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Vaccination with Recombinant Heat Shock Protein 60 from *Histoplasma capsulatum* Protects Mice against Pulmonary Histoplasmosis

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HIS-62 is a glycoprotein that has been isolated from the cell wall and cell membrane fraction of the pathogenic fungus *Histoplasma capsulatum*. It is a target of the cellular immune response to this fungus, and it protects mice against a lethal intravenous inoculum of *H. capsulatum* yeast cells. In this study, we cloned the gene encoding this antigen to reveal its biological nature and studied the immunological activity of recombinant antigen. The amino acid sequences of the NH_2 terminus and internal peptides were obtained by Edman degradation. Degenerate oligonucleotides were used to isolate a gene fragment of HIS-62 by PCR. One 680-bp segment that corresponded to the known peptide sequence was amplified from *H. capsulatum* DNA. This DNA was used to screen a genomic library, and the full-length gene was isolated and sequenced. The deduced amino acid sequence of the gene demonstrated ~70 and ~50% identity to heat shock protein 60 (hsp 60) from *Saccharomyces cerevisiae* and hsp 60 from *Escherichia coli*, respectively. A cDNA was synthesized by reverse transcription PCR and was expressed in *E. coli*. Recombinant protein reacted with a monospecific polyclonal rabbit antiserum raised against native HIS-62, with monoclonal HIS-62-reactive T cells, and with splenocytes from mice immunized with viable yeast cells. Moreover, vaccination with the recombinant protein conferred protection in mice against a lethal intranasal inoculation with yeast cells. Thus, HIS-62 is a member of the hsp 60 family, and the recombinant hsp 60 is protective against pulmonary histoplasmosis in mice.

In previous work, we reported that a cell extract from the cell wall and cell membrane (CW/M) of *Histoplasma capsulatum* yeast cells induced cell-mediated immune responses both in vivo and in vitro in C57BL/6 mice. When used as a vaccine, CW/M conferred protection against an intravenous challenge with yeast cells in C57BL6/J mice (8). Subsequently, a 62-kDa protein, termed HIS-62, was isolated from CW/M by electroelution and its immunobiological activity was analyzed. In vitro, HIS-62 stimulated *H. capsulatum*-specific polyclonal and monoclonal T cells derived from three strains of mice, C57BL/6 ($H\text{-}2^b$), BALB/c ($H\text{-}2^d$), and CBA/J ($H\text{-}2^k$). In vivo, it elicited significant delayed-type hypersensitivity responses in immune but not in naive, control mice. Immunization with HIS-62 induced protection against a lethal intravenous inoculum of *Histoplasma* yeast cells in each strain of mice (10). Moreover, HIS-62 was a target of the human response to *H. capsulatum*, since it stimulated proliferation by peripheral blood mononuclear cells and by T-cell clones from humans exposed to this fungus (11).

In the present study, we sought to identify the biological nature of HIS-62. To accomplish this objective, the gene encoding HIS-62 was cloned and sequenced. HIS-62 was found to be highly homologous with members of the heat shock protein 60-chaperonin 60 (hsp/cpn 60) family. The gene was then expressed and tested for immunological activity. Recombinant antigen was recognized by antisera raised to the native protein and by HIS-62-specific T cells. Vaccination with recombinant protein provided protection against a lethal intranasal challenge with yeast cells in BALB/c mice. Thus, HIS-62 is a member of the hsp/cpn 60 family, and the recombinant protein exerts immunological activity which is similar, if not identical, to that of the native protein.

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MATERIALS AND METHODS

Animals. BALB/c ($H\text{-}2^d$) mice were purchased from Jackson Laboratory, Bar Harbor, Maine. Female New Zealand White rabbits were purchased from Myrtle Rabbitry, Thompson's Station, Tenn.

Antigen. HIS-62 was isolated from CW/M as described previously (10).

Antibodies. To prepare anti-HIS-62 antibody, a rabbit was initially phlebotomized to obtain preimmune control serum. The rabbit was then given an intradermal injection of 50 μg of HIS-62 emulsified in Titermax (Cyt-Rx Corp., Norcross, Ga.). Four weeks later, the immunization was repeated in the absence of adjuvant. Serum was collected and tested for reactivity against HIS-62. Monospecific antibody was prepared as described by Smith and Fisher (25); 50 μg of HIS-62 was electrophoresed in a sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–PAGE) gel and electroblotted onto a nitrocellulose membrane. The membrane was stained with Ponceau S, and the band corresponding to HIS-62 was excised. Subsequently, the nitrocellulose strip was blocked with 5% dried milk in Tris-buffered saline (pH 7.4), air dried, and then incubated with the rabbit polyclonal serum for 24 h at 4°C. The strip was washed three times with 0.1% Tween 20 in Tris-buffered saline, and the bound antibody was eluted with 2 ml of a 200 mM glycine buffer, pH 3.0. Eluted antibody was neutralized with 0.1 volume of 1 M Tris, pH 7.4. Anti-GroEL antibody was a kind gift of Roger Hendrix, University of Pittsburgh.

Amino acid sequence. Ten micrograms of HIS-62 was electrophoresed in a 7.5% polyacrylamide gel and electroblotted onto a polyvinylidene difluoride (Schleicher & Schuell) membrane in a buffer of 10 mM 3-[(cyclohexylamino)-1-propane-sulfonic acid] (CAPS) (pH 11)–10% methanol at 250 mA for 1 h (19).

To determine internal peptide sequences, 100 μg of HIS-62 was lyophilized, resuspended in 70% formic acid, and cleaved with 200 μg of cyanogen bromide for 24 h. The sample was then lyophilized, and the protein fragments were resolved in an SDS–12.5% PAGE gel and transferred to polyvinylidene difluoride membranes. The membranes were stained with 0.3% Coomassie blue and destained with 50% methanol–10% acetic acid, with a final wash with absolute methanol (19). Bands migrating at 11, 13, 17, 19, and 25 kDa as well as the uncleaved HIS-62 were excised and subjected to gas-phase microsequencing (model 475A; Applied Biosystems, Foster City, Calif.).

Isolation of DNA and RNA from *H. capsulatum*. *H. capsulatum* yeast cells were grown in 50 ml of Ham's F-12 at 37°C in a shaking incubator for 48 h to late log phase. Yeast cells were harvested and resuspended in 10 ml of 10 mM Tris (pH 8.0) containing 1 mM EDTA (TE). SDS was added to a 1% final concentration.

