BACTERIAL PATHOGENICITY

Recombinant GroES in combination with CpG oligodeoxynucleotides protects mice against *Mycobacterium avium* infection

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The *groES* gene of *Mycobacterium avium* strain 485 was cloned and expressed in *Escherichia coli* and the recombinant GroES protein was purified by affinity chromatography. The GroES preparation showed high purity by electrophoresis and immunoblotting. Immuno-electron microscopy showed that GroES was located both in the cytoplasm and on the surface of the mycobacterial cells and thus is readily available to interact with the host immune system. BALB/c mice were immunised intranasally with recombinant GroES, alone or in combination with a synthetic oligodeoxynucleotide containing unmethylated CpG motifs, and tested for protection against infection with *M. avium*. Neither GroES nor CpG alone provided any protection against subsequent challenge with *M. avium*, whereas a combination of the two significantly protected the lungs and spleen against colonisation by *M. avium* after intranasal challenge with a low dose of the organism. This indicates that intranasal administration of GroES and CpG oligodeoxynucleotides increases the resistance of BALB/c mice to *M. avium* infection.

Introduction

Organisms belonging to the *Mycobacterium avium* complex are environmental mycobacteria that can cause disseminated infections in AIDS patients and pulmonary disease in individuals without predisposing conditions [1]. *M. avium* infections are thought to occur by colonisation of the respiratory and gastrointestinal tracts [2, 3], e.g., from aerosols generated by showers and from natural waters in which *M. avium* can reach concentrations of up to 1000 cfu/ml [4]. Cellular responses play a role in the development of immunity to *M. avium* infections but only a few antigens of the organism have been cloned and characterised. One of these is GroES, a 10-kDa heat-shock protein (HSP 10) which interacts functionally with GroEL (HSP 65) in protein folding and assembly [5, 6]. *M. tuberculosis* GroES is known to be produced

extracellularly because it can be recovered from the conditioned medium of bacterial cultures [7, 8].

In man, GroES appears to be an immunodominant antigen as it induces antibodies and T-cell proliferation in patients with leprosy and tuberculosis [9]. However, much less is known about the potential role of GroES proteins as protective immunogens. For instance, intradermal immunisation of mice with recombinant *M. leprae* GroES, emulsified in Freund's incomplete adjuvant, has been shown to protect animals against multiplication of *M. leprae* [10]. Furthermore, mucosal immunisation with a recombinant *Helicobacter pylori* GroES-like protein and cholera toxin has been shown to protect mice against *H. pylori* infection [11].

In recent studies, synthetic oligodeoxynucleotides containing an unmethylated CpG dinucleotide flanked by two 5'-purines and two 3'-pyrimidines have been reported to be potent adjuvants for intranasally administered proteins [12, 13]. CpG-containing sequences induce Th1-like responses and can be superior to cholera toxin in inducing immune responses [14].

However, no studies have been reported on the protective activity of mycobacterial proteins combined with CpG adjuvants. For this reason, the *groES* gene of *M. avium* was cloned and expressed in *Escherichia coli* and the recombinant GroES protein and a CpG oligodeoxynucleotide were investigated for their ability to protect mice against *M. avium* infection.

Materials and methods

Micro-organism

M. avium strain 485 was isolated from the blood of an AIDS patient [15]. Smooth transparent colonies, which are known to be associated with high virulence and resistance to antimicrobial agents, were used [1].

Cloning of the *M. avium groES* gene

M. avium cells were grown in Middlebrook 7H9 broth (Difco) at 37°C, with agitation, to an optical density of 0.2 at 500 nm (OD₅₀₀) and used for the preparation of chromosomal DNA [16]. A pair of degenerate primers corresponding to residues 8–14 and 79–85 of the *M. tuberculosis* GroES protein [17] were used to amplify a portion of the *M. avium groES* gene by PCR. The PCR product (234 bp) was sequenced on both strands by the dideoxy-chain termination method [18] to verify its specificity and was used as a probe in Southern hybridisation experiments [16]. When *M. avium* genomic DNA was digested with restriction endonuclease *Pvu*II (New England Biolabs), a single positive signal at c. 800 bp was obtained. Accordingly, *Pvu*II-restricted chromosomal DNA was cloned in the *Pvu*II site of the phagemid Bluescript SK+ (Stratagene) and used to transform *E. coli* XLI competent cells (Stratagene). Recombinant plasmids harbouring the fragment of interest were identified by colony hybridisation with the 234-bp PCR product. One clone (pMAC1), containing a 817-bp insert, was sequenced on both strands.

