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Effects of Heat Shock on Macrophage

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Abstract: The macrophage plays an important role in lipopolysaccharide (LPS)-induced fever. Fever formation is attributed to macrophage activity. The purpose of this study was to examine whether induced fever has the characteristics of self-restriction during the process of a fever response. The result of methyl-[^{3}H]-thymidine incorporation in RAW 246.7 cells showed that the proliferation of the macrophage was suppressed by a heat load of 39℃ for 2 hr. Enzyme-linked immunosorbent assay (ELISA) showed that the activity of IL−1 secretion induced by LPS was also suppressed by heat load. Suppression of activities of the macrophage including proliferation and secretion suggest that negative feedback loops exist where the end product, heat, may act back on the macrophage and finally result in the subsiding of a fever. The induction of heat shock protein (hsp 70) by heat shock in the macrophage was detected by Western-blot and Northern-blot analysis. In U−937 cells, the increase of hsp 70 band appears to be related to the extent of inhibition of IL−1 secretion. It is assumed that IL−1 secretion from the macrophage which effects the induction of fever, has a close relationship to the induction of hsp 70.

Key words: LPS-induced fever, Interleukin−1 (IL−1), Heat shock protein (hsp), Heat shock transcription factor (HSTF), Heat shock element (HSE)

INTRODUCTION

It became apparent about 30 years ago that lipopolysaccharide (LPS)−induced fever is caused by a heat-unstable substance−endogenous pyrogen found in the blood. Since then many papers have described that LPS activates the macrophage in the blood to induce synthesis of endogenous pyrogen and releases it into the blood, and fever is caused (Wolff *et al.*, 1965; Snell *et al.*, 1967; Morimoto *et al.*, 1986). The macrophage is the first line of defense in the immune system, which takes charge of the function of antigen presentation in the immunological reaction (Unanue and Allen, 1987). Interleukin−1 (IL−1) is the principal substance among endogenous pyrogens, which causes varient physiological regulations including a fever response. IL−1 stimulates organum vasculosum laminae terminalis (OVLT) which exists on the sidewall of third ventricle in the brain, and causes the release of prostaglandin E_2 (PGE_2) there. The nerve impulse conduction from the PGE_2 receptor penetrates

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OVLT, reaches to preoptic area and anterior hypothalamus (PO/AH), causes a shift of the set-point, and thus triggers a fever response. The secretion of IL−1 is an initial response that intervenes and promotes many other immune responses (Duff and Durum, 1982). The reaction as follows is important during bacterial infection: LPS−macrophage−IL−1−PGE_2−fever. One might wonder why even extremely high fevers seldom exceed 41℃ or 42℃. It is postulated that there is a self-limiting mechanism, such as a negative feedback loop. However, the existence of a negative feedback loop has not yet been proved. It would thus be quite important to investigate whether or not fever is in charge of this role.

On the other hand, it is well-known that the heat shock protein (hsp 70) can be induced by heat stress (Hatayama *et al.*, 1985, 1986; Ohtsuka and Kano, 1986). Many reports have discussed about the relationship between heat tolerance and hsp 70 including our previous reports (Landry, 1982; Lee *et al.*, 1990, 1991). However, whether hsp 70 relates to a febrile response or not has been seldom discussed (Ciavarra and Simeone, 1990). The present study, therefore, investigated influences of heat shock on macrophage proliferation and IL−1β secretion, studied the induction of hsp 70 in macrophage, and examined the correlation between them.

