Brief Definitive Report

Two Monoclonal Antibodies Generated against Human hsp60 Show Reactivity with Synovial Membranes of Patients with Juvenile Chronic Arthritis

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Summary

Heat-shock proteins have been shown to be critical antigens in a number of autoimmune diseases. In human arthritis and in experimentally induced arthritis in animals, disease development was seen to coincide with development of immune reactivity directed against not only bacterial hsp60, but also against its mammalian homologue. We have developed murine monoclonal antibodies after immunization with recombinant human hsp60. Antibodies with unique specificity for mammalian hsp60, not crossreactive with the bacterial counterpart (LK1), and antibodies recognizing both human and bacterial hsp60 (LK2) were selected. Both antibodies recognize epitopes located between amino acid positions 383 and 447 of human hsp60. In immunogold electron microscopy, the mitochondrial localization of hsp60 in HepG2 cells was shown. Furthermore, both LK1 and LK2 showed a raised level of staining in light microscopy immunohistochemistry of synovial membranes in patients with juvenile chronic arthritis. The increased staining for LK1, with a unique specificity for mammalian hsp60, thus unequivocally demonstrates that this is due to a raised level of expression of endogenously produced host hsp60 and not to deposition of bacterial antigens.

Heat-shock proteins (hsp) are induced in prokaryotic and eukaryotic species under various conditions of stress (1, 2). The hsp's are grouped into families of homologous proteins based on their molecular masses. The 60-kD hsp family, which retained a uniquely high level of sequence conservation during evolution (3), is a focus of interest as a potential antigen in autoimmune diseases. The experimental model of adjuvant arthritis (AA) has provided the initial evidence for an important role of hsp60 in the pathogenesis of autoimmune disorders (4–6). In this model, arthritis is induced by immunization with heat-killed *Mycobacterium tuberculosis* in oil. The mycobacterial hsp60 turned out to be the antigen recognized by T cell clones that could transfer disease into naive animals (4, 5). There is now experimental evidence that responses to hsp60 are subject to regulatory T cell control (6). This may explain the observation that preimmunization with mycobacterial hsp60 induces protection against subsequent development of AA (4, 7), as well as arthritis induced by streptococcal cell walls (8), type II collagen (9), or pristane (10).

These data from experimental animal models have stimulated the search for T cell reactivity against the mycobacterial hsp60 in the synovial fluid (SF) of patients with active arthritis (11–13). It has been suggested that T cell reactivity is directed, at least partly, against the mammalian endogenous hsp.

A human mitochondrial protein, originally designated P1, has been described as the human homologue of the mycobacterial hsp60 (14, 15), and >45% of the protein has sequence identity with its bacterial homologue. Recently, we have succeeded in producing substantial amounts of the human 60-kD homologue by expression of the gene in *Escherichia coli* (16).

Compelling evidence for T cell recognition of "self-endogenous hsp" comes from a study from Hermann et al. (17). They showed that from the joint of a patient with reactive arthritis after infection with *Yersinia enterocolitica*, a *Yersinia-*

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specific T cell line and clone could be isolated that was cross-reactive with recombinant human hsp60 as well as with heat-stressed autologous cells. Also, T cells obtained from SF of children with juvenile chronic arthritis (JCA) responded to the human hsp60 (16).

Previous studies, using anti-mycobacterial hsp60 antibodies that are crossreactive with human hsp60, have shown increased expression of hsp60 in inflamed tissue (16–20). However, because of this crossreactivity, it was impossible to distinguish between the expression of hsp60 from bacterial origin (e.g., after a bacterial infection) or endogenous self-hsp60. To resolve this issue we have set out to generate mAbs with unique specificity for human hsp60. In the present study we describe the characterization of two murine mAbs called LK1 and LK2 derived from mice immunized with recombinant human hsp60. LK1 is only specific for mammalian hsp60, while LK2 shows crossreactivity with bacterial hsp60. Synovial biopsies from patients with JCA stained with LK1 and LK2 revealed an increased hsp60 expression in these patients as compared with controls. Because of the unique specificity of LK1 for mammalian hsp60, these findings have demonstrated a raised synthesis of self-hsp60 in lining cells of the inflamed synovium.

