Leporine Experimental Arthritis Induced by Whole Synovial Fluid from Patients with Rheumatoid Arthritis and Osteoarthritis

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Summary
The value of experimental arthritis models in animals depends on their morphological and immunological similarities to rheumatoid arthritis even if they are not exact counterparts of the human diseases. Many uncertainties regarding rheumatoid arthritis pathogenetic mechanisms persist, including unknown factors and the liberation of arthritogenic antigens. The purpose of the study was to compare experimental arthritis in rabbits induced by intra-articular injections of whole synovial fluid obtained from patients with active rheumatoid arthritis and osteoarthritis, which followed sensitization with synovial fluid in complete Freund's adjuvant. The results showed that rheumatoid synovial fluid-induced arthritis is very reproducible and strikingly similar to the rheumatoid arthritis where the arthritis exacerbations and immunological processes occur all the way from cellular immunity to persistent chronic arthritis.

Introduction
Immunological mechanisms play an important role in the induction and maintenance of rheumatoid arthritis (RA) and other inflammatory chronic joint diseases *(Ceponis et al, 1998)*. Therefore obtaining a suitable RA model is difficult and different immunological models are used in animals (*Graham & Shannon 1972a; Andreis et al, 1975; Goldlust et al, 1978; Geiler et al, 1994*). Chronic experimental arthritis (EA) depends on the persistence of a sufficient antigen in the joint tissues as well as on the models in which development of adequate delayed hypersensitivity to the antigen occurs *(Tak et al, 2000)*. For this reason, animal inflammatory arthritis produced using immunological mechanisms may be useful for studying human joint diseases *(Bendele et al, 1999; Hu et al. 2003)*. The value of such models depends on the severity and chronicity of joint inflammation that closely resembles RA histologically. The antigens used to induce EA stimulate the local production of specific antibodies in the synovial tissue and the accumulation of complement-binding antigen-antibody complexes in the damaged tissues *(Graham & Shannon 1972b; Neidhart et al, 2003; Matulis et al, 1987)*. Local immunoglobulin synthesis by the inflamed synovium is a unique feature of this experimental model as EA antigen does not induce a systemic immune response to the injected antigen, and which suggests some possible differences between the pathogenesis of EA and RA *(Fell & Julb, 1977).*
Several authors indicate the possibility of transmitting an active unknown agent from cell-free homogenates of synovial tissues from RA patients to mice *(Warren et al, 1969; Paliard et al, 1991; Pitzalis et al, 2001; Aidinis et al, 2003).* A single subcutaneous (s.c.) intra-articular injection (i.a.i.) of diluted synovial fluid from RA patients in mice can induce arthritis, which was followed histologically for at least 18 months *(Partsch et al, 1986).* The authors concluded that a transmissible and

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rather slow-acting agent exists in the synovial tissue and fluid of RA patients. These preliminary observations were later supported by other authors by i.a.i. undiluted synovial fluid *(Saxne et al, 1988).* Identical results were obtained by implanting synovial tissue derived from a RA patient into severe combined immunodeficiency (SCID) mice either s.c. or under the renal capsule *(Crocker et al, 1974)*. A pannus-like formation with proliferating synovial fibroblast-like cells that invaded the cartilage was found and messenger RNA for cathepsin D, which showed evidence of destruction, was detected in the subsynovial area after 105 days. Chronic arthritis with exacerbations (i.e. of increasing severity) was induced in SCID mice by grafting on purified fibroblasts isolated from the synovial tissue of RA patients *(Lehmann et al, 2000).* The effect of the synovial fluid from RA patients that was injected into bovine nasal cartilage confirms the presence of mediators in synovial fluid that stimulate chondrocytes in both activating degradation and reducing biosynthesis of the proteoglycans *(Saxne et al, 1988)*.

The purpose of the study was to compare experimental arthritis in rabbits induced by i.a.i. of whole synovial fluid (SF) obtained from patients with active rheumatoid arthritis (RSF) and osteoarthritis (OSF), which followed s.c. sensitization with SFs (RSF or OSF) in complete Freund's adjuvant.

Materials and Methods

Sixty female Chinchila rabbits with a mean age of 2.5 months and weighing 2200 - 2500g were obtained from the Breeding Unit of the Institute of Immunology in Vilnius and kept in polymeric cages under the standard housing conditions in the Vivarium of the Institute of Experimental and Clinical Medicine of Vilnius University. The animals received standard chow and water *ad libitum* and were kept in climate-controlled environment with 12-h light/dark cycles. After 5 days of acclimatization all studies were performed in accordance with the European Union regulations for the handling and use of laboratory animals. The research protocol was reviewed and approved by the Lithuanian Ethics Committee under the State Food and Veterinary Service.

