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Expression of 65- and 67-Kilodalton Heat-Regulated Proteins and a 70-Kilodalton Heat Shock Cognate Protein of *Leishmania donovani* in Macrophages

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Heat shock protein (HSP) expression was examined in murine bone marrow-derived macrophages infected with stationary-phase promastigotes of *Leishmania donovani*. Immunoblotting performed with a rabbit polyclonal antiserum raised against HSP60 from *Heliothis virescens* (moth) revealed the de novo appearance of 65- and 67-kDa proteins in leishmania-infected macrophages. A third protein of 60 kDa, which represented murine HSP60, was also detected, and its expression did not change in response to infection. In contrast, expression of the novel 65- and 67-kDa proteins in infected cells was coordinately regulated and, at 24 h of infection, reached maximal levels of 52 to 100% increases above initial levels determined at 3 h. Proteins which had identical electrophoretic mobilities and were similarly regulated in response to heat were also detected in promastigotes. The appearance of these proteins in macrophages was specific to leishmania infection in that neither protein was detected in noninfected cells either in the basal state or following several treatments, including (i) infection with *Yersinia pseudotuberculosis*, (ii) phagocytosis of *Staphylococcus aureus*, (iii) $NaAsO_2$ treatment, and (iv) heat shock. Expression of the 65- and 67-kDa heat-regulated *Leishmania* proteins was also observed to be selective, in that as their concentration was increasing, the abundance of the *Leishmania* surface protease gp63 in infected cells was noted to decrease. Murine HSP60 but not the *Leishmania* heat-regulated proteins was also recognized by a distinct rabbit antiserum raised against human HSP60, suggesting the presence of specific determinants within these *Leishmania* proteins. A monoclonal antibody that recognizes both mammalian HSP70 and HSP70 from plasmodia detected single isoforms of both *Leishmania* and murine HSP70 in infected cells, and the level of neither protein changed during infection. Moreover, although a murine HSP of 73 kDa was induced in response to both heat shock and $NaAsO_2$ treatment, it was not induced to detectable levels by infection. The rapid and relatively high level of expression of inducible HSP60-related proteins of *L. donovani* and *Leishmania* HSP70 in infected macrophages suggests that these proteins are involved in pathogenesis and may be important targets of the immune response.

Heat shock proteins (HSPs) are a ubiquitous, highly conserved set of molecules that have become a focus of intense interest, particularly within the fields of immunology and infectious diseases (13, 43). Increased expression of some but not all HSPs by individual cells may be observed under conditions of stress, such as exposure to high temperatures, chemical agents, oxidative injury, and inflammation (13, 22, 43). Microbial invasion also represents a form of stress to the host, and infections represent particularly interesting situations, since invading pathogens have the potential not only to induce HSP expression by host cells but also to elicit immune responses to their own HSPs (13, 24, 43).

Numerous investigations on the role of HSPs during host-pathogen interactions have revealed that HSPs of microbial origin are potent immunogens. For example, members of the HSP70 family are among the most abundant and immunogenic proteins present within pathogenic organisms (7, 10, 13, 16, 27, 32, 34). Members of the HSP60 family have also been found to be predominant immunogens in a diverse range of prokaryotic, intracellular infections, including leprosy, tuberculosis, Q fever, syphilis, Lyme disease, and Legionnaires' disease, and during infection with *Chlamydia* spp. (reviewed in reference 13). In both human leprosy and tuberculosis, mycobacterial HSP65 has been found to be a principal antigen recognized by T cells (14, 43), and a form of HSP65 was observed to be expressed in vivo by peritoneal macrophages from mice infected with *Toxoplasma gondii* (28). Induction of the latter protein correlated with protection against infection, but it was not possible to determine whether it was of pathogen or host origin. Taken together, these findings provide substantial evidence to implicate the HSP60 and HSP70 families as important elements in the pathogenesis of intracellular infection.

Leishmania spp. are dimorphic organisms that spend part of their life cycle in the alimentary tract of a sandfly vector as flagellated promastigotes and the remainder as obligate intracellular, nonflagellated amastigotes within mononuclear phagocytes of a mammalian host. When exposed to high temperatures (in the range from 34 to 37°C), diverse species of *Leishmania* promastigotes have been shown to respond by synthesizing HSPs (11, 20, 21, 36, 37, 40–42), and this response is postulated to be a principal event in bringing about the accompanying morphologic transformation of promastigotes to amastigotes (21, 41, 42). In fact, raising the temperature of cultured *Leishmania* promastigotes was found to induce a morphological change from the flagellated form to an amastigote-like, nonflagellated form in vitro (21, 38, 42). It is presumed but not proven that when *Leishmania*

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promastigotes are transferred from the ambient temperature of the poikilothermic insect vector to the mammalian host (37°C), analogous changes in the expression of *Leishmania* HSPs accompany morphologic conversion to the amastigote form within macrophages. Although little is known about the expression of *Leishmania* HSPs in infected cells, these findings suggest that during in vivo infection, these molecules may be expressed at high levels and become important immunogens. This inference is supported by the finding that HSP70 of *Leishmania donovani* was recognized by more than 50% of serum samples obtained from patients with visceral leishmaniasis (23).

To investigate further the potential role of HSPs in the pathogenesis of infections with *Leishmania* spp., the expression of HSP60 and HSP70 during in vitro infection of murine bone marrow macrophages (BMM) with *L. donovani* was examined. There were no detectable changes in the levels of murine HSP60 or HSP70 in response to infection. In contrast, the abundant, regulated expression of 65- and 67-kDa heat-regulated *Leishmania* proteins and the expression of one constitutive isoform of *Leishmania* HSP70 were observed.