The mixture was incubated for 1 h at room temperature and then subjected to three extractions with phenol-chloroform, ethanol precipitated, and washed with 70% ethanol. The nucleic acid extract was resuspended in TE containing 10 μg of RNase A per ml and incubated for 2 h at 37°C. The DNA was extracted again with phenol-chloroform, precipitated, and resuspended in TE.

Total RNA was isolated by the method of Chomczynski and Sacchi (4). *H. capsulatum* poly(A)$^+$ RNA was isolated from yeast cells by the Fast-Track system (Invitrogen Corp., San Diego, Calif.).

Amplification of a gene fragment by PCR. Genomic DNA (0.1 μg) was incubated with the following degenerate primers (1 μM each): NH_2 terminus sense, 5′-AAA GA(A,G) CT(I/C) AA(A,G) TT(C,T) GG(A,G,C,T) GT-3′; and 13-kDa antisense, 5′-TG (I/C)GC (C,T)TT (A,G,C,T)GT (A,G)TC (A,G,C,T) GT-3′. The sequences in parentheses indicate that more than one base was present. The conditions for the PCR were 94°C for 1 min, 48°C for 1 min, and 72°C for 3 min for 40 cycles. The DNA fragment was blunt ended, ethanol precipitated, and gel purified with 1% low-melting-point agarose (FMC Corp., Rockland, Maine). DNA was cloned into an *Eco*RV site in pBluescript SK$^-$ (Stratagene, La Jolla, Calif.) and transformed into *Escherichia coli* XL-1 blue (Stratagene).

DNA sequencing. DNA was sequenced by using modified T7 DNA polymerase (Sequenase; U.S. Biochemical Corp., Cleveland, Ohio). To sequence the entire gene, nested deletions were generated by using exonuclease III (Erase-a-Base system; Promega, Madison, Wis.) according to the manufacturer's instructions.

Radiolabeling of the PCR product. Fifty nanograms of PCR product was incubated with 1 μM (each) NH_2-terminal sense and 13-kDa antisense primers, 150 μCi of [α-^{32}P]dCTP, and 1 μM (each) dATP, dTTP, and dGTP in a volume of 50 μl. The conditions for PCR were 94°C for 1 min, 48°C for 1 min, and 72°C for 3 min for 20 cycles.

Colony screening. An *H. capsulatum* plasmid library, constructed by complete *Hin*dIII digestion of genomic DNA ligated into pBluescript SK$^-$, was kindly provided by Millie Schaefer and Gary Dean. A total of 10^5 colonies were plated and replicated onto nitrocellulose filters (Schleicher & Schuell), which were then incubated in hybridization solution consisting of 5× saline sodium citrate (SSC) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt's solution, 0.1% SDS, 0.1 M $NaPO_4$, and 50% formamide at 42°C for 16 h with 10^6 cpm of radiolabeled PCR product per ml. The filters were washed with 0.2× SSC containing 0.1% SDS at 65°C and autoradiographed at −70°C overnight.

Generation of an intron-free gene by using reverse transcription PCR (RT-PCR). An antisense oligonucleotide encompassing the HIS-62 coding region from bases 692 to 710 was synthesized: 5′-AGACGTATCCGCGGTCGAAT-3′. Four micrograms of total RNA was annealed with 1 pmol of this oligonucleotide and incubated with 500 μM deoxynucleoside triphosphates and 15 U of Superscript reverse transcriptase (Gibco BRL, Grand Island, N.Y.) at 42°C for 1 h. The first-strand cDNA was then PCR amplified with the same antisense primer and a sense primer located at the ATG starting codon of the gene. The sequence of this primer was 5′-TAGAATTCATGCAGCGAGCGGAGCTCTTT-3′. A 5′ *Eco*RI site (underlined) was added to the primer to facilitate cloning into pBluescript. The PCR was performed for 25 cycles of 94°C for 1 min and 72°C for 3 min with 5 U of Vent polymerase (New England Biolabs). The PCR product was cloned, mapped, and sequenced. The intronless cDNA was ligated to the remainder of the coding region of the HIS-62 gene through a unique *Nco*I site, and the construct was termed pHIS-62exp.

Northern (RNA) analysis of HIS-62. Ten micrograms of total RNA or 0.1 μg of poly(A)$^+$ RNA was electrophoresed on denaturing 1% agarose-formaldehyde gels, transferred onto a nitrocellulose membrane by capillary blotting, and hybridized with a ^{32}P-labeled probe that contained 600 bp of the 5′ end of pHIS-62exp. The membrane was washed at 50°C in 0.2× SSC–1% SDS and autoradiographed at −70°C overnight.

Protein expression. To generate recombinant protein, the intron-free pHIS-62exp was incubated with the following set of primers (the positions of the bases are given in relation to the ATG starting codon): NH_2-terminal sense (bases 1 to 18), 5′-CAAGCATATGAAGGAGCTCAAATTCGGCGT-3′; and carboxy-terminal antisense (bases 1802 to 1820), 5′-TTAAGCTTAGAACATGCCACCGC CCA-3′. The sense primer introduces an *Nde*I site (underlined), and the carboxy-terminal antisense primer introduces a *Hin*dIII site (underlined). The material was amplified by PCR using the following conditions: 94°C for 45 s and 72°C for 3 min for 25 cycles with Vent polymerase. The gene product was gel purified and cloned into the *Nde*I and *Hin*dIII sites of pET19b (Novagen, Madison, Wis.). The plasmids were transformed into BL21(DE3) lysogen host cells.

To express the recombinant protein, transformed *E. coli* cells (500 ml) were grown in Luria-Bertani medium at 37°C in a shaking incubator until the optical density at 600 nm reached 0.6. Subsequently, isopropylthiogalactose (IPTG) was added to cultures to a final concentration of 1 mM, and the cultures were incubated for an additional 2 to 3 h. Cells were harvested by centrifugation at 5,000 × *g*. *E. coli* pellets were resuspended in a buffer consisting of 5 mM imidazole, 500 mM NaCl, and 20 mM Tris-Cl (pH 7.9) and lysed by a freeze-thaw cycle followed by sonication. Soluble and insoluble fractions were separated by centrifugation at 20,000 × *g*. The insoluble pellet fraction was found to contain the recombinant product when analyzed by SDS-PAGE.