Sensitization was conducted with mixtures of RSF (30 rabbits) or OSF (30 rabbits) emulsified in the proportion 10:1 in complete Freund's adjuvant that contained 2 mg of *Mycobacterium tuberculosis*. Every animal of each group was sensitized at days 0 and 10 by subcutaneous injection of 1 ml emulsified synovial fluid (prepared as above) at ten sites (0.1ml/site) of both sides rabbit's back. Active immunization was started ten days after the last sensitization. Weekly repeated 2 ml i.a.i. (3 times) of RSF (first group) and OSF (second group) were administered into the rabbits' right knee joint. The control left knee joint received 2 ml i.a.i. of physiological saline. Both knee joints were evaluated for swelling, range of motion, and joint stability. 3, 6, and 12 weeks after the last active immunization, 10 rabbits from each group were euthanized with an overdose of Nembutal. Synovial tissue and articular hyaline cartilage from both knee joints were obtained for microscopic investigation.

The material was fixed in ethanol-formaldehyde fixative and embedded in paraffin. Hematoxylin-eosin, toluidine blue pH 4.5, Safranin O, van Gieson, methyl green-pyronine according to Brachet's method, and periodic acid – Schiff 's (PAS) reaction were used to stain the tissue slices. The grading of the pathological changes in the synovium and articular cartilage after the complete immunization cycles with RSF (30 rabbits) and OSF (30) during the development (at 3, 6, and 12 weeks) of the arthritis was performed using an Olympus BX51 (Japan) light microscope.

Results

The volume diameter (swelling) of the right knee joints of all 30 rabbits had significantly increased after the active first intra-articular immunization with RSF and remained stable during all the immunization cycles from 3 weeks on. Gradual decrease in the volume diameter of the knee joint was observed from 6 weeks until the end of the experi-

ment. Joint motions were markedly limited. Joint swelling post i.a.i. of OSF was slighter and joint effusion gradually diminished at 6 weeks. The control left knee joints injected with physiological saline showed no macroscopical changes with at most slightly swollen knee joints being seen.

RSF-induced Arthritis

Synovium

The strongest inflammation in the synovial membrane of the right knee joint was observed at 3 weeks after the last immunization (Fig 1 A), was less pronounced at 6 weeks, and was slight elevated again at 12 weeks (Fig. 1 B) after the development of the EA.

Intense villous proliferation, hyperplasia of the type-A synoviocytes (up to 10 layers), fibrinoid oedema, macro-focal or follicular lymphoid infiltrations in the upper subsynovial area and vasculitis in the early period (third week) in the synovial tissue were seen (Fig.1 A, B). The inflamed tissue was heavily infiltrated with lymphocytes, immature plasma cells, histiocytes, and polymorphonuclears (PMN). Some subsynovium sites were infiltrated by giant macrophages (Fig.1 C), macrophages-siderophages, and eosinophils. The lymphoid follicles consisted of lymphocytes with plasma cells on the periphery and were located near the lining area. Plasmacellular (chronic) vasculitis coincided with the fibrotic vasculopathy or thrombovasculitis of the small vessels (after 6 weeks) with necrotizing vasculitis of the small arteries (6-12 weeks) being observed less frequently. Thus histopathological signs of chronic synovitis began to develop after 6 weeks. Subsynovial oedema, micro-focal and diffuse inflammation of the subsynovial area, venular stasis, fibrous vasculopathy or necrotizing vasculitis of the small arteries, angiomatosis, and fibrolipomatosis were found later. A prevalence of fibrous vasculopathy with an obstruction of the lumen and sclerosis of the vessel walls was observed at 12 weeks.

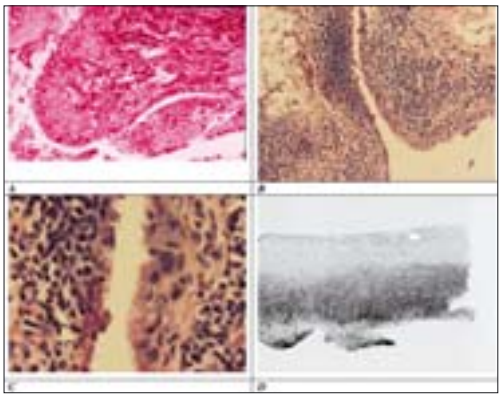

Figure 1. Hyperplasia of villi, subsynovial edema and plasmacellular infiltrations on 3 weeks duration (A, Brachet, X 200), large inflammatory infiltrations, pseudofollicular arrangement of inflammatory cells in subsynovium (B, Hematoxylin eosin, X 200) and focal proliferation of synoviocytes-A with giant macrophages in synovium lining on 6 weeks duration (C, Hematoxylin eosin, X 400) of RSF-induced chronic arthritis. Active pannus on the surface of cartilage (g) with lysis of chondrocytes and disappearance of proteoglycans in the surface and middle zones on 6 weeks duration of RSF-induced arthritis in rabbits (D, Hematoxylin eosin, X 200).

