

Chlamydial Hsp60-2 Is Iron Responsive in *Chlamydia trachomatis* Serovar E-Infected Human Endometrial Epithelial Cells In Vitro[∇]

Richard W. LaRue,¹ Brian D. Dill,^{1*} David K. Giles,¹ Judy D. Whittimore,¹ and Jane E. Raulston^{1,2†}

Departments of Microbiology¹ and Pathology,² James H. Quillen College of Medicine,
East Tennessee State University, Johnson City, Tennessee

Received 13 September 2006/Returned for modification 23 October 2006/Accepted 10 February 2007

Chlamydial 60-kDa heat shock proteins (cHsp60s) are known to play a prominent role in the immunopathogenesis of disease. It is also known that several stress-inducing growth conditions, such as heat, iron deprivation, or exposure to gamma interferon, result in the development of persistent chlamydial forms that often exhibit enhanced expression of cHsp60. We have shown previously that the expression of cHsp60 is greatly enhanced in *Chlamydia trachomatis* serovar E propagated in an iron-deficient medium. The objective of this work was to determine which single cHsp60 or combination of the three cHsp60 homologs encoded by this organism responds to iron limitation. Using monospecific polyclonal peptide antisera that recognize only cHsp60-1, cHsp60-2, or cHsp60-3, we found that expression of cHsp60-2 is responsive to iron deprivation. Overall, our studies suggest that the expression of cHsp60 homologs differs among the mechanisms currently known to induce persistence.

Studies have consistently shown that there is a correlation between the production of chlamydial 60-kDa heat shock protein (cHsp60) antibodies in chlamydia-infected patients and adverse disease consequences. These observations appear to be universal for *Chlamydia* species and their disease presentations. Early studies of whether cHsp60 plays a role in immunopathogenesis involved analyses of serum antibodies from female patients presenting with *Chlamydia trachomatis*-associated tubal infertility (7, 52); elevated levels of anti-cHsp60 in the sera of these patients were significantly associated with disease. A separate group of investigators described the contribution of cHsp60 in a guinea pig model of trachoma; an intense mononuclear cell inflammatory response was observed after conjunctival inoculation of cHsp60 following resolution of a primary ocular infection with *Chlamydia psittaci* GPIC (37). Patients with coronary artery disease often have serological evidence of previous infection by *Chlamydia pneumoniae* (45), and cHsp60 has been directly identified in human atherosclerotic tissue (31). Most recently, *C. pneumoniae* and *Chlamydia pecorum* cHsp60s have been implicated in urogenital tract disease in koalas (*Phascolarctos cinereus*), leading to infertility and death; chlamydiae are the most commonly recognized disease agents in the threatened koala population (23, 24).

Studies to determine the role of cHsp60 and immunopathogenesis are still being performed. Recently, workers have examined the initial interactions of cHsp60 with host cells that induce an inflammatory response. For example, cHsp60 interacts with Toll-like receptor 4, which stimulates the proliferation of human vascular smooth muscle cells (47), activates

macrophages, and activates endothelial cells (8). The interaction between cHsp60 and Toll-like receptor 4 also leads to apoptosis in primary human trophoblasts, placental fibroblasts, and a trophoblast cell line by both caspase-dependent and -independent pathways (18). cHsp60 and other microbial ligands can also activate mononuclear cells by binding to CD14, the monocyte receptor for lipopolysaccharide (32). Although cHsp60 clearly plays a prominent role in chlamydial pathogenesis, it is not the only molecule involved. The genetically linked protein cHsp10, encoded by the *groES* gene upstream of *groEL-1*, is also associated with disease complications (6, 23, 27, 33). Moreover, several studies have demonstrated that genetic predisposition plays a significant role in chronic chlamydial disease (10, 12, 38). Perhaps most interesting is the fact that cHsp60 has been used in a human trial involving women at high risk for *C. trachomatis* infection; cHsp60 was used to stimulate the patients' peripheral blood mononuclear cells to produce gamma interferon (IFN- γ), and the results indicated that a protective response against incidental infection developed (11).

Our laboratory is involved in identifying and analyzing *C. trachomatis* proteins that respond to iron restriction, as well as the mechanisms involved (42, 43, 55); cHsp60 is one of several proteins whose expression increases significantly during iron limitation in vitro (43). It is known that iron sources and the availability of iron fluctuate in menstruating women due to the cyclic pressures of estrogen and progesterone (1, 29); active or persistent *C. trachomatis* organisms in the reproductive tract are therefore likely to respond to this dynamic environment using transcriptional, translational, or posttranslational mechanisms to alter the production of specific chlamydial proteins. While we are not involved in direct studies of persistent chlamydiae, which have been defined as viable but nonculturable organisms (3), iron deprivation is one of several modes for induction of persistent chlamydiae (22, 39, 43). In women with tubal factor subfertility, cHsp60 is a serological marker for persistence (15) along with chlamydial proteasome/protease-

* Corresponding author. Mailing address: Departments of Microbiology and Pathology, James H. Quillen College of Medicine, East Tennessee State University, P.O. Box 70579, Johnson City, TN 37614-1708. Phone: (423) 439-7011. Fax: (423) 439-8044. E-mail: bddill@yahoo.com.

† This paper is dedicated to the memory of our friend and colleague, Jane E. Raulston (12 February 2007).

[∇] Published ahead of print on 16 February 2007.

like activity factor (48). However, the results of recent studies with *C. psittaci* (22) and *C. pneumoniae* (39) indicate that cHsp60 is not a general marker for persistence.

When the complete sequence of the *C. trachomatis* serovar D chromosome became available, one of many surprises was that there are three open reading frames (ORF) that code for *groEL*-related proteins (49). These ORF are located in separate regions of the chromosome and designated as follows: CT110 or *groEL-1*, encoding cHsp60-1; CT604 or *groEL-2*, encoding cHsp60-2; and CT755 or *groEL-3*, encoding cHsp60-3. Only *groEL-1* is preceded by *groES*. Matching cHsp60s in different *Chlamydia* species appear to be conserved in the sequences that are currently available. For example, the predicted level of amino acid sequence identity between cHsp60-1 in *C. trachomatis* serovar D and cHsp60-1 in *C. pneumoniae* AR39 is 91%. However, there are considerable differences between cHsp60-1, cHsp60-2, and cHsp60-3 in a given species or serovariant. In *C. trachomatis* serovar D, the levels of amino acid identity and similarity between cHsp60-1 and cHsp60-2 are 23 and 19%, respectively; the levels of amino acid identity and similarity between cHsp60-1 and cHsp60-3 are 18 and 20%, respectively; and the levels of amino acid identity and similarity between cHsp60-2 and cHsp60-3 are 17 and 15%, respectively (28, 35, 49).