Metal chelate purification of recombinant HIS-62 (rHIS-62). pET19b adds 10 histidines to the NH_2 terminus. This modification allows purification of recombinant protein on nickel columns. The insoluble pellet fraction was resuspended by sonication in a denaturing solubilization buffer consisting of 6 M urea, 500 mM NaCl, 5 mM imidazole, and 20 mM Tris (pH 7.9). Solubilized material was recovered in the supernatant after a centrifugation at 20,000 × *g*, and the supernatant was filtered through a 0.22-μm-pore-size cellulose acetate membrane. The material was then loaded into an Ni^{2+}-Sepharose affinity column (His-Bind; Novagen) and washed with 20 mM imidazole, and the recombinant product was eluted with 1 M imidazole in the same buffer. Eluted material was dialyzed against 20 mM Tris (pH 7.4)–200 mM NaCl containing decreasing concentrations of urea. The eluate was concentrated by ultrafiltration. The protein concentration was determined by the Bradford method (3).

The histidine tag can be cleaved from the protein by using enterokinase. All studies of HIS-62 were conducted with recombinant protein containing the tag, since cleavage did not alter its immunological activity (9).

Western blot (immunoblot) analysis. One microgram of native HIS-62 or rHIS-62 was electrophoresed by SDS–10% PAGE, electroblotted onto nitrocellulose membranes, and incubated overnight at 4°C with either a 1:800 dilution of normal rabbit serum or a 1:800 dilution of polyclonal anti-GroEL rabbit antiserum. In other experiments, rHIS-62 was incubated as described above with a 1:1,000 dilution of either preimmune rabbit serum or monospecific, polyclonal anti-HIS-62 antibody. Nitrocellulose strips were incubated with a 1:2,000 dilution of an alkaline phosphatase-labeled goat anti-rabbit antibody (Kirkegaard & Perry, Gaithersburg, Md.) and then with 3.3 mg of nitroblue tetrazolium per ml and 1.5 mg of 5-bromo-4-chloro-3-indolylphosphate (BCIP) per ml.

Preparation of *H. capsulatum* and injection of yeast cells. *H. capsulatum* yeast cells were prepared as described previously (10). BALB/c mice were immunized with viable yeast cells by being injected subcutaneously with 10^6 yeast cells and 2 weeks later being injected intravenously with 6×10^5 yeast cells. After 3 weeks, mice were inoculated intraperitoneally with 5×10^6 yeast cells. In studies of protective immunity, mice were challenged intranasally with 1.2×10^7 yeast cells.

Immunization of mice with rHIS-62. Groups of mice were immunized subcutaneously with 200 μg of rHIS-62 suspended in MPL plus TDM plus CWS emulsion (Ribi ImmunoChem, Hamilton, Mont.). Two doses were given, and they were separated by 2 weeks. Controls simultaneously received bovine serum albumin (BSA) emulsified in adjuvant.

Preparation of splenocytes and proliferation assay. Splenocytes from mice were isolated as described elsewhere (9), and the proliferation assay was performed as described previously (10).

Antigen recognition by T-cell hybridomas. T cells were suspended in RPMI 1640 supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, 1% sodium pyruvate, 50 μM 2-mercaptoethanol, and 10 μg of gentamicin per ml. Equal numbers of T cells (3×10^4) and LB 27.4 (*H-2$^{b/d}$*) cells (as antigen-presenting cells) were added to each well of a microtiter plate in a final volume of 200 μl. Decreasing concentrations of native HIS-62 or rHIS-62 were added to cells in a volume of 50 μl. After 24 h of incubation at 37°C in 5% CO_2, 100 μl of the supernatants was removed and tested for interleukin 2 activity.

Assay for interleukin 2 activity. Test supernatants were added to 4×10^3 CTLL-2 cells in 0.1 ml of medium and cultured at 37°C in 5% CO_2 for 28 h. Four hours before being harvested, cells were pulsed with 1 μCi of [^{3}H]thymidine, and the incorporated radioactivity was measured.

Statistics. The log rank test was used to analyze survival between groups.

Nucleotide sequence accession number. The sequence of the entire HIS-62 gene is deposited in GenBank. Its accession number is L11390.

RESULTS

Amino acid sequencing and identity of HIS-62. Peptide sequences from the NH_2 terminus and from cyanogen bromide-generated polypeptides are shown in Table 1. The 62-kDa NH_2 terminus and the 11-kDa fragment peptide sequences were similar. Likewise, the 25- and the 19-kDa sequences were nearly identical.

A search of the Swiss-Prot protein bank with the available sequences was performed. Although there was weak homology with several proteins in the database, the NH_2 terminus and the 13-kDa peptide demonstrated relatedness with several members of the hsp 60 family. Therefore, we aligned the peptide sequences with the amino acid sequence of hsp 60 from *Saccharomyces cerevisiae*. The NH_2 terminus and the various fragments of HIS-62 revealed 60 to 75% identity to *S. cerevisiae* hsp 60. This result strongly suggested that HIS-62 was a member of the hsp 60 family.

HIS-62 demonstrates serological cross-reactivity with GroEL. To support the finding that HIS-62 was related to hsp 60, electroeluted HIS-62 was tested by immunoblot analysis with a rabbit antiserum against GroEL (*E. coli* hsp 60). Anti-

TABLE 1. Amino acid sequences of the NH_2 terminus and peptide fragments of HIS-62 cleaved with cyanogen bromide

Peptide[a]	Amino acid sequence[b]
NH_2 terminus	d y K E L K F G V E G w A A L L K G d e
11-kDa fragment	G l K E L K F G V E G I A A L L K G I D T L
	A I V
13-kDa fragment	A F K E G Y V S P Y F I T D T k A Q k V x F a k
	E d
19-kDa fragment	L G S T G S I T I T K E D T I x l N x
25-kDa fragment	x G S T G S I T I T K E D T i x l N G S G

[a] Peptides are designated by their apparent molecular masses in SDS-PAGE gels.
[b] Single-letter code used to denote amino acids. If more than one amino acid was obtained during a cycle, the amino acids are listed in a column. Ambiguous or doubtful amino acids (lowercase letters), an unreadable cycle (x), and the amino acids used for sense and antisense primers (arrows) are indicated.

GroEL antibody but not normal rabbit serum recognized electroeluted HIS-62 (Fig. 1).