Materials and Methods

Antigens. The human hsp60 gene was obtained from Dr. R. A. Young (Whitehead Institute, Cambridge, MA) as a phage λ-gt11 clone from a cDNA library prepared from Hela cells by Dr. R. S. Gupta (Health Science Centre, Hamilton, Ontario, Canada) (14). Through intermediate pEX2-derived plasmids, an SphI restriction site was introduced at the position of the start codon, resulting in plasmid pRH710 expressing the complete human hsp60 as a β-galactosidase fusion protein. The gene was then subcloned as an SphI/HindIII fragment into vector pRH901. The HindIII site downstream of the gene originated from pEX2. pRH901 was constructed from expression vector pNGS20(+) by SalI digestion followed by filling in with T4 polymerase to adjust the reading frame close before the SphI site in the multiple cloning region. Subcloning of the human hsp60 gene into pRH901 thus resulted in plasmid pRH1011, from which hsp60, preceded by a short leader sequence of 31 amino acid residues derived from the cloning vector, could be expressed with isopropyl-b-D-thiogalactopyranoside (IPTG) induction (17).

Deletion mutants of human hsp60 were constructed from plasmid pRH710. Followed by religation, fragments of the hsp60 gene were deleted by cleavage with NsiI/PstI (resulting in plasmid pRH2002 expressing residues 1–447), with EcoRI (pRH2012: 238–573; and pRH2015: 1–239), with StyI (pRH2026: 1–125/450–573; and pRH2028:1–125), and with SpeI (pRH2030: 1–383). Plasmid pLuc1 (residues 356–531) was obtained by Taq-polymerase-facilitated amplification of the corresponding DNA sequence using primers containing restriction sites to allow cloning of the fragment into expression vector pEX2. All constructs were checked by sequencing. The β-galactosidase fusion proteins were obtained from recombinant *E. coli* strains as described (4).

The recombinant hsp65 protein of *Mycobacterium bovis* BCG was prepared after expression from plasmid pRIB1300 and purified as previously described (4, 21). Deletion mutants of the mycobacterial hsp65 were prepared as described (4).

Cell extracts of different bacterial strains were obtained as previously described (22). The strains used are *Borellia*, *E. coli*, *Streptococcus pyogenes*, *Yersinia entercolitica*, *Salmonella typhimurium*, *Treponema hyodysenteriae*, and *Treponema innocense*. A crude extract of *Trichinella spiralis* was obtained as previously described (23).

mAb Production. Mice (BALB/c females; Iffa Credo-Broekman B. V., Brussels, Belgium) were immunized with 100 μl of 1 mg/ml recombinant human hsp60 in PBS mixed with 100 μl monophosphoryl lipid A+trehalose dimycolate emulsion (Ribi; ImmunoChem Research, Inc.) intraperitoneally. 2 mo after immunization, the mice were boosted intravenously with 50 μg human hsp60 in PBS. After 4 d, spleen cells were fused with mouse myeloma cells (Sp2/0) using polyethylene glycol 4000. Anti-hsp60 and anti-mycobacterial hsp60 antibody producing hybridomas were selected by ELISA (24) and Western blotting, and subcloned in limiting dilution.

SDS/PAGE and Immunoblotting. Cell extracts of either vertebrate or bacterial cells were made in Laemmli buffer containing β-mercaptoethanol, and equivalent amounts of protein (0.5 μg) from various extracts were subjected to electrophoresis in 10% SDS gels, as described earlier (22, 25). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets was performed as described (25). The blots were blocked with 0.5% Tween 20 in saline (10 mM Tris hydrochloride, pH 7.4, 155 mM NaCl) and subsequently treated with second antibodies and their corresponding horseradish peroxidase second antibodies. Binding of the conjugate was visualized by using dioctyl sodium sulfasuccinate/tetramethylbenzidine (DONS/TMB) in 0.01 M citrate phosphate buffer, pH 5, and hydrogen peroxide 0.02%.

