American Journal of Pathology, Vol. 137, No. 5, November 1990

Rapid Communication

Antibodies to the Mycobacterial 65-kd Heat-shock Protein Are Reactive with Synovial Tissue of Adjuvant Arthritic Rats and Patients with Rheumatoid Arthritis and Osteoarthritis

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The expression of 65-kd mycobacterial heat-shock protein (HSP)-related antigens in synovial membrane from rats and humans with arthritis was studied using three monoclonal antibodies and one polyclonal antiserum directed to antigens of mycobacteria. The antibodies labeled synovial tissue sections from both adjuvant arthritis (AA) rats and from patients with either rheumatoid arthritis (RA) or osteoarthritis (OA); especially the synovial lining cells appeared to be positive. The cytoplasmic staining patterns in rats and humans were essentially the same and were not related to the extent of inflammation ie, the size of lymphoid infiltration. In control tissues no cytoplasmic staining was observed. The results suggest a role for a 65-kd HSP or a cross-reactive molecule in the immunopathologic process of arthritic disease. (Am J Pathol 1990, 137:1013–1017)

Arthritis can be induced experimentally in rats by immunization with heat-killed mycobacteria. This experimental disease, called adjuvant arthritis, has close histopathologic similarities to human rheumatoid arthritis (RA) and serves as a model for RA.[1] T-cell clones from immunized rats act as regulatory effector cells in the disease[2] and are directed against the 65-kd mycobacterial heat-shock protein (HSP).[3] Heat-shock proteins are extremely well-conserved proteins present in virtually all bacterial species and also in mammalian cells. Their presence in both exogenously invading organisms[1] and the endogenous self[2] and their raised expression in stress conditions, eg, inflammation,[4,5] has led to the suggestion that HSPs may be candidate target molecules in autoimmune disease.

A number of observations suggest such a role for the 65-kd HSP in RA. T cells from RA patients, especially obtained from the synovial fluid, proliferate *in vitro* specifically to the mycobacterial antigens that contain 65-kd HSP.[6-9] This phenomenon is more pronounced in early stages of the disease than in more advanced disease.[6] Furthermore sera from RA patients have been shown to contain raised levels of antibodies directed against the 65-kd mycobacterial HSP.[10] Finally cloning and subsequent sequencing of a human homologue of the mycobacterial 65-kd HSP has revealed extensive sequence homology with the bacterial HSP.[11] This finding suggests that the reactions to mycobacterial HSP in humans may be directed to the mammalian homologue. We studied the expression of molecules antigenically related to the mycobacterial 65-Kd HSP in synovial membranes from rats and humans with arthritis. In both species, expression was observed mainly in the synovial lining cells. This observation supports the possible role of HSPs as target substances in autoimmune arthritic disease, both in the experimental animal model and in human patients.

Accepted for publication September 5, 1990.

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Materials and Methods

Rats

In Lewis rats, adjuvant arthritis was evoked by immunization with 1 mg of heat-killed *M. tuberculosis* H37Ra (Difco Laboratories, Detroit, MI) suspended in 0.1 ml mineral oil, intracutaneously at the base of the tail. Synovial specimens were collected at the time of macroscopically visible arthritis. Twelve synovial specimens were sampled from three of these animals.

Patients

Synovial biopsies from seven patients with RA and three patients with osteoarthritis (OA) were studied (synovial tissue bank, Kennedy Institute, London, UK). For each biopsy, a section was reviewed histologically and the extent of proliferation of synovial lining cells and infiltration by lymphocytes, plasma cells, and histiocytes was scored.

Controls

Three synovial tissue biopsies from three adult patients, three renal biopsies, three liver biopsies, two biopsies of reactive lymphnodes, three biopsies of heart muscle, and three biopsies of skin were used as controls.

Antibody reagents

For detection of 65-kd mycobacterial HSP, three monoclonal antibodies and one polyclonal rabbit antiserum were used (Table 1). The antibody 5H12F8 was raised to the synthetic nonapeptide of the 180–188 sequence of *M. bovis* BCG 65-kd[3] in Ribi adjuvant. Antibodies ML-30 and TB-78, provided by J. Ivanyi (W.H.O. monoclonal bank, Tuberculosis and Related Infections Unit, Royal Postgraduate Medical school, Hammersmith Hospital, London, UK), were generated to whole *M. leprae* and whole *M. tuberculosis*. The rabbit anti-65-kd antiserum 322 was generated after immunization with recombinant *M. bovis* BCG 65-kd protein (R. van der Zee and J. D. A. van Embden, National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). In addition a monoclonal anti-HLA-DR antibody was applied (Becton-Dickinson, Mountain View, CA) on sections of human tissue, and a monoclonal antibody was applied to MHC class II (MRC OX3; Mas 028b, Sera Lab, Crawley Dawn, UK) on sections of rat tissue.

