

Research Article

Molecular and immunological characterization of *Mycobacterium avium* 65 kDa heat shock protein (Hsp65)

VIJAYA NAGABHUSHANAM,* JUDYTA PRASZKIER and CHRISTINA CHEERS

Department of Microbiology and Immunology, University of Melbourne, Victoria, Australia

Summary The heat shock protein Hsp65 has been characterized previously in several mycobacterial species. This is the first report of the complete sequence of the coding region of the *Mycobacterium avium* homologue. The sequence was highly homologous to the Hsp65 of other mycobacterial species, as well as being related closely to the murine and human homologues. Recombinant Hsp65 (rHsp65) was expressed in *Escherichia coli* to high levels and the recombinant protein tested for its immunogenicity in a murine model of *M. avium* infection. Although mice infected with *M. avium* produced antibodies that reacted with rHsp65, they showed low proliferative T-cell responses and no cytokine production in response to the same antigen. However, immunization with rHsp65 in the adjuvant dimethyloctadecylammonium bromide (DDA), induced T cells that responded to native Hsp65 with proliferation and IFN- γ production, indicating that the recombinant and native forms of the protein were antigenically similar. Therefore, the findings indicate that Hsp65 is not a dominant T-cell antigen during *M. avium* infection.

Key words: heat shock proteins, mycobacteria, recombinant antigen.

Introduction

Mycobacterial infections are major causes of morbidity and mortality worldwide. *Mycobacterium tuberculosis* is responsible for 3 million deaths and 8 million new cases a year, while *Mycobacterium leprae* infects 1.8 million people every year.¹ *Mycobacterium avium*, a common environmental organism that was once a rare infection in patients with underlying lung disease, has now emerged as the chief bacterial infection in patients with AIDS.² The live attenuated vaccine, Bacillus Calmette–Guérin (BCG), despite its variable efficacy and the danger of dissemination that it poses in immunocompromised individuals, remains the only available vaccine against mycobacteria to date. Over the past few decades, research has focused on the development of subunit vaccines, encouraged largely by experimental evidence that protective immunity can be achieved in animal models following vaccination with either culture filtrate proteins³ or single purified antigens.^{4,5} Moreover, the extensive antigenic sharing among mycobacteria raises the attractive possibility of subunit vaccines that may protect against more than one species of mycobacteria as well as circumvent the hazards of live vaccines.

Of the mycobacterial antigens, the heat shock protein Hsp65 has been identified previously as an immunodominant antigen eliciting both humoral responses and T-cell responses during mycobacterial infection. Mice immunized with live

BCG or BCG culture filtrate proteins,⁶ or with extracts of *M. leprae* or *M. tuberculosis*,⁷ have been found to produce antibodies that react with Hsp65. Furthermore, splenocytes from mice immunized with killed *M. tuberculosis*,⁸ or immunized with BCG or BCG extracts,⁹ proliferated in response to Hsp65. Mehra *et al.* found that PBMC from patients infected with *M. tuberculosis* or from PPD-positive reactors also proliferated in response to Hsp65.¹⁰ Little is known, however, of the immune response to Hsp65 during *M. avium* infection.

Immunization and evaluation of immune responses to experimental vaccines require the production of large quantities of protein. In addition to strict containment facilities required for the cultivation of pathogenic mycobacteria, these organisms grow very slowly (*M. tuberculosis*) or not at all (*M. leprae*), presenting significant obstacles for the large-scale production of mycobacterial proteins. Apart from the problem of maintaining long-term cultures, isolation of individual antigens demands cumbersome biochemical procedures, added to which the protein yield is very poor. Recombinant DNA technology has offered a viable alternative in that large quantities of well-characterized and reproducible protein may be produced.

The murine model of *M. avium* infection has been well characterized previously. Following intranasal infection, a gradual systemic spread of infection occurs, seeding organisms to liver and spleen, as well as the lung. Four to five weeks later, antigen-specific T cells are activated.¹¹ At this stage and beyond it is possible to study the *in vitro* antigen-specific responses of T cells, which makes the murine model a useful tool for the testing of potential vaccine candidates. Making use of recombinant DNA technology, the present paper describes the cloning and complete sequence of the coding region of the *M. avium* Hsp65, as well as the evaluation of the immune response to recombinant Hsp65 during the mouse infection.

Correspondence: Assoc. Prof. C Cheers, Department of Microbiology and Immunology, University of Melbourne, Vic. 3010, Australia. Email: ccheers@unimelb.edu.au

*Present address: Dr V Nagabhushanam, Division of Infectious Diseases, University of California, Rosalind Russell Arthritis Research Laboratory and Loewenstein Laboratory for Mycobacterial Research, San Francisco General Hospital, San Francisco, CA 94110, USA.