MATERIALS AND METHODS

Reagents and antibodies. Rabbit polyclonal antiserum (SPA 805) raised against *Heliothis virescens* (moth) HSP60 (26) was from Stressgen Victoria, Canada. Mouse monoclonal anti-HSP70 antibody D4F18 (immunoglobulin G1 [IgG1]) raised against a recombinant 72-kDa protein from *Plasmodium falciparum* (1) and known to recognize both mammalian HSP70 and HSP70 from *P. falciparum* was a kind gift from O. Bensaude, Génétique Moléculaire, École Normale Superiore, Paris, France. Mouse monoclonal anti-HSP70 antibody (IgG1) specific for the inducible form of HSP70 was also from Stressgen (SPA 810). Mouse monoclonal antibody (IgG1) to the *Leishmania* surface protease gp63 was kindly provided by W. R. McMaster, University of British Columbia, Vancouver, Canada. Horseradish peroxidase-conjugated goat anti-rabbit IgG was from Bio-Rad, Richmond, Calif., and horseradish peroxidase-conjugated goat anti-mouse IgG was from Tago, Inc., Burlingame, Calif. To control for specificity of the rabbit polyclonal anti-HSP60, separate blotted membranes were probed with normal rabbit serum (Sigma, St. Louis, Mo.), and to control for specificity of the mouse monoclonal antibodies, MOPC 21 (IgG1) (Sigma) was used. As a positive control for HSP60, recombinant mycobacterial HSP65 was used (kindly provided by R. van der See, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands), and recombinant gp63 protein was provided by W. R. McMaster. The size markers used were Rainbow Markers (Amersham).

***Leishmania* strain.** The Sudan strain 2S of *L. donovani* was maintained by serial passage in Syrian hamsters. For experiments, hamster spleens were processed as described before (30), and promastigotes were obtained by culturing amastigotes in Senekjie medium (35) for 10 days (stationary phase) at room temperature. Promastigotes were harvested by centrifugation at 1,200 × *g* for 10 min, washed three times in Hanks' balanced salt solution (HBSS), and resuspended at 1.6×10^7 organisms per ml in RPMI 1640 medium containing 10% fetal calf serum (FCS) plus penicillin (100 U/ml) and streptomycin (100 μg/ml) (RPMI-FCS-P/S).

BMM. Cells were flushed from the tibias and femurs of four 8-week-old female C57BL/6 mice with a 21-gauge needle attached to a 10-ml syringe containing 10 ml of RPMI medium as described before (4). Cells were centrifuged at 800 × *g* for 10 min and then incubated at 4°C in 5 to 10 ml of Tris-NH_4Cl (9 volumes of 0.83% NH_4Cl to 1 volume of 0.17 M Tris [pH 7.2]) to lyse contaminating erythrocytes (30). Cells were washed once in RPMI, and debris and nondispersed tissue were removed by filtering the cell suspension through a 10-ml disposable pipette fitted with a piece of sterile cotton. The cells were then centrifuged, resuspended in 16 ml of RPMI-FCS-P/S with 10% conditioned medium obtained from EMT-6 cells as a source of colony-stimulating factor type 1 as described before (8), and dispensed into a single cell culture dish (100 by 15 mm; Fisher Scientific Co.). After incubation for 24 h at 37°C in a 95% air–5% CO_2 humidified atmosphere, nonadherent cells (~16 ml) were collected in a 50-ml tube, and the cell suspension was brought to a final volume of 48 ml, including 10% EMT-6-conditioned medium. The suspension was divided in equal portions, dispensed into four disposable 75-cm^2 cell culture flasks (Corning) and incubated at 37°C in 95% air–5% CO_2 for 7 days, with the addition of fresh 10% EMT-6-conditioned medium on day 3. At the end of the incubation period (day 8), homogeneous monolayers of approximately 10^7 adherent cells per flask were obtained. The monolayers were washed gently with warm RPMI and incubated for another 24 h in fresh RPMI without EMT-6-conditioned medium. On day 9, phagocytic activity was assessed and found to be >98% when the cells were incubated for 24 h with a 0.002% (wt/vol) suspension of fixed *Staphylococcus aureus* cells as described before (31).

Infection of macrophages. Monolayers were infected with *L. donovani* by adding 12 ml of a suspension of stationary-phase promastigotes at 1.6×10^7 parasites per ml to each flask. After 3 h of incubation at 37°C, noningested organisms were removed by washing each flask three times with warm HBSS. To monitor infection, cytocentrifuged preparations were routinely prepared at this point from cells dislodged from monolayers. These analyses showed that no extracellular promastigotes were present and that all intracellular forms had the morphology of amastigotes. For experiments, cells were then either harvested immediately and processed for Western immunoblotting or allowed to incubate in 12 ml of fresh medium to complete 24 or 48 h of culture. For infection with yersiniae, the YP IIIP$^+$ strain of *Yersinia pseudotuberculosis* was used (kindly provided by B. Finlay, Department of Microbiology, University of British Columbia). Bacteria were grown overnight in Luria broth at 30°C with moderate agitation (250 cycles per min) in a rotary shaker. The concentration of bacteria was adjusted by the optical density at 600 nm (OD_{600}), with an OD_{600} reading of 2 being equal to 1.6×10^9 organisms per ml. Yersiniae were washed three times in warm HBSS, resuspended at a concentration of 0.8×10^8 per ml in RPMI-FCS-P/S, and used to infect macrophage monolayers at an approximate ratio of 100 bacteria per cell.

Sodium arsenite treatment and heat shock. Macrophage monolayers were washed with warm RPMI and then incubated for 3 h in RPMI-FCS-P/S with $NaAsO_2$ (40 μM) (Fisher Scientific). After incubation, the cells were washed and then incubated for another hour in RPMI-FCS-P/S but without $NaAsO_2$. Control cells were processed in parallel but without addition of $NaAsO_2$. Treated and untreated cells were harvested and prepared for Western blotting. For heat shock treatment, macrophage monolayers were gently washed in warm RPMI, replenished with fresh RPMI-FCS-P/S, and incubated at 42°C for 20 min. After incubation, cells were allowed to recover at 37°C for 1 h and then processed

for the Western blotting. Control cells were prepared in parallel but were incubated at 37°C instead of 42°C.

Immunoblotting. After the indicated treatments and incubations were completed, monolayers of BMM were placed on ice for 10 min, dislodged with a disposable cell scraper (Costar Corporation, Cambridge, Mass.) into 10 ml of HBSS, and centrifuged at 800 × *g* for 10 min. Cells were then resuspended in HBSS and washed three times by microcentrifugation (Microcentrifuge E; Beckman) for 30 s. Before the final wash, sample aliquots were taken to determine cell number, intensity of infection (Diff Quik stain; American Scientific Products), and protein concentration as indicated below. Cells were then suspended in Laemmli sample buffer (17) at a concentration of 2×10^7 cells per ml and boiled at 95 to 100°C for 5 min. Samples were either used immediately or stored at −20°C. For immunoblotting, 60 μg of cell protein was separated by electrophoresis on 10% polyacrylamide–sodium dodecyl sulfate slab gels and then transferred to a nitocellulose membrane (Bio-Rad) with a Multiphor II electrophoresis unit (LKB, Bromma, Sweden). After transfer, the membrane was washed briefly in phosphate-buffered saline with 0.1% (wt/vol) Tween and then processed according to the manufacturer's instructions for enhanced chemiluminescence (ECL) (Amersham). Briefly, the membrane was blocked for nonspecific binding with 5% skim milk (Difco Laboratories, Detroit, Mich.) for 1 h and then probed with the indicated primary antibody for 1 h. After extensive washing, an appropriate secondary antibody was added for 40 min; the membrane was then washed again, and the ECL signal was detected with ECL film according to the manufacturer's instructions.

