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Characterization of the Nucleotide Sequence of the *groE* Operon Encoding Heat Shock Proteins Chaperone-60 and -10 of *Francisella tularensis* and Determination of the T-Cell Response to the Proteins in Individuals Vaccinated with *F. tularensis*

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The *groE* operon of *Francisella tularensis* LVS, encoding the heat shock proteins chaperone-10 (Cpn10) and Cpn60, was sequenced and characterized, and the T-cell response of LVS-vaccinated individuals to the two proteins and the third major chaperone, Ft-DnaK, was assayed. The *cpn10* and *cpn60* genes were amplified by PCR with degenerate oligonucleotides derived from the N-terminal sequence of the two proteins. The sequence analysis revealed the expected two open reading frames, encoding proteins with estimated M_rs of 10,300 and 57,400. The deduced amino acid sequences closely resembled Cpn10 and Cpn60 proteins of other prokaryotes. The genes constituted a bicistronic operon, the *cpn10* gene preceding the *cpn60* gene. Upstream of the *cpn10* gene, an inverted repeat and motifs similar to −35 and −10 sequences of σ^{70}-dependent but not of σ^{32}-dependent promoters of *Escherichia coli* were found. The inverted repeat of the operon resembled so-called hairpin loops identified in other characterized prokaryotic *groE* operons lacking σ^{32}-dependent promoters. Primer extension analysis disclosed one and the same transcription start, irrespective of the presence or absence of heat or oxidative stress. After separation of lysates of the *F. tularensis* LVS organism by two-dimensional gel electrophoresis, DnaK, Cpn60, and Cpn10 were extracted and used as antigens in T-cell tests. When compared to those from nonvaccinated individuals, T cells from individuals previously vaccinated with live *F. tularensis* LVS showed an increased proliferative response to DnaK and Cpn60 but not to Cpn10. The present data will facilitate further studies of the involvement of the heat shock proteins in protective immunity to tularemia.

Tularemia is a zoonotic disease caused by the facultative intracellular bacterium *Francisella tularensis*. Two clinical forms predominate: ulceroglandular tularemia, which is vector-borne or caused by direct contact with an infected animal, and respiratory tularemia, which results from inhalation of contaminated dust. Irrespective of form, the disease presents with high fever and regional lymph node enlargement. Histopathologically, accumulation of mononuclear leukocytes with granuloma formation is seen, similar to the picture of mycobacterial disease (35). No potent toxin has been shown to be produced by *F. tularensis* (32). The organism seems to cause disease due to its capacity to proliferate to high numbers before an effective host defense is mounted (36).

Tularemia results in the induction of protective immunity in the mammalian host. Protection is likewise afforded by vaccination with the attenuated live vaccine strain *F. tularensis* LVS (5). The development of an effective host resistance is paralleled by the appearance of *F. tularensis*-induced immunospecific T cells (36). The T cells recognize a variety of membrane proteins of *F. tularensis* (23, 30, 34).

Besides the membrane proteins, there is little knowledge about T-cell-stimulating antigens of *F. tularensis*. Among putative antigens are cytoplasmic stress proteins such as the heat shock proteins. These proteins serve as molecular chaperones in the folding, unfolding, and translocation of polypeptide chains. Several chaperones have been identified and brought together in families on the basis of sequence similarities. Homologs of the *Escherichia coli* heat shock proteins DnaK (70 to 75 kDa), GroEL (chaperone-60 [cpn60]; 60 to 65 kDa), and GroES (Cpn10; 10 kDa) have been thoroughly studied for various intracellular pathogens and found to be involved in the induction of a protective cell-mediated immune response (1, 3, 9, 12, 15, 17, 28, 40).

In vitro exposure of *F. tularensis* to heat or hydrogen peroxide leads to increased synthesis of several proteins (4, 8), including the DnaK, Cpn60, and Cpn10 heat shock proteins. The sequence of the gene encoding the DnaK homolog of *F. tularensis* (DnaK) has recently been determined (44). The deduced protein showed high similarity to other characterized DnaK homologs. In the present study, the *groE* operon encoding Cpn10 and Cpn60 was sequenced and characterized. We also determined the response to the three chaperone proteins of T cells from humans vaccinated with *F. tularensis* LVS and from nonvaccinated individuals.

MATERIALS AND METHODS

Bacterial strains, culture conditions, and plasmids. The vaccine strain *F. tularensis* LVS was supplied by the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Md. It was stored at −70°C and cultivated on modified Thayer-Martin agar containing GC medium base (22). For each experiment, bacteria were grown overnight at 37°C in the synthetic liquid Chamberlain medium (6). After dilution in the same medium, bacteria were grown to exponential phase and harvested. *E. coli* DH5α has been previously described (11).

