[Jpn. J. Parasitol., Vol. 42, No. 6, 461–467, December, 1993]

Correlation Between Heat Shock Protein Expression and the Acquisition of Protective Immunity to *Toxoplasma gondii* in Rats

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(Accepted for publication; October 20, 1993)

Abstract

We previously reported that induction of HSP65 on host macrophage correlates with protection against *Toxoplasma gondii* in mice infected with the high- or the low-virulence strain of the parasite. In contrast to mice, rats are resistant to infection even against a high-virulence strain of *T. gondii*. To investigate whether the correlation between expression of HSP65 and protection against *T. gondii* exists regardless of host species, experiments using rats were performed. Normal rats completely resolved infection with a high-virulence strain of *T. gondii* and HSP65 was strongly expressed on their peritoneal exudate cells (PEC). However, only half of LEC rats which are genetically deficient in $CD4^+$ T cells survived an infection with *T. gondii*. This low resistance coincides with low HSP65 expression on LEC rat PEC. Furthermore, all of the *T. gondii*-inoculated athymic nude rats died of acute infection and HSP65 was scarcely expressed on their PEC. These results indicate that in rats expression of HSP65 also correlates closely with protection against infection with this protozoon. Moreover, T cells, especially $CD4^+$ T cells, are important for acquiring of resistance to *T. gondii* and in the expression of HSP65.

Key words: heat shock protein, toxoplasmosis, protective immunity

Introduction

Exposure of cells to a variety of stimuli including elevated temperature, oxygen metabolites, or infection results in increased synthesis of a family of polypeptides termed heat shock or stress proteins (HSPs) (Lindoquist, 1986). Although they have important roles in protection against harmful stimuli (Schlesinger, 1990), recent studies have revealed that these proteins serve vital functions in the absence of stress (Goloubinoff *et al.*, 1989. Vanbuskirk *et al.*, 1989. Cheng *et al.*, 1990). HSPs have been identified as major immunogens in certain infectious states, for example, leprosy and tuberculosis (Young *et al.*, 1988), malaria (Bianco *et al.*, 1986), Chagas' disease (Engman *et al.*, 1990), filariasis (Rothstein *et al.*, 1989) and schistosomiasis (Hedestrom *et al.*, 1987). T cells reactive to HSP65 derived from pathogen exhibit cytotoxicity to macrophages expressing host-derived HSP65 (Koga *et al.*, 1989), suggesting that HSP contributes to the elimination of intracellular pathogens as target antigens on infected cells. Furthermore, HSP65 is known as one of the ligands of γδ T cells (O'Brien *et al.*, 1991), which participate in protection against early phase of infections with intracellular pathogens (Hiromatsu *et al.*, 1992. Raziuddin *et al.*, 1992). Thus, interaction between microbial HSP and mammalian HSP may modulate ability of protection of host or virulence of pathogens.

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Grant support: Ministry of Education, Science and Culture, Japan (03670192, 03202102).

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Toxoplasma gondii is an obligate intracellular protozoan parasite that infects humans and wild and domestic animals (Krahenbuhl and Remington, 1982). Although *T. gondii* is an uncommon cause of disease in individuals with a normal functional immune system, immunocompromised hosts such as patients with AIDS are at high risk of developing severe toxoplasmosis, especially toxoplasmic encephalitis as opportunistic infections (Sibley *et al.*, 1992). We reported previously that both $CD4^+$ and $CD8^+$ T cells play critical roles in acquisition of resistance in mice susceptible to *Toxoplasma* infection (Nagasawa *et al.*, 1991). However, it remains to be determined whether protective mechanisms against *T. gondii* are different between resistant and susceptible host species.

Our previous investigation showed that induction of HSP65 on host peritoneal exudate cells (PEC) closely correlates with an ability to protect mice from infection with *T. gondii* (Nagasawa *et al.*, 1992). We also obtained lines of evidence showing that T cells may contribute to expression of HSP65 in experiments using *Toxoplasma*-infected normal mice which had been depleted of T cell subsets by monoclonal antibodies to T cell antigens or using nude and SCID mice to which each T cell subset had been transferred (submitted for publication). In this work, we investigated whether the correlation of the expression of HSP65 with protection against *T. gondii* exists not only in mice but also in rats by the use of mice, normal rats and nude rats which genetically lack T cells, and a mutant strain of rats (LEC) which is selectively defective in $CD4^+$ T cells (Agui *et al.*, 1990).

