Induction of heat shock and stress proteins in promastigotes of three *Leishmania* species

(protein synthesis/hyperthermia/protozoan parasite/sodium arsenite/actinomycin)

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**ABSTRACT** The induction of heat shock proteins in three species of *Leishmania*, *L. tropica*, *L. enrietti*, and *L. donovani* is reported. When cultures of promastigotes are shifted from 26°C to 37°C or 40°C, the synthesis of proteins with apparent molecular weights of 88,000, 74,000, and 54,000 is stimulated. Actinomycin D added just prior to the shift prevented the appearance of these proteins but had no effect when present 30 min after the transfer onward, suggesting that the regulation of leishmanial heat shock proteins occurs at the transcriptional level. Exposure of *L. tropica* promastigotes to sodium arsenite elicits the synthesis of three major and four minor polypeptides. Their apparent molecular weights are, respectively, 94,000, 78,000, and 56,000 and 70,000, 45,000, 22,000, and 18,000. The response of *Leishmania* organisms to heat shock and to sodium arsenite is similar to that of other organisms, but some of the proteins identified as stress proteins in the parasite differ in size. The heat shock proteins might play a role in cytodifferentiation during the life cycle of the parasite and also in cellular adaptation to higher temperatures.

The heat shock response first observed in *Drosophila* has been studied extensively (1-3). It involves the induction of the synthesis of a small set of polypeptides referred to as heat shock proteins (HSPs) and the concomitant repression of synthesis of most other proteins (1) and is regulated at both the transcriptional and the translational levels (4). In addition to hyperthermia, a variety of other stress conditions such as exposure to chelating agents, transition state metals, sulphydryl group reagents, and sodium arsenite have been shown to induce HSPs (1, 3, 5, 6).

The physiological role and the mechanism of induction of these proteins have not yet been elucidated but in some cases heat shock may induce tolerancerence and resistance to ultraviolet light (8) and may be involved in resistance to antibiotics (9). In several cases, heat shock treatment was shown to play a role in differentiation; for instance, when differentiating cells of *Naegleria gruberi* were subjected to temperatures between 37°C and 39°C instead of 25°C, the average number of flagella per amebo-flagellate was significantly increased (10). A possible relationship of heat shock response to morphogenesis was also put forward in the case of the dimorphic pathogenic fungus *Histoplasma capsulatum* (11).

The heat shock response has been described in lower eukaryotes, including yeast, and in higher, differentiated eukaryotes such as *Dictyostelium discoideum*, plants, chicken embryo, and mammalian and insect cell lines. De-repression of synthesis of several proteins as a result of raising the growth temperature was also observed in bacteria.

*Leishmania* are protozoan parasites causing cutaneous leishmaniasis in man (*L. tropica*) and guinea pigs (*L. enrietti*) and of kala-azar or visceral leishmaniasis in man (*L. dono-

vanii). During their life cycle these parasites occur in two morphologically distinct states. The promastigotes are flagellar forms living freely in the alimentary tract of the vector. When a sandfly bites the mammalian host, the injected promastigotes become rounded, losing most of the flagellum. This amastigote form is found usually in the host's macrophage.

All these observations and the fact that inoculation of promastigotes cultivated at 26°C into laboratory animals (e.g., mice) or into macrophage cell lines cultivated at 34°C (12) is similar to heat shock prompted us to investigate the response of leishmanial promastigotes to heat.

Our results show that the synthesis of three proteins is stimulated by the temperature shift in all three species. Sodium arsenite, another known stress protein inducer, also elicits the synthesis of a set of polypeptides in *Leishmania*.

**MATERIALS AND METHODS**

*Growth of Organisms.* *L. tropica* (strain LRC L32), *L. donovani* (strain LRC L52), and *L. enrietti* (strain LRC L327) originated from the strain collection of the World Health Organization's International Reference Center for *Leishmania* (WHO–LRC) and is maintained at the Department of Medical Protozoology, the Hebrew University–Hadassah Medical School, Jerusalem. Promastigotes of the three species were grown in medium composed of 45% Dulbecco's modified Eagle's medium, 45% RPMI 1640 medium containing 25 mM Hepes (pH 7.4), 10% heat-inactivated fetal calf serum, streptomycin at 5 μg/ml, penicillin at 5 units/ml, and kanamycin at 5 μg/ml. Growth medium components were provided by Biopro (Mulhouse, France). Logarithmic-phase promastigotes (1 × 10⁷/ml) cultivated at 26°C in 25-ml Nunccon flasks (5 ml of culture per flask) were transferred to a water bath at 37°C or 40°C to induce heat shock. Stress was also induced by adding sodium arsenite *(NaAsO₃)*, 25 μM final concentration, to the promastigote cultures at 26°C. Labeling with L-[³⁵S]methionine was performed at the indicated times and at the indicated temperatures by adding it at 200 μCi/ml (1020 Ci/mmol; 1 Ci = 37 GBq; Amersham) for 30 min. In some experiments, actinomycin D (P-L Biochemicals) at 40 μg/ml was added to the cultures. After labeling, the promastigotes were harvested by centrifugation in a IEC refrigerated centrifuge at 5000 x g for 20 min at 10°C. The pellet was washed in phosphate-buffered saline and then suspended in 300 μl of solubilization buffer [0.1 M Tris Cl, pH 7/2% NaDodSO₄, 10% (vol/vol) glycerol, 3% 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride] and heated in a boiling water bath for 3 min. The samples were electrophoresed in 10% acrylamide gels as described by Laemmli (13). Gels were stained with Coomassie blue, dried under vacuum, and autoradiographed with Kodak X-Omat films for 15–20 days. Films were develop-

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Abbreviations: HSP, heat shock protein; DMEM, Eagle's medium modified by Dulbecco.
opened as suggested by the manufacturer.