Production of the GroES recombinant protein

To express the *M. avium* GroES protein in *E. coli*, the *M. avium groES* gene was amplified by PCR from the pMAC1 clone with *Pyrococcus furiosus* DNA polymerase (Stratagene) and inserted into pDS56 (forward primer 5'-GGGAAAGGATCCGTGGCGAAGGTGAA CATC-3' and reverse primer 5'-GGGAAAAGCTTA CTTGGAGACGACAGC-3') and pQE60 (forward primer 5'-GGGAAACCATGGCGAAGGTGAACATCA-3' and reverse primer 5'-GGGAAAAGATCTCTTGGAG ACGACAGCCAG-3') vectors (Qiagen) to place the 6× histidine (His)-affinity tag at the amino (N)-terminal or at the carboxy (C)-terminal of the fusion protein, respectively. Recombinant plasmids were sequenced (forward primer 5'-CGGATAACAATTTCA CACAG-3' and reverse primer 5'-GTTCTGAGGTCAT TACTGG-3') to verify that the correct open reading

frame had been maintained and used to transform *E. coli* M15 (pREP4) competent cells for the isopropyl-β-D-thiogalactoside (IPTG) inducible expression of the His-tagged GroES proteins [19]. *E. coli* M15 cells were grown in the presence of ampicillin 100 mg/L and kanamycin 50 mg/L. When the culture reached an OD₆₀₀ of 0.7, IPTG was added to a final concentration of 1.5 mM to induce expression. *E. coli* M15 cells transformed with the pQE56 plasmid encoding the mouse (His)-tagged dihydrofolate reductase protein were used as a control. To follow the time-course of the protein production, 1-ml samples of *E. coli* M15 cultures were collected at various times by centrifugation and boiled for 3 min in loading buffer. A 20-μl volume of each sample was subjected to SDS-PAGE and proteins were visualised by staining with Coomassie Blue (see below).

For GroES purification, *E. coli* M15 cells expressing the recombinant GroES protein were collected after incubation for 5 h with IPTG. The bacteria were disrupted by adding lysozyme and by repeated freeze-thaw cycles. After centrifugation to remove cell debris, the supernates were applied to a nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography column [19], which has a remarkable selectivity for proteins containing 6-histidine residues when charged with nickel ions. All purification steps were performed in native (non-denaturing) conditions and recombinant proteins were eluted with 500 mM imidazole. Recombinant GroES protein preparations were analysed for endotoxin content by the *Limulus* amoebocyte lysate assay (Sigma) and were shown to contain <0.1 endotoxin units/ml.

Preparation of anti-GroES serum

New Zealand White rabbits (Charles River) were inoculated intravenously on days 0, 7, 14 and 21 with a mixture containing 0.5 mg of recombinant GroES in 1 ml of phosphate-buffered saline (PBS, pH 7); the mixture was emulsified with 1 ml of incomplete Freund's adjuvant (Difco) and 2 ml of Tween 80 2% v/v. Animals were exsanguinated on day 28; the blood was allowed to clot and serum was stored in aliquots at -80°C. The antibody titre of the polyclonal anti-GroES serum was determined by ELISA as described previously [20], with minor modifications.