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Materials and Methods

Bacteria and vectors

Mycobacterium avium, serovar 8, is a virulent strain isolated from an AIDS patient at Fairfield Hospital (Melbourne, Vic., Australia). The bacteria were grown in Middlebrook 7H9 broth (Difco, Detroit, MI, USA) and stored in 1 mL aliquots at -70°C . Before use, the thawed bacteria were sonicated for 10 s to disperse clumps. *Listeria monocytogenes* EGD (obtained from RV Blanden, Australian National University, Canberra, ACT, Australia) from 24-h cultures on horse blood agar (HBA) was suspended in saline and the concentration adjusted by turbidity. All doses were checked by retrospective viable counts. *Escherichia coli* K12 strain JM101¹² was used for propagating bacteriophage vectors M13tg131B and M13tg130B (derivatives of M13tg131 and M13tg130,¹³ containing a unique *Bgl*III site in the polylinker), and strain JP3438 (*thr-1 leuB6 thi-1 lacY1 gal-351 supE44 tonA21 hsdR4 rpoB364 recA56*) was used for propagating derivatives of plasmid p2GEX-2T.¹⁴ *Escherichia coli* B strain BL21¹⁵ was used for expression and purification of fusion proteins from these plasmids.

Bacterial antigen and extracts

Mycobacterium avium lysate (ML) was prepared as described elsewhere.¹⁶ Heat-killed *Listeria* were prepared by heating bacterial suspensions at 60°C for 1 h and residual viable bacteria checked by sampling 1 mL onto HBA.

Recombinant DNA techniques

Plasmid and bacteriophage DNA were isolated and manipulated as described by Sambrook *et al.*¹⁷ DNA was sequenced using a Model 373 DNA Sequencer and ABI Big Dye Terminator kits (Perkin-Elmer Corp., Norwalk, CT, USA).

Extraction of *Mycobacterium avium* DNA

Mycobacterium avium was grown to mid-log phase in 200 mL Middlebrook 7H9 broth at 37°C with continuous stirring. Cycloserine (Sigma, St Louis, MO, USA) was added to a concentration of 1 mg/mL and the culture incubated for a further 48 h. The bacteria were washed thoroughly in PBS, resuspended in 4 mL of lysis buffer (15% sucrose; 0.05 mol/L Tris, pH 8.5; 0.05 mol/L EDTA; 1 mg/mL lysozyme) and incubated for 30 min at 37°C . Proteinase K (Sigma) and SDS (Sigma) were added to a final concentration of 100 $\mu\text{g}/\text{mL}$ and 4%, respectively, and the volume made up to 10 mL with TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0). The suspension was incubated at 37°C for 30 min, then at 70°C for 5 min, extracted with phenol-chloroform and precipitated with ethanol. The pellet was resuspended in TE buffer, treated with RNaseA (Worthington Biochemical Corp., Lakewood, New Jersey, USA) and Proteinase K (Sigma), then extracted with phenol-chloroform three times and precipitated with ethanol.

Design of primers for the amplification of the gene encoding Hsp65

The sequences of *hsp65* genes from various mycobacterial species were obtained from GenBank, aligned using Clustal V and used to design primers spanning the predicted coding region of the *M. avium* *hsp65*. The forward primer, 5'CCGAGATCTATGGCCAAGACAATTGCGTACGAC-3', introduces a *Bgl*III restriction site (underlined) upstream of the ATG initiation codon (shown in bold), whereas the reverse primer, 5'TCCGAATTC TCAGAARTCCATRCRCCCATG-3' introduces an *Eco*RI site downstream of the stop

codon. Because of sequence heterogeneity at the 3' ends of the *hsp65* gene of mycobacterial species, the antisense primer was designed with 'wobbles' (where 'R' represents either G or C) at three positions in the sequence.

Construction of recombinant plasmid p2GEX-hsp65

The open reading frame of the *hsp65* gene of *M. avium* was amplified by PCR using 1 μg of *M. avium* chromosomal DNA, Taq polymerase (Pharmacia Biotech, Boronia, Vic., Australia) and the forward and reverse primers described earlier. Following incubation at 94°C for 5 min and then 25 cycles of 94°C for 15 s, 50°C for 15 s, and 72°C for 3 min, the PCR products were precipitated with ethanol and purified by gel electrophoresis in low-melting agarose. The purified PCR product was inserted into the *Eco*RI-*Bgl*III site of M13tg131B. Two clones each, from three independent PCR reactions, were sequenced and four found to be identical. The insert from one of these four clones was re-cloned into M13tg130B, which facilitated sequencing of the complementary strand of the insert. The *Bgl*III-*Eco*RI fragment containing the coding sequence of the *hsp65* gene was moved from the M13tg130B derivative into the *Bam*HI/*Eco*RI site of p2GEX-2T so that the *hsp65* reading frame was fused in-frame to the 3' end of one of the two copies of the glutathione-S-transferase (GST) of *Schistosoma japonicum*.

Expression and purification of Hsp65

Escherichia coli strain BL21, transformed with the recombinant plasmid p2GEX-hsp65, was grown in Luria broth supplemented with 100 $\mu\text{g}/\text{mL}$ ampicillin at 30°C to an OD_{600} of 0.8. Expression of the GST fusion was induced by the addition of 0.1 mmol/L isopropylthiogalactoside (IPTG; Sigma). After 4 h, bacteria were harvested, washed in PBS and lysed by sonication. The cell lysate was cleared by centrifugation and the separated cell pellet and supernatant analysed on a 12% SDS-PAGE.¹⁸ The supernatant, which contained the majority of the fusion protein (GST-Hsp65) was filtered through a 0.22 μm filter (Millipore; Millipore Corp., Bedford, MA, USA) and the GST fusion protein purified by binding to glutathione agarose beads.¹⁹ To cleave Hsp65 from GST, the beads were incubated overnight at room temperature with thrombin (Pharmacia) (10 U/mg of protein) in 10 mL of PBS and then pelleted. The supernatant was collected and the protein content was estimated spectrophotometrically using extinction at 205 nm, 260 nm and 280 nm.