Immunohistochemistry

All tissue samples were snap frozen and stored at temperatures less than −20° before analysis. Frozen tissue sections of 6 μm were fixed in acetone. A three-step indirect immunoperoxidase method was applied using rabbit immunoglobulins to mouse immunoglobulins conjugated to horseradish peroxidase in the second step, and swine immunoglobulins to rabbit immunoglobulins conjugated to horseradish peroxidase in the third step (antibodies from Dakopatts, Copenhagen, Denmark). For the rabbit anti-HSP antibody, only a second step with swine anti-rabbit conjugate was applied. Staining was performed using 3-3'diaminobenzidine tetrahydrochloride with H_2O_2 as substrate. The sections were counterstained with hematoxylin.

Results

Conventional Histology

Rats

The synovial biopsies showed signs of mild to moderate synovitis, with some proliferation of synovial lining

Table 1. *Antibodies Against Epitopes of the 65-kd Mycobacterial HSP*

Antibody	Raised in	Ig class	Reactivity	Origin
5H12F8 Monoclonal	Mice	IgM	180–188 peptide of 65-kd mycobacterial HSP (arthritis-related epitope)	Dept. Veterinary Immunology, State Univ. Utrecht The Netherlands
ML-30 (IL-7) Monoclonal	Mice	IgG1	65-kd mycobacterial HSP epitope 280–303 area	W.H.O.-monoclonal bank
TB-78 (IT-13) Monoclonal	Mice	IgG1	65-kd mycobacterial HSP (epitope in 170-234 area, including arthritis-related epitope (3))	W.H.O.-monoclonal bank
322 Polyclonal	Rabbit		65-kd mycobacterial HSP	Dept. Microbiol., Natl. Inst. Public Health and Environmental Protection, Bilthoven, The Netherlands

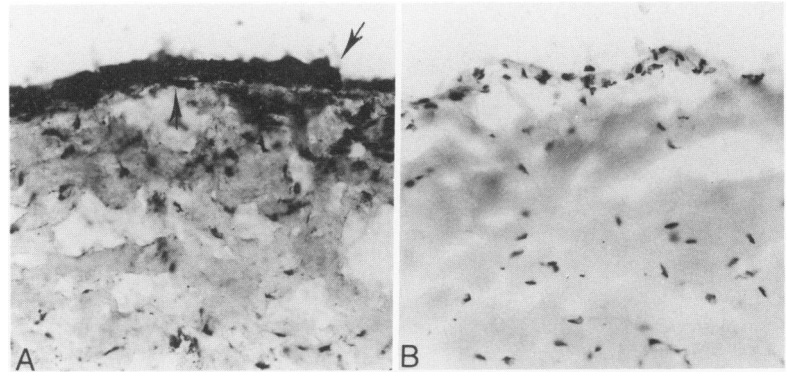

Figure 1. *Synovial frozen tissue section from a rat with adjuvant arthritis, labeled by antibody ML-30* **(A)** *and control with omission of this antibody* **(B)**. *The synovial lining layer in specific labeling is intensely stained (arrows) (×400).*

cells, and infiltration by lymphocytes mainly around blood vessels (data not shown).

Patients

All patients showed mild to moderate synovitis. None of the biopsies showed organization of the infiltration in follicles and interfollicular areas. When present, the infiltrate was localized around blood vessels, and in case of extensive proliferation also diffusely in the connective tissue beneath the synovial lining layer. The numbers of polymorphonuclear neutrophilic granulocytes were negligible when compared to that of lymphoid elements (data not shown).

Immunohistology

Rats

The synovial membranes from the adjuvant arthritis rats showed staining for MHC class II in the synovial lining cells, and in cells scattered throughout the interstitium. All anti–65-kd HSP antibodies predominantly labeled cells of the lining layer and to some extent endothelial cells of blood vessels. Positive cells also were observed scattered throughout the interstitium. The staining pattern was cytoplasmic. The strongest intensity was observed for antibody ML-30 (Figure 1). This reagent also gave low-intensity staining of the connective tissue.