SDS-PAGE and immunoblotting

M. avium sonicate was prepared by treatment of mycobacteria at 4°C for 25 min (1-min cycles with 1-min cooling intervals) with a VCX 400 W ultrasonic disintegrator (Sonics & Materials, Danbury, CT, USA). Recombinant GroES and *M. avium* sonicate were subjected to SDS-PAGE in separating gels containing acrylamide (BioRad) 15% w/v and the proteins were visualised by staining with Coomassie Blue as described previously [20]. Separated proteins were

transferred on to nitrocellulose in a Mini-Trans-Blot module (BioRad). Nitrocellulose strips were soaked for 1 h at 37°C in Tris-HCl buffered saline (NaCl 0.9% w/v, 0.01 M Tris, pH 7.4) containing bovine serum albumin 3% w/v (Tris-BSA) and incubated for 2 h at room temperature with the anti-GroES serum diluted 1 in 2000 in Tris-BSA containing Tween 20 0.05% v/v (Tris-BSA-Tween). After washing in Tween 20 0.05% v/v in saline, the strips were incubated for 1 h at room temperature with goat anti-rabbit IgG alkaline phosphatase conjugate diluted 1 in 5000 with Tris-BSA-Tween and washed in Tween 20 0.05% v/v in saline. Binding of the enzyme-labelled antibodies was detected with the AP colour development reagent and fast red (BioRad).

Cellular distribution of GroES

M. avium cells were grown either to late logarithmic phase (4 days) or to stationary phase (7 days) and then either heat-shocked at 48°C for 1 h or not. Immunoelectron microscopic observations were made with anti-GroES serum (diluted 1 in 10) and gold-conjugated protein A (5 nm diameter, diluted 1 in 50). Both unfixed, unembedded bacteria and chemically fixed, resin-embedded cells were examined. In the first case, cells were harvested by centrifugation, incubated with the anti-GroES serum or the pre-immune rabbit serum, washed in PBS (pH 7.4) twice, labelled with protein A-gold and observed without any further treatment. This method ensured the best antigenic preservation but allowed labelling of only the surface components. To explore whether an internal labelling could occur, ultrathin sections were prepared. Bacteria were harvested by centrifugation, washed twice in PBS and suspended in the same buffer containing paraformaldehyde 0.5% w/v and glutaraldehyde 1% w/v. Cells were embedded in Lowicryl HM20 resin (Taab) and labelled by the post-embedding method [21].

Protection experiments in mice

Phosphorothioate CpG [12] was synthesised (M-Medica, Florence, Italy) based on a previously published sequence (TGACTGTGAACGTTTCGAGATGA) [22]. Male BALB/c mice were immunised intranasally on day 0, 14 and 28 with 40 µl (20 µl per nostril) of PBS containing 10 µg of recombinant GroES, 10 µg CpG, 10 µg GroES + 10 µg CpG, or with PBS alone. Animals were sedated by intraperitoneal administration of 10 µl/g body weight of a solution containing ketamine 5 mg/ml and xylazine 0.3 mg/ml. On day 42, mice were challenged intranasally with 10³ or 2 × 10⁴ cfu of *M. avium*. On day 84, viable bacterial numbers in the spleens and lungs were determined. Organs were collected aseptically and ground in homogenisers in Middlebrook 7H9 broth (6 ml final volume). Four 0.5-ml volumes, either undiluted or diluted 10-fold with distilled water containing Tween 80 0.05% v/v, were plated on to

Middlebrook 7H10 agar (Difco). After incubation for 10–14 days in a humidified atmosphere containing CO₂ 5%, colonies were counted and the numbers of cfu per whole organ were determined.

Statistical analysis

The statistical significance of the data was determined by Student's *t* test; *p* values <0.05 were considered significant.

Results

Production of GroES recombinant protein

Sequence analysis of the 817-bp *PvuII*/*PvuII* fragment in clone pMAC1 displayed the entire *groES* gene (nucleotides 212–511) plus the first 72 codons of the *cpn60.1* gene (nucleotides 602–817), a *groEL* homologue (Fig. 1). The predicted amino acid sequence of the *groES* gene gave a 100-amino acid protein with a deduced molecular mass of 10.7 kDa. The 817-bp fragment is deposited in the EMBL GenBank under accession no. AF079544.

After IPTG induction of *E. coli* M15 cells transformed with the *groES* gene, the C-terminally His-tagged GroES protein appeared to be expressed efficiently (Fig. 2). On the other hand, only a slight expression of the N-terminally His-tagged GroES protein was observed (data not shown). A 100-ml volume of *E. coli* M15 culture grown to an OD₆₀₀ of 0.7 before induction yielded *c.* 1.5 mg of purified GroES protein.

