

AUTOANTIBODIES TO THE CONSTITUTIVE 73-kD
MEMBER OF THE hsp70 FAMILY OF HEAT SHOCK
PROTEINS IN SYSTEMIC LUPUS ERYTHEMATOSUS

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Systemic lupus erythematosus (SLE) is characterized by the development and persistence of multiple autoantibodies to self-constituents. Detection of the presence of certain autoantibodies in patient serum is of considerable diagnostic utility, e.g., anti-dsDNA and antibody to the Smith antigen, Sm (1). Autoantibodies in SLE also have been useful as probes to define the function of their target antigens in normal biological processes. For example, the observation that autoantibodies to U1 ribonucleoprotein inhibit mRNA splicing in vitro (2) provided some of the earliest evidence that U1 ribonucleoprotein is essential for splicing of mRNA precursors.

The hsp70 family (3, 4) consists of five distinct, but structurally and immunologically related, proteins: (a) a constitutive 73-kD/pI 5.5 protein; (b) a stress-inducible 72-kD/pI 5.6 protein; (c) a stress-inducible 73-kD/pI 6.3 protein; (d) a constitutive 74–78-kD protein present in the endoplasmic reticulum (glucose-regulated protein); and (e) a 75-kD member present in the mitochondria. Although the biologic function of proteins in the hsp70 family is incompletely understood, the 73-kD/pI 5.5 protein has been shown to uncoat the clathrin lattice from endocytotic vesicles in an ATP-dependent manner during intracellular protein transport (5). Because certain immunodominant proteins of infecting microorganisms are homologues of heat shock proteins in eukaryotes (6, 7), the present evidence for the existence in SLE of a previously undescribed autoantibody to the 73-kD/pI 5.5 constitutively expressed member of the hsp70 family is consistent with the concept of molecular mimicry (8) as a mechanism for autoantibody induction in this disorder.

Materials and Methods

Patients and Serum. Serum was obtained from 34 adult patients with SLE who met the revised American Rheumatism Association classification criteria (9), from 10 normal subjects, and from patients with various other rheumatologic or viral diseases, including polymyositis, childhood onset SLE, rheumatoid arthritis, ankylosing spondylitis, recent onset infectious mononucleosis, and acute hepatitis A.

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Cells. Normal human PBMC were separated from heparinized blood by flotation on LSM (Litton Bionetics, Kensington, MD), and, for some experiments, stimulated in culture for 3 d with PHA (Burroughs Wellcome Co., Research Triangle Park, NY) as described previously (10). Cell lines included MOLT-4, HSB-2, JURKAT, HUT 78, SB, IMR-32, SK-N-SH, and chick embryo fibroblasts.

mAbs. N27F3-3 (N27), which reacts with both the constitutive form of hsp70 (73 kD/pI 5.5) and a highly homologous, stress-induced 72-kD/pI 5.6 protein (11), and C92F3A-5 (C92), which recognizes only the stress-inducible 72-kD/pI 5.6 protein (12), were used to identify SLE autoantibody-reactive target antigens.

Preparation of Cell Lysates. This was performed using 0.5% Nonidet P-40 as described previously (10), except that protein concentrations were determined by a modified Lowry procedure using bicinchoninic acid (Sigma Chemical Co., St. Louis, MO) instead of Folin-Ciocalteu reagent.

Electrophoresis and Immunoblotting. One-dimensional (1D) electrophoresis (reducing conditions, 7.5% polyacrylamide gels) and immunoblotting were performed as described previously (10), except that Tris-buffered saline, pH 7.4 (TBS), containing 5% non-fat dry milk was used for all washing steps and for dilution of second antibodies. Two-dimensional (2D) electrophoresis (13) was performed with a mini-slab system (Bio-Rad Laboratories, Richmond, CA) using Biolyte (Bio-Rad Laboratories) at final concentrations of 0.4% for 3–10 pH range and 1.6% for 5–7 pH range. Standard 1:10 (for IgM antibodies) or 1:20 (for IgG antibodies) dilutions of SLE serum normalized to IgM or IgG concentrations of 1 or 10 mg/ml, respectively, provided optimum sensitivity and minimum nonspecific staining. Normal human sera and mouse myeloma proteins were used as controls. Alkaline phosphatase-conjugated goat antibodies to human IgG (Cappel Laboratories, Cochranville, PA), human IgM (Cappel Laboratories), and mouse IgG (Zymed Laboratories, San Francisco, CA) were used as second antibodies. BCIP and NBT (Bio-Rad Laboratories) were used for color development according to the manufacturer's instructions. For some experiments, lymphocytes were intrinsically labeled with [³⁵S]methionine (Amersham Corp., Arlington Heights, IL) in methionine-free RPMI 1640/dialyzed FCS. Labeled proteins were analyzed by fluorography.