Protein measurements. The protein content of samples was determined by the DC Protein Assay System (Bio-Rad) for measurement of protein content in the presence of detergent with an enzyme-linked immunosorbent assay plate reader (Multiscan MC; Titertek, Helsinki, Finland) at a wavelength of 690 nm.

RESULTS

Regulated expression of novel 65- and 67-kDa proteins in *Leishmania*-infected macrophages. Immunoblots performed with a rabbit polyclonal antibody to moth HSP60 (SPA 805) demonstrated that infection of BMM with *L. donovani* resulted in the expression of 65- and 67-kDa heat-regulated proteins (Fig. 1, lanes 2, 3, and 4) that were not present in control cells. These molecules were coordinately expressed in a time-dependent manner (Fig. 1 and Table 1). Both proteins, which were detectable at 3 h after infection (Fig. 1, lane 2), were induced to higher levels of expression at 24 h (52 and 100% increases above 3-h levels, respectively, for the 65- and 67-kDa proteins) and were coordinately somewhat diminished at 48 h (Fig. 1, lanes 3 and 4, respectively). The anti-HSP60 reagent SPA 805 also recognized an additional protein in both noninfected and infected BMM of 60 kDa (Fig. 1, lanes 1 to 4), which presumably represented murine HSP60. The expression of murine HSP60 was constitutive but, in contrast to the 65- and 67-kDa proteins, did not change with infection (Table 1).

Further experiments indicated that the 65- and 67-kDa proteins detected in infected cells were heat-regulated *Leishmania* proteins. Both of these proteins were observed to be constitutively expressed in promastigotes cultured in vitro at room temperature (Fig. 2, lane 4) and were increased in abundance when promastigotes were subjected to heat shock in culture for either 3 or 24 h at 37°C (Fig. 2, lanes 5 and 6, respectively; Table 2). Because promastigotes cultured at 37°C for more than 24 h became nonviable, it was not possible to follow the expression of these proteins at 48 h and to compare this with expression in infected BMM at 48 h. Nevertheless, both in infected macrophages (Fig. 2, lanes 2 and 3) and in *Leishmania* promastigotes, the expression and modulation of the 65- and 67-kDa heat-regulated proteins was coordinate with respect to both time and relative abundance (Tables 1 and 2).

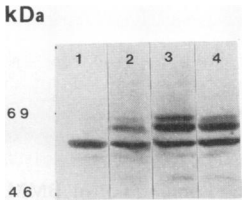

FIG. 1. Anti-HSP60 immunoblot of *Leishmania*-infected BMM. Cells were either uninfected (lane 1) or infected with stationary-phase promastigotes of *L. donovani* at a ratio of 20 extracellular organisms per macrophage and incubated at 37°C for either 3, 24, or 48 h (lanes 2 to 4, respectively). After incubation, the cells were detached with a cell scraper. Diff-Quik-stained cell samples indicated an intensity of infection of 94 to 97%, with 8.7 ± 3.6 (mean ± standard deviation) amastigotes per macrophage. Cells were lysed in Laemmli sample buffer, electrophoresed, and transferred to nitrocellulose membranes. Membranes were probed with polyclonal rabbit antibodies to HSP60 (Stressgen) and developed by ECL (Amersham). The positions of molecular size markers are shown in the left margin. The data shown are from one of three independent experiments which yielded similar results.

To provide further evidence that these two heat-inducible proteins were in fact leishmanial in origin and that they were not macrophage derived in a nonspecific stress response to infection, parallel cultures of BMM were infected with viable *Y. pseudotuberculosis* or allowed to ingest inactivated *S. aureus* cells. As shown in Fig. 2, neither infection of macrophages with *Y. pseudotuberculosis* (lane 7) nor phagocytosis of *S. aureus* (lane 10) brought about expression of the 65- and 67-kDa proteins detected in *Leishmania*-infected BMM (Fig. 2, lanes 2 and 3). Of interest, infection of cells with *Y. pseudotuberculosis* resulted in the appearance of a relatively abundant protein (Fig. 2, lane 7, upper band) which was not present in either control or *Leishmania*-

TABLE 1. Expression of HSP60-related proteins in BMM infected with *L. donovani*[a]

Protein (kDa)	Mean OD units ± SD at:		
	3 h	24 h	48 h
67	0.9 ± 0.3	1.8 ± 1.3	1.6[b]
65	2.1 ± 0.7	3.2 ± 1.3	2.4 ± 1.4
60	3.3 ± 0.7	2.9 ± 0.7	2.9 ± 1.0

[a] Monolayers of BMM were infected with stationary-phase promastigotes of *L. donovani* at a ratio of 20 parasites per macrophage. Infected cells were incubated at 37°C for either 3, 24, or 48 h, detached mechanically, and assessed for intensity of infection as indicated in the legend to Fig. 1. Infected cells were washed with warm HBSS and solubilized in Laemmli sample buffer, and proteins (60 μg per lane) were separated by electrophoresis. Separated proteins were then transferred to nitrocellulose membranes and immunoblotted with polyclonal rabbit antibodies to HSP60. The resulting bands were scanned for determination of OD units (Video Densitometer 620; Bio-Rad). The level of expression of the 60-kDa protein (HSP60) in control noninfected cells was 3.1 ± 0.7 OD units. The data shown are the mean values obtained in three independent experiments with different batches of BMM.

[b] Average of two values.