Although the majority of previous studies clearly involved cHsp60-1, as confirmed by sequence analysis, certain studies, including our study (43), generated new questions concerning the extent to which each cHsp60 responds to a given microenvironment, especially a microenvironment leading to chlamydial persistence. Thus, the purpose of this study was to determine which cHsp60 is iron responsive in *C. trachomatis* serovar E.

MATERIALS AND METHODS

Bacterial strains, eukaryotic host cells, and growth. Stock inocula of *C. trachomatis* serovar E/UW-5CX EB were generated in McCoy cell fibroblasts and titrated to determine their infectivity. Polarized human endometrial epithelial cells (HEC-1B) were used as host cells in iron deprivation experiments and were maintained in Eagle's minimal essential medium containing 2 mM glutamine and 5% (vol/vol) heat-inactivated fetal bovine serum at 37°C. For induction of iron deprivation, chlamydia-infected cultures were allowed to grow until 36 h post-inoculation (hpi), and one-half of the samples were exposed to 500 μ M Desferal for 30 min and 1 and 2 h.

Escherichia coli LMG194(pBAD/HisA) was used to engineer and overexpress each cHsp60. The recombinants expressing cHsp60-1, cHsp60-2, and cHsp60-3 were designated *E. coli* LMG194(pJER516), LMG194(pJER517), and LMG194(pJER518), respectively. Each recombinant *E. coli* was grown in reduced medium (Invitrogen, Carlsbad, CA) containing 0.2% (wt/vol) glucose and 100 μ g/ml ampicillin (Sigma-Genosys, The Woodlands, TX) to the mid-log phase (A_{600} , 0.4 to 0.6) at 37°C. Cultures were subsequently centrifuged, washed, and resuspended in prewarmed glucose-free medium. Arabinose was then added to each culture for 4 h of induction. Maximum expression of cHsp60-1 and cHsp60-2 in *E. coli* LMG194(pJER516) and LMG194(pJER517) required 0.002% (wt/vol) arabinose, whereas maximum expression of cHsp60-3 in *E. coli* LMG194(pJER518) required 20% (wt/vol) arabinose.

DNA amplification, cloning, and sequence analysis. The primers used for PCR amplification of the chlamydial *groEL* genes were designed using the genome sequence of *C. trachomatis* serovar D (49). The reactions were carried out with an Expand High Fidelity PCR system kit (Roche, Nutley, NJ) in the presence of 0.5 pmol of forward, 0.5 pmol of reverse primer, and 10-fold (1:10 to 1:1,000) dilutions of *C. trachomatis* serovar E DNA template. After 35 cycles of amplification, the PCR products were cleaned up using a QIAquick PCR purification kit (QIAGEN, Germantown, MD); the sizes and concentrations of the purified products were monitored by agarose gel electrophoresis in the presence of ethidium bromide and by determining the optical density, respectively. All PCRs were done in duplicate to reduce introduction of errant nucleotides. The PCR products were then directionally cloned into the pBAD/HisA vector (Invitrogen,

Carlsbad, CA) under the control of the *araC* promoter with an N-terminal six-histidine tag for recombinant protein detection and used to transform *E. coli* LMG194 by the traditional CaCl_2 method (46). For each *groEL* gene, the recombinant plasmids from three clones were purified using a Concert nucleic acid purification kit (Invitrogen) and then sequenced to verify in-frame cloning and to determine the complete nucleic acid sequences.

Peptide antibodies. The predictive amino acid sequences of *C. trachomatis* serovar E Hsp60-1, Hsp60-2, and Hsp60-3 were aligned using the EditSeq and MegAlign software from DNASTar, Inc. (Madison, WI). Peptides that were 17 to 21 residues long, described by Giles et al. (21), were commercially synthesized, the purity was assessed by analytical high-pressure liquid chromatography and mass spectroscopy, and each peptide was subsequently used to immunize two female New Zealand White rabbits (Sigma-Genosys, The Woodlands, TX). The results of enzyme-linked immunosorbent assays were provided by the manufacturer to ensure reactivity.

The majority of antiserum from each bleed was immediately stored at -20°C upon receipt. One milliliter of antiserum from each bleed was kept at 4°C to determine the Western blot reactivities of the crude preimmune and immune sera against total protein from HEC-1B cells and *E. coli* LMG194 as controls and arabinose-induced recombinant *E. coli* LMG194(pJER516), LMG194(pJER517), and LMG194(pJER518). Immune sera exhibiting the most selective reactivity with the intended Hsp60 homolog were then placed on protein A columns (ImmunoPure immobilized protein A; Pierce, Rockford, IL) to purify immunoglobulin G, and Western blotting was performed to determine the degree of monospecificity and the reduction in the cross-reactivity with other *E. coli* proteins. Antiserum against the peptide from cHsp60-3 required a further step of adsorption against whole cells of arabinose-induced *E. coli* LMG194(pJER516) expressing cHsp60-1. A monoclonal antibody reagent (Sigma-Genosys, The Woodlands, TX) against the polyhistidine tag was also used in this study.

Protein quantitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, and chemiluminescence. The total protein concentrations of samples were determined using the Micro BCA assay (Pierce, Rockford, IL). Cell pellets were resuspended in lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 mg/ml lysozyme, 0.1 mg/ml DNase I, 10 mM CaCl_2) and subjected to three freeze-thaw cycles. After the final thaw, samples were centrifuged at $8,000 \times g$ for 10 min, and each supernatant was combined with denaturing sample buffer and heated at 100°C for 5 min. The proteins were resolved in small-format 4 to 12% bis-Tris NuPAGE gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes for Western blotting. Preliminary separations were conducted in large-format 12.5% polyacrylamide gels loaded with 1 mg of protein to accommodate multiple blots for screening and titrating antisera.