Antigenic properties and cellular distribution of GroES

The immune serum produced in rabbits immunised with the recombinant GroES showed an ELISA titre of 256 000. The recombinant GroES of *M. avium* showed a high degree of purity, as assessed by the presence of a single major band and the lack of extra bands by SDS-PAGE and immunoblotting (Fig. 3a and b, lane 2); the specificity of the immune serum was proved by the observation that whole-cell ultrasonicates gave only one band in immunoblotting (Fig. 3a and b, lane 1). Moreover, absorption of the serum with recombinant GroES completely removed this reaction (data not shown). These observations indicated that the recombinant GroES preparation represented a homogeneous protein suitable for further studies. The size of the recombinant GroES appeared to be slightly greater than that of native GroES because of the presence of six histidine residues in the recombinant protein.

It was observed by immuno-electron microscopy that expression of GroES was markedly enhanced after heat-shock treatment of the logarithmic phase *M. avium* cells. GroES was abundant on the surface of the intact bacteria, associated with capsular material (Fig. 4b),

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1  CAGCTGATCGCCGACGGCAACACACGAGAAGCAG 34
35  CGGAGTGGCCCTTTGAGTGCTAGCACTCGCATCTATAGAGTGTCTAGGTGGCAATCGACCA 93
94  AGCCCGGTGTTCGGCACCCGCGACGACGCGCTCGGGCATGGACGAATGCCGACACCTGA 152
153  TTATTTCGGCATGTTTCGGGGCGCGCCCCGACCCGACCCAAACACTggaggGCTCCAATC 211
212  GTG GCG AAG GTG AAC ATC AAG CCA CTC GAG GAC AAG ATT CTC GTG 256
      V  A  K  V  N  I  K  P  L  E  D  K  I  L  V
257  CAG GCC AAC GAG GCC GAG ACC ACG ACC GCA TCC GGT CTG GTC ATT 301
      Q  A  N  E  A  E  T  T  T  A  S  G  L  V  I
302  CCC GAC ACC GCC AAG GAG AAG CCG CAG GAA GGC ACC GTC GTC GCC 346
      P  D  T  A  K  E  K  P  Q  E  G  T  V  V  A
347  GTC GGT CCC GGC CGG TGG GAC GAC GAC GGC GCG AAG CGC ATT CCG 391
      V  G  P  G  R  W  D  D  D  G  A  K  R  I  P
392  CTG GAC GTC TCG GAA GGC GAC ACC GTC ATC TAC AGC AAG TAC GGC 436
      L  D  V  S  E  G  D  T  V  I  Y  S  K  Y  G
437  GGC ACC GAG ATC AAG TAC AAC GGC GAG GAG TAC CTG ATC CTG TCG 481
      G  T  E  I  K  Y  N  G  E  E  Y  L  I  L  S
482  GCG CGC GAC GTG CTG GCT GTC GTC TCC AAG TAACAACCGTGTCGCCCCC 530
      A  R  D  V  L  A  V  V  S  K
531  GCGGATCCCCGCGCCCCACGCGGGTGATTTCCGGGGCGGCATGCGTTACAGCCCGGTT 589
590  TGAaggagCCTGATG AGC AAG ATC ATT GAG TAC GAC GAG ACC GCG CGC 637
      M  S  K  I  I  E  Y  D  E  T  A  R
638  CGC GCC ATC GAG GCC GGC GTC AAC ACG CTT GCC GAC GCG GTC CGG 682
      R  A  I  E  A  G  V  N  T  L  A  D  A  V  R
683  GTG ACG CTG GGT CCG CGG GGC CGG CAT GTG GTG CTG GCC AAG GCA 727
      V  T  L  G  P  R  G  R  H  V  V  L  A  K  A
728  TTC GGC GGC CCG GCC GTC ACC AAC GAC GGC GTC ACC GTC GCG CGG 772
      F  G  G  P  A  V  T  N  D  G  V  T  V  A  R
773  GAA ATC GAC CTG GAG GAC CCG TTC GAG AAC CTG GGC GCC CAG CTG 817
      E  I  D  L  E  D  P  F  E  N  L  G  A  Q  L