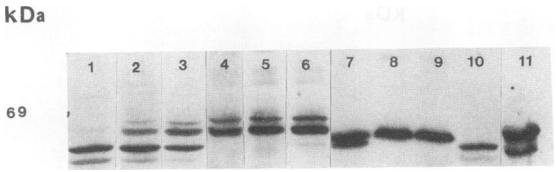

FIG. 2. Anti-HSP60 immunoblot of BMM, promastigotes of *L. donovani*, and *Y. pseudotuberculosis*. Macrophages were uninfected (lane 1), infected with stationary-phase promastigotes of *L. donovani* and incubated for either 3 or 24 h (lanes 2 and 3, respectively), infected for 3 h with an overnight culture of *Y. pseudotuberculosis* (lane 7), or exposed to a suspension of fixed *S. aureus* (lane 10). Stationary-phase *L. donovani* promastigotes were cultured at either room temperature or 37°C for 24 h (lanes 4 and 6, respectively) or incubated at room temperature for 21 h and at 37°C for a final 3 h (lane 5). Broth cultures of *Y. pseudotuberculosis* were incubated overnight at 30°C (lane 8) or incubated for the final 3 h at 37°C (lane 9). Lane 11 contains recombinant mycobacterial HSP65 as a standard. For other details, see the legend to Fig. 1. The positions of molecular size markers are shown in the left margin. The data shown are from one of two independent experiments that yielded similar results.

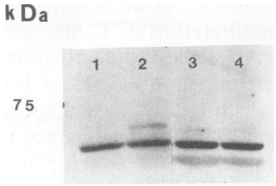

FIG. 3. Anti-HSP60 immunoblot of BMM. Cells were either untreated (lane 1), infected with *Leishmania* promastigotes for 24 h (lane 2), exposed to $NaAsO_2$ (40 μM for 3 h) (lane 3), or heat shocked (42°C for 20 min) (lane 4). After treatment, cells were allowed to recover at 37°C for 1 h and then processed for Western blotting as indicated in the legend to Fig. 1. The positions of molecular size markers are shown in the left margin. The data shown are from one of two independent experiments that yielded similar results.

infected cells. This protein appeared to represent a constitutive form of *Yersinia* HSP60, as it was detected in cultures of *Y. pseudotuberculosis* alone and its expression was not temperature dependent (Fig. 2, lanes 8 and 9).

Additional evidence, shown in Fig. 3, indicated that the 65- and 67-kDa heat-regulated proteins that appeared de novo in infected macrophages were not the result of a nonspecific stress response. Neither of these proteins was detected when noninfected macrophages were exposed to either heat shock or $NaAsO_2$ (Fig. 3). Western blot analysis with the rabbit polyclonal anti-moth HSP60 antiserum detected murine HSP60 in all treatment groups. A protein of 65 kDa was also detected in *Leishmania*-infected macrophages (Fig. 3, lane 2) which corresponded to the smaller and more abundant of the two heat-regulated proteins expressed during *Leishmania* infection in other experiments. However, the 65-kDa protein was not detected in cells exposed to either $NaAsO_2$ or heat shock (Fig. 3, lanes 3 and 4, respectively), even though both treatments brought about increased expression of murine macrophage HSP60 (Fig. 3, lanes 3 and 4).

Comparative expression of *Leishmania* 65- and 67-kDa heat-regulated proteins and *Leishmania* gp63 in infected BMM. Experiments were carried out to examine whether the abundant, regulated expression of the two *Leishmania* heat-regulated proteins was a specific event or was a general phenomenon applicable to other (nonstress) *Leishmania* proteins. As shown in Fig. 4, comparison of the 65- and 67-kDa *Leishmania* heat-regulated proteins with the abundant *Leishmania* surface protease gp63 in BMM demonstrated that expression of the heat-inducible proteins was selectively upregulated during infection. In this particular experiment, only the smaller, more abundant 65-kDa *Leishmania* protein was observed to be expressed in a time-dependent manner in infected BMM (Fig. 4A, lanes 2 to 4). When extracts from the same cells were subjected to immunoblotting with a monoclonal antibody specific for *Leishmania* gp63, initial low-level expression of the *Leishmania* surface protease was apparent (Fig. 4B, lanes 2 to 4). In contrast to the heat-regulated 65-kDa protein of *L. donovani*, however, expression of gp63 decreased markedly by 24 h and was no longer detectable at 48 h, at a time when expression of the 65-kDa *Leishmania* protein was still high. Time-dependent downregulation of gp63 expression within infected cells correlated with a negligible decrease in the expression of gp63 by promastigotes subjected to heat shock

TABLE 2. Expression of HSP60-related proteins in promastigotes of *L. donovani*[a]

Protein (kDa)	Avg OD units at:		
	0 h	3 h	24 h
67	1.8	3.0	2.7
65	3.3	4.3	4.0

[a] Stationary-phase promastigotes of *L. donovani* growing in Senekjie medium were harvested, washed three times in HBSS, resuspended in RPMI-FCS-P/S, and then incubated for either 3 or 24 h at 37°C. After incubation, motile promastigotes were adjusted to a concentration of 4×10^8/ml and solubilized in Laemmli sample buffer. Electrophoresis, Western blotting, and densitometry were carried out as indicated in Table 1, footnote *a*. The results shown are the averages of values obtained in two separate experiments.

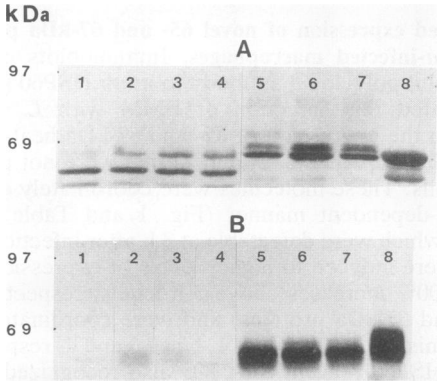

FIG. 4. Anti-HSP60 (A) and anti-gp63 (B) immunoblots of *Leishmania*-infected BMM and promastigotes of *L. donovani*. Macrophages were either uninfected (lanes 1, both panels) or infected with stationary phase promastigotes and then incubated for either 3, 24, or 48 h (lanes 2 through 4, respectively, both panels). Stationary-phase promastigotes were incubated for 24 h at either room temperature (lanes 5, both panels) or 37°C (lanes 6, both panels) or incubated at room temperature for 21 h and for a final 3 h at 37°C (lane 7, both panels). Lane 8 contained either recombinant mycobacterial HSP65 (A) or recombinant *Leishmania* gp63 (B). The remainder of the details are described in the legend to Fig. 1. The positions of molecular size markers are shown in the left margin. The data shown are from a single experiment.