Immunoelectron Microscopy. Cells from the human hepatoma cell line HEPG2 were fixed in a mixture of 2% paraformaldehyde and 1% acrolein in 0.1 M phosphate buffer, pH 7.4, for 24 h at 37°C. Ultrathin cryosections were immunogold labeled for the demonstration of hsp60 as previously described by Slot et al. (26). Briefly, after fixation, cells were washed with PBS, containing 0.15 M glycine. After embedding in 10% gelatin, the gelatin slab was kept at 4°C. Small pieces of gelatin were infiltrated with 2.3 M sucrose and mounted at 4°C. Ultrathin cryosections were immunogold labeled with mouse mAb LK1 or LK2, and control mAb (R73: mouse anti-rat TCR) and 10-nm gold particles, complexed to protein A. Rabbit anti-mouse Ig (HPLC purified, kindly provided by Dr. J. Borst, Netherlands Cancer Institute, Amsterdam) was used as an intermediate step between the mAb and the gold probe. Sections were examined with an electron microscope at 80 kV (1200 EX; Jeol).

Histology and Immunohistochemistry. For immunohistochemistry, a synovial biopsy of a patient with JCA and a non-JCA control were used. The patient had an oligoarticular onset of JCA, as defined by the European League Against Rheumatism. The duration of the disease was 2.0 yr and the duration of the current arthritis was 0.4 yr. As a control, a patient with persistent arthralgia was used. In the case of the JCA patient, the synovial membrane biopsy sample was taken in a phase of active arthritis during arthroscopy of the knee. The synovial biopsy sample of the control was taken during arthroscopy as well, for diagnostic reasons. For histology, part of the samples was fixed in buffered formalin, and embedded in paraffin. Sections of 8 μm were stained with hematoxylin and eosin. The sections were reviewed histologically for the degree of synovitis: mild synovitis in case of the JCA patient and absent synovitis in case of the control. For immunohistochemistry another part was snap-frozen and stored at −70°C until analysis. For detection of human hsp60 or related molecules, LK1 (1:250) and LK2 (1:1,000) were used. In addition, an anti-HLA-DR mAb was applied as a positive control (Becton Dickinson & Co., Mountain View, CA). Staining with a nonrelevant monoclonal of the same isotype (R73)