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Plasmid Bluescript SK^+ was purchased from Stratagene, and pGEX-KG was kindly provided by K. L. Guan and J. E. Dixon (West Lafayette, Ind.).

Vaccination protocol. The vaccinees included in the study were healthy adults. All gave an informed consent. Vaccination was performed 2 to 20 years before the present study by scarification following the instructions of the manufacturer (Salk Institute, Swiftwater, Pa.). Approximately 7.0×10^7 bacteria in 0.1 ml were administered intradermally.

DNA manipulations. All DNA manipulations, including purification, digestion with restriction endonucleases, ligations, and gel electrophoresis, were performed according to methods described by Sambrook et al. (21). Purification of DNA fragments was done by using the Gene-Clean kit (Bio 101, La Jolla, Calif.) or by β-agarase treatment (Boehringer, Mannheim, Germany) following the instructions of the manufacturers. Southern blot analysis was carried out on nylon membranes (Hybond-N; Amersham International, Little Chalfont, United Kingdom) as described by Southern (33). Double-stranded DNA probes were labeled with α-^{32}P (Amersham International) by use of random hexanucleotide primers (Pharmacia AB, Uppsala, Sweden) according to the manufacturer's instructions.

DNA sequencing. On the basis of the previously sequenced N-terminal parts of *F. tularensis* Cpn60 and Cpn10 (8), the following degenerate oligonucleotide primers were designed: AAT(C) ATA(CT) A(C)GA(TCG) CCA(T) T(C)T A(CGT)C AA(G)G AT and ACA CCA TCT(AC) AA(G) CAT T(C)T TT(A)G C. A third primer, CAT A(T)C CA(T)C CCA TA(GT)C CA(T)C C, was based on the sequence GGMGGM, frequently occurring in the C termini of cpn60 homologs.

DNA fragments were amplified by PCR and sequenced in both directions by the dideoxynucleotide chain-termination method with the AmpliCycle sequencing kit (Perkin-Elmer, Norwalk, Conn.). Universal primers and primers based on the sequenced DNA were synthesized by Pharmacia AB. [α-^{32}P]dATP was used for labeling. To obtain sequence information about regions adjacent to the *cpn10* and *cpn60* genes, chromosomal DNA of *F. tularensis* was double digested with *Sac*I and *Cla*I or *Cla*I and *Eco*RI. The cleaved DNA was separated on an agarose gel, blotted on nylon membranes, and probed by a radiolabeled PCR-amplified DNA fragment complementary to the *cpn10* gene. DNA in the size range of the fragments that hybridized was excised and ligated into Bluescript SK^+ vector DNA. From each ligation mixture, several fragments were amplified with one primer complementary to the Bluescript vector DNA and the other complementary to various regions of the *cpn10* and *cpn60* genes.

The software package GCG, developed by Genetics Computer Group (University of Wisconsin, Madison), was used to assemble and analyze the sequences. The FASTA program of the GCG package was used for homology searches in the GenBank and SWISS Protein databases. The free energy (ΔG) of a hairpin loop structure was calculated with the FOLDRNA program of Zuker and Stiegler (45).

Primer extension analysis and Northern blotting. Total RNA was prepared from *F. tularensis* LVS as described by Chomczynski and Sacchi (7). Oligonucleotides complementary to positions 407 to 386 and 899 to 877 of the nucleotide sequence of the *groE* operon were used as probes. The gene probes were end labeled with 50 μCi of [γ-^{32}P]dATP in the presence of 1 μl of T4 kinase (Gibco BRL, Grand Island, N.Y.), following the instructions of the manufacturer. Twenty micrograms of RNA in 15 μl of 0.1 M Tris-HCl buffer, pH 8.2, containing 1.5 M KCl and 10 mM EDTA, was mixed with 0.2 pmol of labeled primer, incubated at 90°C for 5 min, cooled slowly to 45°C, kept at this temperature for 60 min, and finally incubated at 72°C for 5 min. The primer extension reaction mixtures were subjected to electrophoresis next to a sequencing ladder in 5% polyacrylamide gels, with the same oligonucleotide as sequencing primer.

For Northern blotting, 20 μg of RNA samples was separated on a 1.5% agarose gel containing formaldehyde and transferred to a nylon membrane by capillary transfer in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Prehybridization and hybridization were performed as described elsewhere for Southern blotting (31) in an aqueous solution at 47°C.