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Fig. 1. Nucleotide sequence of the 817-bp *PvuII/PvuII* fragment (pMAC1). In the upstream region (nucleotides 1–211), the σ^{70} RNA polymerase –35 and –10 promoter sequences (nucleotides 46–51 and 69–74) are in bold type; the σ^{32} RNA polymerase –35 and –10 specific sequences for HSPs (nucleotides 41–51 and 60–74) are underlined; the CIRCE (controlling inverted repeats of chaperone expression) sequence (nucleotides 53–61 and 71–79), consisting of a 9-bp inverted repeat separated by a 9-bp region unique to HPS-coding operons, is in italics [25, 26]; a Shine-Dalgarno sequence (nucleotides 198–202) is in lower case. The fragment includes the entire *groES* gene (nucleotides 212–511), a region containing a Shine-Dalgarno sequence (nucleotides 593–597), and the first 72 codons (nucleotides 602–817) of the *cpn60.1* gene, a *groEL* homologue [23, 24]. The deduced amino acid sequences of the GroES protein and of part of the Cpn60.1 protein are shown below the corresponding nucleotide sequence.

Fig. 2. Whole-cell protein profiles (SDS-PAGE) of *E. coli* M15 expressing (His)₆-GroES protein at the time of IPTG addition (lane 1) and at 1, 2, 3 and 4 h (lanes 2–5, respectively) after induction. **M**, molecular mass markers; the arrow indicates the GroES band.

Fig. 3. (a) SDS-PAGE of sonicate of *M. avium* (5 μ g protein, lane 1) and the recombinant His-tagged GroES protein (0.5 μ g, lane 2). M, molecular mass markers. (b) Immunoblot, with the rabbit anti-GroES serum, of the sonicate of *M. avium* (1 μ g protein, lane 1) and the recombinant His-tagged GroES protein (0.1 μ g, lane 2). M, molecular mass markers.

and both in the cytoplasm and on the surface of sectioned cells (Fig. 4d). No relevant labelling was detected when cells were not heat-shocked (Fig. 4a and c), or when they reached the stationary phase (data not shown). No labelling was seen with cells treated with the pre-immune rabbit serum.

Protection experiments in mice

In preliminary experiments, BALB/c mice immunised subcutaneously with recombinant GroES plus incomplete Freund's adjuvant were not protected against infection after intraperitoneal challenge with 10^5 cfu of *M. avium* (data not shown). On the basis of this observation, and considering that the nasal mucosa is a probable entry point of *M. avium* into its host, the use of a mucosal adjuvant and a mucosal (intranasal) route of immunisation was investigated for protection of mice against intranasal challenge with *M. avium*.

When mice were challenged intranasally with 2×10^4 cfu, *M. avium* was recovered on day 1 in the lungs ($1.6 \text{ SD } 0.2 \times 10^3$ cfu) but not in the spleen. The

organism multiplied efficiently in both organs, as determined by the increase in cfu in non-immunised control mice by day 42 after challenge (Fig. 5a). At this time, mice immunised with GroES plus CpG showed significantly fewer organisms in the spleen when compared with non-immunised controls, unlike mice immunised with GroES or CpG alone. Under these experimental conditions, the lungs were not protected against the challenge dose.

To mimic more natural conditions of infection, a lower challenge dose (10^3 cfu) was then administered by the nasal route. *M. avium* was recovered on day 1 in the lungs ($1.2 \text{ SD } 0.2 \times 10^2$ cfu) but not in the spleens. Again, the organism had multiplied both in the lungs and spleens of the control group by day 42 of the infection (Fig. 5b), although to a lower extent than in mice challenged with 2×10^4 cfu. However, while GroES or CpG alone did not induce any protection, significantly lower numbers of *M. avium* were observed both in the lungs and spleens of mice immunised with GroES plus CpG, in comparison with control, GroES and CpG groups. No animal died during the course of the experiment.

Discussion

M. avium infects the mucosal surfaces of the respiratory and gastrointestinal tracts. Thus, development of mucosal vaccines against this organism is desirable and intranasal administration of a combined vaccine containing the recombinant GroES with a CpG adjuvant was tested for its ability to confer resistance to intranasal infection with *M. avium* on mice.