Materials and Methods

Animals. Female BALB/c ($H\text{-}2^d$) mice, F344 ($RT1^l$) rats, F344 nu/nu ($RT1^l$) rats were purchased from Shizuoka Laboratory Center (Shizuoka, Japan). LEC ($RT1^u$) rats and LEA ($RT1^u$) rats were bred in the Institute for Animal Experimentation, The University of Tokushima (Tokushima, Japan). LEC rats lack mature $CD4^+CD8^-$ T cells because of the defect in thymic T cell maturation from $CD4^+CD8^+$ to $CD4^+CD8^-$ T cells but they have functional $CD4^-CD8^+$ T cells (Agui *et al.*, 1990). LEA rat is a congenic strain of LEC rat with normal lymphopoiesis. All animals were used for experiments at 10–12 weeks of age.

Parasites. The low-virulence Beverley strain (Ito *et al.*, 1976) and the high-virulence RH strain (Sabin, 1941) of *T. gondii* were used in these studies. Bradyzoites of Beverley strain were prepared from cysts isolated from brains of chronically infected mice by discontinuous density gradient centrifugation with gum arabic solution (Sigma Chemical Co., St. Louis, MO) (Nagasawa, 1984). RH strain was maintained by serial intraperitoneal passages in mice. To prepare tachyzoites of RH strain, peritoneal exudates obtained from infected mice were passed through CF-11 (Whatman BioSystems Ltd., Maidstone, England) column to eliminate host cells, and live parasites in filtrates were counted. Mice were inoculated with 1×10^4 bradyzoites of Beverley strain or 1×10^2~5×10^7 tachyzoites of RH strain intraperitoneally.

Western blotting. To detect HSP65, western blotting was performed as previously described (Nagasawa *et al.*, 1992). Briefly, protein extracts of PEC from infected rats and mice were separated by SDS-PAGE, following by electroblotting onto PVDF membrane (Millipore Co., Bedford, MA). The murine IgG mAb IA10 specific for an epitope located between amino acids 172 and 224 of mycobacterial HSP65 (provided by J. DeBruyn, Institute Pasteur de Brabant, Belgium), which is kown to recognize mammalian HSP65 (Koga *et al.*, 1989), was used with 1:200 diluted culture supernatant as the primary antibody, and goat peroxidase-labelled anti-mouse IgG (Pierce, Rockford, IL) as the secondary antibody. Bound antibodies were detected by Konica immunostaining horseradish peroxidase kit (Konica, Japan).

Results

Difference in resistance to infection with T. gondii between rats and mice. Mice are known to be highly susceptible to the infection with RH strain of *T. gondii*. Mice infected with 1×10^7 tachyzoites of RH strain died within 5 days and even with a low inoculum of 1×10^2 tachyzoites, all mice died within 11 days (Fig. 1). But they are relatively resistant to certain strains, one of which is the low-virulence Beverley strain. When mice were infected with a

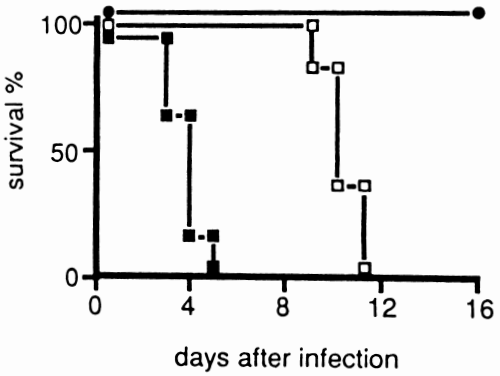

Fig. 1 Difference in resistance to *T. gondii* between rats and mice. F344 rats were infected with 1×10^7 tachyzoites of the RH strain (closed circle). BALB/c mice were infected with 1×10^7 (closed square) or 1×10^2 (open square) tachyzoites of the RH strain. Results represent the survival rate in each group in which six animals were used.

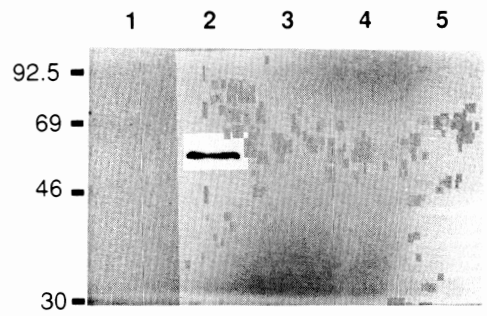

Fig. 2 Expression of HSP65 on PEC from mice infected with *T. gondii*. Western blot analysis of PEC from BALB/c mouse 10 days after infection with 1×10^2 bradyzoites of the Beverley strain (lane 2) or 1×10^2 tachyzoites of the RH strain (lane 3) and from uninfected mouse as negative control (lane 1). Lysates of tachyzoites of the RH strain (lane 4) and bradyzoites of the Beverley strain (lane 5) were also examined. Irrelavant murine IgG mAb against I-Ab (AF6-120.1.2) did not react this protein (data not shown). Two μg of protein was loaded on each lane. Standard molecule weight markers are shown on the left (in kilodaltons).

sublethal dose (1×10^2) of bradyzoites of this strain, they transiently showed acute symptoms of infection. Thereafter, these mice resolved the infection (data not shown). In contrast, rats inoculated with 1×10^7 tachyzoites of RH strain showed no sign of wasting diseases and controlled the infection effectively, indicating that rats are extremely resistant to *T. gondii* infection compared with mice (Fig. 1). Injection with 1×10^8 tachyzoites, however, resulted in 100% death of these resistant rats (data not shown). Thus, 1×10^8 tachyzoites of RH strain was defined as a lethal dose.