Two-dimensional electrophoresis and protein extraction. An overnight culture (10 ml) of bacteria was diluted in a total volume of 100 ml of Chamberlain medium and grown to an optical density of 0.7 at 540 nm. To enhance the synthesis of the chaperone proteins, hydrogen peroxide was then added at a final concentration of 5 mM and the bacteria were grown for 1 h at 37°C, harvested by centrifugation, and suspended in 0.5 ml of distilled H_2O. The suspension was concentrated into a minimal volume in a Speed Vac concentrator (Savant Instruments, Holbrook, N.Y.) and mixed with 500 μl of lysis buffer (9 M urea, 2% [vol/vol] Ampholine [pH 3.5 to 10.0] [Bio-Rad Laboratories, Richmond, Calif.], 2% [vol/vol] Triton X-100, and 8 mM phenylmethylsulfonyl fluoride) for storage at −80°C. After thawing, 5 μl of lysed material was added to 30 μl of sample buffer (8 M urea, 2% [vol/vol] β-mercaptoethanol, 2% [vol/vol] Ampholine [Bio-Rad Laboratories] [pH 3.5 to 10.0], 0.5% [vol/vol] Triton X-100) and subjected to two-dimensional electrophoresis according to a modification (10) of the description by O'Farrell (19). Isoelectric focusing was performed by use of strips of a prefabricated dried gel (Immobiline DryPlate [pH 4.0 to 7.0]; Pharmacia LKB Biotechnology) according to recommendations of the manufacturer. The strips were equilibrated in a solution containing 6 M urea, 10% (vol/vol) Tris-HCl (pH 6.8), 30% (vol/vol) glycerol, and 2% (wt/vol) sodium dodecyl sulfate (SDS) and applied on a uniform 14% acrylamide gel for electrophoresis as described by Laemmli (13).

After two-dimensional electrophoresis, the gels were equilibrated in 0.19 M Tris-HCl–0.1% (wt/vol) SDS (pH 8.8) for 30 min. Before excision of gel pieces containing appropriate proteins, polypeptides were visualized by treating the gel with 0.3 M $CuCl_2$ for 30 min and thereafter washed briefly in water (14). Gel pieces were destained by soaking for 10-min periods, three times in 0.25 M EDTA–0.25 M Tris-HCl (pH 9.0) and once in elution buffer (20 mM Tris-HCl, 0.01% [wt/vol] SDS, 150 mM glycine). Proteins were eluted for 4 h with the model 422 Electro-Eluter (Bio-Rad Laboratories) as recommended by the manufacturer. The eluted proteins were dialyzed against 10 mM Tris-HCl buffer, pH 8.0, and concentrated in a Speed-Vac. Protein concentrations were determined by silver staining of 2-μl portions of the eluted proteins added to nitrocellulose filters, with various concentrations of bovine serum albumin (Sigma, Stockholm, Sweden) as reference. Results roughly correlated with protein determinations with the DC protein assay (Bio-Rad Laboratories) performed according to the instructions of the manufacturer.

Western blot analysis. For Western blot analysis, proteins of acrylamide gels were transferred to nitrocellulose filters and probed as previously described (8). An alkaline phosphatase-conjugated secondary antibody system was used. Some filters were probed by use of the enhanced chemiluminescence Western blotting protocol RPN 2108 (Amersham International). Autoradiography was performed by exposure of the nitrocellulose filters to Hyperfilm-MP (Amersham International) for 1 to 3 days at −80°C. All tularemia patients from which sera were obtained for Western blot analysis demonstrated significant antibody response in convalescent sera as determined by enzyme-linked immunosorbent assay.

Assay of proliferative T-cell response. Blood samples were obtained from 15 healthy adults vaccinated several years previously with *F. tularensis* LVS and from 12 adults with no previous history of tularemia or tularemia vaccination. Peripheral blood mononuclear cells were prepared from heparinized blood by centrifugation on a Ficoll-Metrizoate gradient (Lymphoprep; NYCOMED AS, Oslo, Norway), and cultures were established. Each culture (200 μl) contained 3×10^5 mononuclear cells. The culture medium consisted of RPMI-HEPES (GIBCO Laboratories, Grand Island, N.Y.), supplemented with 15% pooled human serum, 100 μg of gentamicin per ml, and 2 mM L-glutamine. As stimulating agents, DnaK (0.001 μM), Cpn60 (0.002 μM), or Cpn10 (0.01 μM) purified by extraction from two-dimensional gels; Cpn60 from *E. coli* (0.02 μM; StressGen, Victoria, British Columbia, Canada); heat-killed *F. tularensis* (10^6/ml); or purified protein derivative (PPD) (10 μg/ml; Statens Seruminstitut, Copenhagen, Denmark) was used. In preliminary experiments, these antigen concentrations were found to be optimal. To estimate the proliferative response, cultures were incubated at 37°C for 6 days, pulsed for 6 h with 1 μCi of [^{3}H]thymidine, and harvested. For statistical evaluation, Student's *t* test was used.