Cloning and sequencing of the gene encoding HIS-62. Degenerate primers based on the amino acid sequences of the NH_2 terminus and the 13-kDa peptide were used in a PCR (Table 1). A single, 680-bp PCR product was isolated, cloned, and sequenced. The deduced amino acid sequence matched the available peptide sequence. The 680-bp segment was radiolabeled and used to screen an *H. capsulatum* genomic plasmid library. From 10^5 colonies screened, five candidate colonies were picked, replated, and rescreened; 18 positive colonies were grown, and the plasmids were purified and tested by Southern analysis. Two positive plasmids of 11.5 kb hybridized with the probe, and one, termed pHIS-62, was chosen for further analysis. By mapping, Southern analysis, and partial sequencing, a *Hin*dIII-to-*Ssp*I segment of ~2,400 bp was found to contain the complete gene encoding HIS-62. The segment was subcloned, and both strands were sequenced. The complete sequence and its deduced amino acid sequence are shown in Fig. 2. The two introns depicted in the figure were predicted by gaps in the homology of the sequence between HIS-62 and hsp 60 from *S. cerevisiae*. The presence of these introns was confirmed by RT-PCR and sequence analysis of the resultant cDNA.

Homology of HIS-62 to hsp 60. The deduced amino acid sequence of HIS-62 shows 50 to 70% identity with other members of the hsp 60 family (Fig. 3). There is a highly conserved glycine- and methionine-rich stretch at the carboxy terminus of the molecule.

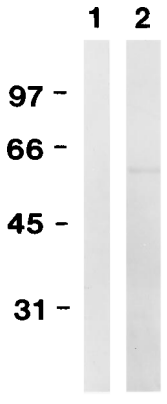

FIG. 1. HIS-62 is immunoreactive with anti-GroEL antiserum. One microgram of native HIS-62 was electrophoresed in a 10% polyacrylamide gel and transferred to nitrocellulose. Strips were excised and incubated with a 1:800 dilution of normal rabbit serum (lane 1) or with a 1:800 dilution of anti-GroEL antiserum (lane 2). Molecular weight markers (in thousands) are indicated on the left.

Northern analysis of HIS-62. Total and poly(A^+) RNAs were analyzed by Northern blot with a radiolabeled 600-bp probe from the HIS-62 coding region. A single band 2.1 kb in size hybridized with the probe (Fig. 4).

Expression of rHIS-62. To express HIS-62 in a bacterial system, it was necessary to construct an intronless version of the coding area. Taking advantage of the fact that the two introns present in the HIS-62 gene were clustered near the 5′ end of the gene, RT-PCR was used to generate a cDNA version of the 5′ end of the coding region. Subsequently, it was ligated to the remainder of the gene by using a unique *Nco*I site. The constructed gene was then cloned into pET19b and expressed in *E. coli*. The expressed protein was evident on Coomassie blue staining of polyacrylamide gels (Fig. 5, lane 2). After sonication, the expressed protein was detected in the insoluble pellet fraction (Fig. 5, cf. lanes 3 and 4). The expressed protein was isolated by metal chelate affinity chromatography. SDS-PAGE analysis of the affinity-purified protein revealed a single band of ~62 kDa (Fig. 5, lane 6).

To verify that the gene we cloned and expressed was HIS-62, rHIS-62 was subjected to Western blot analysis with antiserum raised to the native, electroeluted protein. rHIS-62 reacted with monospecific polyclonal antibody but not with preimmune serum (Fig. 6).

As an additional confirmation that HIS-62 and rHIS-62 were identical, we tested the T-cell hybridoma B4IID6, which was known to recognize native HIS-62 (2), with rHIS-62 and electroeluted HIS-62. B4IID6 responded to the native and recombinant antigens in a dose-dependent fashion, and the dose-response curves were nearly superimposable (Fig. 7).

Since the gene was expressed in *E. coli*, it was important to exclude the possibility that the recombinant protein was GroEL. Therefore, rHIS-62 was subjected to digestion with the proteinase AspN and chromatographed through reverse-phase high-performance liquid chromatography. One peak was selected (retention time, 65.75) and subjected to amino acid sequencing. The sequence of the fragment was DYEKEKLQE RLAKLSGGVAVIKVGGASEVEV. In GroEL, the corresponding region was DYDREKLQERVAKLAGGVAVIKVG AATEVEMK (Fig. 3). Thus, rHIS-62 was from *H. capsulatum*, not *E. coli.*

Immunobiological activity of rHIS-62. Earlier work had indicated that native HIS-62 was a target of the cell-mediated immune response to *H. capsulatum* and that it induced this response in mice and conferred protection (10). In this series of studies, we sought to determine if rHIS-62 exerted similar immunological activity. Two important properties were exam-

