocytes and, to a limited extent, with NK cells. Since the cells with the surface marker of the latter were virtually absent, it seems that the synovium was infiltrated by cytotoxic lymphocytes. They could accumulate in response to some factor released from the site of transplantation, either directly, or via stimulated synovial macro-

phages. Articular chondrocytes produce numerous cytokines upon stimulation with IL-1β[1, 42], but specific chemokines selectively attracting rat T CD8$^+$ have not been described as yet[27, 35, 59]. Nevertheless, the appearance of CD8$^+$ cells indicates that some chemokine is released from the site of transplantation in an amount sufficient to affect the synovial membrane and suggests that the study of the latter should be included in observations of the behavior of intracartilaginous transplants.

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