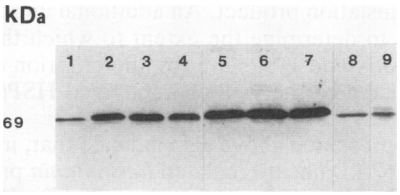

FIG. 5. Anti-HSP70 immunoblot of *Leishmania*-infected BMM and promastigotes of *L. donovani* performed with monoclonal antibody D4F18, specific for a constitutive form of HSP70, as described in Materials and Methods. Macrophages were either untreated and cultured for 48 h (lane 1) or incubated with stationary-phase promastigotes and then cultured for either 3, 24, or 48 h (lanes 2 through 4, respectively). Stationary-phase promastigotes were incubated for 24 h at either room temperature (lane 5) or 37°C (lane 7) or incubated for 21 h at room temperature and for a final 3 h at 37°C (lane 6). Lane 8 shows BMM exposed to fixed *S. aureus*, and macrophages exposed to $NaAsO_2$ (40 μM for 3 h) are shown in lane 9. Integrated OD units for lanes 1 to 9 were, respectively, 1.8, 4.0, 4.0, 3.5, 5.2, 6.0, 6.2, 1.9, and 1.8. The remainder of the details are described in the legend to Fig. 1. The positions of molecular size markers are shown in the left margin. The data shown are from a single experiment.

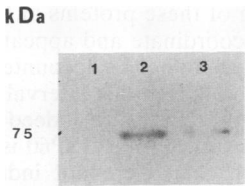

FIG. 6. Anti-HSP70 immunoblot of stressed macrophages. Cells were untreated (lane 1) or exposed to either $NaAsO_2$ (40 μM for 3 h, lane 2) or heat shock (42°C for 20 min, lane 3). The remainder of the details are given in the legend to Fig. 1. The anti-HSP70 monoclonal antibody (SPA 810; Stressgen) specific for the inducible form of HSP70 was used. The positions of molecular size markers are shown in the left margin. The data shown are from one of three independent experiments that yielded similar results.

during culture at 37°C for either 3 or 24 h (Fig. 4B, lanes 5 to 7).

Expression of HSP70 in *Leishmania*-infected BMM. To examine whether other *Leishmania* HSPs are also expressed during infection, lysates of infected BMM were analyzed by immunoblotting with murine monoclonal antibody D4F18, raised against a recombinant 72-kDa protein from *P. falciparum* (1) and known to recognize both mammalian HSP70 and HSP70 from plasmodia (4a). This antibody recognized a protein with an apparent subunit M_r of 70,000 in control cells (Fig. 5, lane 1), which presumably represented a constitutive form of mammalian HSP70. Of interest, in comparison to its reactivity in control cells, antibody D4F18 showed markedly increased reactivity with a protein with an approximate subunit M_r of 70,000 in BMM that were infected with *L. donovani* for 3, 24, or 48 h (Fig. 5, lanes 2 to 4). The expression of this protein appeared to be maximal at 3 h, was stable through 24 h, and appeared to decrease slightly by 48 h postinfection.

Three findings indicated that this protein likely represented *Leishmania* HSP70 and not an inducible form of mammalian HSP70. First, antibody D4F18 also detected a protein with an identical subunit M_r which was expressed constitutively in promastigotes cultured at 25°C and which appeared to be only modestly upregulated (approximately 20%) during promastigote culture at 37°C for either 3 or 24 h (Fig. 5, lanes 5 to 7). Second, when noninfected BMM were stressed either by phagocytosis of inactivated *S. aureus* or by treatment with 40 μM $NaAsO_2$ for 3 h (Fig. 5, lanes 8 and 9, respectively), there was no change in signal intensity generated with antibody D4F18 in comparison to its intensity in control cells. Third, when BMM were stressed by either heat shock or $NaAsO_2$ treatment and lysates were examined with an anti-HSP70 monoclonal antibody (SPA 810; Stressgen) that specifically recognizes an inducible form of the HSP70, a protein with a higher apparent subunit M_r of 73,000 was detected (Fig. 6, lanes 2 and 3, respectively). This 73-kDa protein was not detected either in control cells in the basal state (Fig. 6, lane 1) or in *Leishmania*-infected macrophages (data not shown) and presumably represents the inducible form of mammalian HSP70, which is known to be about 72 kDa in size.

DISCUSSION

During infections of mammalian hosts with either intracellular or extracellular pathogens, HSPs become principal targets of the immune response (7, 13, 16, 23, 24, 43). Specifically with respect to intracellular organisms, including, for example, *Leishmania* spp., *Plasmodium* spp., *Trypanosoma cruzi*, and *Mycobacterium* spp., immune responses to HSP at both the B-cell and T-cell levels have been described (7, 16, 23, 43). Notwithstanding the apparent importance of these responses, there is relatively little direct information available about the expression of either host- or pathogen-derived HSPs within infected cells. For example, it has been observed that both the GroEL and DnaK proteins of *Salmonella* spp. are expressed within infected macrophages (2). It has also been shown that normal murine BMM contain a 68- to 70-kDa protein that is recognized in Western blots by monoclonal antibody IA10, specific for mycobacterial HSP65 (15). Furthermore, when BMM were infected with *Mycobacterium bovis*, expression of mycobacterial HSP65 was also detectable in infected cells and HSP65 could be distinguished from the slightly larger, cross-reactive murine macrophage protein of 68 to 70 kDa. Whether the expression of either mycobacterial HSP65 or the corresponding host protein was regulated during infection was not examined. In addition, the same mycobacterial HSP65-specific monoclonal antibody, IA10, was also shown to recognize a 65-kDa protein in peritoneal macrophages from *Toxoplasma*-infected mice but not in cells from corresponding control animals (28). It was not possible from these studies, however, to determine whether this protein was of host or *Toxoplasma* origin.

