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Immortalized B-lymphocytes from rheumatoid synovial tissue show specificity for bacterial HSP 60

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Abstract Several studies indicate a pathogenetic role of T-lymphocytes with specificity for heat shock proteins (HSP) in rheumatoid arthritis (RA). Surprisingly, there are no experimental data for B-lymphocytes with specificity for HSP. To investigate whether B-lymphocytes from rheumatoid synovial tissue show a specificity for HSP 60 we immortalized synovial tissue B-lymphocytes by the electrofusion technique and tested the specificity of the B-cell clones for HSP 60 by ELISA. Tissue samples from four patients with classic, active RA were used in this study. The isolated cells were electrofused in strongly hypo-osmolar medium with cells either of the mouse strain X63-Ag8-653 (Ag8) or the heteromyeloma strain HAB-1. Clones positive for IgG, the IgG fraction of the supernatant of the isolated synovial cells and the IgG of the serum of the patients were tested in an ELISA for reactivity to the recombinant HSP 60 of *Yersinia enterocolitica*, which shows great homology with mycobacterial HSP 65 and human HSP 60. The expression of this HSP 60 was studied in normal and rheumatoid synovial tissue using a polyclonal rabbit serum against HSP 60 from *Y. enterocolitica* (Ye HSP 60). In this way we investigate differences in the expression of HSP 60 and compared the pattern of this HSP60 with the pattern of mycobacterial HSP65 and human HSP 60 described by others. In three of four patients 10 IgG secreting B-cell clones showing a specificity for HSP 60 were detected. IgG specific for HSP 60 was also detected in the supernatant of the isolated synovial cells before fusion and in the serum of these patients. HSP 60 was demonstrated immunohistochemically within the rheumatoid synovial tissue and showed stronger expression with a different distribution when compared with the expression in normal synovial tissue. B-cell clones from rheumatoid synovial tissue thus exhibit a specificity for bacterial HSP 60, and a monospecific rabbit serum against this HSP shows strong reactivity within the rheumatoid synovial tissue. It may be postulated that a humoral HSP 60 response, initially directed against an infectious agent, could react with cross-reactive epitopes of rheumatoid synovial tissue or with self-HSP perpetuating the local inflammatory process.

Key words Rheumatoid arthritis · Electrofusion · Synovial B-lymphocytes · HSP 60 · Human monoclonal antibodies

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease involving the synovial tissue in an intense immunological activity. B-lymphocytes, which show a distinct follicular organization within the synovial tissue during active disease, are assumed to be involved in the pathogenesis of RA by producing rheumatoid factors (RF) [2, 3, 15, 17, 19, 22] and a variety of monoreactive and polyreactive autoantibodies showing reactivity to tetanus toxoid, thyroglobulin and DNA [19]. In all these studies the specificity of immortalized synovial B-lymphocytes has been analyzed, but the pathogenetic relevance of these antibodies in inducing or maintaining RA is not understood. In recent years evidence has accumulated that different forms of heat shock proteins (HSPs), expressed within the synovial tissue [4, 23], play a central role in the pathogenesis of RA by exhibiting structural mimicry of self- and non-self compontents [11]. The remarkable conservation of aminoacid sequence between bacterial and human HSPs [8] may explain why immune responses initially directed against the HSPs of an infectious agent would have the potential to initiate or perpetuate autoimmune disease. HSPs therefore provide a link be-

Table 1 Patient data and specificity of IgGs for HSP 60

Patient (series)	Age	Sex	ESR	RF	Local symptoms			HSP 60 specificity of IgG		
					Swelling	Warmth	Effusion	B-cell clones	Supernatant	Sera
R.J. (53)	42	Female	15/35	+	+	+	+	0/3	+	+
K.E. (54)	55	Female	41/67	+	++	++	−	4/19	+	+
R.E. (58)	70	Female	35/60	+	++	++	++	2/25	+	+
K.A. (71)	54	Male	10/32	+	++	++	−	4/20	+	+

tween immunity to bacterial infections to autoimmune disease: the experimental transfer of T-lymphocytes specific for bacterial HSP 65 results in a pernicious inflammation in the joints of the animals tested [25]. Additionally, synovial fluid T-lymphocytes from patients with RA can be activated in vitro by human HSP 60 [5] as well as bacterial HSP 65 [21], and peripheral T-lymphocytes with a specificity for HSP 60 are detected during active disease, but not during clinical remission of RA [5, 6]. These experiments and observations suggest that HSP T-cell reactivity is a relevant mechanism in RA.