Materials and Methods

Cells and cell culture: RAW 246.7 cells (mouse macrophage cell line) were obtained from Dr. J. Unkeless (Rockefeller University). They were cultivated in growth medium, RPMI 1640 medium containing 10% fetal bovine serum, antibiotics, and glutamine at 37℃ and 5% CO_2. When they were grown to confluence in a 5㎝ Falcon flask, the cell passage (1:4) was accomplished by treatment with 12 mM lidocaine in a growth medium. The culture medium was replaced every 24 to 48 hr. U−937 cells (human monocyte-like histiocytic lymphoma) were obtained from JCRB (Japanese Cancer Research Resources Bank). They were cultivated in RPMI 1640 medium containing 10% fetal bovine serum, antibiotics, and glutamine at 37℃ and 5% CO_2. They were grown from the cell density of 2.0×10^5 cells/ml, and the passage was performed 5 days later at a cell density of about 1.0×10^6 cells/ml.

Materials: Polyclonal anti-hsp 70 antibody was obtained from Dr. K. Ohtsuka (Aichi Cancer Center Research Institute). Human hsp 70 cDNA was obtained from Oncogene Science Inc. LPS, from *Escherichia coli* O127:B8, was obtained from Sigma. All reagents for electrophoresis were electrophoresis pure-grade.

Cell proliferation: RAW 264.7 cells proliferation after exposure to heat were assessed by methyl−[^{3}H]−thymidine incorporation, as described by Kantengwa *et al.* (1990). Five hundred μl of a cell suspension containing 4×10^4 cells for each condition were added to each well of 24−well plates (Falcon), then incubated at 37℃. Subsequently, the medium was changed to a serum-free medium 24 hr before treatments. Cells were heated in a water bath for heat shock (1) at 39℃, 41℃, 42℃, 44℃ for 2 hr and (2) at 39℃, 42℃, 44℃ for 3 hr. After each treatment, the cells were labelled with 0.25 ml/well methyl−[^{3}H]−thymidine.

After incubation at 37℃ for 4 hr, the cells were washed with PBS 1 ml/well for 3 times and 10% TCA for 2 times. Five hundred μl of 2% SDS was added to each well and incubated at 37℃ for 1 hr to lysis. A proper amount of lysate was transferred into vials and counted with scintillation fluid (Ammersham).

Western-blot: Cells, $(10-20)\times10^6$, were treated with heat-shock stimuli at 37℃, 39℃, 41℃, 43℃ for various time durations. The cells were then lysed with 0.1% SDS solution. Proteins were then quantified using the Bio-Rad Protein Assay. Protein, 25μg, from each cell population, was separated by 10% SDS−PAGE (Laemmli, 1970) and electro-blotted on nitrocellulose membrane using ATTO HORIZBLOT. Membranes were then incubated for 2 hr at 37℃ with BLOCK ACE (Dainippon Pharm. Co.) for blocking. Thereafter, a final dilution 1:500 of polyclonal anti-hsp 70 antibody, which recognizes 70 kD and 72 kD hsp, was used (Welch and Suhan, 1986). The proteins on the membranes reacted with biotin/avidin system using NECTASTAIN ABC reagent for antigen detection.

Analysis of mRNA levels: Northern-blot analysis was performed according to the standard procedure (Maniatis *et al.*, 1982). Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Thirty micrograms of total RNA was analyzed by electrophoresis through 1% agarose/formaldehyde gels, followed by Northern blot transfer to Hybond nitrocellulose sheets. Human hsp 70 cDNA (from Oncogene Science Inc.), an oligonucleotide probe, was labelled with [$\gamma-^{32}$P] ATP using T4 polynucleotide kinase. Membranes were pretreated and hybridized in 50% formamide with 50% Denhardt's reagent and washed 3 times, each time for 5 min with 2×SSC (1×SSC: 0.15 mol/l NaCl, 0.015 mol/l Sodium citrate) and 0.1% SDS at room temperature. Finally, they were washed with 0.1×SSC and 0.1% SDS at 37℃ for 30 min. The membranes were exposed for 24 hr at −80℃ with intensifying screens for autoradiography.