The molecular weights of the polypeptides were estimated by co-electrophoresis of standard proteins (Sigma): phosphorylase A, 98,000; bovine serum albumin, 68,000; ovalbumin, 43,000; α-chymotrypsinogen A, 25,000; α-globulin heavy chain, 50,000; cytochrome c, 12,600.

RESULTS

Effect of Temperature on Protein Synthesis in Leishmania Promastigotes. Protein synthesis in control and heat-shocked cells was determined by subjecting the promastigote cultures to higher growth temperature and labeling them with [35S]methionine. General protein synthesis in L. tropica was altered slightly by 2 hr of incubation between 26°C and 37°C but then it fell sharply (Fig. 1). We therefore studied the effect of hyperthermia at two temperatures: 37°C and 40°C. Shifting the promastigote culture of L. tropica from 26°C to 37°C resulted in decreased cell mobility and a change in the protein synthesis pattern. The autoradiogram of NaDdSO₄ gel electrophoresis showed that these modifications appeared rapidly during the first 30 min after temperature elevation. The apparent molecular weights of the three HSPs observed under these conditions were 88,000, 74,000, and 54,000 (Fig. 2A). Additional prominent bands of lower molecular weight were seen in some preparations but they were absent or reduced in others. After 30 min at 40°C, L. tropica promastigotes lost their mobility and became rounded. The rate of protein synthesis decreased severely and while at the beginning of the treatment the three major HSPs were actively synthesized, after 60 min only two high molecular weight HSPs could be visualized (Fig. 2B).

We checked the effect of the heat treatment on the viability of promastigotes. Control cultures run in parallel were returned to 26°C after 2 hr at 37°C and 40°C. Cell shape and mobility were monitored every 2 hr by microscopic examination and the number of promastigotes was counted 24 hr later. In cells exposed to both temperatures, mobility increased with time of incubation at 26°C. After 15 hr, the cells recovered the promastigote shape and mobility and cell number doubled after 24 hr. Identical results were obtained with the three Leishmania species studied. These data clearly show the reversibility of the heat treatment.

Effect of Actinomycin D on the Synthesis of HSPs. When actinomycin D was added to the promastigote culture just before it was shifted to 37°C or 40°C (Fig. 3), there was no increase in HSP synthesis. Addition of actinomycin D 30 min after elevation did not affect the appearance of the protein pattern seen in heat-shocked cells. These results suggest that appearance of the HSPs depends on the translation of new mRNA.

Heat Shock Response in Various Leishmania Species. Shifting the cells from 26°C to 37°C for 2 hr resulted in a similar change in protein synthesis in all three species studied (Fig. 4). The proteins hsp88, hsp74, and hsp54 were observed, the former two being the most prominent. The intensity of the response at 37°C seemed higher for L. tropica and L. enrietti than for L. donovani. In all cases synthesis of new mRNA was needed for the increased synthesis of these proteins.

Effect of Sodium Arsenite on the Protein Synthesis Pattern of L. tropica Promastigotes. In various cells, sodium arsenite treatment produces the same effect on the protein synthesis pattern as does heat shock. Thus, we investigated the effect of this substance on the protein synthesis pattern of the parasite. When Leishmania promastigotes were exposed to sodium arsenite, the pattern of protein synthesis as visualized by the pattern of radiolabeled cellular proteins on NaDdSO₄/polyacrylamide gels changed (Fig. 5). Major proteins of molecular weight 94,000, 78,000, and 56,000 and minor proteins of molecular weight 70,000, 45,000, 22,000, and 18,000 were induced. When the arsenite concentration was held constant and the time of exposure to the molecule was varied, enhanced synthesis of all these proteins began after 90–120 min, except that the protein of molecular weight 22,000 began to appear during the first 30 min of treatment.
**DISCUSSION**

The data are now rather compelling for the existence of a common response to a temperature elevation among all organisms hitherto studied, from *Arachaeobacteria* (14) to mammalian cells. This response is characterized by a rapid increase in the synthesis of a few proteins (1, 5). An altered pattern of protein biosynthesis leading to the stimulation of a similar, if not identical, set of polypeptides can also be provoked by certain chemicals and environmental stress conditions (3, 6). Moreover, a possible relationship of morphogenesis, development, and differentiation to the heat shock response has also been reported recently (10, 11, 15, 16).