Immunoprecipitation with SLE Autoantibodies. After preclearing IgG from SLE or control human serum with protein A-Agarose (Zymed Laboratories), solid-phase IgM was prepared by mixing serum (500 μ l of a 1:10 dilution) with protein A-Agarose (50 μ l) and rabbit anti-human IgM (Cappel Laboratories) (5 μ l). The solid-phase immunoabsorbent was washed with PBS, pH 7.4, and incubated with detergent lysate of PHA-stimulated normal peripheral T cells (500 μ l adjusted to a protein concentration of 1 mg/ml) at 4°C for 4 h with continuous rotation. Adsorbed proteins were analyzed by SDS-PAGE and immunoblotting.

Results and Discussion

Serum from adult patients with SLE contained IgM (13:34) or IgG (5:34) antibodies to a phylogenetically widely expressed \sim 70-kD cellular antigen that comigrated with protein(s) of the hsp70 heat shock protein family in 1D immunoblots prepared with Nonidet P-40 cell lysates of various types (Fig. 1). Autoantibodies to this antigen were absent in 10 normal sera and were observed infrequently in the other disease sera studies, including childhood SLE (2:16), rheumatoid arthritis (0:5), ankylosing spondylitis (0:3), hepatitis A (0:20), and infectious mononucleosis (2:20). Because SLE sera frequently contain anti-DNA antibodies that may bind secondarily to putative cellular target antigens with DNA-binding activity via DNA in serum, cell lysates separated on 1D or 2D gels and electroeluted to nitrocellulose were incubated successively with ssDNA and mouse mAb to ssDNA. Several molecules in the 48–115-kD range, but no members of the hsp70 family, were identified as DNA-binding proteins. Taken together, these data suggested that SLE autoantibodies were binding directly to the \sim 70-kD protein and that this system may be relatively specific for adult SLE.

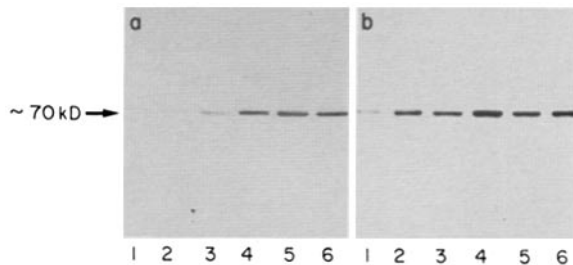


FIGURE 1. Comigration of SLE IgM autoantibody target antigen with a protein of the hsp70 family identified by mAb N27. Proteins in Nonidet P-40 cell lysates were subjected to electrophoresis under reducing conditions on 7.5% SDS polyacrylamide (1D) gels, electroeluted to nitrocellulose, and examined for reactivity with IgM in serum from SLE patient Chr (a) or with mAb N27 (b). Antigen substrates included: chicken embryo fibroblasts (lane 1), heat-shocked chicken embryo fibroblasts (lane 2), unstimulated normal human peripheral T cells (lane 3), PHA-stimulated normal peripheral T cells (lane 4), E6-1 cells (lane 5), and heat-shocked E6-1 cells (lane 6). In experiments not shown here, the SLE autoantibody target comigrated with a ~70-kD antigen recognized by mAb C92 as well.