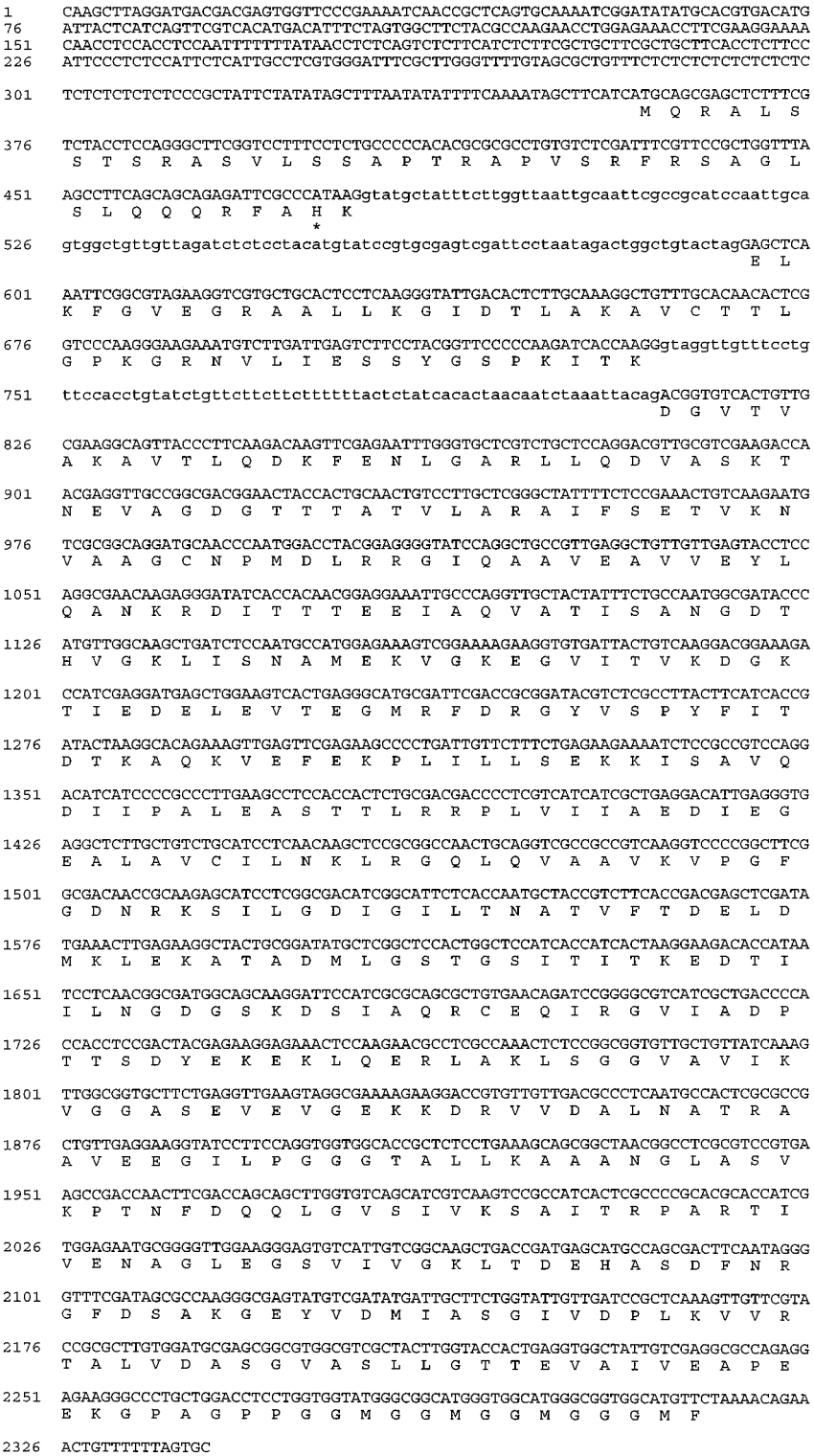

FIG. 2. Gene sequence and deduced amino acid sequence of HIS-62. Base numbers are indicated on the left. Introns (lowercase letters) were confirmed by sequencing cDNA. The start of the mature protein (⋆) was at a histidine and was determined by amino acid sequencing.

ined: (i) recognition by lymphocytes from animals exposed to *Histoplasma* organisms and (ii) protective efficacy. Splenocytes from *H. capsulatum*-immunized mice responded vigorously to rHIS-62, whereas cells from unimmunized controls did not (Fig. 8).

BALB/c mice were injected with a total of 400 μg of rHIS-62 or with an equal amount of BSA emulsified in adjuvant. As an additional control, a group of mice were unimmunized. Two weeks after immunization, mice were challenged with 1.2×10^7 yeast cells intranasally. This route was chosen because

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HC          MQRALSSTSRASVLSSAPTRAPVSRFRSAGLSLQQQRFA-HKELKFGVEG          49
SC          M----------------L.SS.V.S.ATLRP.LRRAYSS.........          33
GROEL       M-----------------------------------AA.DV...NDA          12

HC          RAALLKGIDTLAKAVCTTLGPKGRNVLIESSYGSPKITKDGVTVAKAVTL          99
SC          ..S....VE...E..AA............QPF.P............SIV.          83
GROEL       .VKM.R.VNV..D..KV.........VLDK.F.A.T......S..REIE.          62

HC          QDKFENLGARLLQDVASKTNEVAGDGTTTATVLARAIFSETVKNVAAGCN          149
SC          K.....M..K...E.......A......S....G....T.S.........          133
GROEL       E.....M..QMVKE....A.DA............Q..IT.GL.A....M.          112

HC          PMDLRRGIQAAVEAVVEYLQANKRDITTTEEIAQVATISANGDTHVGKLI          199
SC          .......S.V...K.I.F.S...KE...S..............S.....L          183
GROEL       ....K...DK..T.A..E.K.LSVPCSDSKA....G.....S.ET.....          162

HC          SNAMEKVGKEGVITVKDGKTIEDELEVTEGMRFDRGYVSPYFITDTKAQK          249
SC          AS............IRE.R.L..............FI.......P.SS.          233
GROEL       AE..D..........E..TGLQ...D.V...Q.....L.....NKPETGA          212

HC          VEFEKPLILLSEKKISAVQDIIPALEASTTLRRPLVIIAEDIEGEALAVC          299
SC          .......L........SI...L....I.NQS....L.....VD.....A.          283
GROEL       ..L.S.F...AD....NIREML.V...VAKAGK..L.....V......TA          262

HC          ILNKLRGQLQVAAVKVPGFGDNRKSILGDIGILTNATVFTDELDMKLEKA          349
SC          ........VK.C...A........NTI...AV..GG....E...L.P.QC          333
GROEL       VV.TI..IVK.....A.....R..AM.Q..AT..GG..ISE.IG.E....          312

HC          TADMLGSTGSITITKEDTIILNGDGSKDSIAQRCEQIRGVIADPTTSDYE          399
SC          .IEN...CD...V.....V....S.P.EA.QE.I...K.S.DIT..NS..          383
GROEL       .LED..QAKRVVIN.DT.T.ID.V.EEAA.QG.VA...QQ.EEA.-...D          361

HC          KEKLQERLAKLSGGVAVIKVGGASEVEVGEKKDRVVDALNATRAAVEEGI          449
SC          ..................R..............YD..............          433
GROEL       R......V...A.........A.T...MK...A..E...H.........V          411

HC          LPGGGTALLKAAANGLASVKPTNFDQQLGVSIVKSAITRPARTIVENAGL          499
SC          ........V..S-RV.DE.VVD....K...D.IRK......KQ.I....E          482
GROEL       VA...V..IRV.SK-..DLRGQ.E..NV.IKVALR.MEA.L.Q..L.C.E          460

HC          EGSVIVGKLTDEHASDFNRGFDSAKGEYVDMIASGIVDPLKVVRTALVDA          549
SC          .....I...I..YGD..AK.Y.AS.S..T..L.T..I..F....SG....          532
GROEL       .P..VANTVKGGDGN-Y--.YNA.TE..GN..DM..L..T..T.S..QY.          507

HC          SGVASLLGTTEVAIVEAPEEKGPA-GPPGGMGGMGGMGGGMF                  590
SC          .......A.......D...PPAA.-.AG.MP...P..P.-.M                 572
GROEL       AS..G.MI...CMVTDL.KNDAADL.AA.........-...M                  548
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FIG. 3. Homology of HIS-62 with hsp/cpn 60 proteins from *S. cerevisiae* and *E. coli*. Deduced amino acid sequences were aligned by using PC/GENE (IntelliGenetics, Mountain View, Calif.). HC, *H. capsulatum* HIS-62; SC, hsp 60 from *S. cerevisiae*; GROEL, hsp 60 from *E. coli*. Identical amino acids (dots) are indicated. Amino acid numbers are indicated on the right. Dashes indicate gaps in the alignment.

infection is acquired via the respiratory tract, and this phase of the fungus was used because it is responsible for the clinicopathological manifestations of disease. Survival was monitored on a daily basis for 45 days. All unimmunized mice died by day 18 of infection, and only 1 mouse injected with BSA survived the entire period of observation (Fig. 9). There was no statistical difference between these two groups ($P = 0.224$). In striking contrast, all mice immunized with rHIS-62 survived for 45 days ($P = 0.00016$ versus BSA controls) (Fig. 9).