To develop a better understanding of the nature and extent of HSP expression during intracellular infection, the expression of proteins reactive with antibodies to HSP60 and HSP70 was examined directly in the present study in macrophages infected with *L. donovani*. In immunoblots with a rabbit anti-HSP60 antiserum raised against purified moth HSP60, both control and infected cells were observed to express a single isoform of mammalian HSP60 with an apparent subunit molecular size of 60 kDa. Quantitatively, the amount of this protein detected in infected cells was not affected by infection. In contrast to control cells, two proteins appeared de novo in infected BMM, and these heat-inducible proteins were shown to be leishmanial in origin. In comparison to the amount of mammalian HSP60 present in the same cells, these two proteins, with subunit molecular sizes of 65 and 67 kDa, were relatively abundant. They also appeared rapidly (within 3 h of infection) and were inducible, with maximal expression observed at 24 h. In-

creased expression of these proteins at between 3 and 24 h postinfection was coordinate and appeared to be an inducible event, since it could not be accounted for by increasing intensity of infection during this interval (data not shown).

That these two proteins were indeed *Leishmania* HSPs and not novel forms of murine HSP60 is supported by two observations. First, they were not induced to detectable levels in noninfected cells which had been exposed to several conditions known to bring about the expression of stress proteins. Second, two proteins with identical electrophoretic mobilities and in the same relative abundance were demonstrated in stationary-phase *Leishmania* promastigotes. Furthermore, these two proteins, which were observed to be expressed constitutively in promastigotes cultured at room temperature, demonstrated the same kinetics of inducibility in response to heat shock as did the 65- and 67-kDa proteins detected in infected BMM. Moreover, the specificity of the appearance of the 65- and 67-kDa heat-regulated proteins for *Leishmania* infection was illustrated by the finding that they were not detected in cells that had been exposed to several alternative treatments known to induce stress protein expression, including (i) infection with *Y. pseudotuberculosis*, (ii) phagocytosis of *S. aureus*, (iii) $NaAsO_2$ treatment, and (iv) heat shock. It is also of note that the abundant expression of the 65- and 67-kDa heat-regulated *Leishmania* proteins was selective, in that expression of the *Leishmania* surface protease gp63 within infected cells was observed to decrease at a time when expression of these heat-regulated *Leishmania* proteins was increasing.

Infection of BMM with *L. donovani* was also accompanied by the expression of what appeared to be a minimally inducible *Leishmania* protein belonging to the HSP70 family. This protein had an apparent subunit size of 70 kDa and was thus essentially identical in size to murine HSP70. The latter was also recognized by the anti-HSP70 monoclonal antibody D4F18, raised against HSP70 from *P. falciparum*, and was expressed constitutively in noninfected cells. This murine HSP70 appeared to correspond to the 70-kDa heat shock cognate protein, which is known to be noninducible (13, 22, 43), since its expression did not change significantly in response to either treatment with $NaAsO_2$, phagocytosis of *S. aureus*, or infection with *L. donovani*. Moreover, a larger, approximately 73-kDa protein that was not detectable in the basal state and which was strongly induced in response to both $NaAsO_2$ treatment and heat shock was detected in immunoblots performed with a monoclonal antibody specific for the inducible form of mammalian HSP70, which is known to be about 72 kDa in size. As was the case for the 65- and 67-kDa heat-regulated *Leishmania* proteins, *Leishmania* HSP70 was also expressed constitutively in stationary-phase promastigotes. Unlike the 65- and 67-kDa proteins, however, the 70-kDa *Leishmania* HSP was observed to be only minimally inducible either in promastigotes or in infected BMM (about 15% above the baseline value).

Leishmania HSPs belonging to the HSP60 and HSP70 families have been the subject of considerable interest, and the findings of the present study provide direct evidence that *Leishmania* HSPs are expressed in infected cells. Moreover, they show that two heat-regulated *Leishmania* proteins, which appear to be immunologically related to the HSP60 family of proteins, are expressed and coordinately induced both in promastigotes and in infected cells. In light of these findings, an important objective will be to determine whether the 65- and 67-kDa heat-regulated *Leishmania* proteins represent products of distinct genes, alternative processing of a single gene transcript, or posttranslational modifications of a common translation product. An additional important objective will be to determine the extent to which these 65- and 67-kDa *Leishmania* HSPs are, by the criterion of sequence identity, related to the well-characterized HSP60 family of proteins.

The data presented above also indicate that, in contrast to the 65- and 67-kDa heat-regulated *Leishmania* proteins, host proteins belonging to the 60-kDa and 70-kDa HSP families do not appear to alter their expression in response to infection. This finding is of significant interest, as it is well known, for example, that viral infections frequently bring about increased expression of host HSPs, and it has been postulated that other intracellular infections with bacteria as well as with more complex organisms may do the same (13). At least with respect to *L. donovani* (and, for that matter, *Y. pseudotuberculosis* and HSP60) and members of the murine HSP60 and HSP70 families, this appears not to be the case.

Seven distinct HSP70 genes have been cloned and sequenced either partially or completely from *Leishmania major* (3, 21, 33). All of the mRNAs for these genes were observed to be transcribed constitutively, and four showed markedly increased levels in response to heat. Whether the expression of these genes was regulated further at the posttranscriptional level has not been addressed (21, 33), and the protein products of these genes remain to be characterized. Only one HSP70 gene has been cloned from *L. donovani*, and it was found to be present in the genome on a single chromosome in multiple tandem repeats (23). The corresponding mRNA and protein for this gene were both constitutively expressed and only minimally induced by heat in either promastigotes or amastigotes. The expression of this protein also did not change during conversion from amastigotes to promastigotes in culture. The 70-kDa HSP observed to be expressed in infected BMM in the present study also had limited inducibility and likely corresponds to the HSP70 from *L. donovani* (23). Moreover, in respect to its limited inducibility, this HSP likely represents the *Leishmania* homolog of the 70-kDa heat shock cognate protein described for other organisms. While little is known about the immune response to this protein, its abundant expression in infected cells suggests that it may be of significance, and this is consistent with the finding that serum samples from 50% of patients with visceral leishmaniasis recognized a fusion protein containing HSP70 from *L. donovani* (23).