Several lines of evidence suggested that SLE autoantibodies were directed against the constitutively expressed 73-kD/pI 5.5 protein in the hsp70 family. First, a SLE autoantibody-reactive protein of this estimated molecular mass exhibited a characteristic increase in synthesis in MOLT-4 cells labeled with [³⁵S]methionine 2 h after a 10-min, 45°C heat shock. Second, serum from patients with SLE stained a single protein of M_r and pI apparently identical to the 73-kD/pI 5.5 protein reactive with N27, an mAb specific for both hsp72 and hsp73. Thus, heat-shocked cells or, as illustrated in Fig. 2 a, PHA-activated peripheral T cells, contained four proteins having the approximate molecular mass of the SLE autoantibody target(s): a relatively acidic, constitutively expressed, but stress-inducible 73-kD/pI 5.5 protein (arrow 1); stress-inducible 72-kD/pI 5.6 (arrow 2) and 73-kD/pI 6.3 proteins (not visible) that were barely discernable in preparations of non-heat-shocked cells; and a constitutively expressed, non-heat-inducible 75-kD protein (arrow 3) identified in other experiments as a hsp70 family-related mitochondrial protein (Mizzen, Barells, and Welch, manuscript in preparation). Monoclonal N27 reacted with both the constitutive form of hsp70 (73 kD/pI 5.5) and a highly homologous, stress-induced 72-kD/pI 5.6 protein (Fig. 2 b, arrows 1 and 2, respectively). mAb C92 recognized only the 72-kD/pI 5.6 protein (Fig. 2 c, arrow 2). IgM from SLE patient Chr stained the 73-kD/pI 5.5 protein reactive with N27 (Fig. 2 d, arrow 1), but did not stain the 72-kD/pI 5.6 protein reactive with C92. Different IgM autoantibodies in certain serum specimens from this and other SLE patients also stained a 75-kD hsp70 family protein (Fig. 2 d, arrow 3). Similar evidence for discrete reactivity of SLE Ig with the 73-kD/pI 5.5 protein was obtained in two other patients with SLE. Third, SLE serum specifically immunoprecipitated a ~70-kD protein that reacted in 1D immunoblots with N27, but did not react with C92 (Fig. 3). SLE sera differed in this regard from infectious mononucleosis sera, which recognized both the 73-kD and 72-kD members of the hsp70 family.

In a previous investigation from this laboratory, IgG autoantibodies to hsp90, another major heat shock protein, also were found to occur frequently patients with