Surprisingly, no work has been carried out demonstrating specificity of synovial tissue B-lymphocytes for HSP, although the percentage of synovial B-lymphocytes and plasma cells in active disease is nearly comparable to T-cell levels, and elevated antibody levels to HSP are characteristic in patients with RA [24]. In this study we therefore immortalized B-lymphocytes from rheumatoid synovial tissue and tested the specificity of the immortalized B-cells for HSP 60. A recombinant 60 kD HSP of *Yersinia enterocolitica* was used, which shows 59% amino acid sequence homology with HSP 65 of mycobacterium bovis and 51% homology with human HSP 60 [16].

Evidence of bacterial HSP reactivity of synovial B cells from the affected joint may indicate the pathogenetic relevance of these locally produced antibodies by reacting with rheumatoid synovial tissue HSP or cross-reacting antigens and might demonstrate a link between bacterial infections and the pathogenesis of RA.

In all experiments the electrofusion method was used because it is possible to obtain fusion and hybrid yields about 3–4 orders of magnitude higher attainable with conventional chemical fusion techniques [13, 26, 27] and there is no need for preselection by in vitro stimulation, which normally modifies the B-cell activity pattern [10]. The distribution of this HSP 60 within the synovial tissue was studied immunhistochemically in normal and rheumatoid synovial tissue by using a monospecific polyclonal rabbit serum against the recombinant HSP 60. This identified differences in the expression of HSP 60 in normal and rheumatoid synovial tissue and allowed us to compare the pattern of the recombinant HSP 60 of *Y. enterocolitica* with the pattern of human HSP 60 and mycobacterial HSP 65 described by other authors [4, 23].

Materials and methods

The synovial tissue used in this study was obtained from three women and one man patient with classical seropositive RA (Table 1) defined according to the criteria of the American Rheumatism Association [12]; these patients were undergoing synovectomy at the Orthopädische Universitätsklinik in würzburg. The patients had a mean age of 55 years and a duration of the disease of 2-12 years. All synovial tissue was taken during therapeutic synovectomy (elbow, n=1, knee n=3). All patients were on non-steroidal treatment and three patients were on non-steroidal and steroidal treatment. One patient had moderately active disease and three highly active disease, as assessed by erythrocyte sedimentation rate, rheumatoid factor titer and physical examination of the joints (Table 1). Normal non-inflamed synovial tissue was obtained from two clinically normal joints removed at amputation.

Immediately after synovectomy the tissue was minced and dissociated by treatment with 1 mg/ml of collagenase type 2 (Sigma, St. Louis, Mo., USA) in RPMI 1640 culture medium for 90–180 min at 37° C, followed by three filtrations through metal mesh (pore size 400 µm). The eluted cell suspension was centrifuged 3 times at 500 *g* for 5 min, and finally the cells were resuspended in RPMI 1640-10% fetal calf serum (FCS) culture medium and antibiotics. In order to remove fibroblasts and macrophages by plastic adhesion the cell populations were incubated from 18 h to 2 days at 37° C. The quantities of mononuclear cells used for fusion varied between 6×10^6 and 1.5×10^7.

In order to determine the composition of the isolated synovial tissue cell population immunohistochemical staining was performed. The percentages of CD22 (To15)-positive B lymphocytes, IgG-, IgM-, IgA-positive plasma cells, CD-3-positive T-lymphocytes and CD 68 (KiM6)-positive monocytes were determined in aliquots of 2000 to 5000 cells by the cytospin preparation technique. Aliquots were centrifugated at 200 *g* for 2 min, dried for 2–24 h, treated with acetone and stained with peroxidase-coupled antibodies against CD22 (To15), IgG, IgM, IgA, CD3 and KiM6 (CD68). At least 500 cells were counted in each stained cytospin preparation. The analyzed synovial tissue cell population contained a mixture of other cell types different from leukocytes such as smooth muscle cells, endothelial cells and fibroblasts that been excluded in the evaluation. Therefore, the summation of the percentages of lymphocytes and monocytes shown in Table 3 is smaller than 100%.

As fusion partners we used either the mouse myeloma Ag8 or the heteromyeloma strain HAB-1 [7]. The heteromyeloma strain HAB-1 is very stable when grown under culture conditions and in long-term production of immunoglobulins of the fused hybrids. Both cell lines were grown in complete RPMI 1640 medium containing 10% FCS and harvested as described below.