Western blotting of *Mycobacterium avium* proteins

Following electrophoretic separation on a 12% reducing or non-reducing PAGE, proteins were transferred onto a nitrocellulose membrane (Millipore) using a semidry transfer system (CBS Scientific Company Inc., Del March, CA, USA). The membrane was blocked with 5% skim milk in PBS and then incubated with 1:1000 dilution of anti-Hsp65 monoclonal CS-44 (kindly supplied by Dr J. Belisle, Colorado State University, CO, USA) in 0.5% skim milk in PBS for 1 h. The blot was developed in goat antimouse horseradish peroxidase (HRP) conjugate (Silenus, Melbourne, Vic., Australia) followed by diaminobenzidine (Sigma).

Infection and immunization of mice

Six to eight-week-old BALB/c mice were pedigree bred and maintained under conventional but infection-free conditions at the Department of Microbiology and Immunology, University of

Melbourne (Melbourne, Vic., Australia). They were infected intranasally as described elsewhere²⁰ with 10^5 *M. avium* or 6×10^3 *L. monocytogenes* in 50 μ L PBS. For immunization, dimethyldioctadecylammonium bromide (DDA; Acros Organics, Brussels, Belgium) was made up to a concentration of 25 mg/mL in pyrogen-free water and heated to 80°C to form a gel.²¹ The gel was mixed immediately with an equal volume of antigen at a concentration of 1 mg/mL. Mice were immunized intradermally at the base of the tail with 20 μ L of the mixture, each mouse receiving 10 μ g of antigen and 250 μ g of DDA. Control mice were immunized with 250 μ g of DDA in saline. Mice were sacrificed by CO₂ narcosis 10 days later and the draining inguinal lymph nodes and spleens removed aseptically.

Detection of anti-Hsp65 antibodies during *Mycobacterium avium* infection

Flat-bottomed, 96-well microtitre plates (Nunc, Riskilde, Denmark) were coated with 50 μ L at 5 μ g/mL of either *M. avium* lysates or recombinant Hsp65 in carbonate-coating buffer (pH 9.1) at 4°C overnight. The plates were washed three times in PBS with Tween 20 and blocked with 2% FCS (Life Technologies, Gaithersburg, MD, USA) in PBS for 1 h at 37°C. Serum collected from *M. avium*-infected or uninfected BALB/c mice was added in serial twofold dilutions, in PBS, and the plates incubated for 2 h at 37°C. The plates were washed and antimouse HRP conjugate (Silenus) added at 1:1000 dilution and incubated for 1 h at 37°C. The ELISA was developed using 3,3',5,5'-tetramethylbenzidine (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) and the optical density read at 450 nm using a Multiskan microplate reader (Multiskan, MCC, Helsinki, Finland).

Preparation of lymph node cells

Mediastinal lymph nodes from 6-week-old *M. avium*-infected mice or 7-day-old *Listeria*-infected mice were pooled from five mice per group. Single cell suspensions were centrifuged over Ficoll-Histopaque (Sigma, Castle Hill, NSW, Australia) to isolate viable cells. Cells were washed and adjusted to a concentration of 2×10^6 cells/mL in DMEM (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated FCS.

T-cell proliferation assay

Lymph node cells were cultured in 96-well flat-bottomed plates (Nalgene Nunc Int., Mt Waverly, Vic., Australia) at 2×10^6 /mL in 200 μ L volumes with or without antigen. Cellular proliferation was assayed after 72 h by pulsing cultures with 37 mBq of [³H]thymidine (³HTdR) (Amersham, Buckinghamshire, UK) for the last 6 h of culture. The cells were harvested using a Micro 96 harvester (Skatron Instruments, Tranby, Norway) and the incorporated radioactivity measured using a Packard Matrix direct β counter 9600 (Packard, Meriden, CT, USA). For the inhibition of T-cell subsets, 10 μ g/mL of either anti-CD4 (GK 1.5)²² or anti-CD8 (3.168)²³ mAb were added 30 min before the addition of antigen.

In vitro production of cytokines

Frequency of IFN- γ - or IL-4-producing cells was measured using ELISPOT. Maxisorb plates (Nalgene Nunc), 96 wells, were coated overnight at 4°C with HB170 (anti-IFN- γ) or 11B11 (anti-IL-4) in carbonate-coating buffer, pH 9.1. After washing and blocking, cells were added (2×10^5 , 1×10^5 , 5×10^4 , 2.5×10^4 per well in triplicate) and incubated with antigen for 72 h. The cells were washed off and

bound cytokines detected with XMG 1.2 (anti-IFN γ) or BVD6 (anti-IL-4) conjugated to biotin, followed by streptavidin alkaline phosphatase. ELISPOTS were developed using the substrate 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium (Sigma, Castle Hill, NSW, Australia). All antibodies were used at predetermined optimal dilutions. The spots were counted under a bright light with 10 \times magnification. Alternatively, biologically active IFN- γ released into the supernatant of equivalent cultures was measured by its ability to inhibit the proliferation of WEHI 279 cells.²⁰

Statistical analysis

The statistical significance of experimental data was determined using Student's *t*-test. Differences with $P < 0.05$ were considered significant.