Sequence analysis of a DNA fragment containing the *groES* gene showed that *M. avium*, like other mycobacteria [23, 24], has a *groESL* operon. The fragment contained the *groES* gene and a part of the *cpn60.1* gene, a *groEL* homologue. The operon is preceded by sequences for conventional and HSP-specific promoters and by a controlling inverted repeat (CIRCE) for chaperone expression [25, 26]. *M. avium* GroES showed 97%, 92% and 88% amino acid identity with the GroES of *M. tuberculosis*, *M. bovis* and *M. leprae*, respectively, with only one residue (D 53) unique to *M. avium* (data not shown).

The recombinant GroES preparation showed a high degree of purity, as demonstrated by the presence of a single major band by SDS-PAGE and immunoblotting. The specificity of the immune serum and its suitability for use in immunolocalisation studies were demonstrated by the observation that whole-cell ultrasonicates produced one major band in immunoblotting. The band (native GroES) showed a mol. wt slightly lower than that of recombinant GroES, in accordance with the observation that histidine-tagged proteins run more slowly than the equivalent, untagged proteins [27].

Fig. 4. Electron micrographs showing immunolocalisation of GroES. (a) Cells of *M. avium* grown at 37°C. (b) Cells of *M. avium* after heat shock at 48°C. (c) Ultra-thin sections of *M. avium* grown at 37°C. (d) Ultra-thin sections of *M. avium* after heat-shock at 48°C. Bars, 100 nm. Gold particles measure 5 nm in diameter.

Fig. 5. Protection provided by recombinant GroES in combination with CpG oligodeoxynucleotides. Mice were immunised intranasally with 10 μ g GroES + 10 μ g CpG, 10 μ g GroES, 10 μ g CpG, or PBS (control) and challenged intranasally with 2×10^4 cfu of *M. avium* (a), or 10^3 cfu (b) on day 42. The numbers of cfu in the lungs and spleens of immunised and control mice on day 84 are shown. Data represent the means of three experiments ($n = 6$ mice per group in each experiment). Error bars represent SD. * $p < 0.05$ in comparison with control, GroES and CpG groups.

As HSPs are usually regarded as intracellular proteins involved in protein folding and assembly, in theory they should not be found outside the cells. Nevertheless, *M. tuberculosis* GroES was found extracellularly [7, 8] either as a result of leakage from dead cells or secretion by live bacteria [9]. *M. avium* cells not stressed by heat-shock treatment showed few GroES molecules when examined by immuno-electron microscopy. In contrast, after heat-shock, GroES was present intracellularly and on the bacterial surface and thus would be readily available for interaction with the host immune cells. Other intracellular HSPs such as GroEL and HSP70 are also surface-expressed proteins, possi-

bly functioning as adhesins and having a direct activating effect on various host cell populations [28].

The potential for cross-reactivity with mammalian proteins was thought to preclude a role for HSPs as vaccine candidates. However, immunisation of mice with GroES and GroES-like proteins protected animals against *M. leprae* [10] and *H. pylori* infections [11], respectively. Furthermore, DNA vaccines encoding the *groEL* gene induced very strong antituberculous activity in mice [29]. DNA vaccines are known to derive their immunogenic potential from the adjuvant action of sequences containing CpG motifs [30]. To

date, most of the work with CpG as adjuvant has been performed with parenteral administration to mice [13] and non-human primates [31]. Intraperitoneal administration of CpG oligodeoxynucleotides protects mice against *M. tuberculosis* by inducing a Th1 response [32]. However, several studies have shown that intranasal delivery, with CpG oligodeoxynucleotides as adjuvants, results in strong systemic and mucosal immune responses to co-administered antigens, including hepatitis B surface antigen [14] and influenza virus [33]. Intranasal administration of CpG and interleukin-12 also improves the efficacy of BCG vaccination in mice challenged with *M. tuberculosis* [34].