Electrofusion was used to immortalize the lymphocytes. This technique makes use of the membrane charge of the lymphocytes and the principle of dielectrophoresis to fuse the cells (for a detailed description see [28]). The cell suspensions (lymphocytes, HAB-1 and Ag8) were harvested, washed with 10 ml complete growth medium resuspended in the same volume and mixed in a ratio of 1:1. The cell mixture was washed twice with 75 mosmol fusion medium containing 0.1 mM calcium acetate, 0.5 mM magnesium acetate, 1 mg bovine serum albumine, and appropriate amounts of sorbitol. The total suspension density was adjusted to

Table 2 Electrofusion experiments

Series	Fused cell number	Growing hybrids	Ig-positive clones			Fusion partners	
			IgM	IgA	IgG	Ag8	HAB1
29	1×10^7	2	–	–	–	5×10^6	5×10^6
53	6×10^6	10	–	–	3	3×10^6	3×10^6
54	6×10^6	42	1	2	16	3×10^6	3×10^6
58	1×10^7	54	–	2	23	5×10^6	5×10^6
64	1.5×10^7	3	–	–	–	5×10^6	5.5×10^6
71	6×10^6	80	–	1	19	6×10^6	–

Table 3 Immunophenotyping of isolated synovial tissue cells

Series	CD22 (%)	Plasma cells (%)			CD3 (%)	CD68 (%)
		IgM	IgA	IgG		
29	5	3	7	4	22	46
53	10	2	5	10	28	14
54	11	6	8	3	26	31
58	9	4	7	1	33	38
64	7	3	5	14	25	37
71	12	1	11	2	32	37

2×10^6 cells/ml. Two hundred microliters of this suspension was run into the helical fusion chamber and electrically aligned using the Biojet CF equipment. Thirty seconds after alignment fusion was induced by injection of a field pulse of 30 V amplitude and 15 µs duration, followed by post-alignment with a constant alternating field of the same amplitude and frequency for a further 30 s. Ten minutes later the pulsed cell suspension was removed carefully from the fusion chamber, diluted with complete growth factor and transferred to a 96-well cloning plate. Twenty-four hours later the medium was replaced by HAT selection medium.

After 2–3 weeks the supernatants of the growing clones were screened for production of IgM, IgA and IgG, as well as for lambda- and kappa-chains using a specific enzyme-linked immunosorbent assay (ELISA). The positive primary clones were recloned and tested in the same way again [12, 25, 26].

For the ELISA technique for the reactivity recombinant HSP 60 from *Y. enterocolitica* [16] was coated at a final concentration of 0.146 ng/well (96-well plate) in carbonate buffer (pH 9.2) overnight at 4° C. The staining procedure was performed in PBS (pH 7.4). Serum was diluted 1:100, whereas hybridoma supernatant was applied undiluted. As second antibody, peroxidase conjugated rabbit immunoglobulins to IgG (DAKO) were used with o-phenylendiamin (Sigma) as substrate). The incubation of the primary and secondary antibody was for 2 h at 37° C. The ELISA was developed with 0.03 orthophenyl-diamine and 0.02% hydrogen peroxide in 0.3 M citrate buffer. The absorbance was measured with a wavelength of 492 nm in an ELISA reader. Results were compared with a negative control using an irrelevant related isotype. Since the antibody concentration in the supernatants of the B-cell clone were different, the evaluation is semiquantitative.

Screening for rheumatoid factor reactivity was carried out by the latex agglutination ELISA test. The latex assay was performed by application of 4 µl of human IgG-coated latex particles (Rapi Tex-RF, Behring, Marburg) to 50 µl of unknown supernatants. The agglutination was determined microscopically after 20 min.

To prepare and characterize the rabbit anti-HSP 60 serum β-galactosidase-HSP60 fusion protein (N-terminal 375 amino acids of β-galactosidase) was induced in W3110-pAR11, separated on polyacrylamide gel, cut out, electroluted out of the gel slice and dialyzed against PBS (a paper by A. Roggenkamp on "Cloning and sequencing the HSP60 gene from *Yersinia enterocolitica* serotype 0:3 using a polymerase chain reaction based approach" is in preparation). Two hundred micrograms of the fusion protein was mixed with 2 ml of complete adjuvant and incomplete adjuvant (Sebak Biologische Forschung, Aidenbach, Germany), respectively. Two female New Zealand white rabbits were immunized by intramuscular and subcutaneous injections, beginning with complete adjuvans and continued with the incomplete adjuvans at 4-week intervals for 12 weeks. Specific antibody production was monitored by immunoblotting using *Y. enterocolitica* whole-cell lysates and β-Gal-HSP60 fusionprotein as antigen. The days after last injection rabbits were bled by heart puncture. The serum was identified as anti-YeHSP 60 and stored at –20° C for further analysis. To exclude the possibility that the antibody is also directed against the β-Gal epitopes, Western blots were performed with the fusion protein β-Gal. These Western blots showed no bands (data not shown).