Results

Cloning, sequencing and expression of the *Mycobacterium avium* hsp65

Initial attempts at lysing *M. avium* cells to obtain template DNA using osmotic lysis or caesium chloride were unsuccessful. Mizuguchi and Tokunaga have described the use of the mycobacteriostatic antibiotic, cycloserine, for the disruption of mycobacterial cell walls and the subsequent extraction of DNA from rapid-growing mycobacterial species.²⁴ The *M. avium* culture was therefore grown to mid-log phase and incubated with cycloserine for a further 48 h, which is when the mycobacterial cell wall can be easily disrupted and DNA extracted. Although the extracted DNA was sheared, the major portion being approximately 22 kb, it was sufficiently intact for the amplification of the *hsp65* gene. Using primers based on the published sequences for *M. tuberculosis*,²⁵ *M. paratuberculosis*,²⁶ *M. leprae*²⁷ and *M. bovis BCG*²⁸ with 'wobbles' to account for their differences, the 1.6 kb gene encoding the *M. avium* Hsp65 was amplified using PCR. As Taq polymerase, which was employed in the amplification of *hsp65* from *M. avium*, is known to cause occasional errors, three independent PCR reactions were performed. The PCR products from each reaction were cloned, separately, into M13tg131B and two clones from each reaction were sequenced. The clones from two of the three PCR reactions (reactions 1 and 3) were identical, whereas the clones from PCR reaction 2 had a number of base substitutions. Therefore, the *hsp65* DNA insert from one of the four clones, from reactions 1 and 3, was moved into the bacteriophage vector M13tg130B, which facilitated sequencing of the complementary strand.

The DNA sequence was found to be very similar to those of the four mycobacterial species (*M. tuberculosis*, *M. bovis BCG*, *M. leprae* and *M. paratuberculosis*), sharing 97% identity with the *M. paratuberculosis* homologue (Table 1). Consequently, the predicted 541 amino acid sequence was very similar to the other mycobacterial 65 kDa heat shock proteins, having the characteristic low number of aromatic residues, the total absence of cysteine and the typical Gly-Gly-Met motif at the C-terminus. The protein sequence was found to share a 93% homology with *M. tuberculosis* (Fig. 1). Residues that were different on alignment with *M. tuberculosis* appeared at the corresponding positions in the other mycobacterial species, with the exception of histidine at

Table 1 Comparison of DNA and protein sequences of mycobacterial and mammalian homologues of Hsp65

Organism/species	% DNA homology	% Protein homology	GenBank Accession No.
<i>Mycobacterium paratuberculosis</i>	97	97	U15989
<i>Mycobacterium tuberculosis</i>	90	93	M15467
<i>Mycobacterium bovis</i> BCG	89	92	M17705
<i>Mycobacterium leprae</i>	87	92	M14341
Human Hsp60	41	46	M34664
Murine Hsp60	42	47	X55023

Figure 1 Clustal V alignment of *Mycobacterium avium* and *M. tuberculosis* (M. tuberc) Hsp65 sequences, showing 93% sequence identity. Histidine at position 467 (indicated by Δ) is the only amino acid unique to this strain of *M. avium*; all other substitutions are found in *M. leprae*, *M. bovis* BCG or *M. paratuberculosis* Hsp65. Asterisks between the two sequences indicate sequence identity, whereas dots indicate conservative substitutions. The sequence has been submitted to GenBank (Accession no. AF281650).

		10	20	30	40	50	60
<i>M. avium</i>		MAKTIAYDEEARRGLERGLNALADAVKVTLGPKGRNVVLEKKWGAPTITNDGVSIAKEIE					
<i>M. tuberc</i>		MAKTIAYDEEARRGLERGLNALADAVKVTLGPKGRNVVLEKKWGAPTITNDGVSIAKEIE					

		70	80	90	100	110	120
<i>M. avium</i>		LEDPYEKIGAEVKEVAKKTDVAGDGTTTATVLAQALVREGLRNVAAGANPLGLKRGIE					
<i>M. tuberc</i>		LEDPYEKIGAEVKEVAKKTDVAGDGTTTATVLAQALVREGLRNVAAGANPLGLKRGIE					

		130	140	150	160	170	180
<i>M. avium</i>		KAVEKVTETLLKSAKEVETKDQIAATAAISAGDQSIGDLIAEAMDKVGNEGVITVEESNT					
<i>M. tuberc</i>		KAVEKVTETLLKSAKEVETKDQIAATAAISAGDQSIGDLIAEAMDKVGNEGVITVEESNT					

		190	200	210	220	230	240
<i>M. avium</i>		FGLQLELTEGMRFDKGYISGYFVTDARQEAVLEDPFILLVSSKSVSTVKDLLPLEKVIQ					
<i>M. tuberc</i>		FGLQLELTEGMRFDKGYISGYFVTDARQEAVLEDPYILLVSSKSVSTVKDLLPLEKVIQ					

		250	260	270	280	290	300
<i>M. avium</i>		AGKPLLI AEDVEGEALSTLVVNKIRGTFKSVAVKAPGFGDRRKAMLQDMAIITGGQVIS					
<i>M. tuberc</i>		AGKPLLI AEDVEGEALSTLVVNKIRGTFKSVAVKAPGFGDRRKAMLQDMAIITGGQVIS					