*Leishmania* are dimorphic parasites. The promastigotes (free living form) are cultivated at 26°C. The infection of macrophages in culture or of whole animals takes place at 35°C–37°C. We have demonstrated that the transfer of the promastigote cultures from 26°C to higher temperatures induces a heat shock response in the three *Leishmania* species studied. The apparent molecular weights of the HSPs of *Leishmania* are in the ranges of 80,000–90,000, 69,000–74,000, and 50,000–54,000. The two former proteins are similar in size to those identified in most of the heat-shocked cells. These two proteins and particularly the 70,000–72,000 one seems to be the best conserved during evolution (17, 18). The third leishmanial HSP is similar in size to one of the polypeptides induced upon hyperthermia in mouse embryonal carcinoma (p59) or in BHK-21 cells infected with Herpes simplex virus (p57) (15, 19).

Similar to what happens in chicken cells (6), sodium arsenite elicits the synthesis of a set of proteins in *L. tropica* promastigotes. The molecular weights of the three major and the four minor stress proteins are, respectively, 94,000, 78,000, and 56,000 and 70,000, 45,000, 22,000, and 18,000. These proteins differ in size from those induced by arsenite in *Drosophila* and in chicken cells (6, 20). It seems that similar to their action in these two cell types, arsenite and heat shock alter protein biosynthesis in *Leishmania* in different ways. According to our preliminary results, stress conditions such as methionine starvation or exposure to sublethal doses of sinefungine, an antileishmanial antibiotic, also induce the synthesis of stress proteins in promastigotes. In *Leishmania*, as in *Drosophila*, chicken cells or *Nasca*, actinomycin D prevents the synthesis of HSPs when added prior to the shock. The appearance of these proteins could not be inhibited when actinomycin D was added 30 min after elevating the temperature. These results indicate that, in these parasites also, the regulation of HSP synthesis occurs at the transcriptional level.

The function and the intracellular localization of the HSPs have not been elucidated and may be different in different species. In some cases, hyperthermia has been shown to confer thermotolerance on cells (3, 7, 21). According to Kelley and Schlesinger (22), in chicken embryo fibroblasts

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**FIG. 3.** Requirement for transcription. Autoradiogram of a NaDodSO4/polyacrylamide gel of [35S]methionine-labeled proteins from *L. tropica* promastigotes. Heat shock was carried out at 37°C (A) or at 40°C (B). Cells were labeled at 26°C, 37°C, or 40°C. Lanes: a, heat-treated cells; b, actinomycin D (40 μg/ml) was added at the time of temperature elevation; c, actinomycin D (40 μg/ml) was added 30 min after elevation. The experiment was performed in triplicate.

**FIG. 4.** Heat shock response of three *Leishmania* species. Promastigotes of *L. donovani*, *L. tropica*, and *L. enrietti* were grown at 26°C, then transferred to 37°C for 2 hr. Lanes: a, heat-shocked cells; b, actinomycin D (40 μg/ml) was added at the time of temperature elevation. Labeling was carried out for 30 min after 2 hr of incubation at 37°C. Identical results were obtained in three independent experiments.

**FIG. 5.** Effect of NaAsO2 on the pattern of protein synthesis of *L. tropica* promastigotes. Sodium arsenite (25 μM) was added to cultures at 26°C for various times. Lanes: a, 120 min; b, 90 min; c, 60 min; d, 30 min. Following treatment, the cells were labeled with [35S]methionine at 200 μCi/ml for 30 min. Three independent experiments gave similar results.
hsp95 and hsp76 are major proteins of the cell membrane and they are possibly associated with glucose metabolism and hexose transport. The major HSPs of Leishmania metabolism may also have a function under normal growth conditions, since most are synthesized at detectable levels in the absence of stress. In this respect, it is interesting to mention the work of Fong and Chang (23) on the cytodifferentiation of L. mexicana amazoniensis. According to this work, when amastigotes are shifted from 35°C to 27°C to allow the transformation to promastigotes, the synthesis of three proteins was stimulated. Their molecular weights were 55,000, 70,000, and 90,000. The M₆, 55,000 protein was shown to be tubulin. Thus it seems that temperature decrease also induces the alteration of the protein biosynthesis pattern at least in amastigotes of this species.

It is difficult to speculate on the role of HSPs in Leishmania, but there appears to be a strong relationship between skin temperature and the nature and extent of the lesions caused by these parasites (24). The fact that even after intraperitoneal inoculation of hamsters with L. braziliensis lesions appear in parts of the body with a subcutaneous temperature below 33°C suggests that the parasites have a thermosensitive stage during their development (25). It has also been shown by Sacks et al. (26) that Leishmania strains of the New World are much more sensitive to elevated temperatures than Old World strains. Further characterization of the genes of the various Leishmania HSPs may explain the difference in thermotolerance existing among these parasites and enable the establishment of evolutionary relationships of parasite HSPs to other organisms.

Note Added in Proof. Since we submitted this manuscript, Hunter et al. (27) have shown the induction of seven heat-shock proteins in L. mexicana during temperature-induced in vitro differentiation.

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