IL−1 immunoassay: U−937 cells, 6×10^5, suspended in 1 ml RPMI−1640 medium containing LPS at a final concentration of 500 μg/ml were added into 4−well plates for each condition. LPS was added to the plates at the begining and incubated at 37℃ for the entire 24 hr period. Fifteen hours into the experiment, the plates were subjected to heat shock for 30 min at 39℃, 41℃, 43℃. After this treatment, the plates were returned to the incubator, still at 37℃. After a total of 24 hr incubation, cells were transferred to Eppendorf tubes and centrifuged at $2,000\times g$ for 5 min. Twenty μl of supernatant were taken from each well and IL−1β assay were performed by enzyme-linked immunosorbent assay (ELISA) using Human IL−1β ELISA Assay Kit (Otsuka Pharm. Co.). Absorbances at 492 nm for various conditions of samples were measured, and IL−1β concentrations were determined and compared.

Results

The experiment was performed to test whether heat shock response could effect IL−1β secretion during LPS stimulation in U−937 cells. Control cells cultured without LPS did not secret IL−1β. U−937 cells, 6×10^5, cultured with LPS (500 μg/ml) for 24 hr at 37℃

induced 30 pg/ml of concentration of IL−1β. Quantificaion of IL−1β indicated that at 39℃, 41℃ and 43℃, IL−1β secretion was respectively 92%, 82% and 74% of the level at 37℃ (Fig. 1).

Elevated temperature inhibits proliferation of RAW 246.7 cells. By the assessment of methyl−[^{3}H]−thymidine incorporation, the proliferation of macrophage was suppressed by a heat load of 39℃ for 2 hr (Fig. 2). Conditions of high heat load such as 42℃, and 44℃ were

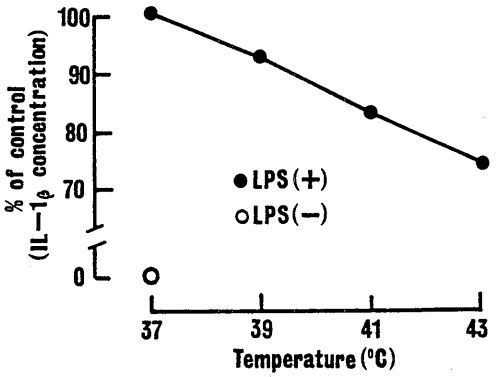

Fig. 1. Effect of heat on LPS induced IL−1 secretion in U−937. U−937 cells were cultured with LPS (500 μg/ml) for 24 hr at 37℃. An interval of 30 min of heat shock at 39℃, 41℃, 43℃ respectively was performed after 15 hr of incubation at 37℃. Quantification of IL−1 indicated that at 39℃, 41℃, 43℃, IL−1 secretion was respectively 92%, 82%, 74% of the level at 37℃.

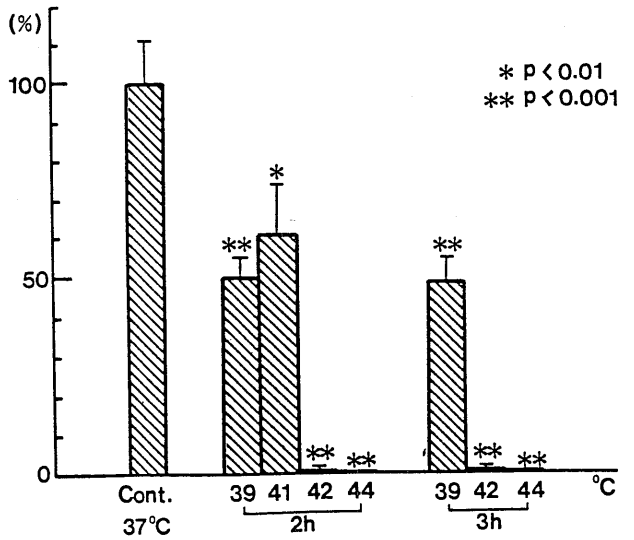

Fig. 2. Inhibition of proliferation by elevated temperature in RAW 246.7 cells. By the assessment of methyl−[^{3}H]−thymidine incorporation, the suppression of proliferation was observed at heat shock of 39℃ for 2 hr. Conditions of high heat load such as 42℃, 44℃ were performed simultaneously for comparison.

performed simultaneously for comparison. As was expected, the proliferation was also obviously suppressed.