		310	320	330	340	350	360
<i>M. avium</i>		EEVGLSLESADISLLGKARKVVVTKDETTIVEGAGDSDAIAGRVAQIRTEIENSDDSYDR					
<i>M. tuberc</i>		EEVGLTLENADLSLLGKARKVVVTKDETTIVEGAGDTDAIAGRVAQIRTEIENSDDSYDR					

		370	380	390	400	410	420
<i>M. avium</i>		EKLQERLAKLAGGVAVIKAGAATEVELKERKHRIEDAVRNAKAAVEEGIVAGGGVALLHA					
<i>M. tuberc</i>		EKLQERLAKLAGGVAVIKAGAATEVELKERKHRIEDAVRNAKAAVEEGIVAGGGVALLHA					

		430	440	450	460	470	480
<i>M. avium</i>		IPALDELKLEGDEATGANIVRVALEAPLKQIAFNNGGLEPGVVAEKVHNSPAGTGLNAATG					
<i>M. tuberc</i>		APTLDLKLKLEGDEATGANIVRVALEAPLKQIAFNNGGLEPGVVAEKVHNSPAGTGLNAATG					
		*.*****Δ* ** *					
		490	500	510	520	530	540
<i>M. avium</i>		EYEDLLKAGVADPVKVTRSALQNAAS IAGLFLTTEAVVADKPEKAAAPAGDPTGGMGMD					
<i>M. tuberc</i>		VYEDLLAAGVADPVKVTRSALQNAAS IAGLFLTTEAVVADKPEKASVPG-GGDMGMD					

position 467, which appears to be unique to *M. avium*, at least to this particular strain. Hsp65 is conserved in evolution and the amino acid sequences of the murine²⁹ and human homologues³⁰ were found to share a 46–47% homology with the *M. avium* Hsp65 (Table 1).

When the expressed protein was analysed on SDS-PAGE, the fusion protein migrated at the expected molecular weight of 91 kDa. The fusion protein was affinity-purified using glutathione agarose beads and the recombinant Hsp65 (rHsp65) cleaved from GST using thrombin. The cleaved protein migrated at the expected molecular weight (Fig. 2).

Comparison of native and recombinant Hsp65

Mycobacterial lysate was used as a source of native Hsp65. When analysed for the presence of Hsp65 by SDS-PAGE followed by western blotting with the CS-44 mAb, ML was

found to contain detectable levels of native Hsp65 (Fig. 3a,b). Furthermore, the native form of the protein migrated at the same molecular weight as the recombinant protein, under both reducing and non-reducing conditions (Fig. 3b).

Immune responses to recombinant Hsp65 during Mycobacterium avium infection

To determine if rHsp65 was recognized by the humoral arm of the immune response, serum from *M. avium*-infected or uninfected BALB/c mice was tested for reactivity with rHsp65 using ELISA. As shown in Fig. 4, serum from mice infected with *M. avium* for different lengths of time developed antibodies that reacted with rHsp65.

Although the humoral response is a means of determining antigenic targets during infection, it does not protect against mycobacterial infection, the control of which requires the

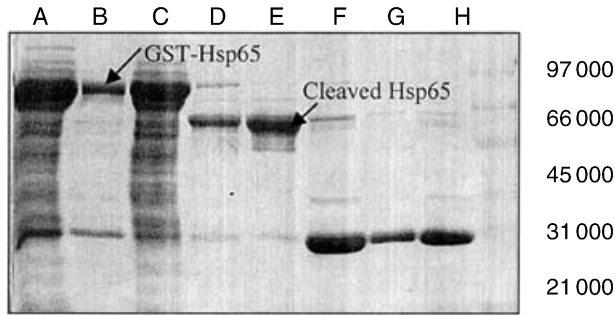


Figure 2 Sodium dodecyl sulfate-PAGE analysis of *hsp65* gene products on induction at 30°C. Lane A, supernatant from *Escherichia coli* lysate; lane B, fusion protein bound to beads (glutathione-S-transferase (GST)-Hsp65); lane C, unbound protein; lane D, Hsp65 cleaved from GST; lane E, residual Hsp65 recovered in wash; lanes F-H, GST, three successive elutions (GST); Lane I, molecular weight markers.

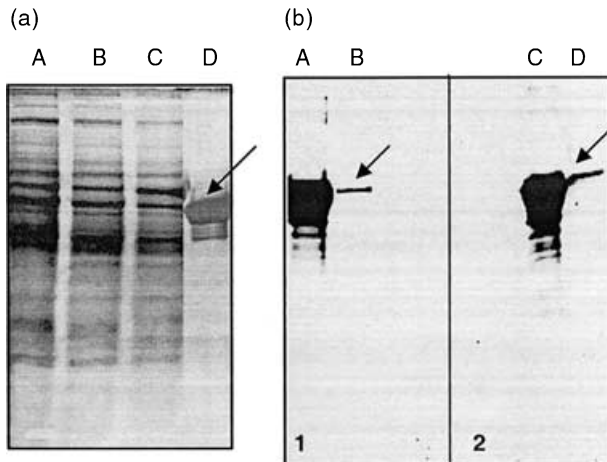


Figure 3 Comparison of native and rHsp65. (a) 12% SDS-PAGE of purified rHsp65 and *Mycobacterium (M.) avium* lysate. Lanes A-C, *M. avium* lysate; lane D, purified rHsp65. The position of Hsp65 is indicated. (b) Western blot with anti-Hsp65 mAb. Lane A, *M. avium* lysate; lane B, rHsp65, 12% reducing SDS-PAGE; lane C, *M. avium* lysate; lane D, rHsp65, 12% non-reducing SDS-PAGE. The position of Hsp65 is indicated.