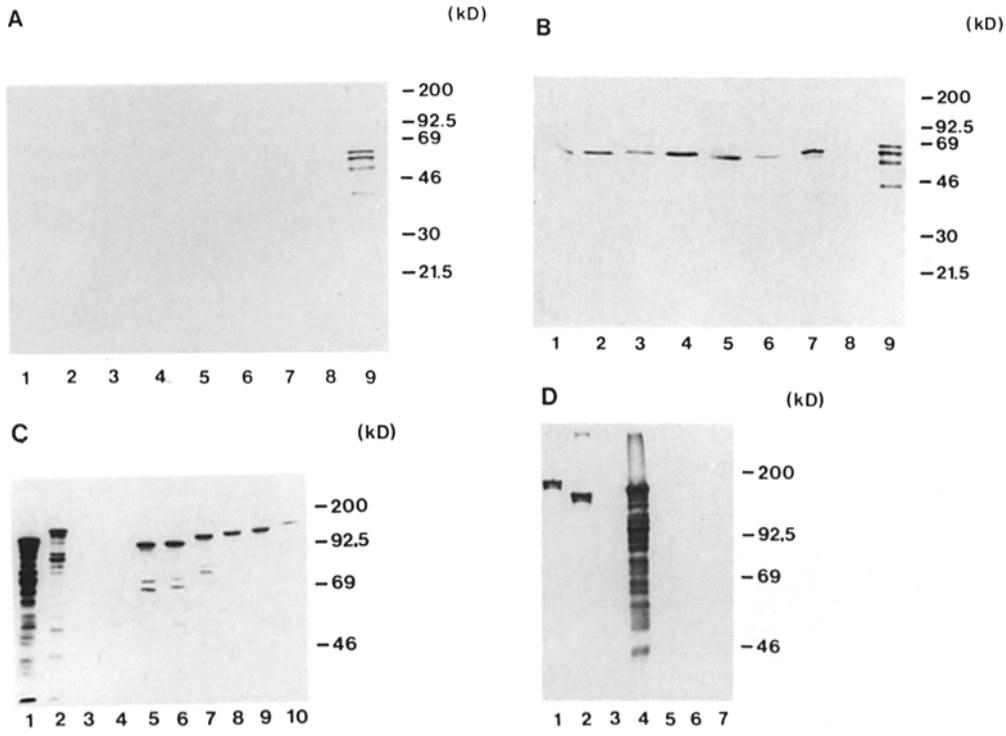

Figure 1. Western blot analysis of LK1 and LK2 mAbs. Cell extracts from various bacterial species were subjected to electrophoresis in SDS-polyacrylamide gels and blotted onto nitrocellulose, and crossreactivity to LK1 and LK2 mAbs was examined. (*A*) mAb LK1: cell extract or antigen. Lane *1*, *Trichinella spiralis* crude extract (500 ng); lane *2*, *Treponema innocence* (500 ng); lane *3*, *Treponema hyodysenteria* (500 ng); lane *4*, *Salmonella typhimurium* (500 ng); lane *5*, *Yersinia enterolitica* (500 ng); lane *6*, *E. coli* (500 ng); lane *7*, recombinant mycobacterial hsp60 (100 ng); lane *8*, control pRH901 (100 ng); and lane *9*; recombinant human hsp60 (100 ng). (*B*) mAb LK2: cell extracts or antigens, as described in *A*, lanes *1–9*. (*C*) mAb LK2: human hsp60 deletion mutants (lanes *1* and *2*) and mycobacterial hsp65 deletion mutants (lanes *3–10*). Lane *1*, pRH2012 (amino acid [aa] residue 238–573, human hsp60); lane *2*, pRH710 (aa residue 1–573, human hsp60); lane *3*, pEX-2 control; lane *4*, pRIB 1463 (aa residue 404–540, mycobacterial hsp60); lane *5*, pRIB 1444 (aa residue 303–540, mycobacterial hsp60); lane *6*, pRIB1451 (aa residue 276–540, mycobacterial hsp60); lane *7*, pRIB 1426 (aa residue 171–540, mycobacterial hsp60; lane *8*, pRIB 1422 (aa residue 109–540, mycobacterial hsp60); lane *9*, pRIB 1424 (aa residue 84–540); lane *10*, pRIB 1404 (aa residue −2–540). (*D*) mAb LK1: human hsp60 deletion mutants. Lane *1*, pRH710 (aa residue 1–573); lane *2*, pRH2012 (aa residue 238–573); lane *3*, pRH2026 (aa residues 1–124 and 450–573); lane *4*, pRH2002 (aa residue 1–447); lane *5*, pRH2015 (aa residue 0–239); lane *6*, pRH2028 (aa residue 1–125); and lane 7, pEX-2 control.

and staining without applying a first antibody served as negative control. Frozen tissue sections of 9 μm were fixed in acetone. A three-step indirect immunoperoxidase method was applied using a first incubation with mAb, followed by rabbit Igs to mouse Igs conjugated to horseradish peroxidase in the second step, and swine Igs to rabbit Igs conjugated to horseradish peroxidase in the third step (antibodies from Dakopatts, Glostrup, Denmark). Staining was performed using 3-3′ diaminobenzidine tetrahydrochloride with hydrogen peroxidase as substrate. The sections were counterstained with hematoxylin.