In agreement with these observations, the present study showed that BALB/c mice immunised intranasally with recombinant GroES protein with the addition of a CpG adjuvant acquired a significantly increased resistance to infection with *M. avium* after intranasal challenge. Protection was related to the mycobacterial burden from the challenge dose. When mice were challenged with a relatively high dose, the lungs were not protected by either GroES alone or GroES plus CpG. This dose gave rise to counts of *c.* 1600 cfu per lung on day 1. Nevertheless, when a lower challenge dose (10^3 cfu) was used, perhaps better resembling environmental conditions of infection [4], both lungs and spleen were significantly protected against *M. avium*. The latter dose gave rise to a count of *c.* 120 cfu per lung on day 1, i.e., a mycobacterial burden which is expected to be partially contained in the lung by the emerging immune response [35].

One of the primary routes of infection by *M. avium* is the intranasal route. Our observation that intranasal administration of a single mycobacterial protein and a CpG adjuvant protects mice against mycobacterial infection by the same route suggests that other recombinant mycobacterial proteins should be investigated.

We are grateful to Pasqualina Crateri and Simona Recchia for their valuable technical assistance. This work was supported in part by the Italian AIDS Project, Istituto Superiore di Sanità, Ministero della Sanità, Grants 50D.5 and 50D.4.

References

- Inderlied CB, Kemper CA, Bermudez LEM. The *Mycobacterium avium* complex. *Clin Microbiol Rev* 1993; **6**: 266–310.
- Horshburg CR. Epidemiology of *Mycobacterium avium* complex. In: Korvick JA, Benson CA (eds) *Mycobacterium avium* complex infection: progress in research and treatment. M. Dekker. 1996:1–22.
- Middleton AM, Chadwick MV, Nicholson AG *et al.* The role of *Mycobacterium avium* complex fibronectin attachment protein in adherence to the human respiratory mucosa. *Mol Microbiol* 2000; **38**: 381–391.
- von Reyn CF, Waddell RD, Eaton T *et al.* Isolation of *Mycobacterium avium* complex from water in the United States, Finland, Zaire and Kenya. *J Clin Microbiol* 1993; **31**: 3227–3230.
- Georgopoulos C, Welch WJ. Role of the major heat shock proteins as molecular chaperones. *Annu Rev Cell Biol* 1993; **9**: 601–634.
- Cobb AJ, Frothingham R. The GroES antigens of *Mycobacterium avium* and *Mycobacterium paratuberculosis*. *Vet Microbiol* 1999; **67**: 31–35.
- Verbon A, Kuijper S, Jansen HM, Speelman P, Kolk AH. Antigens in culture supernatant of *Mycobacterium tuberculosis*: epitopes defined by monoclonal and human antibodies. *J Gen Microbiol* 1990; **136**: 955–964.
- Orme IM, Miller ES, Roberts AD *et al.* T lymphocytes mediating protection and cellular cytotoxicity during the course of *Mycobacterium tuberculosis* infection. Evidence for different kinetics and recognition of a wide spectrum of protein antigens. *J Immunol* 1992; **148**: 189–196.
- Coates ARM. Immunological aspects of chaperonins. In: Ellis RJ (ed) *The chaperonins*. Academic Press. 1996: 267–296.
- Gelber RH, Mehra V, Bloom B *et al.* Vaccination with pure *Mycobacterium leprae* proteins inhibits *M. leprae* multiplication in mouse footpads. *Infect Immun* 1994; **10**: 4250–4255.
- Ferrero RL, Thiberge JM, Kansau I, Wuscher N, Huerre M, Labigne A. The GroES homolog of *Helicobacter pylori* confers protective immunity against mucosal infection in mice. *Proc Natl Acad Sci USA* 1995; **92**: 6499–6503.
- Scheule RK. The role of CpG motifs in immunostimulation and gene therapy. *Adv Drug Deliv Rev* 2000; **44**: 119–134.
- McCluskie MJ, Davis HL. Oral, intrarectal and intranasal immunizations using CpG and non-CpG oligodeoxynucleotides as adjuvants. *Vaccine* 2001; **19**: 413–422.