activation of antigen-specific T cells.³¹ To determine if rHsp65 was recognized by T cells, mediastinal lymph nodes cells from *M. avium*-infected mice were cultured with rHsp65 and assayed in an *in vitro* proliferation test. As mediastinal lymph nodes are not detectable in uninfected mice, mice were infected intranasally with *L. monocytogenes* to obtain control lymph nodes that served as an irrelevant infection for the specificity of responses to rHsp65. The specificity of the two infections was first confirmed by proliferation of mediastinal lymph node cells in response to their respective antigens (Fig. 5a). On stimulation with rHsp65, cells from *M. avium*-infected mice proliferated specifically (Fig. 5b). Although the responses were small in magnitude, they were specific. Those cells that did proliferate appeared to be CD4⁺ T cells, as the preincubation of lymph node cell cultures with anti-CD4

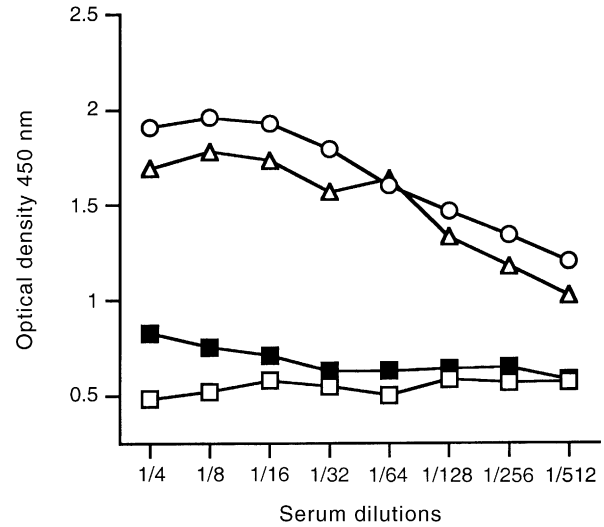


Figure 4 Antibody response during *Mycobacterium avium* infection. Serum was collected from BALB/c mice (five mice per group) at (□) 0 weeks, (■) 6 weeks, (○) 12 weeks and (△) 16 weeks postinfection with 10⁵ *M. avium* and tested for reactivity with rHsp65 by ELISA.

mAb abrogated antigen-induced proliferation to the extent of 50%, whereas anti-CD8 mAb had little effect on proliferation (Fig. 5c). Although the lymph node cells from infected mice proliferated in response to rHsp65, cytokine production (IFN- γ or IL-4) by these cells was undetectable (Fig. 5d).

To determine if native Hsp65 in ML could stimulate mice immunized with rHsp65, inguinal lymph nodes were removed from mice immunized intradermally with 10 μ g rHsp65 in 250 μ g of DDA, a known inducer of Th1 responses.²¹ Lymph node cell suspensions from mice immunized with rHsp65 in DDA proliferated in response to stimulation with ML (Fig. 6a). Additionally, ML recalled significant amounts of IFN- γ from mice immunized with the rHsp65, indicating that ML contained sufficient quantities of native Hsp65 that could stimulate IFN- γ production from T cells primed with rHsp65 (Fig. 6b).

Discussion

This article describes the cloning, expression and immunological properties of the Hsp65 of *M. avium*. The cloning and expression of mycobacterial proteins pose a number of problems. Many are expressed poorly in *E. coli*, which is the organism of choice for the production and purification of recombinant bacterial proteins. The difference in codon usage between mycobacteria and *E. coli* is thought to be at least in part responsible for the low level of expression of mycobacterial genes.³² In addition, because many mycobacterial proteins are characteristically highly hydrophobic, they may not be expressed well or may be toxic to *E. coli*.³³ Examination of the sequence of *M. avium hsp65* showed that its Codon Adaptation Index (CAI) for *E. coli* was 0.324. The CAI is a measure of synonymous codon bias and is thought to give an indication of the likelihood of successful expression of a heterologous gene in *E. coli*. Although the CAI of Hsp65 is lower than the CAI for the highly expressed genes