Nucleotide sequence accession number. The nucleotide sequence encoding the *F. tularensis groE* operon was submitted to the EMBL/GenBank/DDBJ database and given accession no. X98853.

RESULTS

Sequence analysis of the *cpn60* and *cpn10* genes of *F. tularensis* LVS. We have previously sequenced the N termini of the *F. tularensis* Cpn60 and Cpn10 proteins (8). Using this information and the fact that Cpn60 homologs frequently contain the unusual repeat GGMGGM in their C termini, we designed degenerate oligonucleotide primers (A to C in Fig. 2). To minimize the degeneration, sequence information on the codon usage in *F. tularensis* was utilized (30). By using combinations of the three degenerate oligonucleotides, three fragments 0.4, 1.6, and 2.0 kb in size were successfully amplified by PCR (Fig. 2).

The sequence analysis disclosed two open reading frames (ORFs) encoding 95 and 544 amino acids. The estimated M_rs of the deduced Cpn10 and Cpn60 proteins were 10,300 and 57,400, respectively, and their calculated pIs were 5.46 and 4.99, respectively. The amino acid sequence deduced from the 5′ part of the *cpn60* gene was identical to the N-terminal sequence previously determined (8). The previously deduced N terminus of Cpn10 was AAKQVLFSDG, indicating that the formyl-methionine is posttranscriptionally cleaved. The two genes showed homology to *cpn10* and *cpn60* of other bacteria. The A+T content of the *cpn10* and *cpn60* genes of *F. tularensis* was high (61%). This is in accordance with previously obtained sequence information on *Francisella* (30, 44).

Transcriptional and translational organization of the *groE* operon of *F. tularensis*. To obtain sequence information on regions adjacent to the *cpn10* and *cpn60* genes, the 0.4-kb

```
---------+---------+---------+---------+---------+---------+ 60
TGAAAAATAAACTTAATTATTATATATGTTATTTAGCTAGTTTTTTTAATTAAAGTTAAA

---------+---------+---------+---------+---------+---------+ 120
ATCGAGAGCTTGTTTGACAAAAAAACAAAAAAATTTCTTGAAAATTTTTTTTTTGACTCA
                                      -35
---------+---------+---------+---------+---------+---------+ 180
ATATCTAGACTTGCAAGAGCTTGGAACTTTGAGATTGTTCTAAGATGCATACAAATTCAA
  -10       ↑       >>>>>>>>         <<<<<<<<
---------+---------+---------+---------+---------+---------+ 240
AATGCTTAAACAAAAATAATTTAACAAAGGAGTAAGATTGTTATGAACATTCGTCCATTA
                          SD            M  N  I  R  P  L
```

FIG. 1. Nucleotide sequence of the region upstream of the *groE* operon of *F. tularensis* LVS and the start of the ORF encoding Cpn10. DNA bases are numbered and listed to the right of the sequence. The putative Shine-Dalgarno ribosome binding site for the *cpn10* and *cpn60* genes is designated SD and underlined. The inverted repeat is marked by arrowheads below the sequence. The −10 and −35 regions of identified promoter sequences are also underlined. The transcription start mapped by primer extension analysis is indicated by an arrow.

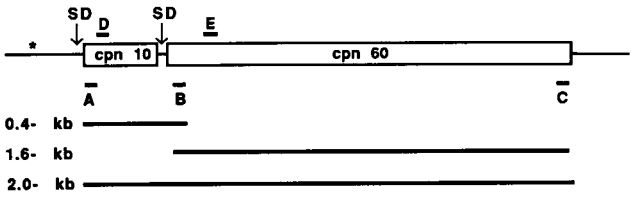

FIG. 2. Physical map of the *groE* operon of *F. tularensis* LVS. The boxes show the ORFs encoding *F. tularensis* Cpn10 and Cpn60, and the asterisk indicates the transcription start of the genes. Shine-Dalgarno sequences are marked SD and indicated with vertical arrows. The locations of oligonucleotides deduced for construction of primers in PCR-based DNA sequencing are shown (A to C) as well as the three DNA fragments generated by use of the primers. D and E indicate the locations of primers used in primer extension analysis.

fragment was used as a probe in Southern blot analysis. A 6.0-kb *Sac*I-*Cla*I fragment and a 7.0-kb *Cla*I-*Eco*RI fragment of chromosomal DNA of *F. tularensis* LVS were shown to hybridize to the probe. The two fragments were ligated into the Bluescript SK^+ vector. By repeatedly using a universal primer of the vector and a primer derived from the previously sequenced DNA, the regions located upstream and downstream of the *cpn60* and *cpn10* genes were amplified and sequenced (Fig. 1).