Articular cartilage.

Distinct structural and metabolic changes in the hyaline cartilage of the right knee joint were obtained at the third week after the last immunization (Fig. 1 D).

An inflammatory pannus (→) covered the articular perichondrial area and the surface of the cartilage was marked by surface and deep erosions (*usurae*) (Fig. 1 D). Mild fibrillation of the matrix with prominent diminution and leaching of the proteoglycans from the middle to the deep layer of the cartilage were prominent at 3 weeks, slightly diminished at 6 weeks, with stabilization of the pathological changes at 12 weeks. These effects were related to the activity of inflammatory pannus and coincided with the activation of the inflammatory cells

in the synovium. Some signs of atrophy of the cartilage developed at 12 weeks.

The chondrocytes of all the cartilage areas were markedly damaged especially at the margins of hyaline cartilage, were arranged in a disorderly manner and were usually present in diminished numbers. Chondrocytes showed a degenerative changes with numerous intracytoplasmic lipid droplets in cytoplasm or empty lacunae and were sometimes homogenized or lysed, especially those in the superficial and middle layers of the cartilage (Fig. 1 D). At 6 and 12 weeks, the accumulations of chondrocytes were stained pale with nuclear and cytoplasmic dyes (chondrolysis) with groups of active chondrocytes only being seen occasionally. So the disturbances in the articular cartilage were related not only to the structural but also to the metabolic changes in the chondrocytes.

OSF-induced Arthritis

Synovium

A histological image of acute synovitis with micro-focal inflammatory vascular reactions and increased vascularity, capillary hyperaemia, and thickening and swelling of the vessel wall was seen at 3 weeks. The subsynovium was infiltrated with both lymphocytes and PMN leucocytes. Thus the acute lympho-leukocytic synovitis induced by the OSF was marked by focal proliferation of the synovial lining cells, fibrin deposition, micro-focal and diffuse inflammatory infiltrations, and a slight activation of the subsynovial fibroblasts (Fig. 2 A, B).

In the later stages of the arthritis (after 6 weeks), both surface fibrin deposits and mild adhesion of fibrinous material between the type-A synoviocytes, with focal thickening of up to three lining cells and some villi enlargement, were seen. Subsynovial oedema with slight fibrosis as well as small and very infrequent foci of lymphocytes and plasma cells were found in the subsynovial layer (Fig. 2 B). Leukocyte thrombi of the small arterioles and solitary PMN infiltrations with some focal proliferation of subsynovial fibroblasts with different intensities were observed. Thus the signs of acute inflammation seen at 3 weeks decreased at 6 weeks. Only slight and circumscribed inflammatory cells (histiocytes and very rarely lymphocytes) were found in the subsynovium with a slight proliferation of the type-A synoviocytes in synovial lining.

The late stage in the development of the EA (12 weeks) was marked by a relatively normal synovial lining with a normal thickness and mild fibrosis of the subsynovium area (Fig.2 B). The pathological changes disappeared and the findings in the synovium were similar to those as in the control left knee joint.

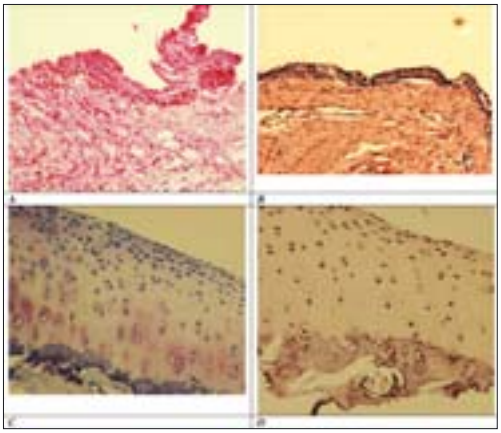

Figure 2. Fibrinous material deposition on the surface of synovium lining and between synoviocytes-A, some focal thickening of lining with villi enlargement, diffuse subsynovial inflammatory infiltrations and slight activation of fibroblasts on 3 weeks duration (A, Brachet, X 200) and subsynovium fibrosis on 6 weeks duration (B, Hematoxylin eosin, X 200) of OSF-induced arthritis in rabbits. Marked destruction of chondrocytes and diminution of proteoglycans in surface and middle zones of hyaline cartilage with zones of proliferation and diminution of chondrocytes on 6 weeks (C, Toluidine blue, X 400), and athrophy of cartilage on 12 weeks duration (D, Hematoxylin eosin, X 400) of OSF - induced arthritis in rabbits.