For Western blotting, membranes were blocked with Blotto-plus (5% [wt/vol] dry nonfat milk in phosphate-buffered saline, 0.1% [vol/vol] Tween 20, 10% [vol/vol] heat-inactivated fetal bovine serum), and washing was performed with phosphate-buffered saline containing 0.1% (vol/vol) Tween 20. Various dilutions were examined for the polyclonal peptide antisera generated against each of the cHsp60s, and a monoclonal antibody against the polyhistidine tag (Sigma-Genosys, The Woodlands, TX) was also used as a control. Specific signals were then detected either (i) by a colorimetric assay with an anti-rabbit alkaline phosphatase-conjugated secondary antibody and Western Blue substrate (Promega, Madison, WI) or (ii) by chemiluminescence using an anti-rabbit horseradish peroxidase conjugate, the SuperSignal West (Pierce) solution, and Kodak X-OMAT AR film.

Electron microscopy. Samples of *C. trachomatis*-infected polarized HEC-1B cells at 36 hpi were exposed to 500 μ M Desferal for 30 min and 1 and 2 h; mock-exposed samples were used as controls. Each sample was immediately washed, fixed, processed, and embedded in Epon-araldite and Lowicryl (Polysciences, Inc.) for high-contrast electron microscopy and immunoelectron microscopy, respectively, as described by Giles et al. (21).

Visualization and image capture were done using a Philips Tecnai 10 transmission electron microscope (FEI Company, Hillsboro, OR) operating at 80 kV.

Nucleotide sequence accession numbers. The sequences determined in this study have been deposited in the GenBank database under the following accession numbers: AY447001 for *C. trachomatis* serovar E *groEL-1*, AY447002 for *C. trachomatis* serovar E *groEL-2*, and AY447003 for *C. trachomatis* serovar E *groEL-3*.

RESULTS

Amplification and nucleotide sequence analysis of *C. trachomatis* serovar E *groEL* ORF. DNA that was the expected size was amplified with all dilutions of the *C. trachomatis* serovar E

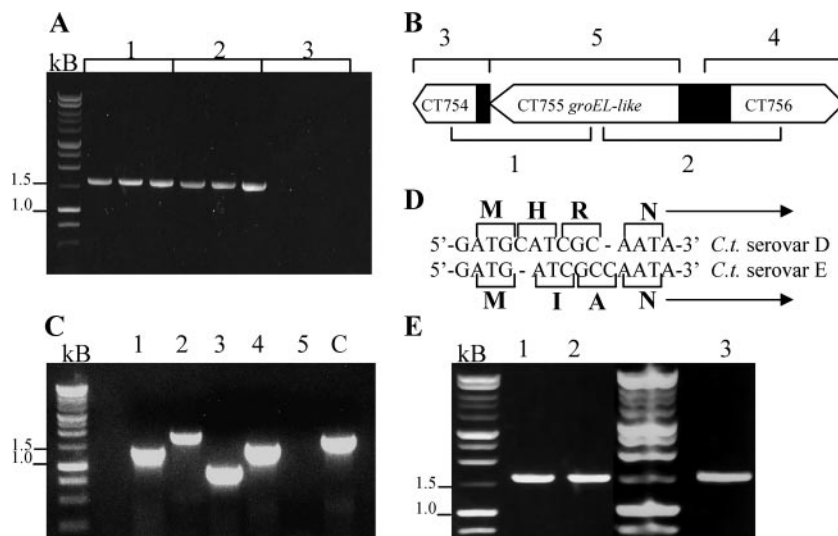


FIG. 1. PCR amplification of *C. trachomatis* serovar E *groEL*. (A) Initial attempt to amplify *groEL-1*, *groEL-2*, and *groEL-3* (lanes 1, lanes 2, and lanes 3, respectively) using a *C. trachomatis* serovar E DNA template and primers based on the sequence of *C. trachomatis* serovar D. (B) Strategy used to amplify *groEL-3* and flanking sequences. (C) Result of amplification of *groEL-3* and flanking sequences. Lanes 1 through 5 contained areas indicated in panel B, and amplification of *groEL-1* was used as a control. (D) Difference in the starting sequences of *C. trachomatis* serovars D and E. (E) Amplification of *C. trachomatis* serovar E *groEL-1*, *groEL-2*, and *groEL-3* with redesigned primers for *groEL-3*.

groEL DNA templates except the *groEL-3*/CT755 template (Fig. 1A, lanes 3). Figures 1B and C show the results of a change in primer strategy, and the data revealed that CT755 was present along with the flanking sequences. Further analysis showed that a missing cytosine residue in the initial sequence of CT755 in serovar E at position 25 was responsible for the lack of primer hybridization and amplification (Fig. 1D). As determined by comparison with the previously published sequence of CT755 in serovar D, a frameshift placed the ORF back into frame by insertion of a cytosine residue at position 30 in serovar E. The final results were confirmed using a new set of primers (Fig. 1E). The nucleotide sequences of *C. trachomatis* serovar E *groEL-1*, *groEL-2*, and *groEL-3* are 99.7, 98.5, and 99.2% identical to their counterparts in *C. trachomatis* serovar D, with only 6-, 25-, and 8-bp differences, respectively.

Specificity of the peptide antisera. Monospecificity was achieved for anti-cHsp60-1 and anti-cHsp60-2 with purification of immunoglobulin G alone (Fig. 2A). Antiserum against cHsp60-3 initially exhibited faint cross-reactivity with Hsp60-1 that was removed by cross-adsorption against whole cells of arabinose-induced *E. coli* LMG194(pJER516) expressing cHsp60-1. A control using an anti-histidine monoclonal antibody (Sigma-Genosys, The Woodlands, TX) was included.

Iron responsiveness of cHsp60s. Next, the peptide antibodies were used to examine *C. trachomatis*-infected cells with or without 500 μ M Desferal, which were exposed for 30 min and 1 and 2 h, beginning at 36 hpi (Fig. 2B). Preliminary experiments using the standard 50 μ M Desferal for 96 h resulted in induction of a persistence-like state (43); these initial experiments indicated that only cHsp60-2 responds to iron deprivation beginning at 36 hpi (data not shown). Therefore, we changed the strategy in a manner analogous to application of heat, cold, or acid shock in other bacteria. Figure 2B confirms that cHsp60-2 is the primary cHsp60 that responds to iron

limitation. Notably, cHsp60-2 is a target of proteolysis during cell lysis even in the presence of protease inhibitors (several combinations of inhibitors were tested). The data also showed that cHsp60-1 is strongly expressed, but there was little or no difference between the expression in the absence of Desferal and the expression in the presence of Desferal; cHsp60-3 expression was delayed, but again, there was little difference between the expression in the absence of Desferal and the expression in the presence of Desferal.