Similar to most other organisms characterized so far, the *groE* operon of *F. tularensis* displayed a transcriptional start upstream of the ORF encoding the cpn10 protein (Fig. 1). This ORF was followed by 40 nucleotides, containing a Shine-Dalgarno ribosome binding site but no promoter sequence, and the *cpn60* gene. Upstream of the *cpn10* gene, motifs similar to −35 and −10 sequences of σ^{70}-dependent promoters of *E. coli* were found, 18 nucleotides apart (Fig. 1). No similarities to the consensus sequences of the σ^{32}-dependent promoter of enterobacterial *groE* operons were found within 350 nucleotides upstream of the *cpn10* gene. An inverted repeat was identified downstream of the −10 region (Fig. 1). Inverted repeats, so-called hairpin loops, have been identified in prokaryotic *groE* operons utilizing σ^{70}-dependent promoters for transcription (2, 16, 18, 24–27, 39). In the repeat here identified, 5 of 7 nucleotides upstream and 6 of 7 nucleotides downstream of the loop were identical to those of a consensus sequence (Table 1). The free energy of the *F. tularensis* hairpin loop was −25.1 kJ/mol. Altogether, the two ORFs and the intergenic region encompassed 1,950 nucleotides.

Primer extension analysis was performed with a probe complementary to the *cpn10* or *cpn60* gene (D and E in Fig. 2). Irrespective of probe used, a transcriptional start was identified 90 nucleotides upstream of the initiation codon of the *cpn10* gene and six bases downstream of the −10 region (Fig. 3). To determine whether the start was operational also during heat or oxidative stress, RNA was prepared from bacteria growing at 42°C or in the presence of 5 mM hydrogen peroxide. The same 5′ end of the transcripts was identified, and this was true irrespective of which of the two probes was used (data not shown).

The estimated overall size of the operon conformed well with the results of Northern blot analysis which detected one message 2.4 to 2.6 kb in size. Altogether, the findings indicated that a region resembling a σ^{70} promoter initiated transcription of the bicistronic operon.

Sequence similarity between Cpn60 and Cpn10 and their homologs in other prokaryotes. The deduced amino acid sequences of Cpn60 and Cpn10 were aligned with those of the homologous heat shock proteins of five other bacterial species, *Legionella pneumophila*, *E. coli*, *Coxiella burnetii*, *Brucella abortus*, and *Mycobacterium tuberculosis. F. tularensis* Cpn60 exhib-

TABLE 1. Comparison of inverted repeat sequences of the *groE* operons of various bacterial species

Operon (reference)	Sequence
Consensus	TTAGCACTC-9 N-GAGTGCTAA
Francisella tularensis groE	CTTGGAAC -9 N- GTTCTAAG
Clostridium acetobutylicum groE (18)	..TGTTAGCACTC-9 N-GAGTGCTAACA
Agrobacterium tumefaciens groE (25)	...TGCTGGCACTC-9 N-GAGTGCTAACA
Bacillus subtilis groE (24)	TTAGCACTC-9 N-GAGTGCTAA
Synechocystis sp. *groE* (39)	ATTAGCACTC-9 N-GAGTGCTAAT
Leptospira interrogans groE (2)	TAAGCACTC-9 N-TAGTGCTAAT
M. leprae groEL (16)	TTGCACTC-9 N-GAGTGCTAA
M. tuberculosis groEL (27)	TTGCACTC-9 N-GAGTGCTAA

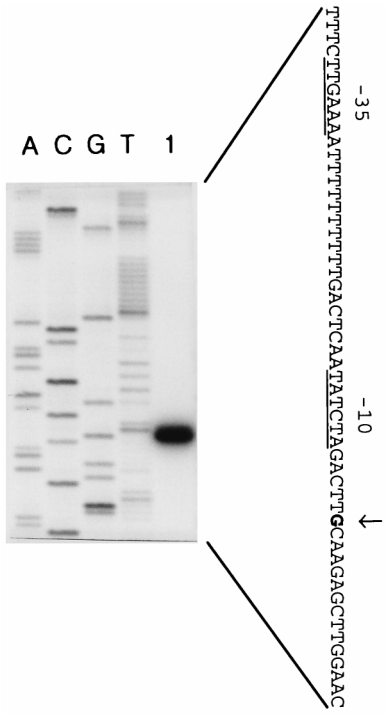

FIG. 3. Mapping of the 5′ end of the *groE* transcript by primer extension analysis. A primer complementary to the 5′ end of *cpn10* was hybridized to total RNA from *F. tularensis* LVS isolated after temperature increase from 37 to 42°C. The primer extension product was analyzed on a sequencing gel (lane 1). G, A, T, and C represent products of the sequencing reactions with each of the primers.