The induction of hsp 70 family by heat shock at 42℃ and 44℃ for 30 min to 6 hr were identified in RAW 246.7 cells by the Western-blot method and the Northern-blot method. Results, indicated in Fig. 3, show immunoprecipitation response using anti-hsp 70 antibody, which is a polyclonal antibody specifically reacting with 70 kD and 72 kD of the hsp 70 family (Ohtsuka, Personal communication). Lane 1, control, incubated at 37℃; lane 2, heat-shocked RAW 246.7 cells for 30 min at 39℃, had a recovery time of 24 hr at 37℃. Lane 1 and lane 2 showed only 72 kD protein, constitutional-type of the hsp 70 family, which responded to this antibody without induction of inducible-type of the hsp 70 family. Heat-shocked cells for 30 min at 42℃ (lane 3), 44℃ (lane 4) and recovery time of 24 hr at 37℃ showed induction of hsp 70. The largest quantities of hsp 70 accumulated at 42℃. Lane 5, induction of hsp 70 in rat liver tissue (Lee *et al.*, 1990) was performed simultaneously for reference. As

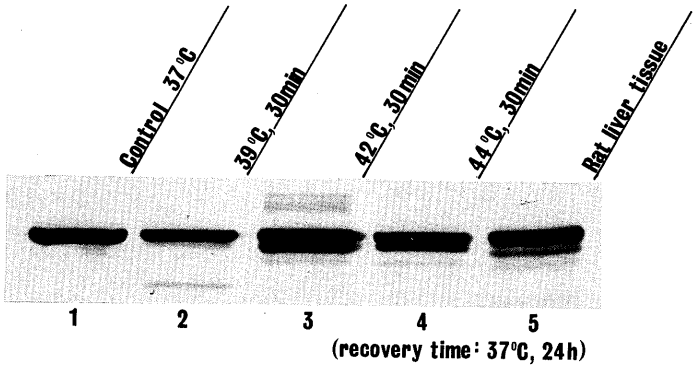

Fig. 3. The induction of hsp 70 by heat shock for 30 min at various temperature. RAW 246.7 cells were subjected to a heat shock for 30 min at 39℃, 42℃, 44℃. The recovery time was 24 hr at 37℃. The largest quantity of hsp 70 accumulated at 42℃.

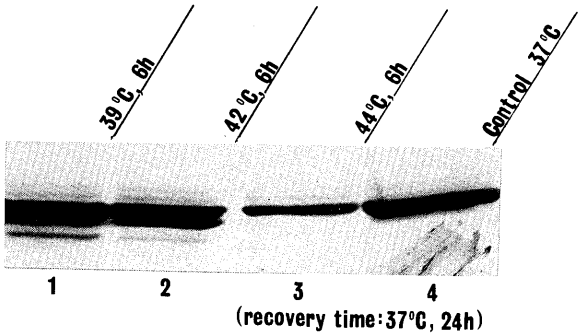

Fig. 4. The induction of hsp 70 by heat shock for 6 hr at various temperature. RAW 246.7 cells were subjected to a long heat load for 6 hr at 39℃, 42℃, 44℃. The recovery time was 24 hr at 37℃. Hsp 70 induction was positively identified at 39℃ and 42℃.