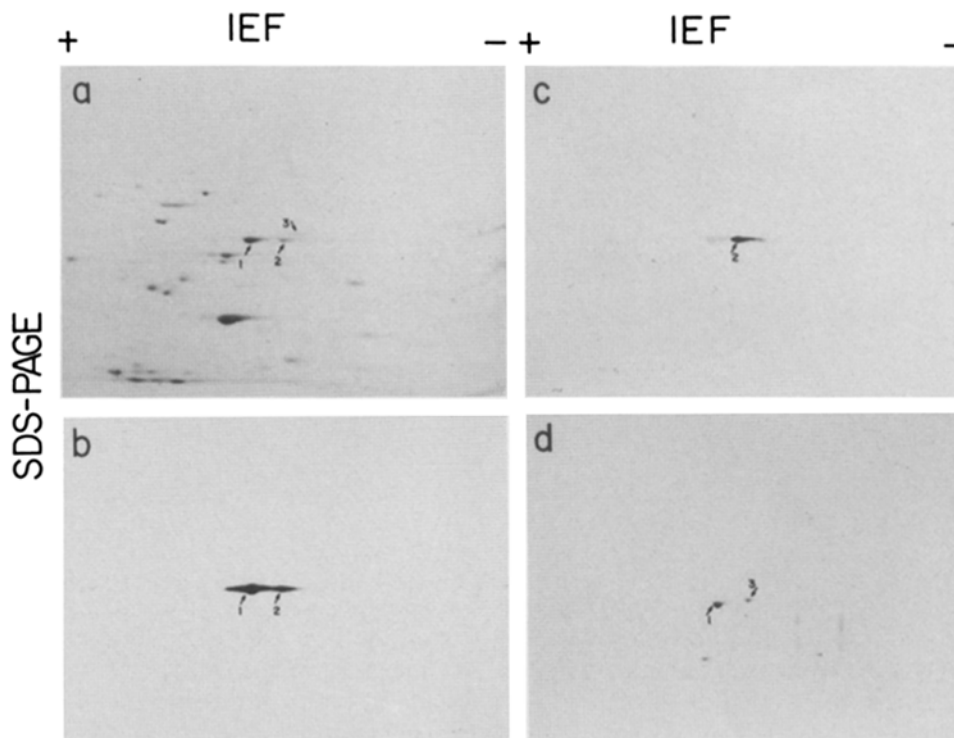


FIGURE 2. 2D immunoblots of PHA-stimulated normal peripheral T cells stained with various mAbs to proteins in the hsp70 family or with SLE IgM autoantibodies. Proteins (70 μ g) were separated by IEF/SDS-PAGE, electroeluted to nitrocellulose, and stained with amido black (a) or for antibody reactivity with N27 (b), C92 (c), or IgM in serum from SLE patient Chr (d). Members of the hsp70 family are identified by arrows.

SLE (14). No particular association of antibodies to hsp90 with antibodies to hsp70 in individual sera was evident, however. A larger systematic survey of patient sera will be required to clarify the issue of linkage of anti-hsp70 antibodies to other types of autoantibodies and to define the relationships of these autoantibodies with disease activity and/or specific disease manifestations.

The four main members of the hsp70 family of heat shock proteins are structurally conserved throughout eukaryotic species, and exhibit a high degree of amino acid sequence similarity throughout their primary structure. Since the SLE autoantibodies identified in the present investigation do not recognize epitopes on the 73-kD/pI 5.5 protein deriving from conserved peptide sequences shared by other hsp70 proteins in this structurally highly homologous family (reviewed in reference 4), a comparison of nonconserved peptide sequences of the 73-kD/pI 5.5 protein with sequences of other proteins should be useful in approaching the question of molecular mimicry (epitope homology) (8, 15) as a basis for microbial "triggering" of autoimmunity in SLE. For example, an immunodominant peptide of *Schistosoma mansoni* has amino acid sequence homology with hsp70, and may be responsible for autoantibodies to hsp70 in individuals infected with this organism (6). A related question concerns the possibility that autoantibodies to hsp70 crossreact with epitopes shared

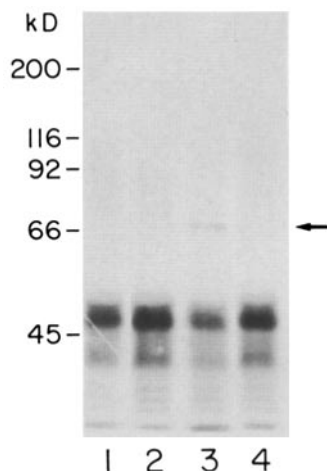


FIGURE 3. SLE IgM autoantibody precipitates a ~70-kD protein recognized by mAb N27. Immunoprecipitation with solid-phase IgM from control human serum (lanes 1 and 2) or SLE serum (patient Chr, lanes 3 and 4) was performed as described in detail in Materials and Methods. 1D immunoblots of adsorbed proteins separated on 7.5% SDS-PAGE under nonreducing conditions were stained with N27 (lanes 1 and 3) or C92 (lanes 2 and 4).

by other self antigens in different cell compartments, e.g., cell surface proteins (10, 16) and nuclear antigens, as has been demonstrated for IgM antibodies to the glycine-alanine repeat region of EBNA-1, which develop in the course of infectious mononucleosis (15). Autoantibodies to hsp70 also may prove to be useful probes for further defining the biologic function of this group of heat shock proteins.

Summary

Serum from patients with systemic lupus erythematosus (SLE) frequently contain IgM and IgG autoantibodies to the constitutively expressed 73-kD/pI 5.5 member of the hsp70 family of heat shock proteins, as determined by one-dimensional (SDS-PAGE) and two-dimensional (IEF/SDS-PAGE) immunoblotting, and by solid-phase SLE Ig immunoprecipitation experiments using hsp70 protein-specific mAbs as probes. Autoantibodies to hsp70 also were detected in a minority of sera from patients with other rheumatic or viral diseases, but not in normal sera. These data may provide additional insight into etiologic and pathophysiologic mechanisms in this and related autoimmune disorders.

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