In contrast to HSP70, relatively little information is available about the HSP60 family in *Leishmania* spp. It is known from metabolic labeling studies of *Leishmania* promastigotes that during exposure to either $NaAsO_2$ or heat shock, increased expression of several proteins, including proteins in the 60- to 70-kDa range, is observed (20, 36, 40). From the findings of the present study, it is clear that at least two HSPs of 65 and 67 kDa are present in *L. donovani* and that both of these are relatively abundantly expressed in infected macrophages. In the context of what is known from other systems of intracellular infection, it seems likely that this has important implications for the immune response to *Leishmania* infection. The most extensive studies concerned with the mammalian immune response to HSP60 during intracellular infection have been carried out with mycobacteria. The results obtained with *Mycobacterium tuberculosis*, *M. bovis*, and *Mycobacterium leprae* indicate that the mycobacterial HSP65 is an immunodominant antigen that elicits both T- and B-cell responses (43). At least six distinct B-cell determinants (6, 12, 25) and a dozen distinct T-cell determinants (18, 19, 39) have been identified within this protein. Some of the latter appear to be recognized by γδ T cells (9, 29), and

this has led to the suggestion that mycobacterial HSP65, and perhaps its analogs in other microbes, is an important immunodominant antigen during the early response to microbial invasion. Thus, the observation that two heat-regulated *Leishmania* proteins of 65 and 67 kDa, which appear to be immunologically related to the HSP60 family of proteins, are rapidly and abundantly expressed in infected macrophages raises the clear possibility that these proteins may be critical targets of the immune response.

Members of individual HSP families from different species have greater than 50% amino acid sequence identity, and this fact has led to the concern that conserved determinants within pathogen- and host-derived HSPs may engender autoimmune disease (reviewed in references 13 and 43). While findings in clinical forms of leishmaniasis do not a priori suggest the presence of autoimmune components directed at HSPs, this remains a possibility to be considered. On the other hand, it may be reasonable to anticipate the existence of *Leishmania*-specific determinants for both T- and B-cell responses within the 65- and 67-kDa *Leishmania* heat-regulated proteins and in *Leishmania* HSP70. Support for this argument is provided by at least two observations. First, notwithstanding the 73% amino acid sequence identity between the HSP70s from humans and *T. cruzi*, serum samples from persons with Chagas' disease were shown to recognize only the latter (7). Second, in the present study, murine HSP60 was recognized by two different rabbit antisera, one raised against purified moth HSP60 (26) and the other raised against human HSP60 (5). Only the former antiserum recognized the 65- and 67-kDa heat-regulated *Leishmania* proteins; the latter antiserum recognized neither (data not shown). Thus, *Leishmania*-specific HSP determinants likely exist, and defining their role in disease pathogenesis is an important objective.

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REFERENCES

1. **Blisnick, T., F. Lema, J. C. Mazie, and L. Pereira Da Silva.** 1988. *Plasmodium falciparum*: analysis of B epitopes of a polypeptide antigen expressed in *E. coli*, using monoclonal antibodies. Exp. Parasitol. **67**:247–256.
2. **Buchmeier, N. A., and F. Heffron.** 1990. Induction of *Salmonella* stress proteins upon infection of macrophages. Science **248**:730–732.
3. **Coulson, R. M. R., and D. F. Smith.** 1990. Isolation of genes showing increased or unique expression in the infective promastigotes of *Leishmania major*. Mol. Biochem. Parasitol. **40**:63–76.
4. **Descoteaux, A., and G. Matlashewski.** 1989. c-*fos* and tumor necrosis factor gene expression in *Leishmania donovani*-infected macrophages. Mol. Cell. Biol. **9**:5223–5227.

4a. **Dubois, P., O. Bensaude, and L. Pereira Da Silva.** Unpublished data.

5. **Dudani, A. K., and R. S. Gupta.** 1989. Immunological characterization of a human homolog of the 65-kilodalton mycobacterial antigen. Infect. Immun. **57**:2786–2793.
6. **Engers, H. D., V. Houba, J. Bennedsen, T. M. Buchanan, S. D. Chaparas, G. Kadival, O. Closs, J. R. David, J. D. A. van Embden, T. Godal, A. S. Mustafa, J. Ivanyi, D. B. Young, S. H. E. Kaufmann, A. G. Khomenko, A. H. J. Kolk, M. Kubin, J. A. Louis, P. Minden, T. M. Shinnick, L. Trnka, and R. A. Young.** 1986. Results of a WHO sponsored workshop to characterize antigens recognized by mycobacterium-specific monoclonal antibodies. Infect. Immun. **51**:718–720.
7. **Engman, D. M., E. A. Dragon, and J. E. Donelson.** 1990. Human humoral immunity to hsp70 during *Trypanosoma cruzi* infection. J. Immunol. **144**:3987–3991.
8. **Gregory, C. J., and A. C. Eaves.** 1978. *In vitro* studies of erythropoietic progenitor cell differention, p. 179–192. *In* B. Clarkson, P. A. Marks, and J. E. Till (ed.), Differentiation of normal and neoplastic hematopoietic cells, book A. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Haregewoin, A., G. Soman, R. Hom, and R. W. Finberg.** 1989. Human γδ-T cells respond to mycobacterial heat-shock protein. Nature **340**:309–312.
10. **Hedstrom, R., J. Culpepper, R. A. Harrison, N. Agabian, and G. Newport.** 1987. A major immunogen in *Schistosoma mansoni* infections is homologous to the heat-shock protein Hsp70. J. Exp. Med. **165**:1430–1435.
11. **Hunter, K. W., C. L. Cook, and E. G. Hayunga.** 1984. Leishmanial differentiation in vitro: induction of heat shock proteins. Biochem. Biophys. Res. Commun. **125**:755–760.
12. **Kaufmann, S. H., U. Vath, J. E. R. Thole, and J. D. A. van Embden.** 1987. Enumeration of T cells reactive with *Mycobacterium tuberculosis* organisms and specific for the recombinant mycobacterial 64 kilodalton protein. Eur. J. Immunol. **17**:351–357.
13. **Kaufmann, S. H. E.** 1990. Heat shock proteins and the immune response. Immunol. Today **11**:129–136.
14. **Kaufmann, S. H. E., B. Schoel, A. Wand-Wurtenberger, U. Steinhoff, M. E. Munk, and T. Koga.** 1990. T-cells, stress proteins, and pathogenesis of mycobacterial infections. Curr. Top. Microbiol. Immunol. **155**:125–141.
15. **Koga, T., A. Wand-Wurttenberger, J. Debruyn, M. E. Munk, B. Schoel, and S. H. E. Kaufmann.** 1989. T cells against a bacterial heat shock protein recognize stressed macrophages. Science **245**:1112–1115.
16. **Kumar, N., Y. Zhao, P. Graves, J. P. Folgar, L. Maloy, and H. Zheng.** 1990. Human immune response directed against *Plasmodium falciparum* heat shock-related proteins. Infect. Immun. **58**:1408–1414.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Lamb, J. R., J. Ivanyi, A. D. M. Rees, J. B. Rothbard, K. Holland, R. A. Young, and D. B. Young.** 1987. Mapping of T cell epitopes using recombinant antigens and synthetic peptides. EMBO J. **6**:1245–1249.
19. **Lamb, J. R., and D. B. Young.** 1990. T cell recognition of stress proteins: a link between infectious and autoimmune disease. Mol. Biol. Med. **7**:311–321.
20. **Lawrence, F., and M. Robert-Gero.** 1985. Induction of heat shock and stress proteins in the promastigotes of three *Leishmania* species. Proc. Natl. Acad. Sci. USA **82**:4414–4417.
21. **Lee, M. G.-S., B. L. Atkinson, S. H. Giannini, and L. H. Van der Ploeg.** 1988. Structure and expression of the hsp 70 gene family of *Leishmania major*. Nucleic Acids Res. **16**:9567–9585.
22. **Lindquist, S.** 1988. The heat-shock proteins. Annu. Rev. Genet. **22**:631–677.
23. **MacFarlane, J., M. L. Blaxter, R. P. Bishop, M. A. Miles, and J. M. Kelly.** 1990. Identification and characterisation of a *Leishmania donovani* antigen belonging to the 70-kDa heat-shock protein family. Eur. J. Biochem. **190**:377–384.
24. **Maresca, B., and L. Carratu.** 1992. The biology of the heat shock response in parasites. Parasitol. Today **8**:260–266.
25. **Mehra, V., D. Sweetser, and R. A. Young.** 1986. Efficient mapping of protein antigenic determinants. Proc. Natl. Acad. Sci. USA **83**:7013–7017.
26. **Miller, S. G.** 1987. Association of a sperm-specific protein with the mitochondrial F1F0-ATPase in *Heliothis*. Insect Biochem. **17**:417–432.