DISCUSSION

Our laboratory is engaged in a program to isolate antigens that contribute to the generation of a cellular immune response and, more importantly, a protective immune response against *H. capsulatum*. In a previous work, we reported that HIS-62 was a target of the cellular immune response and protected mice against an intravenous challenge with yeast cells (10). Among CW/M proteins, we estimated that it constitutes <1% of the total protein content (9), thus suggesting that its abundance does not explain its immunological properties. In this study, we have determined the biological nature of this antigen and have shown that the recombinant form expressed in a prokaryote exerted immunobiological activity similar to that of the native HIS-62.

Two lines of evidence indicated that this antigen is a member of the hsp/cpn 60 family. First, the native protein cross-reacted with antibody to GroEL, the hsp 60 homolog from *E. coli*. Second, we cloned and sequenced the gene of this antigen. Analysis of the gene sequence and its deduced amino acid sequence revealed that HIS-62 manifested a high degree of identity to hsp 60 from *S. cerevisiae* (~71%) and to GroEL (~54%). These data strongly support the finding that HIS-62 is a member of the hsp/cpn 60 family.

It was important to verify that HIS-62 was encoded by the gene that had been isolated. Accordingly, Western blot analysis revealed that a monospecific, polyclonal rabbit antiserum raised against the native HIS-62 recognized the recombinant protein. Moreover, monoclonal *H. capsulatum*-reactive T cells that recognized HIS-62 reacted almost identically to the recombinant protein. Thus, HIS-62 is encoded by the gene that was isolated.

The sequence obtained by Edman degradation exhibited three principal differences from the deduced sequence of the HIS-62 gene. The amino acids in position 2 of the NH_2 terminus and the 11-kDa fragment differ from the deduced amino acid sequence. Moreover, the amino acids determined for positions 1 and 3 of the 13-kDa fragment are alanine and either lysine or glutamic acid, respectively, while in the deduced sequence arginine and aspartic acid were detected. The incongruity may be a result of Edman degradation sequencing mistakes. A second explanation is that there were point mutations in the cloned DNA. However, these regions have been se-

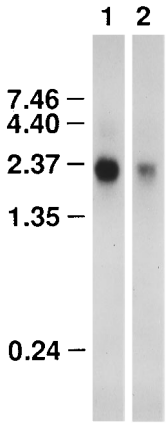

FIG. 4. Northern analysis of HIS-62. Ten micrograms of total RNA (lane 1) or 0.1 μg of poly(A)$^+$ RNA (lane 2) was hybridized with a ^{32}P-labeled probe spanning the first 600 bp of the gene and autoradiographed. Molecular weight markers (in 10^3 bp) are depicted on the left.

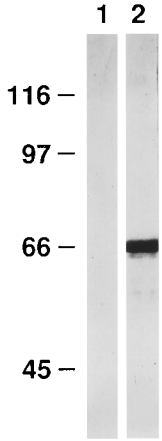

FIG. 6. Western blot analysis of rHIS-62. One microgram of rHIS-62 was electrophoresed in a 10% polyacrylamide gel and electroblotted onto nitrocellulose. Strips were incubated with a 1:1,000 dilution of preimmune rabbit serum (lane 1) or a 1:1,000 dilution of monospecific rabbit anti-HIS-62 antiserum (lane 2). Molecular weight markers (in thousands) are indicated on the left.

quenced from PCR-amplified genomic DNA and PCR-amplified cDNA, and the same nucleotide sequence was apparent in the two DNAs.

Another possibility is that there exists more than one hsp/cpn 60 *H. capsulatum* gene. *Mycobacterium tuberculosis*, for example, encodes two different variants of hsp/cpn 60 genes. The homology between them is 50% at the amino acid level (16). However, this explanation is unlikely, because Southern and Northern analyses suggested the existence of only a single gene and a single message (9). The possibility that *H. capsulatum* expresses two different versions of the hsp/cpn 60 protein family cannot be excluded formally, but it is doubtful since the other amino acids obtained by Edman degradation were identical to that obtained from the gene sequence.

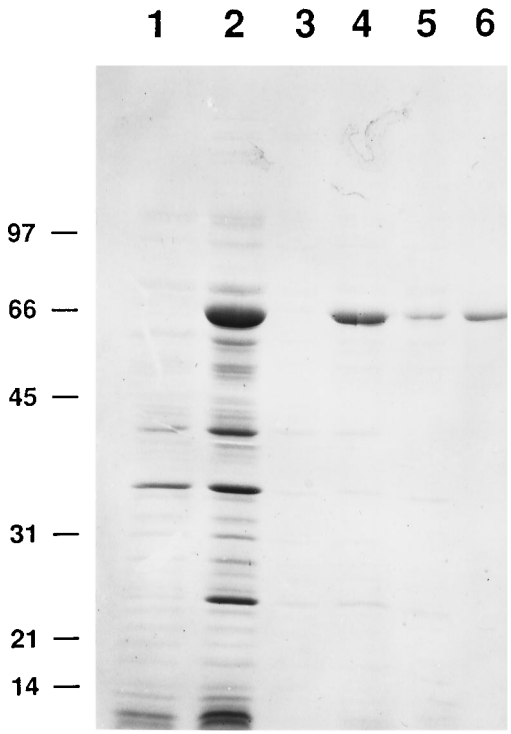

FIG. 5. SDS-PAGE analysis of purification of rHIS-62 by metal chelate chromatography. rHIS-62 was expressed in *E. coli* and purified by Ni^{2+}-Sepharose. Material was electrophoresed in a 10% polyacrylamide gel. Lane 1, preinduction *E. coli* lysate; lane 2, *E. coli* lysate 2 h after induction with 1 mM IPTG; lane 3, supernatant following sonication; lane 4, insoluble pellet incubated in 6 M urea; lane 5, chelate column pass-through; lane 6, rHIS-62 eluted with 1 M imidazole from metal chelate column. Molecular weight markers (in thousands) are indicated on the left.

hsp/cpn 60 is a highly conserved protein that is present in prokaryotes and eukaryotes. Homology at the amino acid level between the *E. coli* and *Homo sapiens* proteins is as high as 50% (22). The presence of the genes encoding these proteins is required for cell growth and survival. Postulated functions of hsp/cpn 60 are to promote folding and assembly of nascent polypeptides within the cell. This protein family also is involved in refolding of polypeptides that have been damaged by environmental stress, and it acts as a transporter of proteins that are targeted to cellular compartments (6, 15, 24).