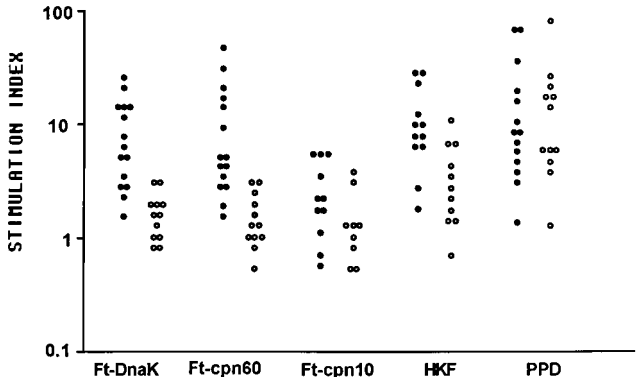

FIG. 4. Lymphocyte proliferative responses to purified *F. tularensis* (Ft) Cpn10, Cpn60, and Cpn70 proteins. Peripheral blood mononuclear cells were prepared from tularemia-vaccinated (filled; left) and nonimmunized (open; right) donors and stimulated with each of the three chaperone proteins (0.01, 0.002, and 0.001 μM, respectively), heat-killed bacteria (HKF; 10^6/ml), or PPD (10 μg/ml). Thymidine incorporation was measured after 5 days in culture and expressed as stimulation index (mean counts per minute of five cultures containing antigen/mean counts per minute of five cultures lacking antigen).

TABLE 2. Lymphocyte responses of *F. tularensis*-vaccinated individuals to the cpn60 chaperonins of *F. tularensis* (Cpn60; 0.002 μM) and *E. coli* (Cpn60; 0.02 μM) and PPD (10.0 μg/ml)[a]

Individual no.	Antigen[b]		
	F. tularensis Cpn60	*E. coli* Cpn60	PPD
1	10.4	4.7	5.6
2	7.4	2.1	2.7
3	14.1	2.8	10.5
4	4.6	2.3	19.0
5	7.6	3.4	11.7
6	6.4	4.0	15.6
7	2.5	2.1	1.8
8	2.7	1.8	2.4
9	2.2	1.7	13.4
10	0.8	0.7	5.1
11	2.2	1.3	10.0
12	1.2	1.7	27.9

[a] Peripheral blood mononuclear cells were pulsed with [^{3}H]thymidine after 5 days of incubation.

[b] Stimulation index = mean counts per minute of five cultures containing antigen/mean counts per minute of five cultures lacking antigen.

ited 57 to 73% and Cpn10 exhibited 39 to 62% similarity with the homologs. Substantially conserved regions (>90%) of the aligned Cpn10 protein comprised amino acids 25 to 123, 246 to 298, and 358 to 421. As in most other bacterial Cpn60 homologs, the C-terminal sequence of Cpn60 contained the unusual repeat GGMGGM, the function of which is unknown.

Proliferative response of T cells to *F. tularensis* DnaK, Cpn60, and Cpn10. The major heat shock proteins *F. tularensis* DnaK, Cpn60, and Cpn10 were extracted from two-dimensional gels and used as antigens in assays of T-cell proliferation. The mean thymidine incorporation after stimulation with DnaK or Cpn60 was higher in T cells from tularemia vaccinees than in those from nonimmune individuals ($P < 0.02$ for each antigen [Fig. 4]). It should be remarked, however, that responses to the chaperone proteins were not strictly immunospecific but occurred at relatively high magnitude also in some of the nonimmune individuals. The magnitude of the response to Cpn70 or Cpn60 was similar to that to heat-killed *F. tularensis* organisms. The T-cell response to Cpn10 was generally poor, with no significant difference between the two groups of individuals ($P > 0.10$). There was no difference between the groups in magnitude of response to PPD (Fig. 4).