As we previously described, mice infected with 1×10^2 tachyzoites of RH strain died of acute infection within 11 days and mice infected with the same number of bradyzoites of Beverley strain survive the infection. Expression of HSP65 was readily detected on PEC of mice 10 days after infection with the low-virulence Beverley strain, but not with the high-virulence RH strain (Fig. 2). Thus, in mice, expression of HSP65 correlates with protection against *T. gondii*.

Expression of HSP65 on PEC of rats infected with T. gondii. When F344 rats were infected with 5×10^6 tachyzoites of the high-virulence RH strain intraperitoneally, they did not suffer from acute symptoms of infection. PEC was prepared from

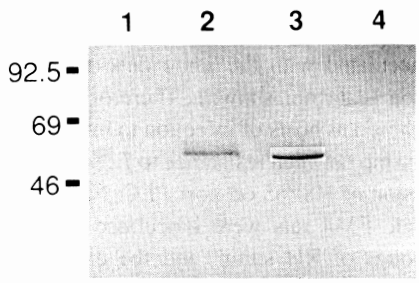

Fig. 3 Western blot analysis of PEC from F344 rat 2 days (lane 2), 5 days (lane 3) and 10 days (lane 4) after infection with 5×10^6 tachyzoites of the RH strain and from naive F344 rat (lane 1). Three μg of protein was loaded in each lane.

these rats at various intervals after infection, and the expression of HSP65 was determined by western blotting. As shown in Fig. 3, HSP65 was readily detected on PEC of rats 5 days after infection and less readily detectable on days 2 and 10 after infection.

Induction of HSP65 on PEC of nude rats infected with T. gondii. Athymic nude rats which lack T cells are highly susceptible to *Toxoplasma* infection (Rid-

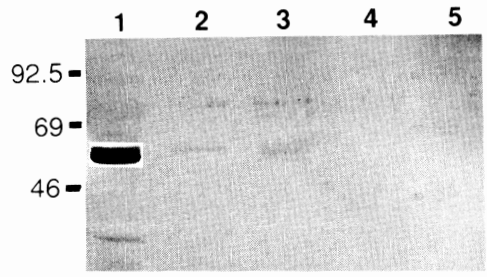

Fig. 4 Western blot analysis to detect HSP65 on PEC from athymic rats infected with *T. gondii*. Extracts of PEC from F344 rats 5 or 10 days after infection with 1×10^7 tachyzoites of the RH strain (lane 1 and 3, respectively), athymic F344 nu/nu rats 5 or 10 days after infection with 1×10^7 tachyzoites of the RH strain (lane 2 and 4, respectively), and from uninfected F344 rat (lane 5) were examined. Five μg of protein was loaded in each lane.

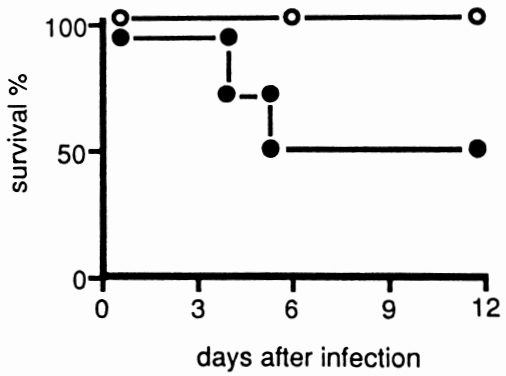

Fig. 5 Impaired resistance to *T. gondii* in LEC rat. Mutant LEC rats (closed circle) and the congenic LEA rats (open circle) were inoculated with 5×10^7 tachyzoites of the RH strain. The values in the y-axis represent the survival percentage in each group in which four animals were used.

dle *et al.*, 1988) compared with euthymic normal rats. In preliminary experiments, we also observed that most F344 nude rats inoculated with 1×10^7 tachyzoites of RH strain died of acute infection within 14 days, whereas all of the euthymic F344 rats inoculated with the same dose resolved the infection (data not shown). Therefore, nude rats were chosen as hosts of infection to investigate the relationship between resistance to *T. gondii* and the expression of HSP65 on host PEC. Nude rats and euthymic F344 rats were inoculated with 1×10^7 tachyzoites of RH strain, and the expression of HSP65 on their PEC was examined. In normal F344 rats, HSP65 was detected on PEC 5 days after infection, whereas this protein was not induced on PEC of nude rats (Fig. 4). These results demonstrate that the expression of HSP65 correlates with resistance to toxoplasmosis and also suggest that T cells are indispensable for the expression of HSP65.