27. **Moser, D., O. Doumbo, and M. Klinkert.** 1990. The humoral response to heat shock protein 70 in human and murine *Schistosomiasis mansoni*. Parasite Immunol. **12**:341–352.
28. **Nagasawa, H., M. Oka, K. Maeda, C. Jian-Guo, H. Hisaeda, Y. Ito, R. A. Good, and K. Himeno.** 1992. Induction of heat shock protein closely correlates with protection against *Toxoplasma gondii* infection. Proc. Natl. Acad. Sci. USA **89**:3155–3158.
29. **O'Brien, R. L., M. P. Happ, A. Dallas, E. Palmer, R. Kubo, and W. K. Born.** 1989. Stimulation of a major subset of lymphocytes expressing the T cell receptor γδ by an antigen derived from *Mycobacterium tuberculosis*. Cell **57**:667–674.
30. **Reiner, N. E.** 1982. Host-parasite relationship in murine leishmaniasis: pathophysiological and immunological changes. Infect. Immun. **38**:1223–1230.
31. **Reiner, N. E., W. Ng, C. B. Wilson, W. R. McMaster, and S. K. Burchett.** 1990. Modulation of *in vitro* monocyte cytokine responses to *Leishmania donovani*. Interferon-γ prevents parasite-induced inhibition of interleukin 1 production and primes monocytes to respond to *Leishmania* by producing both tumor necrosis factor-α and interleukin 1. J. Clin. Invest. **85**:1914–1924.
32. **Rothstein, N. M., G. Higashi, J. Yates, and T. V. Rajan.** 1989. *Onchocerca volvulus* heat shock protein 70 is a major immunogen in microfilaremic individuals from a filariasis-endemic area. Mol. Biochem. Parasitol. **33**:229–236.
33. **Searle, S., A. J. R. Campos, M. R. Coulson, T. W. Spithill, and D. Smith.** 1989. A family of heat shock protein 70-related genes are expressed in the promastigotes of *Leishmania major*. Nucleic Acids Res. **17**:5081–5095.
34. **Selkirk, M. E., D. A. Denham, F. Partono, and R. M. Maizels.** 1989. Heat shock cognate 70 is a prominent immunogen in Brugian filariasis. J. Immunol. **143**:299–308.
35. **Senekjie, H. A.** 1939. Studies on the culture of *Leishmania tropica*. Trans. R. Soc. Trop. Med. Hyg. **33**:267–269.
36. **Shapira, M., J. G. McEwen, and C. L. Jaffe.** 1988. Temperature effects on the molecular processes which lead to stage differentiation in *Leishmania*. EMBO J. **7**:2895–2901.
37. **Shapira, M., and E. Pinelli.** 1989. Heat-shock protein 83 of *Leishmania mexicana amazonensis* is an abundant cytoplasmic protein with a tandemly repeated genomic arrangement. Eur. J. Biochem. **185**:231–236.
38. **Smejkal, R. M., R. Wolff, and J. G. Olenick.** 1988. *Leishmania braziliensis panamensis*: infectivity resulting from heat shock. Exp. Parasitol. **65**:1–9.
39. **Thole, J. E. R., W. C. A. van Schooten, W. J. Keulen, P. W. M. Hermans, A. A. M. Janson, R. R. P. De Vries, A. H. J. Kolk, and J. D. A. van Embden.** 1988. Use of recombinant antigens expressed in *Escherichia coli* K-12 to map B-cell and T-cell epitopes on the immunodominant 65-kilodalton protein of *Mycobacterium bovis* BCG. Infect. Immun. **56**:1633–1640.
40. **Toye, P., and H. Remold.** 1989. The influence of temperature and serum deprivation on the synthesis of heat-shock proteins and α and β tubulin in promastigotes of *Leishmania major*. Mol. Biochem. Parasitol. **35**:1–10.
41. **Ul'masov, K. A., A. Ovezmukhammedov, K. K. Karaev, and B. Evgen'ev.** 1988. Molecular mechanisms of adaptation to hyperthermia in higher organisms. III. Induction of heat-shock proteins in two species of *Leishmania*. Mol. Biol. **22**:1583–1589.
42. **Van der Ploeg, L. H., S. H. Giannini, and C. R. Cantor.** 1985. Heat shock genes: regulatory role for differentiation in parasitic protazoa. Science **228**:1443–1446.
43. **Young, R. A.** 1990. Stress proteins and immunology. Annu. Rev. Immunol. **8**:401–420.