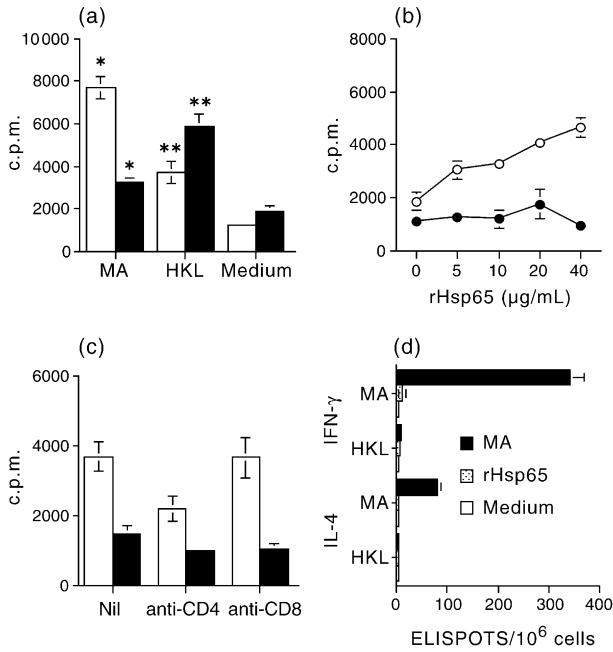


Figure 5 Response of T cells from *Mycobacterium avium*-infected mice. (a) Specificity of T-cell response. Mediastinal lymph nodes from mice infected for 8 weeks with *M. avium* (□) or 10 days with *Listeria* (■) were cultured with 5×10^6 live *M. avium* (MA), 6×10^6 heat-killed *Listeria* (HKL), or without antigen. Proliferation was measured by uptake of ³HTdR after 72 h. Data are expressed as the mean of triplicate cultures. Error bars correspond to \pm SD. *Significant difference in proliferation between lymph node cells from *M. avium*-infected and *Listeria*-infected mice in response to live *M. avium* ($P < 0.002$). **Significant difference in proliferation between lymph node cells from *Listeria*-infected and *M. avium*-infected mice in response to heat-killed *Listeria* ($P < 0.001$). (b) Proliferation of T cells in response to Hsp65. Nodes from mice infected for (○) 8 weeks with *M. avium* or (●) 10 days with *Listeria* were cultured with varying concentrations of rHsp65. Proliferation was measured by uptake of ³HTdR after 72 h. Data are expressed as the mean of triplicate cultures. Error bars correspond to \pm SD. (c) Phenotype of responding cells. Mediastinal lymph node cells (2×10^6 /mL) from mice infected for (□) 8 weeks with *M. avium* or (■) 10 days with *Listeria* were incubated in 200 μ L volumes with 10 μ g/mL anti-CD4 or anti-CD8 mAb, for 30 min. rHsp65 (20 μ g/mL) was added to cultures and proliferation was measured by the uptake of ³HTdR, after 72 h. Data are expressed as the mean of triplicate cultures. Error bars correspond to \pm SD. *Significant inhibition of proliferation with anti-CD4 mAb ($P < 0.002$). (d) Frequency of IFN- γ - and IL-4-producing cells in response to Hsp65. Mediastinal nodes from mice infected for 8 weeks with *M. avium* or 10 days with *Listeria* were cultured with live *M. avium* or 20 μ g/mL rHsp65. The frequency of IFN- γ - or IL-4-producing cells was determined at 72 h by ELISPOT. Data, expressed as ELISPOTS/10⁶ cells, are the mean of triplicate cultures. Error bars correspond to \pm SD.

of *E. coli*, it is noteworthy that most of the rarest codons are not used (AGA, AGG, CGA, AUA) or used infrequently (CGG, GGA, GGG). Furthermore, the Hsp65 protein of *M. avium* is not highly hydrophobic, which may have

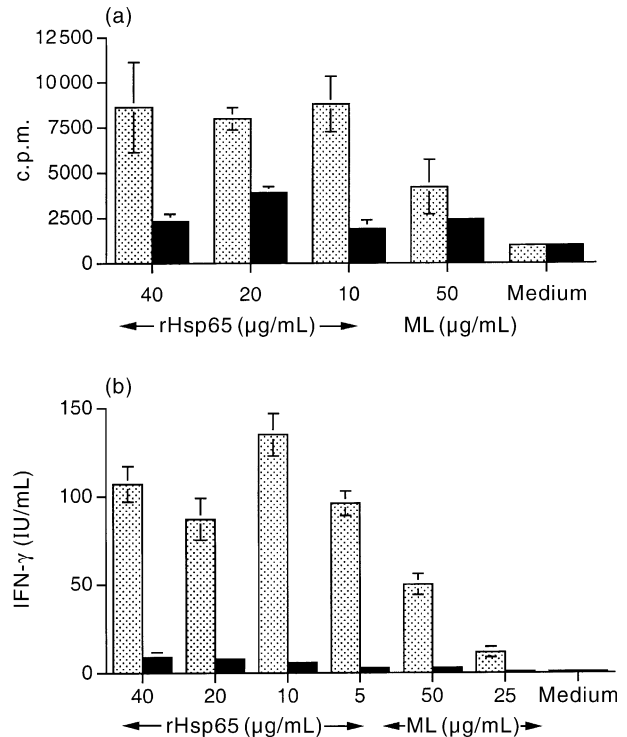


Figure 6 Cross-reactivity between native and rHsp65. (a) Mice were immunized with (□) 10 μ g rHsp65 in 250 μ g dimethyldioctadecylammonium bromide (DDA) or with (■) DDA alone. The inguinal lymph nodes were collected 10 days later and cultured with varying concentrations of rHSP65 or *Mycobacterium avium* lysate. Proliferation was measured by the uptake of ³HTdR after 72 h. Data are expressed as the mean of triplicate cultures. Error bars correspond to \pm SD. (b) Mice were immunized with (□) 10 μ g rHsp65 in 250 μ g DDA or with (■) DDA alone. The inguinal lymph nodes were collected 10 days later and cultured with varying concentrations of rHSP65 or *M. avium* lysate. IFN- γ was detected in culture supernatants 72 h later. Data, expressed as International Units/mL, are the mean of triplicate cultures. Error bars correspond to \pm SD.

contributed to its relatively high level of expression in the GST fusion system described here.