Results and Discussion

Isolation and Specificity of mAbs LK1 and LK2. Several hybridoma cell culture supernatants were screened for binding to the recombinant human hsp60 as well as to the mycobacterial hsp60. We selected and subcloned hybridomas by limiting dilution and have characterized in depth two mAbs, LK1 and LK2. These mAbs were examined on Western blots. Part of the results of these studies are presented in Fig. 1. As can be seen, the LK1 antibody only reacts with recombinant human hsp60 (Fig. 1 *A*, lane *9*), while the LK2 antibody also showed reactivity with recombinant mycobacterial hsp60 as well as corresponding proteins (e.g., groEL protein of *E. coli*; Fig. 1 *B*, lane *6*) present in cell extracts of other bacteria and helminths (Fig. 1 *B*, lanes *1–9*). Because of more downstream internal initiation points, human hsp60 translation products of different sizes were obtained (Fig. 1, *a* and *b*, lanes *9*). In immunoblots with whole mammalian cell extracts, LK1 and LK2 reacted with a single 60-kD band only (data not shown). Thus, no crossreactivity with other human proteins was found. LK2 also showed a strong reaction when tested on extracts of yeast and spinach chloroplasts (data not shown). Subsequently, we tested the mAbs on the pEX deletion mutants from mycobacterial hsp60 or human hsp60 in an immunoblot. As can be seen in Fig. 1 *C*, LK2 recognizes mycobacterial hsp60 protein fragments that lacked the amino acids up to residue 303 (see pRIB 1444; Fig. 1 *C*, lane *5*), but deletions extending to amino acid 404 (pRIB 1463; Fig. 1 *C*, lane *4*) showed no reactivity. A fragment with a deletion extended to amino acid 390 (pRIB 1456) was found to be negative (data not shown). Also, deletion mutants from human hsp60 were tested, and in Fig. 1 *D*, the results are shown for LK1 reactivity. The reaction pattern obtained with LK2 was completely identical to that of LK1, indicating that the epitope(s) recognized by LK1 and LK2 are formed by amino acid residues within the sequence 238–447 of the human hsp60. Recently, we made two more deletion mutants of the human hsp60. Only Luc1 (amino acid residues 356–531) was recognized by both LK1 and LK2, whereas pRH2030 (amino acid residues 1–383) was not (data not shown). Taken together, these data indicate that the epitope recognized by LK1 is located between amino acid positions 383 and 447 of the