In addition to its vital role in cell survival, this family of proteins appears to be a prominent target of the immune response to several pathogenic microbes (for reviews, see references 13, 31, and 32). These pathogens include *Mycobacterium* spp., *Chlamydia trachomatis*, and *Legionella pneumophila*. The 65-kDa antigens from *M. tuberculosis* and *Mycobacterium leprae* are members of the hsp/cpn 60 family, and they are a target of cellular and humoral immune responses to these bacteria (17, 18, 30). A large proportion of T cells (~20%) from mice immunized with killed *M. tuberculosis* cells suspended in adjuvant respond to the 65-kDa antigen (14). T cells from humans exposed to mycobacteria recognize this antigen (18). In addition, a 57-kDa protein that is homologous to hsp/cpn 60 induces a delayed-type hypersensitivity response in the conjunctivas of guinea pigs exposed to *C. trachomatis*. Thus, this antigen appears to be responsible, in part, for the inflammatory response to *C. trachomatis* (20).

Once the identity of HIS-62 was known, it was surprising that a highly conserved antigen was protective against a pathogen. Nevertheless, recent evidence that an hsp/cpn 60 homolog from a bacterium also mediates protection has emerged. The major cytoplasmic membrane protein of *L. pneumophila* is a member of this family of conserved proteins (2). When administered to guinea pigs as a vaccine, it elicits a cellular immune response and protection in animals challenged with a lethal

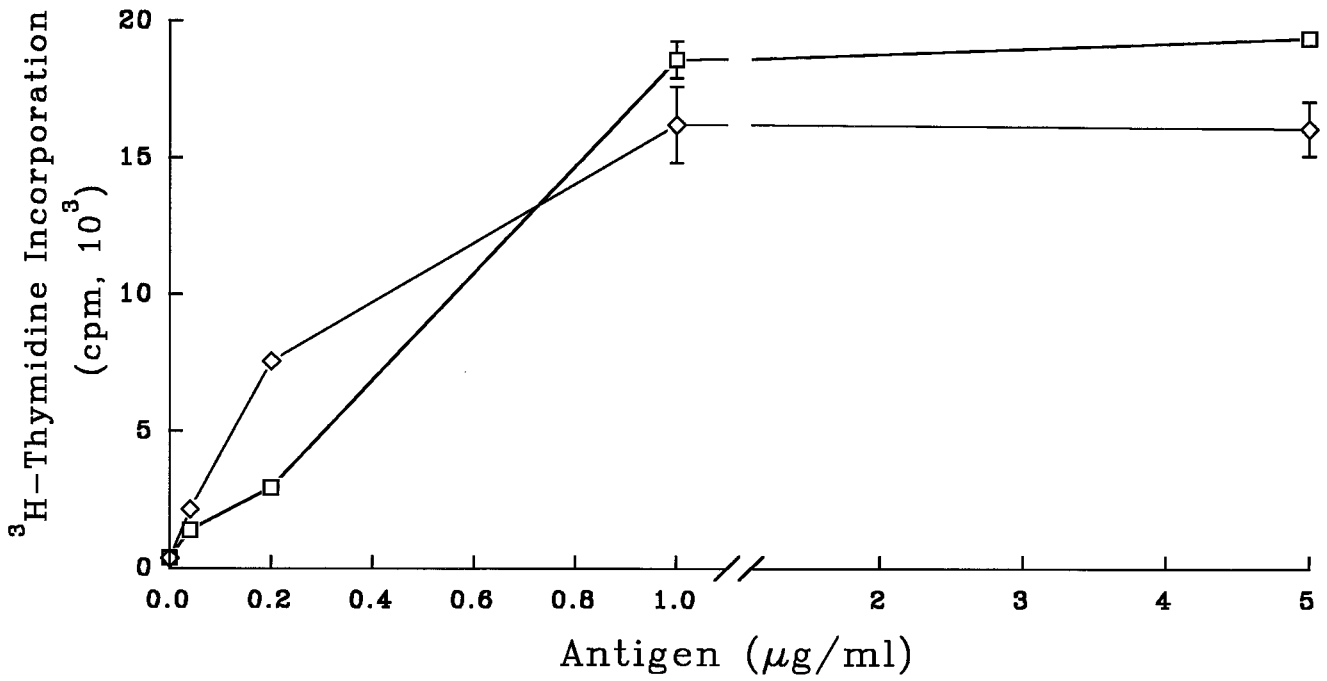

FIG. 7. Native HIS-62 and rHIS-62 stimulate the T-cell hybridoma B4IID6. T cells were incubated with increasing concentrations of native antigen (□) or rHIS-62 (◇), and supernatants were harvested 24 h later. The supernatants were assayed for interleukin 2 activity by using CTLL-2 cells. Data are means ± standard errors of the means of triplicate determinations. Results of one representative experiment of four are shown.

aerosol dose of this bacterium. Therefore, our studies combined with those regarding *L. pneumophila* provide strong evidence that the hsp/cpn 60 antigens can promote a protective immune response.

The dose that was used to vaccinate mice in this study was 400 μg, which is more than the 80 μg of native HIS-62 that was employed previously (10). The larger dose was chosen because we were uncertain whether all molecules of rHIS-62 were folded in a conformation identical to that of the native protein.