shown in Fig. 4, RAW 246.7 cells were subjected to a long heat load treatment for 6 hr at 39℃ (lane 1), 42℃ (lane 2), 44℃ (lane 3). Hsp 70 induction was positively identified at 39℃ (lane 1) and 42℃ (lane 2). Compared to the control at 37℃, the reduction of 72 kD protein was recognized at 44℃, due to the general reduction in protein synthesis (data not shown). For the purpose of examining the induction of hsp 70 in the initial stage, heat shock treatment for 1 hr at various temperatures accompanied by 1.5 hr of recovery time at 37℃ was performed (Fig. 5). It shows phased augmentation of induction of hsp 70 at various temperatures: control at 37℃ (lane 1), 39℃ (lane 2), 42℃ (lane 3), 44℃ (lane 4). Hsp 70 induction was detected at the treatment of 39℃ for 6 hr as shown in Fig. 4, but not detected, at the treatment of 39℃ for less than 3 hr (data not shown), at which supression of cell proliferation was observved. The induction of hsp 70 was also identified in U−937 cells. Fig. 6 shows that hsp 70 was induced by heat shock for 30 min at various temperatures: control at 37℃ (lane 1), 39℃ (lane 2), 42℃ (lane 3), 44℃ (lane 4). The augmentation of hsp 70 mRNA expression in RAW 246.7 cells by heat shock for 30 min at 41℃ (lane 2), 43℃ (lane 3) was further confirmed (Fig. 7).

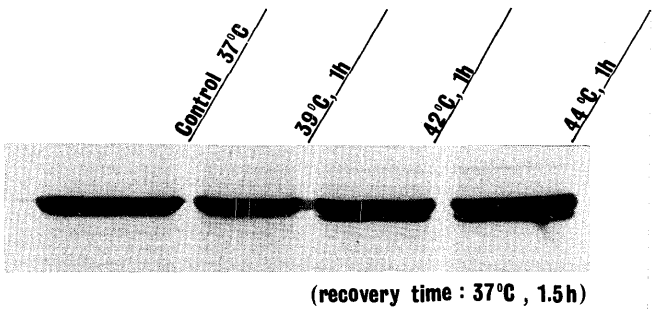

Fig. 5. The induction of hsp 70 by heat shock for 1 hr at various temperature. RAW 246.7 cells were subjected to a heat shock treatment for 1 hr at 39℃, 42℃, 44℃ and had a recovery time for 1.5 hr at 37℃.

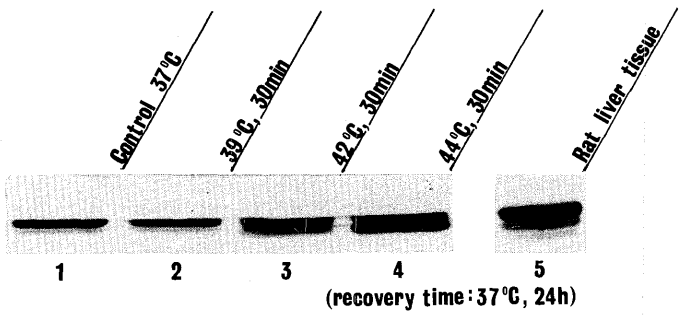

Fig. 6. The induction of hsp 70 by heat shock for 30 min at various temperature. U−937 cells were subjected to a heat shock treatment for 30 min at 39℃, 42℃, 44℃ and had a recovery time for 24 hr at 37℃.

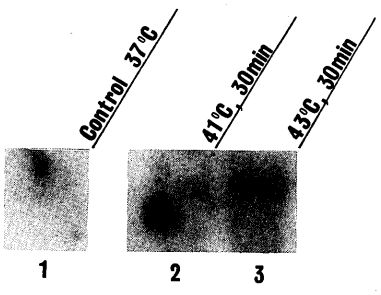

Fig. 7. Northern-blot analysis of hsp 70 mRNA in RAW 246.7 cells. The phase augmentation of hsp 70 mRNA expression in RAW 246.7 cells by heat shock for 30 min at 41℃, 43℃ were identified.