The specificity of the serum was tested by immunoblotting and compared with the affinity purified antiserum (Fig. 1). A negative control was performed using the pre-immune serum, which showed no bands on the blot. A second approach for proving the specificity of the serum was carried out. In order to demonstrate whether HSP60 is more strongly expressed after heat shock *Y. enterocolitica* (serotpye 0:3 strain Y-106) was grown at different temperatures (10°, 27° and 40° C; LB medium), and immunoblots from whole cells lysates were subsequently performed (Fig. 2).

In all immunoblots the size of the protein was determined by a commercial molecular weight standard (Boehringer Mannheim, Premixed Protein Molecular Weight Marker 39.2–200 kD).

Affinity purified Ye HSP60 was prepared by adsorption and elution of antibodies using protein antigens that were immobilized on nitrocellulose blots, as described by [16]. In short, the antiserum was adsorbed to the 60 kDa area of a nitrocellulose sheet to which *Y. eneterocolitica* cell lysates separated by SDS-PAGE had been transferred. Antibodies were eluted from nitrocellulose strips by four successive 45-s washes with glycine-HCl puffer, pH 2.3. This eluate was immediately neutralized by the addition of 0.25 vol of 250 mM sodium phosphate.

For immunocytochemistry all tissue samples were snap-frozen and stored at a temperature of about –80° C before analysis. For immunohistochemical staining air-dried cytospin preparations and serial sections (6 µm) of synovial tissue on poly-L-lysin-coated slides were prepared and stored at –70° C. Immediately before staining, the tissue sections were treated with acetone for 10 min at room temperature. The following immunohistochemical procedures were performed.

The indirect immunoperoxidase technique involved incubation of the slides for 30 min with the primary mAb. The mAb KiM4, CD22 (To15) [20]; IgG, IgM, IgA, CD3, and CD68 (KiM6) were used in dilutions of 1:200, 1:100, 1:200, 1:500, 1:300, 1:500, and 1:400. Incubation was for 30 min with peroxidase-conjugated rabbit anti-mouse immunoglobulins, diluted 1:50 in PBS (pH 7.4) containing 30% AB-Rh-positive human serum. The third step comprised incubation for 30 min with peroxidase-conjugated goat anti-rabbit immunoglobulins, diluted 1:50 in PBS (pH 7.4) containing 30% AB-Rh-positive human serum.

The polyclonal anti HSP 60 *Y. enterocolitica* antibody was diluted 1:200 in PBS (pH 7.4) and was detected with the peroxidase-conjugated goat anti-rabbit antibody, as described above. Prior to immunohistochemistry the slides were treated with rabbit serum to block nonspecific binding sites. Finally, the slides were incubated for 10 min with diaminobenzidine for peroxidase reaction. Negative controls were always obtained on parallel sections by replac-

ing the primary mAb by PBS or with an irrelevant mAb and in the polyclonal serum by preimmunserum. This staining demonstrated no photographically recordable staining reaction (data not shown).

Results

Histopathology of rheumatoid synovial tissue

Paraffin-embedded sections from the synovial tissue of patients with RA were stained with Giemsa and hematoxylin-eosin to determine the stage of inflammation. Four (nos. 53, 54, 58, and 71) of six cases contained synovial villous hypertrophy with an increase in the number and size of synovial lining cells and exhibited an intense infiltration by lymphocytes, plasma cells forming lymphatic follicles. Two (nos. 29, 64) of the cases exhibited chronic granulation tissue and fibrosis with a small non-follicular infiltration of lymphatic cells. Four cases (nos. 53, 54, 58, and 71) were classified as active and two (nos. 29, 64) the chronic, fibrotic stage.

Hybridoma yields

A total of six hybridoma fusions from different patients were performed with isolated synovial tissue cells that had not been stimmulated prior to fusion. One hundred and eighty-six clones were obtained in four (nos. 53, 54, 58, 71) of six fusions using the electrofusion protocol described above. Fifty-four were clones derived from fusion with HAB-1; 132 clones were derived from fusions with Ag8. The IgG ELISA showed that 67 of the primary B-cell clones (45 HAB clones and 22 Ag8 clones) secreted human monoclonal antibodies (humAbs; see Table 2). Three B-cell clones (IgG) were derived from the fusion of series 53 (fused cell number 6×10^6), 19 B-cell clones (1 IgM, 2 IgA, 16 IgG) were derived from the fusion of series 54 (fused cell number 6×10^6), 25 B-cell clones (2 IgA, 23 IgG) from series 58 (fused cell number 1×10^7) and 20 B-cell clones were derived from series 71 (fused cell number 6×10^6). No Ig-producing clones were obtained from the fusions of series 29 and series 64 (Table 2).