Cross-reactivity of T cells from tularemia-vaccinated individuals with *E. coli* cpn60. When peripheral blood mononuclear cells from 12 tularemia-vaccinated individuals were incubated in parallel with Cpn60 and Cpn60 of *E. coli*, cross-reactivity was demonstrated. Of six individuals (no. 1 to 6 in Table 2) who responded to Cpn60 with a stimulation index of >3, all showed a response to *E. coli* Cpn60 of ≥2.1 (Table 2). In contrast, all of a group of six individuals (no. 7 to 12 in Table 2) responding to Cpn60 with an index of <3 showed an index of ≤2.1 in response to Cpn60 of *E. coli*. The two groups of individuals were similar with regard to response to PPD (Table 2), indicating that they did not differ from each other in general ability to respond to T-cell antigens.

Western blot analysis of human serum antibodies to *F. tularensis* DnaK, Cpn60, and Cpn10. After two-dimensional gel electrophoresis of lysates of *F. tularensis* LVS, serum from nonimmune individuals or patients with a history of tularemia or tularemia vaccination was analyzed by Western blotting. Irrespective of category analyzed, there was a readily visible reactivity to *F. tularensis* DnaK and Cpn60, but no visible reactivity to Cpn10 (Fig. 5A). Two patients were analyzed within 1 week of onset of disease as well as 1 to 2 months later. As illustrated in Fig. 5B and C, only a marginally increased antibody reactivity to DnaK and Cpn60 was observed in convalescent-phase serum. However, an induction of antibodies to other, nondefined proteins was seen.

DISCUSSION

Our primary rationale was to provide a basis for studies of the immunogenicity of heat shock proteins of *F. tularensis*. Previous data on individuals undergoing tularemia or tularemia vaccination indicate that the cell-mediated immune response to whole heat-killed *F. tularensis* or membrane proteins of the organism is highly specific. In view of the conserved nature of heat shock proteins and their occurrence as major constituents also under normal conditions (8), we asked whether these proteins were immunospecifically recognized as well. Immunospecificity was demonstrated insofar as *F. tularensis* DnaK and Cpn60 induced a significantly higher proliferative response in T cells from individuals vaccinated with the homologous strain of *F. tularensis* than in T cells from individuals without tularemia or tularemia vaccination. A cross-reactivity was, however, demonstrated insofar as T cells from individuals responding well to *F. tularensis* Cpn60 also showed a relatively strong response to *E. coli* Cpn60. This was in line with a sequence identity of 73% between the two chaperonins. The results were similar to those obtained with DnaK or Cpn60 from mycobacteria and a few other intracellular pathogens (12, 15, 40, 41).

In contrast, *F. tularensis* Cpn10 did not induce a significant T-cell response, either in tularemia-vaccinated or in nonvaccinated individuals. This differs from results with *M. tuberculosis* and *Mycobacterium leprae*, according to which Cpn10 induces a strong immunospecific T-cell response in humans (3, 17). The reason for the poor response to Cpn10 is unknown. Obviously, the conserved nature of chaperone proteins makes it difficult to predict whether they will indeed be recognized by human T cells. Besides, the small molecular size of Cpn10 may be important. Another small protein of *F. tularensis*, the 17-kDa outer membrane protein, has been cloned and sequenced previously (29, 30). Mapping of the 17-kDa protein identified only