Immunoelectron microscopy. To confirm that *C. trachomatis* cHsp60-2 is iron responsive, the final experiment involved examination of thin sections by immunoelectron microscopy. Multiple images were captured, saved as TIFF files, and printed; 2- μ m square grids were used to enclose 10 to 15 randomly selected squares containing chlamydial reticulate bodies (RB) on prints of each sample, and the gold particles in each box were counted (Fig. 3).

Figures 3A, C, and E show chlamydial RB in HEC-1B cells at 36 hpi (mock exposure); Fig. 3B, D, and F show RB at 36 hpi in cells that were exposed to 500 μ M Desferal for 1 h. Consistent with the results of the Western blot analyses, cHsp60-1 was strongly expressed, but there was not a significant difference between mock-exposed chlamydiae and chlamydiae exposed to Desferal (Fig. 3A and B); the numbers of particles in Fig. 3A and B for cHsp60-1 were 19 ± 8 and 18 ± 6 , respectively. Likewise, for weakly expressed cHsp60-3 there was no difference between mock exposure and exposure to Desferal; the numbers of gold particles in both Fig. 3E and F were 5 ± 2 . However, there was a significant difference ($P < 0.001$) between expression of cHsp60-2 after mock exposure and expression of cHsp60-2 after exposure to Desferal (Fig. 3C and D), as determined by Student's two-tailed *t* test; the numbers of gold particles in Fig. 3C and D were 4 ± 2 and 10 ± 3 , respectively.

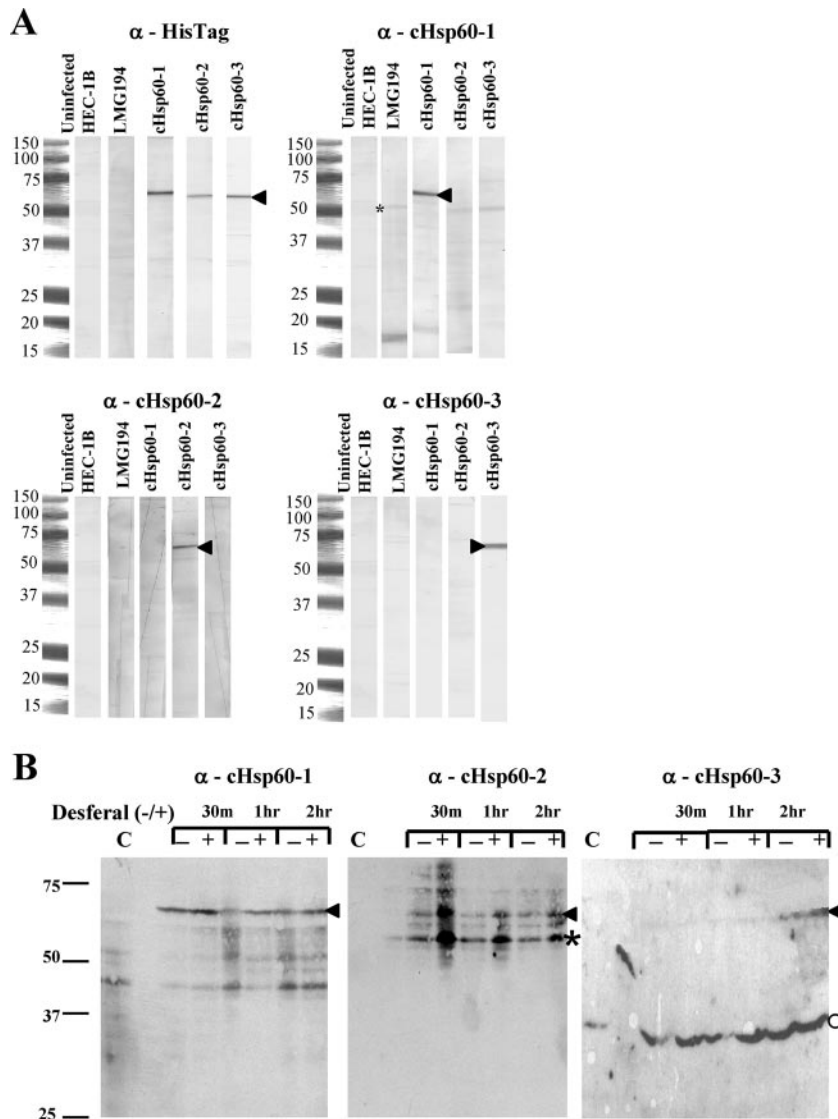


FIG. 2. Specificity of peptide antisera and response of cHsp60-2 to iron deprivation. (A) Samples used for Western blotting included uninfected HEC-1B cells, *E. coli* LMG194 alone, and arabinose-induced recombinants *E. coli* LMG194 (pJER516), LMG194 (pJER517), and LMG194 (pJER518), representing cHsp60-1, cHsp60-2 and cHsp60-3, respectively. An anti-His tag monoclonal antibody was used as a control (upper left panel). (B) Samples included uninfected HEC-1B cells (control) (lanes C), cells mock exposed for 30 min and 1 and 2 h, and cells exposed to Desferal for 30 min and 1 and 2 h. One milligram of protein was loaded onto preparative gels (A), whereas 15 μg was loaded into each lane in panel B. Arrowheads indicate the position of cHsp60. The asterisk indicates the position of a major proteolytic product of cHsp60-2, and the circle indicates the position of a cross-reactive protein in HEC-1B cells.

DISCUSSION

In this report we show conclusively that *C. trachomatis* serovar E cHsp60-2 is the primary cHsp60 that exhibits enhanced expression in response to iron restriction. In a larger context, the specificity of cHsp60-2 expression as a result of iron limitation indicates that the mechanisms for development of chlamydial persistence have unique signatures. This is an emerging concept in the study of chlamydial pathogenesis. The expression of cHsp60s, as determined by either protein expression or transcript analysis, has been examined using several models of persistence (2, 4, 17, 19, 22, 39).