Isolation of genomic DNA from mycobacteria is made difficult by the tough mycobacterial cell wall, which makes the organism resistant to lysis. Harsh mechanical methods, such as sonication, often result in sheared or poor quality DNA. Therefore, we used cycloserine to facilitate enzymatic lysis and extraction of DNA. This method has been used previously to extract DNA from rapid-growing mycobacterial strains and nocardia species, which share the cell wall characteristics of mycobacteria.^{24,34}

The *M. avium* Hsp65 bore a striking similarity to the homologous proteins from other mycobacterial species, showing a 93% amino acid homology with the pathogenic *M. tuberculosis* and a higher 97% similarity to *M. paratuberculosis*, which is now considered to be part of the *M. avium* complex. There was also almost 50% homology with human and murine homologues. Although antibodies to rHsp65 were detected in *M. avium*-infected mice, the T-cell

response was marginal, with some proliferative response but no detectable cytokines, either Th1 (IFN- γ) or Th2 (IL-4).

Hsp65 was chosen for the present study because it has been previously identified as an immunodominant antigen during human tuberculosis infection,¹⁰ as well as in mice immunized with killed *M. leprae* or *M. tuberculosis* or their protein extracts.^{6,8} Therefore, it was surprising to find that mice infected with *M. avium* showed such a poor T-cell response. We considered whether the antigen produced in its native state differs from that expressed in *E. coli*. One possible explanation involved differences in the oligomeric forms of the recombinant and native forms of the antigen. This theory was suggested to explain the fact that, whereas the native *M. tuberculosis* GroES protein stimulated both T-cell proliferation and production of IFN- γ from PBMC obtained from PPD-positive individuals, the recombinant form was not capable of eliciting similar responses.³⁵ However, SDS-PAGE analysis of the *M. avium* rHsp65 did not reveal any difference in migration, which would result from such oligomers. Another possibility rests with the fact that mycobacteria handle post-translational modifications of proteins, such as the addition of carbohydrates or lipids, differently from *E. coli*. Some mycobacterial proteins are glycosylated,³⁶ which is not characteristic of *E. coli* proteins. Deglycosylation of the 45 kDa *M. tuberculosis* antigen significantly reduced its ability to stimulate lymphocytes from *M. tuberculosis*-infected guinea pigs.³⁶ However, as T cells from mice immunized with rHsp65 were able to recognize native Hsp65, there seemed to be no difference in antigenicity between the two. We concluded that few T-cell clones were stimulated during infection, and that Hsp65 is not a dominant T-cell antigen during this infection.

This notion is in agreement with observations made during tuberculosis experiments. The proliferative response to Hsp65 appears to be higher in mice immunized with heat-killed organisms than in infected mice. Proliferative responses to rHsp65 were observed initially by Kaufmann *et al.*, who found that in limiting dilution assay, 10–20% of *M. tuberculosis*-specific cells in mice immunized with killed *M. tuberculosis* recognized Hsp65.⁸ Andersen *et al.* observed that responses by mice immunized with heat-killed *M. tuberculosis* were superior to those by mice infected with the live organisms.³⁷ Orme *et al.* confirmed that rHsp65 did not stimulate strong proliferative responses in mice infected with live *M. tuberculosis*.³⁸ The likely explanation was that Hsp65, being largely cytoplasmic and cell wall-associated, was released by dying organisms and was a marker of bacterial autolysis. Hence, immunization with killed organisms contained larger quantities of rHsp65 and stimulated a larger population of Hsp65-specific T cells. This is the most likely explanation for the low proliferative and IFN- γ responses by lymph node cells obtained from *M. avium*-infected mice to rHsp65 in the present study.

Nevertheless, it is noteworthy that protection against experimental infection has been achieved by immunization with mycobacterial Hsp65 alone. Interestingly, initial attempts to immunize mice with recombinant protein failed to induce protection comparable with that induced by killed *M. leprae*.³⁹ More recent experiments have involved Hsp65 expressed endogenously within a macrophage line J774 which, when injected into mice, afforded strong (100 \times)

protection against experimental tuberculosis.⁴⁰ DNA vaccines encoding Hsp65 also have been found to confer good protection against experimental *M. tuberculosis* infection.⁴¹

Mycobacteria are known for their extensive sharing of antigens,⁴² as we have observed for the *M. avium* 65 kDa antigen. It has been suggested that using such shared antigens would allow for immune responses to be extrapolated from one species to another, such as *M. leprae* and *M. avium*. However, the use of Hsp65 as a vaccine candidate must be viewed with caution. In the sequence analysis of the *M. avium* we found that the predicted protein sequence of Hsp65 shared 46% and 47% sequence similarity with the human and murine homologues, respectively. The conserved nature of the protein suggests that immunization with Hsp65 has the potential to generate autoreactive T cells and trigger auto-immune responses.⁴³ Therefore, while evidence that immunization with Hsp65 protects against experimental murine tuberculosis suggests that it is a convenient antigen to use for the study of new adjuvants and immunization strategies, its potential role in autoimmunity implies that its practical use is limited. Any role would be dependent on the use of protective antigenic epitopes. We are currently in the process of defining relevant epitopes on Hsp65.

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