Articular cartilage.

Some pathological changes in the articular hyaline cartilage of the right knee joint were found at 3 weeks. Slight oedema of the cartilage surface (Fig.2 C) and small foci of fibrous pannus were limited to the perichondrial area. Solitary surface fissures and fibrillation of the matrix in the superficial layers developed together with a decreased chondrocyte density and a thinning throughout the entire hyaline cartilage. The chondrocytes in the superficial layer were stained intensively with nuclear dye. At 6 weeks, mild alterations in the chondrocytes in the superficial layer and foci of proliferated chondrocytes, especially in the middle area, were seen (Fig. 2 C). The cartilage maintained its normal structure with the exception of some fibrillation in the matrix. The proliferated chondrocytes gradually diminished in number and acquired some degenerative changes (reduced staining of nuclear chromatin). Such changes disappeared at 12 weeks and the hyaline cartilage acquired a normal histological appearance (Fig. 2 D).

Discussion

A good deal of data indicates that the antigen or tissue factors which induce the formation of antigen-antibody complexes with complements in a sequestered state persist in patients with RA and are regarded as part of the reason for the development of chronic arthritis. In this respect, inducing experimental arthritis by immunizing rabbits with unfiltered and undiluted synovial fluid from patients with active RA by repeated i.a.i. into the knee joints after sensitization of the rabbits with the RSF in complete Freund's adjuvant is very attractive. Experimental arthritis induced by whole synovial fluid from patients with chronic OA was used as a control.

The histological study of the group with repeated i.a.i. of RSF to the sensitised animals showed the presence of acute erosive arthritis at 3 weeks which had become chronic arthritis at 6-12 weeks. Synovial hyperplasia and hyperaemia, proliferation of the lining cells, infiltration of subsynovial area with large foci of lymphocytes, plasma cells, and macrophages, and vasculitis of the small vessels with a proliferation of subsynovial fibroblasts were predominant early synovial features of the right knee joints. A gradual decrease in the inflammation at 6 weeks and subacute synovitis at 12 weeks showed distinct signs of chronic inflammation associated with arthritis exacerbations and cellular and humoral immune mechanisms. A thickening of the subsynovial area and a proliferation of the fibroblasts, and subsequently synovial pannus overgrowths on both sides of perichondrial regions of the articular cartilage, were evident. Thus subsynovial fibroblasts were activated to give aggressive proliferation and the formation of a pannus with increased production of collagens. The formation of a pannus at the surface with aggressive penetration into the cartilage and subchondral bone shows the development of erosive arthritis which mostly resembled RA according to its morphological signs. Only slight alterations were observed in the synovial tissue of the rabbits receiving the OSF (control group). The synovial membrane showed much less thickening and oedema of subsynovial area with only a slight enlargement of the villi. Leukocyte thrombi were observed only sporadically in the small vessels. Signs of acute inflammation decreased at 3 weeks with lymphocytes and plasma cells seldom being found. No pannus formations on cartilage and bone erosions were observed.

The inflammatory cells were not themselves a cause of the immune mechanisms involved in the destruction of the cartilage in the group with OSF-induced arthritis. Changes in the synovial tissue should be associated with a local response and may be regarded as a secondary reaction to the OSF.

The data suggest that there are considerable differences between the two models of experimental arthritis. Chronicity of the arthritic process is dependent on the development of a cellular memory to repeated antigen stimuli accompanied by a specific humoral immune response *(Hu et al, 2003; Pitzalis et al, 2001)*. Only a transient inflammatory response may be induced in animals receiving an antigen in the absence of the development of a

memory responsiveness to that antigen. The RSF may induce immunologically mediated memory responsiveness to an unknown superantigen that is localized in the synovial fluid of RA patients. Despite intensive efforts, no autoantigen or superantigen that causes arthritis has been found. Although the initiation of the arthritis appears to be T cell dependent, once it is established, a complete depletion of the T cells has no effect on the course of the arthritis. Researchers have proposed a new hypothesis and are shifting the focus from T cells to fibroblasts as a key effector cell in RA. Rheumatoid fibroblasts appear to be mutated at the so-called oncogene p53 site, lose the ability to achieve apoptotic death, and became aggressive in their behaviour towards the cartilage and bone *(Tak et al, 2000; Aupperle et al, 1998).*

The model of EA induced in rabbits by RSF represents an experimental model of chronic arthritis with exacerbations of synovitis in rabbits that can be reproduced easily. It may be useful in both studying the local mechanisms of an immune inflammation of the synovium and the destruction of cartilage and in suppressing erosive arthritis by various immunomanipulations.

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