Belland et al. (4) conducted a comprehensive microarray study of *C. trachomatis* serovar D transcription and compared standard

growth and growth of IFN-γ-mediated persistent chlamydiae in HeLa 229 cells. None of the *groEL* transcripts varied significantly for the first 24 h; however, by 48 hpi, transcription of *groEL-1* had increased 2.8-fold due to tryptophan depletion by IFN-γ. Tryptophan is an essential amino acid for *C. trachomatis*. A separate group of investigators examined transcription using quantitative real-time PCR for three distinct modes of persistence, exposure to IFN-γ, penicillin G, and iron depletion, in *C. psittaci* growing in HEp-2 cells (22). At 24 hpi, *groEL-1* was upregulated only in the penicillin G model of persistence; IFN-γ persistence actually resulted in significant downregulation of *groEL-1*. Downregulation of *groEL-1* was also observed by 48 hpi for *C. psittaci* persistence induced by iron deprivation; *groEL-2* was not examined in this

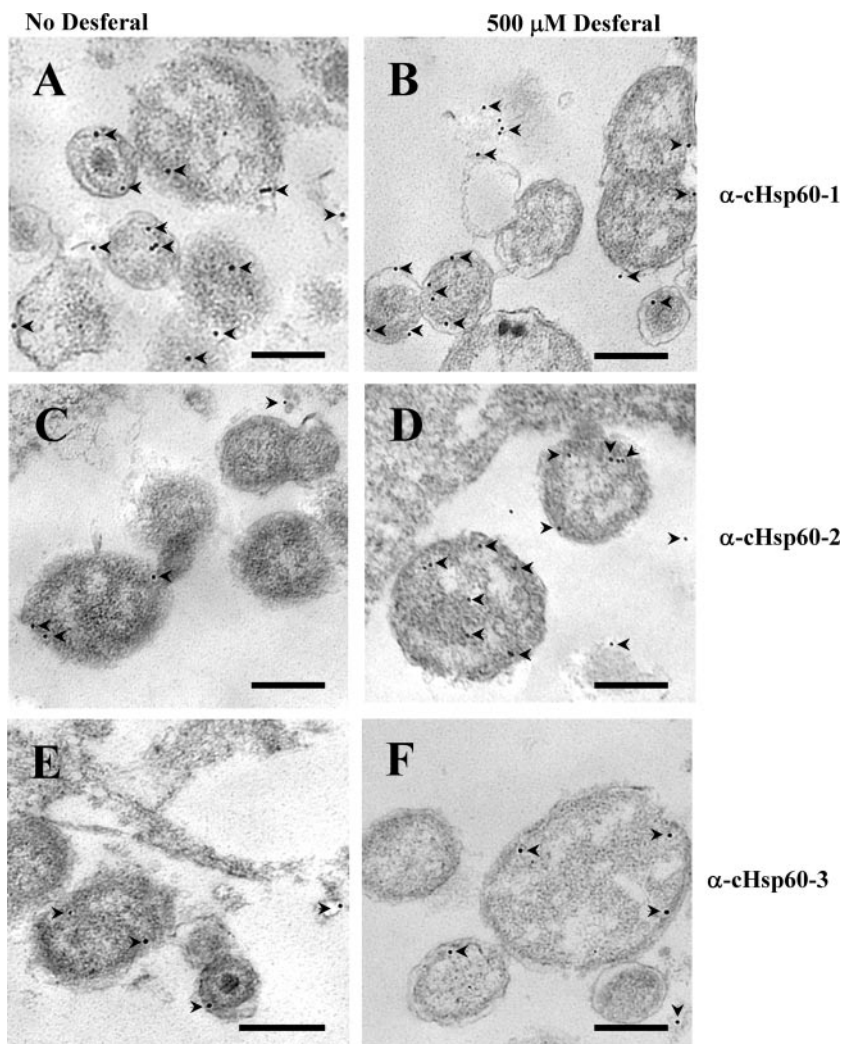


FIG. 3. Immunolabeling transmission electron microscopy showing the response of cHsp60-2 to iron limitation. Chlamydia-infected HEC-1B cells at 36 hpi were either not exposed to Desferal (A, C, and E) or exposed to 500 μ M Desferal (B, D, and F) for 1 h and labeled using a 1:100 (vol/vol) dilution of anti-cHsp60-1 (A and B), a 1:20 (vol/vol) dilution of anti-cHsp60-2 (C and D), or a 1:40 (vol/vol) dilution of anti-cHsp60-3 (E and F). A 15-nm gold-conjugated anti-rabbit serum (Amersham Biosciences) was used at a 1:200 (vol/vol) dilution for visualization. Bars = 0.5 μ m. The arrowheads indicate gold particles.

study. Using a different stress environment, Karunakaran and colleagues (28) examined transcription using a heat shock model. HeLa 229 cells were infected with *C. trachomatis* serovar D for 18 h and subsequently subjected to a 10-min heat pulse at 45°C. mRNA was quantified using a microarray procedure, and the results showed that there was a >5-fold increase in *groEL-1* transcripts; the quantities of the *groEL-2* and *groEL-3* transcripts did not change. In studies of protein expression, expression of *C. trachomatis* serovar A cHsp60-1, as determined using Western blotting and an anti-cHsp60-1 monoclonal antibody, was enhanced in an in vitro model of IFN- γ -mediated persistence (2). For *C. pneumoniae* cHsp60-1, there was a twofold increase in expression at 48 hpi with the following three different models of persistence and/or stress: (i) IFN- γ exposure, (ii) iron deprivation, and (iii) heat shock (39).

Our findings are more consistent with results reported by Gerard and colleagues (20). These investigators quantified mRNA for each *groEL* homolog in *C. trachomatis* serovar K

using real-time reverse transcription-PCR with the following systems: (i) active infection in HEp-2 cells, (ii) persistent infection in human monocytes, and (iii) synovial tissue from patients with *Chlamydia*-associated arthritis. In active HEp-2 cell infection, all *groEL* transcripts were present beginning at 8 hpi, and the levels increased throughout chlamydial development; *groEL-3* was transcribed at the highest levels. In the monocyte persistence model, the levels of *groEL-1* and *groEL-3* transcripts were low, whereas the level of the *groEL-2* transcripts increased threefold over 3 days as the organisms entered the persistent state. Findings for the synovial tissues also showed that the levels of *groEL-2* transcripts were high. Comparisons of our model with this model of *C. trachomatis* serovar K persistence in monocytes may not be entirely legitimate because our model involves *C. trachomatis* serovar E, a less invasive organism, in epithelial cells, but the observations are intriguing nonetheless.