DISCUSSION

The results of methyl−[^{3}H]−thymidine incorporation and ELISA analysis showed that IL−1β secretion and the proliferation of the macrophage were suppressed upon heat shock. Simultaneously, induction of hsp 70 by heat shock was identified by the results of Western-blot and Northern-blot performed in this study. The regulation of hsp 70 induction is at the mRNA expression level. The results presented above indicate that LPS−induced fever may act as a negative feedback control on the macrophage's activity. It is assumed that hsp 70 may play a role correlated to this fever response. The release of IL−1 from macrophage is an important step which triggers a series of LPS−fever response. A variety of inducing signals suggests that negative regulatory mechanisms must exist to limit IL−1 bio-synthesis but as yet, these are poorly understood. One possibility is that negative feedback loops exist where the IL−2 bioactivity act back on production. Such a role has been postulated for PGE_2, which is in response to IL−1α and β (Knudsen, 1986). Schimidt and Abdulla (1988) reported that the synthesis of LPS induced IL−1β precursor protein, p35 (and its mRNA), were down-regulated by heat shock, compared with control cells at 37℃. From this study, it was identified that not only the biosynthesis of pre−IL−1, p35, but also the release of the mature form IL−1β was inhibited by heat shock. During heat stress the redistribution of heat-shock trancription factor (HSTF) to heat shock element (HSE) (Wu *et al.*, 1990) is accompanied by binding to many additional chromosomal sites and may act as a repressor of normal gene activity (Westwood *et al.*, 1991). Therefore, it may possible that HSTF binds to IL−1β gene and represses its gene expression. It may possibly be one of the reasons why IL−1β secretion is inhibited by heat shock. However this hypothesis needs more experimental evidence in order to be confirmed.

The effect of proliferation suppression was observed at the heat shock 2 hr into the experiment at 39℃, at which the so-called repairing protein, hsp 70, had not yet been detected within the cells. The correlation between DNA replication and hsp 70 has not yet been really clarified. However, it was reported that adding blood plasma to a culture of Hela cells in which blood plasma had been first withdrawn and induced into the stationary phase of the

cell cycle, increased hsp 70 mRNA parallel to the synthesis of DNA (Morimoto, 1989). The suppression of the macrophage proliferation by heat shock may be due to a mechanism other than the active role of hsp 70.

From the viewpoint of Q_{10} value (temperature dependence of chemical reaction), the optimal temperature of enzymatic and chemical reaction activities is at 37℃ *in vivo*; therefore, in the present study, the activities of IL−Iβ secretion and cell proliferation were set at 37℃ as 100%. Thus, the responses of the range around 36℃ to 38℃ must be precisely determined in future studies.

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REFERENCES

1) Chomczynski, P. & Sacchi, N. (1987): Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., 162, 156−159.

2) Ciavarra, R.P. & Simeone, A. (1990): T lymphocyte stress response. I. Induction of heat shock protein synthesis at febrile temperatures is correlated with enhanced resistence to hyperthermic stress but not to heavy metal toxicity or dexamethasone-induced immunosuppression. Cell. Immunol., 129, 363−376.

3) Duff, G.W. & Durum, S.K. (1982): Fever and immunoregulation: Hyperthermia, interleukin 1 and 2, and T−cell proliferation. Yale J. Biol. Med., 55, 437−442.

4) Ferris, D.K., Bellan, A.H., Morimoto, R.I., Welch, W.J. & Farrar, W.L. (1988): Mitogen and lymphokine stimulation of heat shock proteins in T lymphocytes. Proc. Natl. Acad. Sci. USA, 85, 3850−3854.

5) Hatayama, T., Fujio, N., Honda, K., Kano, E. & Yukioka, M. (1986): Biochemistry of heat shock proteins: Induction mechanism and function. Jpn. J. Hyperthermic Oncol., 2, 104−114. (in Japanese with English abstract)

6) Hatayama, T., Kano, E. & Yukioka, M. (1985): Cell biology of heat shock response: Induction mechanism and role of heat shock proteins. Jpn. J. Hyperthermic Oncol., 1, 115−129. (in Japanese with English abstract)

7) Kantengwa, S., Capponi, A.M., Bonventre, J.V. & Polla, B.S. (1990): Calcium and the heat-shock response in the human monocytic line U−937. Am. J. Physiol., 259, C77−C83.