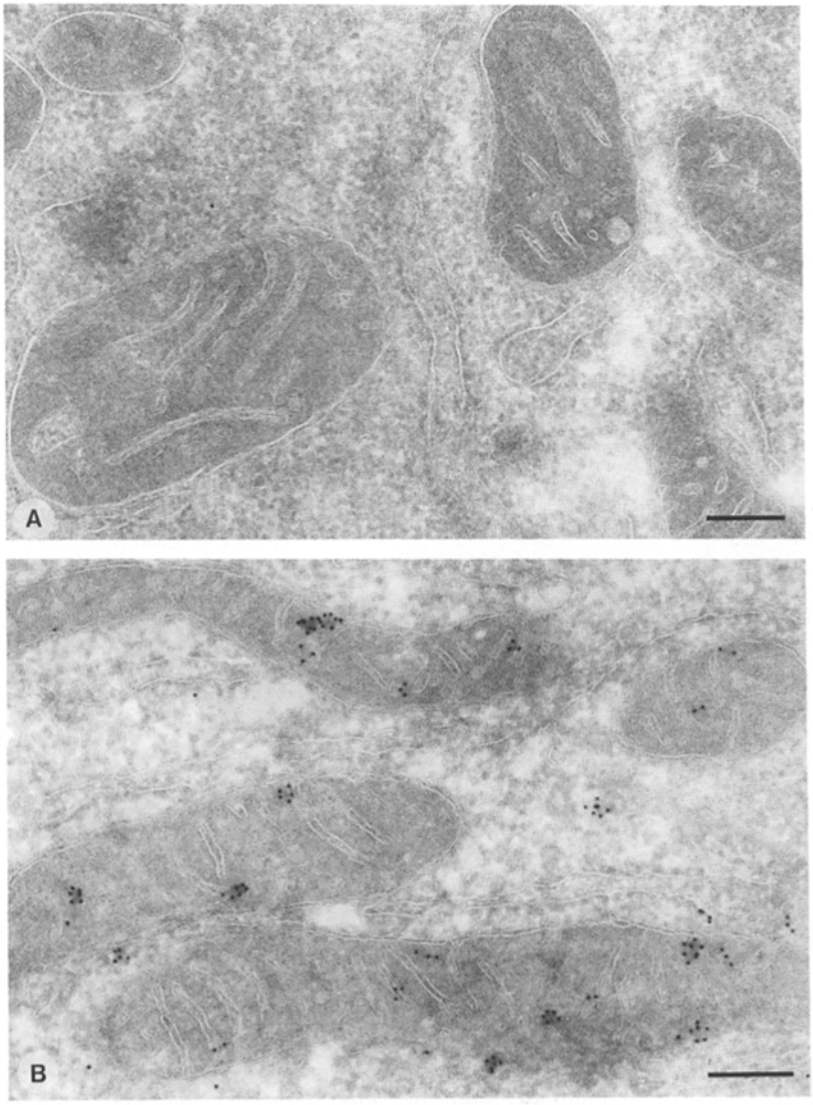

Figure 2. Immunoelectron microscopy of HepG2 cells. Immunogold labeling of hsp60 in ultrathin cryosections of fixed HepG2 cells. (*A*) Section labeled with control R73 isotype-identical irrelevant mAb. The mitochondria show no label. Only one background particle can be seen. The mitochondria are negative. ×51,000. (*B*) Section labeled with LK1 antibody. The mitochondria show abundant labeling for human hsp60. ×55,200.

human sequence. The epitope recognized by LK2 is located between amino acid residues 383 and 419 of the human hsp60 sequence (which corresponds with amino acid residues 356–393 of the mycobacterial hsp60 sequence). Because the LK2 antibody crossreacts with bacterial hsp, this antibody, in contrast to LK1, most probably reacts to an epitope in the molecules that is conserved between bacterial and mammalian hsp.

Upon examination of the human cell line HepG2 by immunoelectromicroscopy (Fig. 2), LK1 reactivity was observed exclusively in the mitochondria (Fig. 2 *B*). No gold particles were observed in other structures. This finding confirms earlier evidence for the mitochondrial localization of human hsp60 (14). No mitochondrial staining was obtained with an isotype-matched (γ1) control mAb of irrelevant specificity (R73; Fig. 2 *A*).

The Level of Expression of Human hsp60 Is Raised in Arthritic Synovium. Because of previous reports documenting increased staining with anti-mycobacterial hsp60 antibody in inflamed tissues (18–20), we tested LK1 and LK2 in a similar manner. Fig. 3 shows examples of staining with the human hsp60-specific antibody LK1 in synovial biopsy samples of a JCA patient and a non-JCA control child. A significant difference in staining intensity was observed between the JCA patient and the non-JCA control for LK1, LK2, and HLA-DR (Fig. 3, *A–F*). No specific staining was observed when the primary antibody was omitted or an irrelevant isotype-matched first antibody was used (Fig. 3, *G* and *H*). The staining pattern for LK1 and LK2 was cytoplasmic. Positive cells were observed in the synovial lining layer, the endothelial cells of blood vessels, and cells scattered throughout the interstitium. However, the staining was most prominent in the synovial lining layer.

One hypothesis for the induction of autoimmune arthritis is based on molecular mimicry, first described by Oldstone (27). Epitopes shared between viral or bacterial infectious agents and self-antigens present in the host are recognized by specific cells during the immune response. The ensuing