From the standpoint of immunopathogenesis, the impor-

tance of cHsp60s in disease has been the subject of several excellent reviews (9, 13, 14, 16, 26, 30, 34, 40). Our previous work, performed with the antisera generated in this study, showed that cHsp60-2 and cHsp60-3, but not cHsp60-1, escape from chlamydial inclusions via vesicle eversion, a process that is exacerbated by exposure to azithromycin (21, 44). The vesicles are thought to interact with host cell antigen presentation and to contribute to the inflammatory response. Studies of heat shock proteins, in general, are being performed since heat shock proteins carry antigens and deliver peptides to the major histocompatibility complex, thus priming the adaptive immune response by inducing specific B and T cells in the absence of adjuvants (41). Heat shock proteins also participate in the innate immune response by stimulating the production of chemokines (41). In one study, cHsp60 serum antibodies were shown to be the best predicting factor for tubal factor infertility (51). Mapping of cHsp60-1 peptide epitopes in human sera has been done (50, 54); it may be worthwhile to investigate whether peptides of cHsp60-2 and/or cHsp60-3 contribute to the generation of specific immunoglobulins.

GroEL proteins are essential for bacterial growth and ensure that newly synthesized proteins are functional; expression of GroEL proteins increases in response to a variety of stresses, including heat shock and nutrient deprivation (56). Structural studies of cHsp60s have shown that although the primary amino acid sequences of cHsp60s differ from the primary amino acid sequences of other organisms, amino acid residues involved in binding polypeptides are conserved (28). It is also clear that cHsp60-1 is negatively regulated by the interaction of a negative regulator, HcrA, with a CIRCE element in the operator regions of the *groES-groEL-1* and *dnaK* operons; HrcA does not appear to regulate *groEL-2* or *groEL-3* (53). Although the results of studies of *C. trachomatis* serovar K persistence and synovial fluid support the hypothesis that there is regulation of chlamydial *groEL-2* at the level of transcription, there appears to be no Fur/DcrA binding site in upstream sequences. There is only a partial Fur box approximately 300 bp downstream in *C. trachomatis* serovar E *groEL-2*; determining whether DcrA binds to *groEL-2* sequences is part of a separate project in our laboratory. A likely alternative mechanism for enhanced expression of cHsp60-2 may involve small RNAs that regulate genes posttranscriptionally or by stabilization of mRNA; numerous iron-responsive proteins in other bacteria are known to be regulated in this fashion (25). Chlamydiae code for several small RNAs (5). Finally, the increased level of cHsp60-2 might also involve protein stability. For example, when *E. coli* GroEL is bound to an unfolded substrate in vitro, the complex remains stable at 25°C for more than 2 weeks; at 43°C, the half-life is 1.5 h (36).

Overall, the results of this study and our previous work (21, 43) strongly indicate that cHsp60-2 should be considered a potential mediator of immune-mediated damage, and they mechanistically indicate that not all modes of chlamydial persistence are identical.

ACKNOWLEDGMENTS

This work was supported by grant RO1AI40915 from the NIAID, National Institutes of Health, to J.E.R.

Our appreciation is extended to Priscilla B. Wyrick and Robert V. Schoborg for helpful suggestions and advice.