B-cell clones and specificity

In three of four patients B-cell clones from synovial tissue B-lymphocytes showed specificity to recombinant HSP60 from *Y. enterocolitica* using the ELISA technique. In patient no. 54, 4 of 19 clones showed specificity for HSP 60; in patient 58 this was true for 2 of 25 clone and in patient 71 for 4 of 20 clones (Fig. 1). All HSP-60-positive B-cell clones belonged to the isotype IgG. In patient 53 none of the 3 B-cell clones was positive for HSP60.

In sera and supernatants of cultured synovial cells from all patients (nos. 53, 54, 58, 71) specificity to recombinant HSP60 from *Y. enterocolitica* was detected by

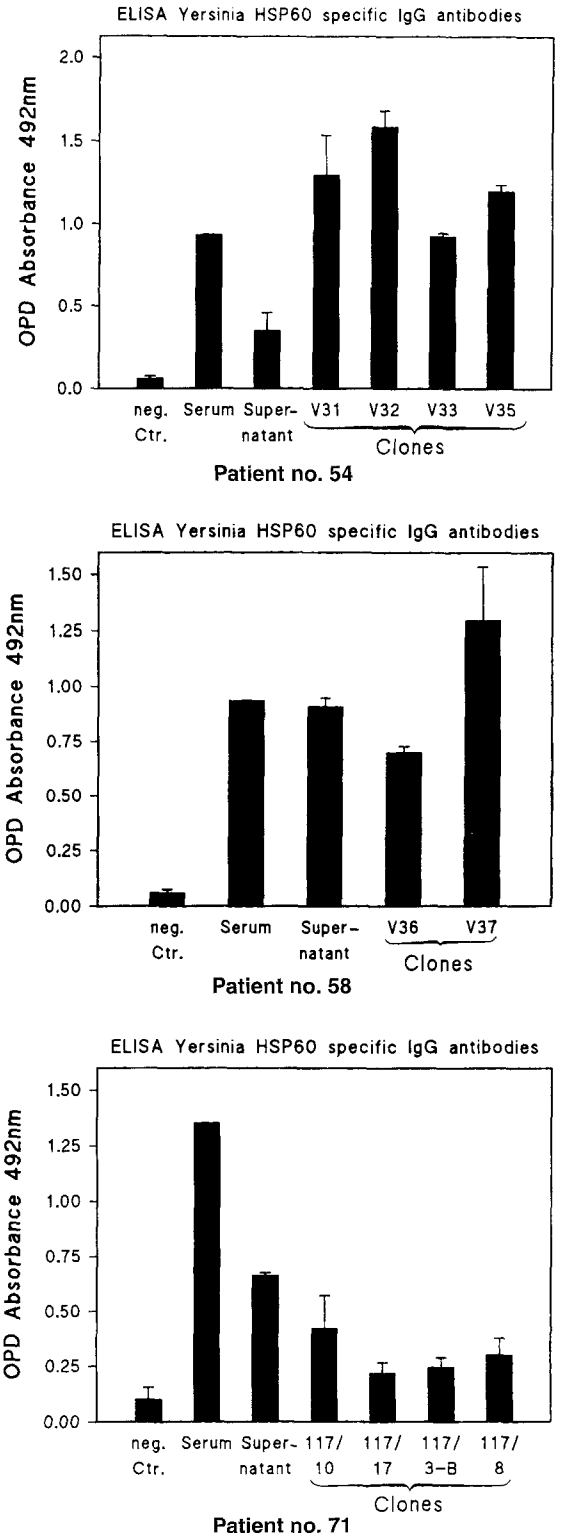

Fig. 1 ELISA of IgG from serum, supernatant of cultured synovial cells and producing B-cell clones (patient nos. 54, 58, and 71) for reactivity to the recombinant HSP 60 from *Yersinia enterocolitica*. The absorbance was measured with a wavelength of 492 nm in an ELISA reader. The results were compared with a negative control using an irrelevant related isotype

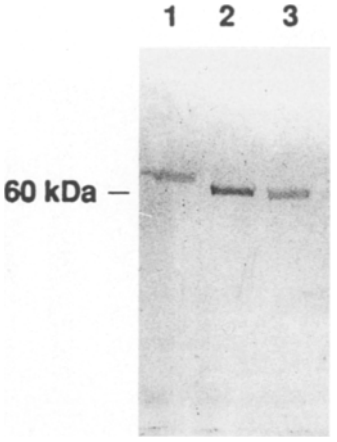

Fig. 2 Specificity of antiserum anti-Ye HSP 60 in immunoblots (*lanes 2 and 3*). Proteins in homogenates of *Y. enterocolitica* whole-cell lysates were probed with affinity-purified anti-Ye HSP 60 (*lane 3*) and original anti-Ye HSP 60 (*lane 2*). In *lane 1* mycobacterial HSP 65 was detected with a monospecific HSP 65 antibody in a protein homogenate of *Myocobacteria bovis*