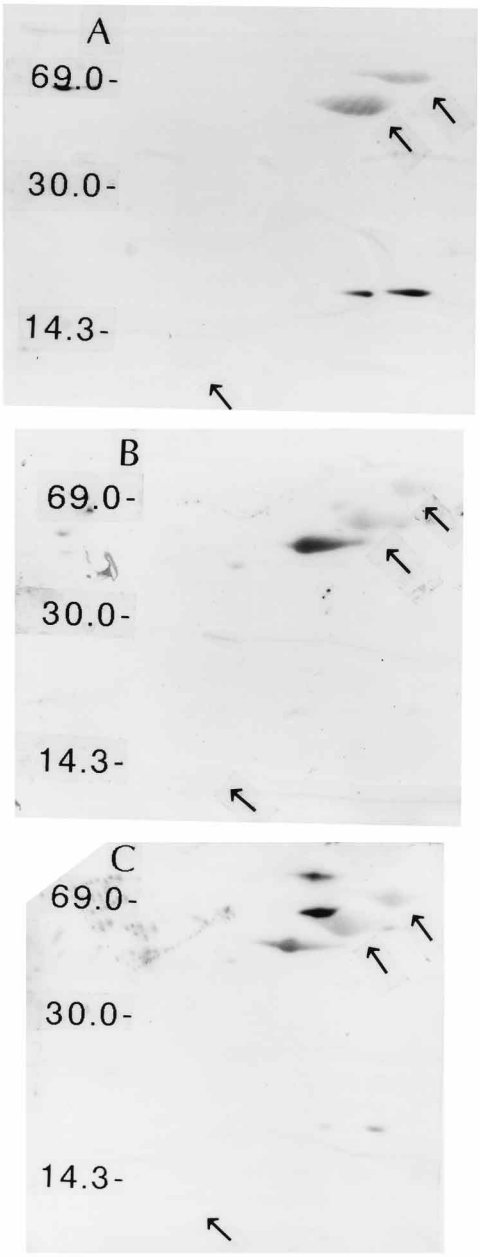

FIG. 5. Antibody response of human sera to proteins of *F. tularensis* LVS. Cell extracts were separated by isoelectric focusing in the first dimension (pH 4 to 7) and by SDS-polyacrylamide gel electrophoresis in the second dimension. Gels were blotted onto nitrocellulose filters and probed with human sera diluted 1/800. (A) Reactivity of serum from nonimmune individual. (B) Reactivity of serum from a patient 4 days after onset of tularemia. (C) Reactivity of serum from the same individual as in panel B obtained 2 months after onset of disease. Numbers on the left indicate molecular mass in kilodaltons. The locations of Cpn10, Cpn60, and DnaK of *F. tularensis* are indicated with arrows.

two T-cell epitopes, and in T-cell assays, each immunized individual recognized at the most one of them. A total of 4 of 10 individuals tested did not recognize the 17-kDa protein at all (30). By analogy, a paucity of T-cell epitopes might be a reason for the poor response to Cpn10.

The magnitude of the T-cell responses to *F. tularensis* DnaK and Cpn60 varied widely among different individuals. This variation was similar to that found in studies of several membrane proteins of *F. tularensis* LVS (23, 30). Similar to the membrane proteins, the two chaperone proteins thus seem to be T cell reactive without being immunodominant. The results are in line with the view that the T-cell response of *F. tularensis*-vaccinated individuals is based on the utilization of a wide variety of bacterial antigens and that the strength of the immune response will depend on the sum of T-cell specificities involved (37). However, before the chaperone proteins can be placed in the same category of T-cell antigens as the membrane proteins of *F. tularensis*, the phenotype of the responding cells has to be defined. Membrane proteins of *F. tularensis* stimulate predominantly αβ T cells (30). For mycobacteria, there is controversy regarding the subpopulations stimulated by the chaperone proteins, particularly in respect to Cpn60 (20, 38).

The heat shock response of *groE* operons of *E. coli* and other enterobacteria is mediated by a σ^{32} factor. No consensus sequence of σ^{32}-dependent promoters was here found in the *groE* operon of *F. tularensis*. In contrast, the *groE* operon of this gram-negative bacterium seemed to be regulated in accordance with that of certain gram-positive bacteria, purple bacteria, and cyanobacteria, i.e., exclusively under control of a σ^{70} factor (2, 16, 17, 24, 25, 27, 39). In the latter bacteria, the operon includes a hairpin loop structure, and such a structure with similar sequence and localization was here found in *F. tularensis* LVS. The conserved nature of the repeat and the fact that it has been found only in *groE* operons exclusively dependent on σ^{70} indicate an important role in heat shock control. In fact, studies of *Bacillus subtilis* have shown that the presence of the repeat shortens the half-life of the *groE* transcript and also represses the transcription (42). The latter effect was subsequently demonstrated to be mediated by ORF39, a gene product of the *B. subtilis dnaK* operon, possibly by direct interaction with the inverted repeat (43).

Northern blot analysis demonstrated that, as in most other *groE* operons studied, the two *groE* genes of *F. tularensis* are cotranscribed. The transcription start was located 6 nucleotides downstream of the −10 sequence, a distance typical of σ^{70}-dependent promoters. Moreover, primer extension analysis revealed that transcription was initiated from the same position irrespective of the presence or absence of heat shock. In this respect, the regulation of the *groE* operon of *F. tularensis* resembled that of other prokaryotes possessing a σ^{70}-dependent heat shock control (2, 18, 24, 25).

Together with the recent elucidation of the *dnaK* gene of *F. tularensis* (44), the present determination of the nucleotide sequence and the organization of the genes encoding Cpn60 and Cpn10 will facilitate studies of the involvement of the three predominant heat shock proteins in protective immunity to tularemia. *F. tularensis* DnaK and Cpn60, but not Cpn10, stimulated T cells from individuals previously vaccinated by intracutaneous injection of viable homologous organisms. The phenotype of the responding T cells remains to be studied before it can be stated that these proteins are handled by the immune system in the same manner as previously shown for a variety of T-cell-reactive membrane proteins of the organism.

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