REFERENCES

- Andrews, N. C. 2000. Iron homeostasis: insights from genetics and animal models. *Nat. Rev.* **1**:208–216.
- Beatty, W. L., G. I. Byrne, and R. P. Morrison. 1993. Morphologic and antigenic characterization of interferon gamma-mediated persistent *Chlamydia trachomatis* infection in vitro. *Proc. Natl. Acad. Sci. USA* **90**:3998–4002.
- Beatty, W. L., G. I. Byrne, and R. P. Morrison. 1994. Repeated and persistent infection with *Chlamydia* and the development of chronic inflammation and disease. *Trends Microbiol.* **2**:94–98.
- Belland, R. J., D. E. Nelson, D. Virok, D. D. Crane, D. Hogan, D. Sturdevant, W. L. Beatty, and H. D. Caldwell. 2003. Transcriptome analysis of chlamydial growth during IFN- γ -mediated persistence and reactivation. *Proc. Natl. Acad. Sci. USA* **100**:15971–15976.
- Belland, R. J., L. Rose, and Y. AbdelRahman. 2006. Promoter mapping of coding and non-coding RNAs using an intergenic microarray for *Chlamydia trachomatis*, p. 17–20. In M. Chernesky, H. Caldwell, G. Christiansen, I. N. Clarke, B. Kaltenboeck, C. Knirsch, C.-C. Kuo, J. Mahony, R. G. Rank, P. Saikku, J. Schachter, W. E. Stamm, R. S. Stephens, J. T. Summersgill, P. Timms, and P. B. Wyrick (ed.), *Proceedings of the 11th International Symposium on Human Chlamydial Infections, Niagara-on-the-Lake, Ontario, Canada. International Chlamydia Symposium, San Francisco, CA.*
- Betsou, F., J. M. Sueur, and J. Orfila. 2003. Anti-*Chlamydia pneumoniae* heat shock protein 10 antibodies in asthmatic adults. *FEMS Immunol. Med. Microbiol.* **35**:107–111.
- Brunham, R. C., I. W. Maclean, B. Binns, and R. W. Peeling. 1985. *Chlamydia trachomatis*: its role in tubal infertility. *J. Infect. Dis.* **152**:1275–1282.
- Bulut, Y., E. Faure, L. Thomas, H. Karahashi, K. S. Michelson, O. Equils, S. G. Morrison, R. P. Morrison, and M. Arditì. 2002. Chlamydial heat shock protein 60 activates macrophages and endothelial cells through Toll-like receptor 4 and MD2 in a MyD88-dependent pathway. *J. Immunol.* **168**:1435–1440.
- Cohen, C. R., and R. C. Brunham. 1999. Pathogenesis of *Chlamydia* induced pelvic inflammatory disease. *Sex. Transm. Infect.* **75**:21–24.
- Cohen, C. R., J. Gichui, Rukaria, S. Sinei, L. K. Gaur, and R. C. Brunham. 2003. Immunogenetic correlates for *Chlamydia trachomatis*-associated tubal infertility. *Obstet. Gynecol.* **101**:438–444.
- Cohen, C. R., K. M. Koochesfahani, A. S. Meier, C. Shen, K. Karunakaran, B. Ondondo, T. Kinyari, N. R. Mugo, R. Nguti, and R. C. Brunham. 2005. Immunoepidemiologic profile of *Chlamydia trachomatis* infection: importance of heat shock protein 60 and interferon-gamma. *J. Infect. Dis.* **4**:591–599.
- Conway, D. J., M. J. Holland, A. E. Campbell, R. L. Bailey, P. Krausa, R. W. Peeling, H. C. Whittle, and D. C. Mabey. 1996. HLA class I and II polymorphisms and trachomatous scarring in a *Chlamydia trachomatis*-endemic population. *J. Infect. Dis.* **174**:643–646.
- Darville, T. 2000. *Chlamydia* spp., p. 229–261. In J. P. Nataro, M. J. Blaser, and S. Cunningham-Rundles (ed.), *Persistent bacterial infections*. ASM Press, Washington, DC.
- Debattista, J., P. Timms, J. Allan, and J. Allan. 2003. Immunopathogenesis of *Chlamydia trachomatis* infections in women. *Fertil. Steril.* **79**:1273–1287.
- den Hartog, J. E., J. A. Land, F. R. M. Stassen, A. G. H. Kessels, and C. A. Bruggeman. 2005. Serological markers of persistent *C. trachomatis* infections in women with tubal factor subfertility. *Hum. Reprod.* **20**:986–990.
- Di Felice, V., S. David, F. Cappello, F. Farina, and G. Zummo. 2005. Is chlamydial heat shock protein 60 a risk factor for oncogenesis? *Cell. Mol. Life Sci.* **62**:4–9.
- Drees-Werrigloer, U., I. Padubrin, B. Jurgens-Saathoff, A. P. Hudson, H. Zeidler, and L. Kohler. 2000. Persistence of *Chlamydia trachomatis* is induced by ciprofloxacin and ofloxacin in vitro. *Antimicrob. Agents Chemother.* **44**:3288–3297.
- Equils, O., D. Lu, M. Gatter, S. S. Witkin, C. Bertolotto, M. Arditì, J. A. McGregor, C. F. Simmons, and C. J. Hobel. 2006. *Chlamydia* heat shock protein 60 induces trophoblast apoptosis through TLR4. *J. Immunol.* **177**:1257–1263.
- Gerard, H. C., L. Kohler, P. J. Branigan, H. Zeidler, H. R. Schumacher, and A. P. Hudson. 1998. Viability and gene expression in *Chlamydia trachomatis* during persistent infection of cultured human monocytes. *Med. Microbiol. Immunol.* **187**:115–120.
- Gerard, H. C., J. A. Whittum-Hudson, H. R. Schumacher, and A. P. Hudson. 2004. Differential expression of three *Chlamydia trachomatis* Hsp60-encoding genes in active vs. persistent infections. *Microb. Pathog.* **36**:35–39.
- Giles, D. K., J. D. Whittimore, R. W. LaRue, J. E. Raulston, and P. B. Wyrick. 2006. Ultrastructural analysis of chlamydial antigen-containing vesicles everting from the *Chlamydia trachomatis* inclusion. *Microb. Infect.* **8**:1579–1591.
- Goellner, S., E. Schubert, E. Liebler-Tenorio, H. Hotzel, H. P. Saluz, and K. Sachse. 2006. Transcriptional response patterns of *Chlamydia psittaci* in different in vitro models of persistent infection. *Infect. Immun.* **74**:4801–4808.
- Higgins, D. P., S. Hemsley, and P. J. Canfield. 2005. Association of uterine