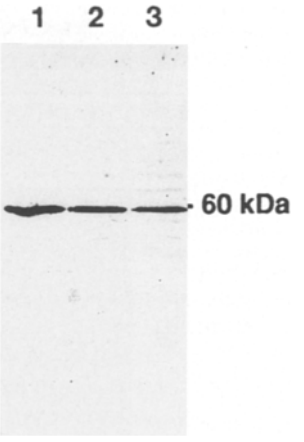

Fig. 3 Specificity of antiserum anti-Ye HSP 60 in immunoblots (*lanes 1–3*): in order to prove that cells after heat shock show a stronger reaction on the Western blot, whole-cell lysates from *Yersinia enterocolitica* grown at different temperatures (*lane 1* 40° C, *lane 2* 27° C, *lane 3* 10° C) were produced. Subsequently performed Western blots revealed a stronger reaction after heat shock (*lane 1*). The higher the growth temperature, the more HSP 60 was detectable

the ELISA technique (Fig. 1). All HSP-60-positive antibodies were from the isotype IgG.

Rheumatoid factors producing B-cell clones

Two B-cell clones (1 IgM of series 54, 1 IgG of series 58) exhibited rheumatoid factor (RF) activity when tested by the latex agglutination ELISA test. All B-cell clones that showed specificity for HSP60 exhibited no RF activity.

Specificity of rabbit anti-HSP 60 serum

The specificity of the anti-HSP 60 serum was demonstrated by immunoblotting in comparison with the affinity-purified antiserum (Fig. 2) and in an immunoblot of *Y. enterocolitica* whole-cell lysates grown at different temperatures (heat shock experiment). This immunoblot demonstrated a higher expression of HSP60 after heat shock; the higher the growth temperature, the more HSP60 was detectable (Fig. 3).

Immunocytochemical findings

Immunoperoxidase staining of CD22 (To15) showed a follicle-like distribution of CD22-positive B-cells (Fig. 4D) in four (nos. 53, 54, 58 and 71) out of six RA cases. Some of the positive cells were located in the vicinity of vessels, and others were diffusely distributed within the synovial tissue, showing no distinct pattern.

Immunoperoxidase staining of KIM$_4$, which is specific for follicular dendritic reticulum cells (FDC) [18], showed two forms of distribution FDC in the rheumatoid synovial tissue in four (nos. 53, 54, 58 and 71) of six cases: a follicular network-like distribution within the follicles and a dispersion of single positive cells which were mainly located in the interfollicular area and within the synovial linig cells (Fig. 4C).

Localisation of HSP60: We have focused on evaluating 3 different compartments in the rheumatoid synovial tissue: The synovial lining cells, the inflammatory infiltrate and the blood vessels. The rheumatoid synovial tissue exhibited an intense cytoplasmic staining of the synovial lining cells in all cases. Intense staining was found within the lymphatic follicles: HSP 60 was found to be expressed in the cytoplasma of lymphatic cells as well as in scattered cells forming a reticular staining pattern. Endothelial cells of blood vessels exhibited a fainter staining. Intense staining was observed in large mononuclear cells distributed diffusely in the synovial tissue probably representing macrophages (Fig. 4A). No staining could be observed within the walls of large vessels. The expression of HSP60 within normal synovial tissue was restricted to the synovial lining cells and endothelial cells and was nearly absent in the subsynovial area (Fig. 4B).

The evaluation of the immunohistochemistric staining of the isolated synovial cells from series 29, 53, 54, 58, 64 and 71 for CD22 (To15), IgG, IgA, IgM, CD3 and CD68 (KiM6) are shown in Table 3.

Discussion

The specificity of human lymphocytes for HSP 60 and 65 has been demonstrated for cells originating from synovial fluid [14, 15] and blood [21] in patients suffering