Patients

Staining for HLA-DR served as a positive control. All samples revealed a strong cytoplasmic staining of synovial lining cells, blood vessels, and cells scattered throughout the interstitium. The 65-kd HSP staining was most pronounced in the synovial lining cells (Figures 2 and 3). This pattern of staining was seen in all biopsies from RA patients and in two of the three OA biopsies, using either one of the four reagents. There were gradual differences in staining between the antibodies. The highest prevalence of positivity and strongest staining intensity were observed with antibody ML-30 (Figure 2). Using this antibody, some staining for 65-kd HSP also was seen in endothelium and pericytes of blood vessels. This staining of endothelium was not seen for all antibodies. In addition, antibody ML-30 gave, in some biopsies, diffuse low-intensity staining of the connective tissue, especially when the synovial lining cells showed intense staining. In all analyses a negative control was included. In these the primary antibody was omitted or replaced by an irrelevant antibody of the same

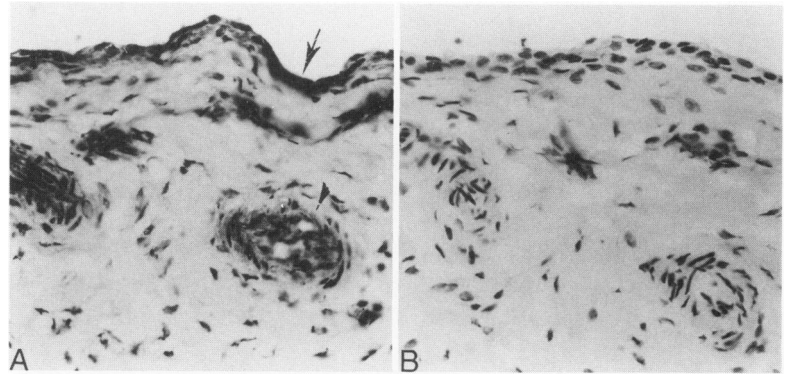

Figure 2. *Synovial frozen tissue section from an RA patient labeled by antibody ML-30* **(A)** *and corresponding part of the biopsy in control incubation with an irrelevant MAb* **(B)**. *In the specific labeling, the lining layer is stained (arrows) as is the endothelium underneath (×400).*

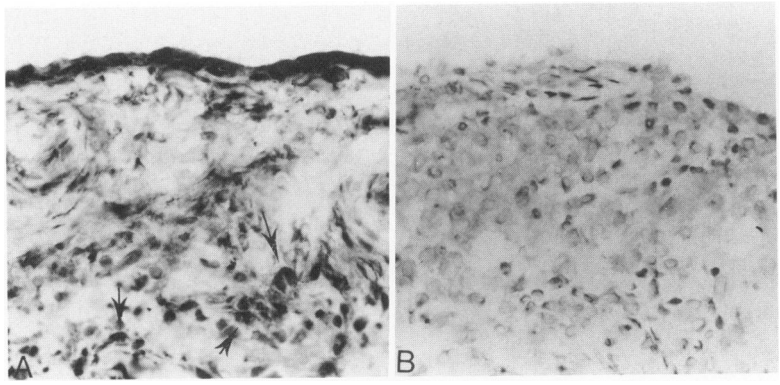

Figure 3. *Synovial frozen tissue section from an RA patient labeled by antibody 5H12F8* **(A)** *and corresponding part of the biopsy in control incubation with an irrelevant MAb* **(B).** *In the specific labeling, the lining layer is stained as are some scattered cells in the connective tissue (×400).*

subclass or normal rabbit serum; the section was otherwise processed in the same way for immunohistology. No staining was seen in these sections. The immunohistochemical data on the presence of 65-kd protein were related to the histologic features. There was no correlation, neither for lining cell proliferation nor for the density and location of lymphoid infiltration.

Controls

In the control tissues no labeling with anti-HSP antibodies was observed. For the antibodies 5H12F8, ML-30, and to some extent for TB-78 a diffuse low intensity staining was seen in tubular epithelium of normal kidney, liver parenchyma, and heart muscle. There was no staining for the polyclonal antiserum 322. In sections of reactive lymph node there was no staining for any of the anti–65-kd HSP antibodies (Table 1). In normal skin we observed for ML-30 a very low intensity of cytoplasmic staining in the epidermis in one of the three biopsies. No staining was seen for any of the other antibodies.

Histologically normal synovial membranes show scattered positive cellular staining for the antibodies 5H12F8, ML-30, and TB-78. The intensity, however, was low to very low (Figure 4).