- and salpingeal fibrosis with chlamydial Hsp60 and Hsp10 antigen-specific antibodies in *Chlamydia*-infected koalas. *Clin. Diag. Lab. Immunol.* **12**:632–639.
24. Jackson, M., N. White, P. Giffard, and P. Timms. 1999. Epizootiology of *Chlamydia* infections in two free-range koala populations. *Vet. Microbiol.* **65**:255–264.
 25. Kadner, R. J. 2005. Regulation by iron: RNA rules the rust. *J. Bacteriol.* **187**:6870–6873.
 26. Kalayoglu, M. V., Indrawati, R. P. Morrison, S. G. Morrison, Y. Yuan, and G. I. Byrne. 2000. Chlamydial virulence determinants in atherogenesis: the role of chlamydial lipopolysaccharide and heat shock protein 60 in macrophage-lipoprotein interactions. *J. Infect. Dis.* **181**:S483–S489.
 27. Karinen, L., A. Pouta, A. L. Hartikainen, A. Bloigu, M. Paldanius, M. Leinonen, P. Saikku, and M. R. Jarvelin. 2004. Antibodies to *Chlamydia trachomatis* heat shock proteins Hsp60 and Hsp10 and subfertility in general population at age 31. *Am. J. Reprod. Immunol.* **52**:291–297.
 28. Karunakaran, K. P., Y. Noguchi, T. D. Read, A. Cherkasov, J. Kwee, C. Shen, C. C. Nelson, and R. C. Brunham. 2003. Molecular analysis of the multiple GroEL proteins of chlamydiae. *J. Bacteriol.* **185**:1958–1966.
 29. Kelper, M. E., A. Kaul, B. Nowicki, W. E. Findley, T. W. Hutchens, and N. Nagamani. 1996. Estrogen regulation of lactoferrin expression in human endometrium. *Am. J. Reprod. Immunol.* **36**:243–247.
 30. Kinnunen, A., J. Paavonen, and H. M. Surcel. 2001. Heat shock protein 60 specific T-cell response in chlamydial infections. *Scand. J. Immunol.* **54**:76–81.
 31. Kol, A., G. K. Suhkova, A. H. Lichtman, and P. Libby. 1998. Chlamydial heat shock protein 60 localizes in human atheroma and regulates macrophage tumor necrosis factor- α and matrix metalloproteinase expression. *Circulation* **98**:300–307.
 32. Kol, A., A. H. Lichtman, R. W. Finberg, P. Libby, and E. A. Kurt-Jones. 2000. Cutting edge: heat shock protein (HSP) 60 activates the innate immune response: CD14 is an essential receptor for HSP60 activation of mononuclear cells. *J. Immunol.* **164**:13–17.
 33. LaVerda, D., L. N. Albanese, P. E. Ruther, S. G. Morrison, R. P. Morrison, K. A. Ault, and G. I. Byrne. 2000. Seroreactivity to *Chlamydia trachomatis* Hsp10 correlates with severity of human genital tract disease. *Infect. Immun.* **68**:303–309.
 34. LaVerda, D., M. V. Kalayoglu, and G. I. Byrne. 1999. Chlamydial heat shock proteins and disease pathology: new paradigms for old problems? *Infect. Dis. Obstet. Gynecol.* **7**:64–71.
 35. Lipman, D. J., and W. R. Pearson. 1985. Rapid and sensitive protein similarity searches. *Science* **227**:1435–1441.
 36. Melkani, G. C., G. Zardeneta, and J. A. Mendoza. 2005. On the chaperone activity of GroEL at heat-shock temperature. *Int. J. Biochem. Cell. Biol.* **37**:1375–1385.
 37. Morrison, R. P., K. Lyng, and H. D. Caldwell. 1989. Chlamydial disease pathogenesis. Ocular hypersensitivity elicited by a genus-specific 57-kDa protein. *J. Exp. Med.* **169**:663–675.
 38. Mozzato-Chamay, N., O. S. Madhi, O. Jallow, D. C. Mabey, R. L. Bailey, and D. J. Conway. 2000. Polymorphisms in candidate genes and risk of scarring trachoma in a *Chlamydia trachomatis*-endemic population. *J. Infect. Dis.* **182**:1545–1548.
 39. Mukhopadhyay, S., R. D. Miller, E. D. Sullivan, C. Theodoropoulos, S. A. Mathews, P. Timms, and J. T. Summersgill. 2006. Protein expression profiles of *Chlamydia pneumoniae* in models of persistence versus those of heat shock stress response. *Infect. Immun.* **74**:3853–3863.
 40. Peeling, R. W., and D. C. Mabey. 1999. Heat shock protein expression and immunity in chlamydial infections. *Infect. Dis. Obstet. Gynecol.* **7**:72–79.
 41. Qazi, K. R., M. R. Qazi, E. Julian, M. Singh, M. Abedi-Valugerdi, and C. Fernandez. 2005. Exposure to mycobacteria primes the immune system for evolutionarily diverse heat shock proteins. *Infect. Immun.* **73**:7687–7696.
 42. Rau, A., S. Wyllie, J. Whittimore, and J. E. Raulston. 2005. Identification of *Chlamydia trachomatis* genomic sequences recognized by chlamydial divalent cation-dependent regulator A (DcrA). *J. Bacteriol.* **187**:443–448.
 43. Raulston, J. E. 1997. Response of *Chlamydia trachomatis* serovar E to iron restriction in vitro and evidence for iron-regulated chlamydial proteins. *Infect. Immun.* **65**:4539–4547.
 44. Raulston, J. E., T. R. Paul, S. T. Knight, and P. B. Wyrick. 1998. Localization of *Chlamydia trachomatis* heat shock proteins 60 and 70 during infection of a human endometrial epithelial cell line in vitro. *Infect. Immun.* **66**:2323–2329.
 45. Saikku, P., M. Leinonen, K. Mattila, M. R. Ekman, M. S. Nieminen, P. H. Huttunen, and V. Valtonen. 1988. Serological evidence of an association of a novel *Chlamydia*, TWAR, with chronic coronary heart disease and acute myocardial infarction. *Lancet* **ii**:983–986.
 46. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY.
 47. Sasu, S., D. LaVerda, N. Qureshi, D. T. Golenbock, and D. Beasley. 2001. *Chlamydia pneumoniae* and chlamydial heat shock protein 60 stimulate proliferation of human vascular smooth muscle cells via Toll-like receptor 4 and p44/p42 mitogen-activated protein kinase activation. *Circ. Res.* **89**:244–250.
 48. Sharma, J., F. Dong, M. Pirbhaj, and G. Zhong. 2005. Inhibition of proteolytic activity of a chlamydial proteasome/protease-like factor by antibodies from humans infected with *Chlamydia trachomatis*. *Infect. Immun.* **73**:4414–4419.
 49. Stephens, R. S., S. Kalman, C. Lammel, J. Fan, R. Marathe, L. Aravind, W. Mitchell, L. Olinger, R. L. Tatusov, Q. Zhao, E. V. Koonin, and R. W. Davis. 1998. Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science* **282**:754–759.
 50. Sziller, I., S. S. Witkin, M. Zeigert, Z. Csapo, A. Ujhazy, and Z. Papp. 1998. Serological responses of patients with ectopic pregnancy to epitopes of the *Chlamydia trachomatis* 60kDa heat shock protein. *Hum. Reprod.* **13**:1088–1093.
 51. Tiitinen, A., H. M. Surcel, M. Halttunen, S. Birkelund, A. Bloigu, G. Christiansen, P. Koskela, S. G. Morrison, R. P. Morrison, and J. Paavonen. 2006. *Chlamydia trachomatis* and chlamydial heat shock protein 60-specific antibody and cell-mediated responses predict tubal factor infertility. *Hum. Reprod.* **21**:1533–1538.
 52. Toye, B., C. Laferrrie, P. Claman, P. Jessamine, and R. Peeling. 1993. Association between antibody to the chlamydial heat shock protein and tubal infertility. *J. Infect. Dis.* **168**:1236–1240.
 53. Wilson, A. C., C. C. Wu, J. R. Yates III, and M. Tan. 2005. Chlamydial GroEL autoregulates its own expression through direct interactions with the HrcA repressor protein. *J. Bacteriol.* **187**:7535–7542.
 54. Witkin, S. S., M. Askienazy-Elbhar, J. Henry-Suchet, J. Belaisch-Allart, J. Tort-Grumbacj, and K. Sarjdine. 1998. Circulating antibodies to a conserved epitope of the *Chlamydia trachomatis* 60 kDa heat shock protein (hsp60) in infertile couples and its relationship to antibodies to *C. trachomatis* surface antigens and the *Escherichia coli* and human Hsp60. *Hum. Reprod.* **13**:1175–1179.
 55. Wyllie, S., and J. E. Raulston. 2001. Identifying regulators of transcription in an obligate intracellular pathogen: a metal-dependent repressor in *Chlamydia trachomatis*. *Mol. Microbiol.* **40**:1027–1036.
 56. Young, R. A., and T. J. Elliot. 1989. Stress proteins, infection, and immune surveillance. *Cell* **59**:5–8.