8) Knudsen, P.J., Dinarello, C.A. & Strom, T.B. (1986): Prostaglandins posttranscriptionally inhibit monocyte expression of interleukin 1 activity by increasing intracellular cyclic adenosine monophosphate. J. Immunol., 137, 3189−3194.

9) Laemmli, U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680−685.

10) Landry, J., Bernier, D., Chretien, P., Nicole, L.M., Tanguay, R.M. & Marceau, N. (1982): Synthesis and degradation of heat shock proteins during development and decay of thermotolerance. Cancer Res., 42, 2457−2461.

11) Lee, J.M., Kosaka, M., Yang, G.J., Amador, J., Matsumoto, T., Ohwatari, N., Tsuchiya, K. & Nakamura, K. (1991): Physiological analysis of weak thermo−tolerance in pika rabbits. pp. 96−97. *In* M. Yoshikawa, M. Uono, H. Tanabe, & S. Ishikawa (ed.). New Trends In Autonomic Nervous System Research. Elsevier Science Publishers B.V., Amsterdam.

12) Lee, J.M., Yang, G.J., Kosaka, M., Matsumoto, T., Ohwatari, N., Takashi, I. & Hatayama, T. (1990): Physiological analysis of heat tolerance in pika. pp. 316−317. *In* M. Saito (ed.). Hyperthermic Oncology In Japan '89. The Japanese Society of Hyperthermic Oncology, Tokyo, Japan.

13) Maniatis, T., Fritsch, E.F. & Sambrook, J. (1982): Molecular cloning. A laboratory manual. p. 545. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

14) Morimoto, A., Murakami, N., Ono, T. & Watanabe, T. (1986): Dehydration enhances endotoxin fever by increased production of endogenous pyrogen. Am. J. Physiol., 251, R41−R47.

15) Morimoto, R.I. (1989): Transcriptional regulation of human heat shock genes. Exp. Med., 7(17), 1970−1981.

16) Ohtsuka, K. & Kano, E. (1986): Biochemical characterization of heat shock proteins and their relation with thermotolerance. Jpn. J. Hyperthermic Oncol., 2, 90−103. (in Japanese with English abstract)

17) Schmidt, J.A. & Abdulla, E. (1988): Down-regulation of IL−1β biosynthesis by inducers of the heat-shock response. J. Immunol., 141 (6), 2027−2034.

18) Snell, E.S. & Atkins, E. (1967): Interactions of gram-negative bacterial endotoxin with rabbit blood *in vitro*. Am. J. Physiol., 212, 1103−1112.

19) Unanue, E.R. & Allen, P.M. (1987): The basis for the immunoregulatory role of macrophages and other accessory cells. Science, 236, 551−557.

20) Welch, W.J. & Suhan, J.P. (1986): Cellular and biochemical events in mammalian cells during and after recovery from physiological stress. J. Cell. Biol., 103, 2035−2352.

21) Westwood, J.T., Clos, J. & Wu, C. (1991): Stress-induced oligomerization and chromosomal relocalization of heat-shock factor. Nature, 353, 822−827.

22) Wolff, S.M., Mulholland, J.H. & Ward, S.B. (1965): Quantitative aspects of the pyrogenic responses to rabbits of endotoxin. J. Lab. Clin. Med., 65, 268−276.

23) Wu, C., Zimarino, V., Tsai, C., Walker, B. & Wilson, S. (1990): Transcriptional regulation of heat shock genes. p. 429−442. *In* R.I. Morimoto, A. Tissieres & C. Georgopulos (ed.). Stress Proteins In Biology And Medicine. Cold Spring Harbor Laboratory Press, NY.