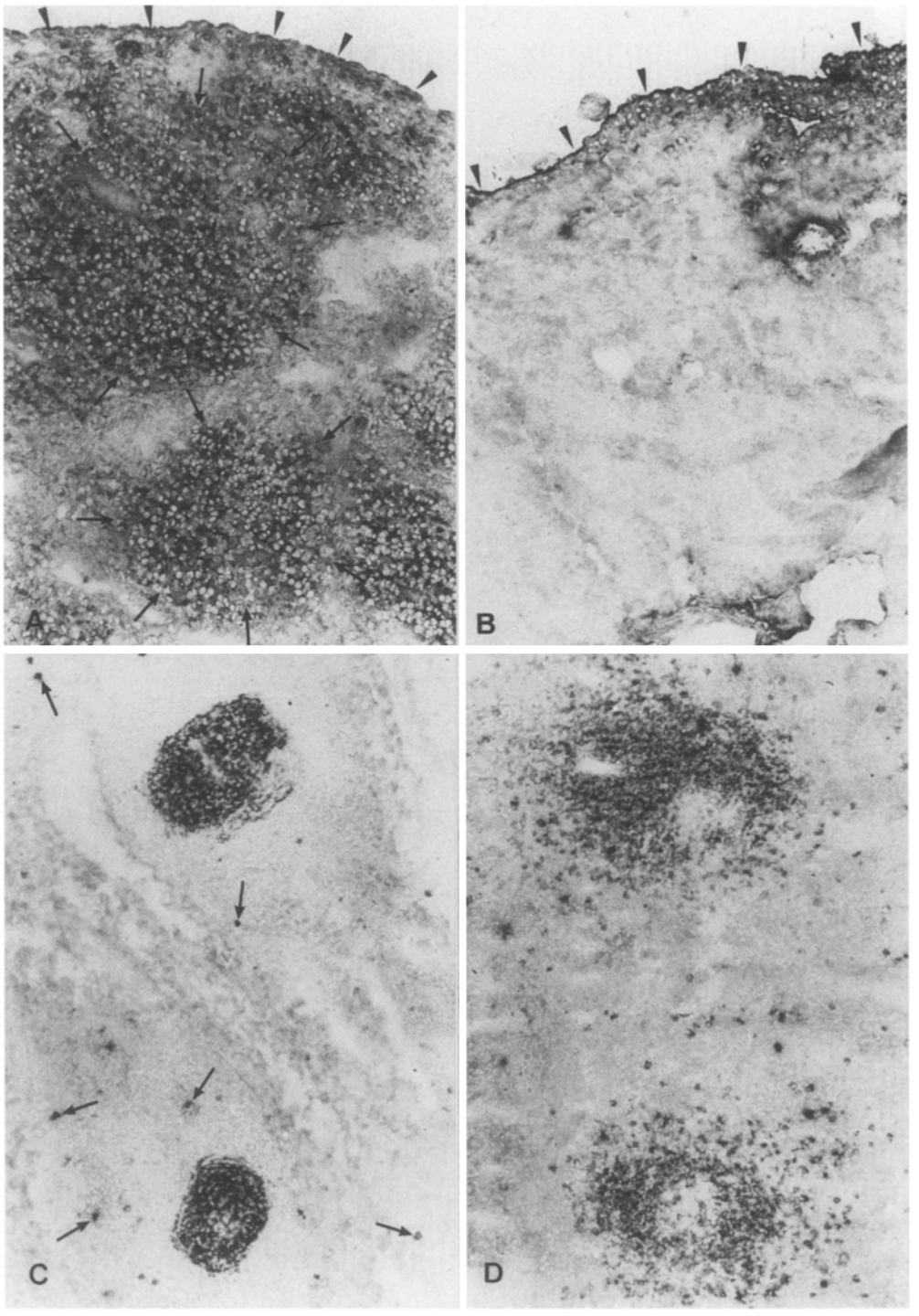

Fig. 4A–D Immunohistochemical analysis of synovial tissue. Immunohistochemical staining with polyclonal rabbit serum against HSP 60 of *Yersinia enterocolitica* in rheumatoid synovial tissue (**A**) of patient 58 and in normal synovial tissue (**B**). Arrowheads point to the synovial lining cells; *arrows* point to endothelial cells (cryostatscections, ×160, ×230). **C** Immunohistochemical staining of follicular dendritic reticulum cells (FDCs) with the Mab KiM4; *arrows* point to single and extrafollicular located FDCs (×90). **D** Immunohistochemical staining of B-lymphocytes with Mab CD22 in rheumatoid synovial tissue (×90)

from RA. Our experiments demonstrate that lymphocytes originating from rheumatoid synovial tissue show specificity for HSP 60. In three of four cases B-cell clones with specificity for HSP60 were identified. The fact that in one case no HSP 60 specific B-cell clone could be found may be explained by the very small number (three) of B clones produced. Immortalization of B-lymphocytes with specificity for HSP60 from synovial tissue, which is the most important pathogenetic compartment in RA, stresses the significance of these humAbs in the pathogenesis of the local inflammatory response.

All of the Mabs showing specificity for HSP 60 are IgG and are therefore different from "natural antibodies", which mostly belong to the IgM classes. Since these Mabs showed no RF activity, it appears that these Mabs are different from the polyreactive rheumatoid factors that have been described by other authors [19]. The fact that the production of IgG is a T-cell-dependent process [14] suggests that the production of humAbs with

specificity for HSP 60 is part of the specific immune response in RA. The synovial tissue used for immortalization showed B-cell follicles with follicular dendritic cells in the germinal centers (Fig. 4C, D) where antigen triggered B-cell maturation is expected to take place [1].