- McCluskie MJ, Davis HL. CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. *J Immunol* 1998; **161**: 4463–4466.
- Fattorini L, Xiao Y, Ausiello CM *et al.* Late acquisition of hyporesponsiveness to lipopolysaccharide by *Mycobacterium avium*-infected human macrophages in producing tumor necrosis factor- α but not interleukin-1 β and -6. *J Infect Dis* 1996; **173**: 1030–1034.
- Ausubel FM, Brent R, Kingston RE *et al.* Current protocols in molecular biology. New York, John Wiley & Sons. 1995.
- Mande SC, Mehra V, Bloom BR, Hol WGJ. Structure of the heat shock protein chaperonin-10 of *M. leprae*. *Science* 1996; **271**: 203–207.
- Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 1977; **74**: 5463–5467.
- Qiagen. The Qiaexpressionist, 3rd edn. Hilden, Germany, Qiagen. 1997.
- Wagner B, Fattorini L, Wagner M *et al.* Antigenic properties and immunoelectron microscopic localization of *Mycobacterium fortuitum* beta-lactamase. *Antimicrob Agents Chemother* 1995; **39**: 739–745.
- Molinari A, Calcabrini A, Meschini S *et al.* Detection of P-glycoprotein in the Golgi apparatus of drug-untreated human melanoma cells. *Int J Cancer* 1998; **75**: 885–893.
- von Hunolstein C, Mariotti S, Teloni R *et al.* The adjuvant effect of synthetic oligodeoxynucleotide containing CpG motif converts the anti-*Haemophilus influenzae* type b glycoconjugates into efficient anti-polysaccharide and anti-carrier polyvalent vaccines. *Vaccine* 2001; **19**: 3058–3066.
- Kong TH, Coates ARM, Butcher PD, Hickman CJ, Shinnick TM. *M. tuberculosis* expresses two chaperonin-60 homologs. *Proc Natl Acad Sci USA* 1993; **90**: 2608–2612.
- Rinke de Wit TF, Bekelie S, Osland A *et al.* Mycobacteria contain two groEL genes: the second *Mycobacterium leprae* groEL gene is arranged in an operon with groES. *Mol Microbiol* 1992; **6**: 1995–2007.
- Zuber U, Schumann W. CIRCE, a novel heat shock element involved in regulation of heat shock operon dnaK of *Bacillus subtilis*. *J Bacteriol* 1994; **176**: 1359–1363.
- Hecker M, Schumann W, Volker U. Heat-shock and general stress response in *Bacillus subtilis*. *Mol Microbiol* 1996; **19**: 417–428.
- Schon U, Schumann W. Construction of His6-tagging vectors allowing single-step purification of GroES and other polypeptides produced in *Bacillus subtilis*. *Gene* 1994; **147**: 91–94.
- Lewthwaite J, Skinner A, Henderson B. Are molecular chaperones microbial virulence factors? *Trends Microbiol* 1998; **6**: 426–428.
- Lowrie DB, Tascon RE, Bonato VL *et al.* Therapy of tuberculosis in mice by DNA vaccination. *Nature* 1999; **400**: 269–271.

30. Tighe H, Corr M, Roman R, Raz E. Gene vaccination: plasmid DNA is more than just a blueprint. *Immunol Today* 1998; **19**: 89–97.
31. Jones TR, Obaldia N, Gramzinski RA *et al.* Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenicity of a peptide malaria vaccine in Aotus monkeys. *Vaccine* 1999; **17**: 3065–3071.
32. Juffermans NP, Leemans JC, Florquin S *et al.* CpG oligodeoxynucleotides enhance host defense during murine tuberculosis. *Infect Immun* 2000; **70**: 147–152.
33. Moldoveanu Z, Love-Homan L, Huang WQ, Krieg AM. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. *Vaccine* 1998; **16**: 1216–1224.
34. Freidag BL, Melton GB, Collins F *et al.* CpG oligodeoxynucleotides and interleukin-12 improve the efficacy of *Mycobacterium bovis* BCG vaccination in mice challenged with *M. tuberculosis*. *Infect Immun* 2000; **68**: 2948–2953.
35. Kelly BP, Furney SK, Jessen MT, Orme IM. Low-dose aerosol infection model for testing drugs for efficacy against *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 1996; **40**: 2809–2812.