Discussion

We show that antibodies to the 65-kd mycobacterial HSP label synovial membranes from both rats with an AA and from patients with RA and OA, especially synovial lining cells. In only some sections of control synovial tissues a diffuse low-intensity staining was observed for one or two of the four applied antibodies. The labeling was performed using a sensitive three-step immunoperoxidase technique. The staining patterns observed were remarkably similar for both the experimental animal model and the human disease situation, and for the four antibodies applied. The antibodies differed in intensity of staining; some were negative when another reagent gave intense positive staining. This phenomenon may be due to the sensitivity, dependent on the avidity of the antibodies. Antibody ML-30 manifested the optimal staining properties, both in frequency of positive samples and in staining intensity. This is in accordance with Western blot analysis, where the most intense labeling of the mammalian 65-kd HSP was observed for this antibody (R. Van der Zee, personal communication). This Western blot confirmation of reactivity to the 65-kd mammalian HSP and the identical staining patterns in immunohistochemistry strongly suggests the presence of epitopes of the 65-kd mammalian HSP in rat and human synovium under arthritic conditions. However it cannot be excluded that a different molecule,

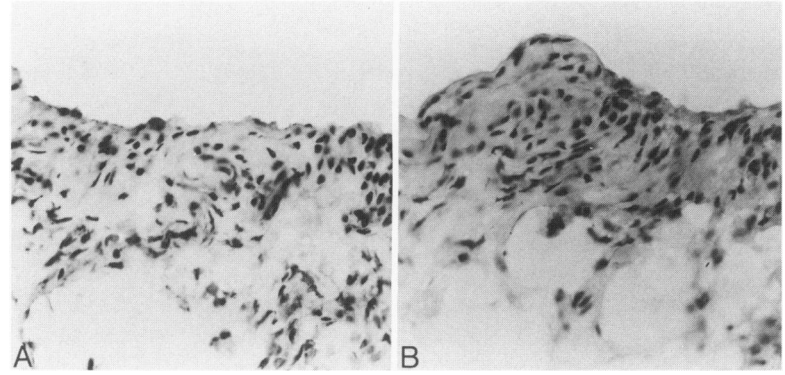

Figure 4. *Synovial tissue from a patient without arthritic disease, labeled by antibody ML-30* **(A)** *and 5H12F8* **(B).** *No labeling is observed (×400).*

immunologically related to HSP is being labeled. The antibody 5H12F8 may exemplify this phenomenon. This reagent was generated to the 180–188 arthritis-related nonapeptide of the 65-kd mycobacterial HSP. As defined by the rat arthritogenic T-cell clone A2B, this epitope shows a structural mimicry with a cartilage-derived proteoglycan epitope.[12] The antibody does not bind the intact 65-kd mycobacterial antigen in enzyme-linked immunoassays; it is possible that the immunohistochemical staining reflects the presence of the proteoglycan-associated epitope in arthritic synovium. Although the tissue location of binding of 5H12F8 makes such a suggestion very unlikely, the phenomenon of cross-reactivity of the antibodies with a non-HSP protein remains possible.

Proliferative responses of synovial T lymphocytes from patients with RA to the 65-kd mycobacterial HSP have been reported.[6-9] The present finding of the expression of the 65-kd HSP, or of cross-reactive molecules in the inflamed synovium, locates the HSP antigen at a site that might enable recognition by T lymphocytes. Further investigations, including inflamed tissue, are required to give information on whether the expression of 65-kd HSP is associated with the initiation of the arthritic process *in situ.* Another possibility is that the 65-kd HSP or related molecules act as target antigen in tissue destruction, or only reflect the presence of inflammation. As an argument against the latter possibility, it can be stressed that the expression of 65-kd HSP does not appear to be related to that of MHC class II molecules, which was almost constant in all biopsies, as the reflection of the inflammatory state. Furthermore the labeling intensity observed was not related to the extent of inflammation as apparent from the size of lymphoid infiltration nor to that of synovial lining cell proliferation.

In conclusion, our data suggest the expression of a 65-kd HSP or an immunologically related molecule in synovial membranes in similar locations for rats with adjuvant arthritis and for patients with RA and OA. Recently Karlsson-Parra et al[13] showed a strong reactivity of the anti-HSP antibody (ML-30) in the cartilage-pannus junction in RA and in rheumatoid nodules, but not in normal joints or in normal or inflamed kidney or liver. However our findings are essentially different because we found strong anti–65-kd reactivity of synovial lining cells both in rats with AA and in patients with RA and OA. The location of anti–65-kd reactivity on a site that is critical to development of arthritis requires further studies on the putative role of the 65-kd HSP in arthritic diseases.

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Acknowledgments

The authors thank PJS van Kooten for generating monoclonal antibody 5H12F8; Dr. E. J. M. Hogervorst for supplying synovial specimens of rats with adjuvant arthritis; Dr. J. Ivanyi, Dr. R. van der Zee and Dr. J. D. A. van Embden for supplying monoclonal antibodies. They also thank the Arthritis and Rheumatism Council for their continuing support of research at the Kennedy Institute of Rheumatology, London, where part of the immunohistologic work was performed.