Taking these data together, and keeping in mind the fact that the isolated B-lymphocytes had not been stimulated with EBV [3] or mitogens [19] before fusion and that high levels of HSP 60 Mabs could be detected in the supernatant of the cultivated synovial cells before fusion and in the serum of the patients, we conclude that this specificity for HSP 60 is part of the specific and immediate immune response of synovial B-lymphocytes in RA. Our work shows the benefits of electrofusion immortalizing small amounts of B-lymphocytes without prior in vitro expansion or stimulation. In four out of six fusions 67 Ig secreting B-cell clones were produced. The two fusions (29, 64) that produced no secreting B-cell clones stemmed from clinically inactive RA, and the synovial tissue showed a more chronic fibrotic stage, with a non-follicular distribution of lymphocytes and plasma cells. Synovial tissue from active RA provides the best source for the generation of B-cell clones.

The specificity of the anti-HSP 60 antiserum used for immunohistochemistry was demonstrated by immunoblot in comparison with affinity-purified antiserum (Fig. 2) and by the demonstration of a temperature-dependent expression of HSP 60 in *Y. entercolitica* grown at different temperatures (Fig. 3). Immunohistochemically, the HSP 60 or cross-reactive molecules in the rheumatoid synovial tissue exhibited a characteristic pattern (Fig. 4A, B), which shows remarkable similarities with the distribution of human HSP 60 [23] and mycobacterial HSP 65 [4]. This may be explained by the extensive homologies between mammalian and microbial HSP [8]. Compared with normal, non-inflammed synovial tissue HSP60 was expressed in rheumatoid synovial tissue at a very high level and exhibited a different pattern of expression (Fig. 4A, B). As the rheumatoid synovial tissues showed high percentages of inflammatory cells (Table 3), it appears that the level of HSP60 expression is influenced by the quantity of inflammatory cells that had immigrated into the the synovial tissue. These findings are in accordance with the findings of authors who have demonstrated a different expression for mycobacterial HSP65 in normal and rheumatoid synovial tissue [4]. However, other authors [23] detected only minor differences in the expression of human HSP60 in normal and rheumatoid synovial tissue. This discrepancy may be explained by the fact that in most of our experiments synovial tissue with dense, follicular infiltrates of inflammatory cells of patients with active RA was used.

We postulate that HSP60 or cross-reactive, immunologically related molecules in the rheumatoid synovial tissue may act as a target substance for the locally produced HSP60 Mabs driving a local autoreactive inflammatory process. This postulated mechanism is supposed to be a reaction to an enigmatic synovial tissue disorganizing and stressing event since HSP is only expressed in response to extreme conditions (shortage of nutrients, increase in temperature, bacterial infections and other forms of cellular stress). Consequently, the reaction of the HSP 60 Mabs with endogenous tissue HSP 60 is not a triggering event, but has a perpetuating and amplifying function in the pernicious inflammatory process within the rheumatoid synovial tissue.

The demonstration of B-cell clones with specificity for bacterial HSP suggests a pathogentic role of exogenic antigens in the immunopathogenesis of RA: a humoral HSP 60 immune response initially directed against an infectious bacterial agent would have the potential to perpetuate RA by reacting with self-HSP or cross-reactive epitopes of the rheumatoid synovial tissue. This agrees with clinical experience where bacterial infection often precedes the onset of episodic flares of rheumatic disease. It is not clear whether the specificity of the HSP 60 humAbs is restricted to the recombinant HSP 60 of *Y. enterocolitica*. The majority of the humAbs with specificity for the recombinant HSP 60 showed further specificity for other bacterial HSP 60 when tested in an ELISA (Hsp 60 from *Mycobacteria bovis* and from *Escherichia coli*; 3 Mabs from series 54 and 4 Mabs from series 58; data not shown). It therefore appears that these synovial B-cell clone respond to shared epitopes of *Y. enterocolitica, E. coli* and mycobacterial HSP 65. Similar behavior was demonstrated for *Y. enterocolitica* HSP-60 specific T-cell clones from reactive arthritis, which showed cross-recognition of human and recombinant HSP65 [9].

We are currently analyzing whether a synthetic peptide that contains shared epitopes of HSP60 from *Y. enterocolitica, M. bovis* and from *E. coli* shows reactivity with the HSP 60 humAbs. Such a protein could be used for experimental inhibition or induction of arthritis in animals and could therefore be helpful in understanding the molecular mechanism of RA.

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