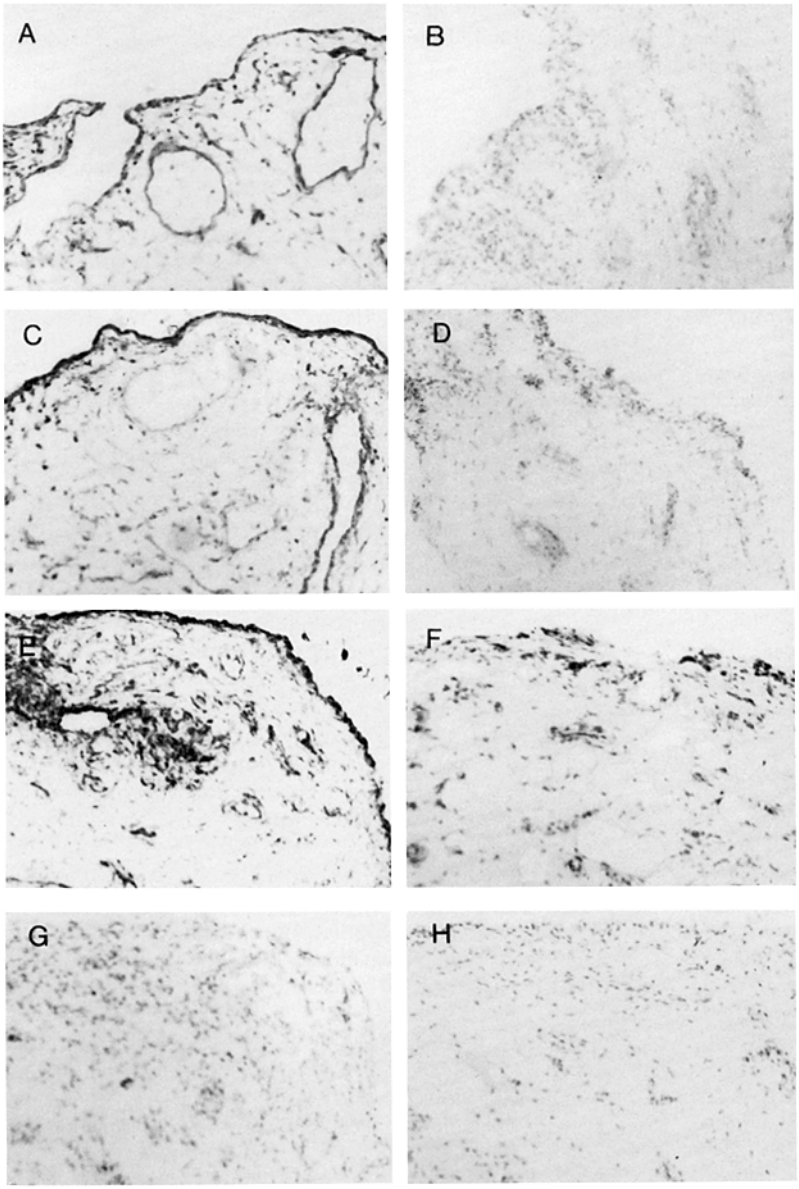

Figure 3. Immunohistochemistry of synovial membrane biopsy samples with mAbs LK1, LK2, and HLA-DR. Comparison of a patient with an oligoarticular onset of JCA (*left*) and a non-JCA control child (*right*). (*A* and *B*) Sections labeled with LK1: a significant difference in cytoplasmic staining of the synovial lining cells can be observed in the JCA patient in comparison with the non-JCA control child. ×132. (*C* and *D*) Sections labeled with LK2: a similar difference in staining pattern as for LK1 between the JCA patient and the non-JCA control child. ×132. (*E* and *F*). Sections labeled with HLA-DR: the staining is more extensive in the JCA patient than in the non-JCA control child. ×132. (*G* and *H*) No specific staining is observed in the negative control section (i.e., omitting or irrelevant isotype-matched primary antibody) for the JCA patient and non-JCA control child. ×132.

crossreactive response results in an attack on the foreign invader but also on self structures. Because of their extensive evolutionary conservation, heat-shock proteins are likely candidates for such a process and are therefore increasingly mentioned as target antigens in autoimmune diseases (1, 2, 5, 10). There is already evidence supporting the hypothesis that an immune response originally triggered by bacterial hsp might induce hsp-specific T cells that recognize an epitope common to bacterial and self-hsp (2, 5, 17).

Recently, we documented that T cells from synovial fluid of patients with JCA showed proliferative responses to human hsp60 (16). Whether the presence of such hsp60-specific T cells is the consequence of stimulation by locally deposited bacterial hsp antigens or by endogenously expressed human hsp60 remains to be established. The findings of staining with LK1 mAb uniquely specific for mammalian hsp60 on synovial cells is clearly in support of the latter possibility. To substantiate this, follow-up studies are necessary and immunohistochemical measurement of the expression of endogenous hsp60 or related molecules using mAbs LK1 and LK2 as well as monitoring of the T cell reactivity against human recombinant hsp60 might be important tools in testing this hypothesis.

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