Since mammalian and microbial hsp/cpn 60 proteins may be 50% identical at the amino acid level, there is a legitimate concern that immunization with hsp/cpn 60 proteins may induce autoimmune diseases. This potential problem has been suggested by several pieces of evidence. T cells reactive to the

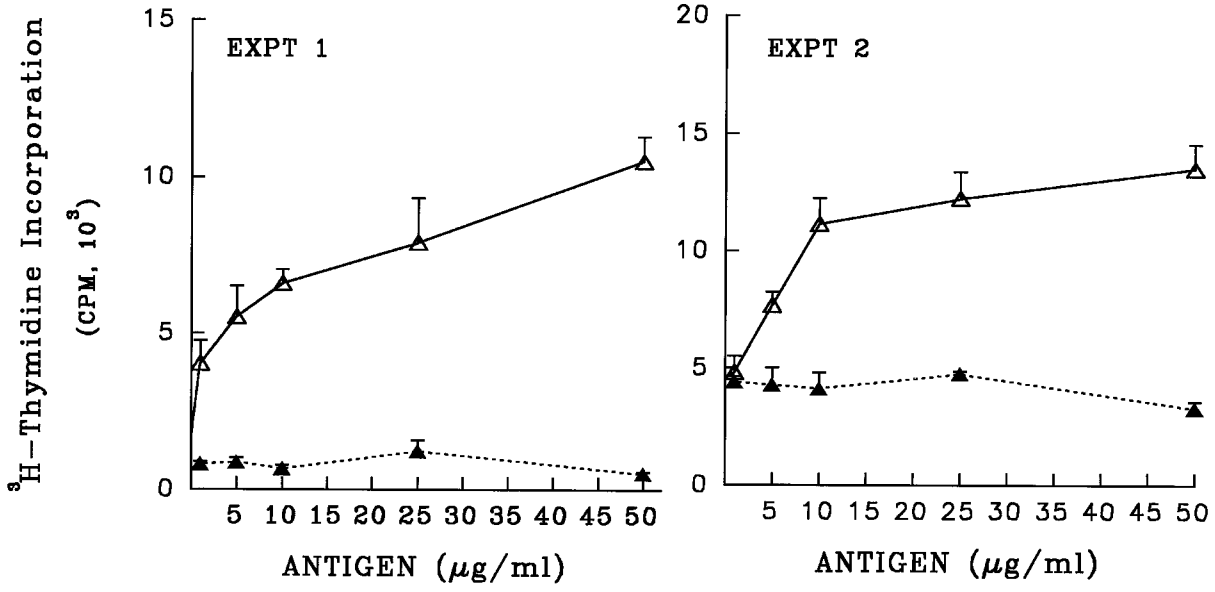

FIG. 8. rHIS-62 is a target of the cellular immune response to *H. capsulatum*. rHIS-62 was incubated with 4×10^5 splenocytes from normal mice (▲) or mice immunized with viable yeast cells (△). [^{3}H]thymidine incorporation was measured after 6 days of culture. Data are means ± standard errors of the means of triplicate determinations. Results of two of five experiments are illustrated.

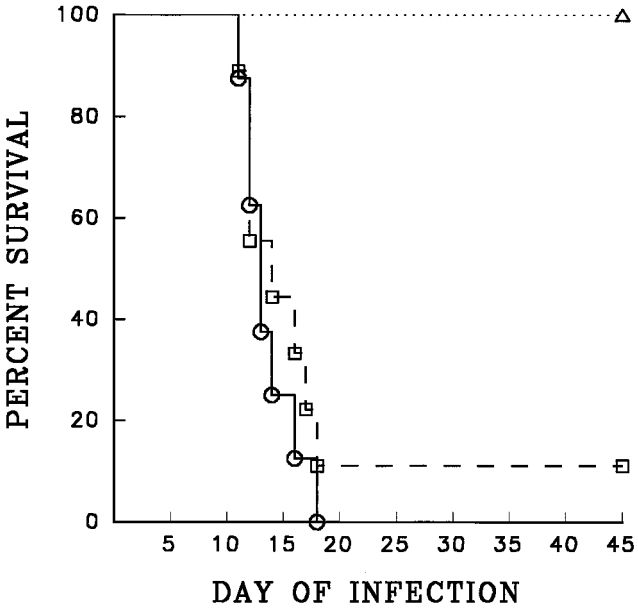

FIG. 9. Protective efficacy of rHIS-62. Groups of BALB/c mice were immunized with 400 μg of rHIS-62 ($n = 10$) (△) or with an equal amount of BSA ($n = 9$) (□). As an additional control, a group of mice ($n = 8$) (○) were not immunized. Two weeks after the last injection of antigen, the animals were challenged with 1.2×10^7 yeast cells intranasally. Survival was monitored for 45 days.

mycobacterial hsp 65-kDa antigen have been found in the synovial fluids of patients with rheumatoid and reactive arthritis (12, 23). Moreover, a T-cell clone that recognizes an epitope from the mycobacterial hsp 65-kDa antigen transfers severe adjuvant-induced arthritis to rats (28).

In contrast, abundant literature suggests that the hsp 60 family does not produce autoimmune disease. Injection of recombinant *M. leprae* hsp 60 does not cause arthritis (1). In fact, immunization with the mycobacterial hsp 65-kDa antigen protects rats from adjuvant-induced and streptococcal cell wall-induced arthritis (27, 29). Vaccination with the human 65-kDa hsp protects nonobese diabetic mice from developing autoimmune diabetes (5). Another study has demonstrated that T cells from healthy subjects respond in vitro to epitopes from human hsp 60 (21). This result suggests that an autoimmune state is not a prerequisite for recognition of self-epitopes. We have failed to observe any evidence of arthritis in animals immunized with native HIS-62 or with rHIS-62, although observation has been for only up to 3 months.

There is an apparent contradiction between the finding that HIS-62 was isolated from the water-insoluble CW/M fraction and the fact that the predicted cellular location of hsp/cpn 60 is in the cytosol in bacteria or in the mitochondrial inner space in eukaryotic cells (15, 24). The mycobacterial 65-kDa antigen is located in the cytosol when studied by immunoelectron microscopy (7), but it is associated with the water-insoluble fraction when cells are disrupted by sonication (26). In *L. pneumophila*, major cytoplasmic membrane protein is either detected in the cytosolic fraction or associated with the inner membrane (2). One explanation for the finding that hsp/cpn 60 proteins are found in the water-insoluble fraction is that they associate noncovalently with a variety of intracellular structures before and after cell disruption and thus may appear as components of several cell subfractions.

In conclusion, we have cloned, sequenced, and expressed the gene encoding a protective immunogen from *H. capsulatum* yeast cells. Furthermore, it has been demonstrated that the immunological activity of the recombinant protein is similar to that of the native protein. Future studies will endeavor to identify the smallest protein fragment that expresses the immunological activity.

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