Resistance to T. gondii in CD4$^+$ T cell-deficient mutant (LEC) rats. LEC rats are a mutant strain that is defective in thymic T cell differentiation and consequently lack mature CD4$^+$ T cells (Agui *et al.*, 1990). To examine influence of CD4$^+$ T cell defect on resistance to *Toxoplasma* infection and HSP65 expression, these mutant rats were infected with 5×10^7 tachyzoites of RH strain. Only 50% of infected LEC rats survived, whereas all of the congenic LEA rats resolved the infection (Fig. 5). HSP65 was

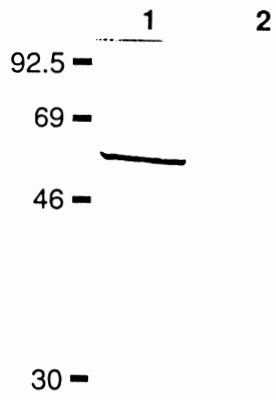

Fig. 6 Comparison of the expression of HSP65 between LEC and LEA rat infected with *T. gondii*. Forty million of tachyzoites were inoculated into each LEA rat (lane 1) and LEC rat (lane 2). PEC was prepared from each group 5 days after infection. Ten μg of protein was loaded in each lane.

expressed strongly on PEC of LEA rats, while only faintly on those of LEC rats (Fig. 6). Thus, T cells, especially CD4$^+$ T cells, appear to play a crucial role in the protection against infection with *T. gondii* and the expression of HSP65 in rats.

Discussion

T. gondii is an obligate intracellular parasite. They survive and replicate intracellularly after being phagocytized by macrophages of susceptible animals (Krahenbuhl and Remington, 1982). The major host-defense mechanisms against this parasite are cell-mediate immunity. Our previous results with mice infected with either the low-virulence Beverley strain or the high-virulence RH strain demonstrated that the expression of HSP65 on host macrophages clearly correlates with the potential of protective immunity in mice infected with *T. gondii* (Nagasawa *et al.*, 1992). Furthermore, we elucidated that γδ-T cells as well as $CD4^+$ αß-T cells play a crucial role in the expression of HSP65 on host macrophages in mice which have acquired protective immunity against infection with *T. gondii* (submitted for publication). It is of importance to understand whether HSP65 expressed on host macrophages contributes to host defense and whether a relationship exists between the level of HSP65 expression and the protective activity across the barrier of species of hosts.

In the present study, we intended to clarify the correlation between the resistance to *T. gondii* and the expression of HSP65 on host macrophages by comparing these two parameters in mice and various strains of rats including athymic nude rats and $CD4^+$ T cell-deficient rats. In contrast to mice, rats were strongly resistant to infection with *T. gondii*. Lethal dose of the highly virulent RH strain was less than 10^2 parasites for mice, while it is 10^8 for rats. All of the normal (F344 and LEA) rats inoculated with 5×10^7 tachyzoites of RH strain controlled the infection, whereas all of athymic nude rats (data not shown) and about 50% of the $CD4^+$ T cell-deficient LEC rats injected with the same inoculum died of acute infection. Thus, in rats, T cells, especially $CD4^+$ T cells, appear to contribute to protection against the infection with the RH strain of *T. gondii*, although they are extremely resistant as compared with mice. HSP65 was strongly expressed on PEC of normal (F344 and LEA) rats infected with RH tachyzoites but only weakly expressed on those of LEC rat and barely detectable on those of nude rat infected with this protozoon. HSP65 was not expressed on either *T. gondii* themselves or PEC of uninfected rats. Therefore, this protein must have been synthesized in macrophages of hosts resistant to infection with RH tachyzoites. T cells, especially $CD4^+$ T cells, seem to participate in rendering hosts capable of resolving infection with intracellular pathogens and inducing the expression of HSP65 on host macrophages.

Mammalian cells may synthesize HSPs in response to infections and/or physiological stimulation. Phagocytes protect themselves from noxious molecules that they produce, such as the highly reactive oxygen metabolites. Indeed, phagocytosis and physiological activators of the oxidative burst induce HSP synthesis in macrophages (Polla and Kantengwa, 1991). However, *Toxoplasma* parasites can survive and replicate within macrophages after phagocytosis, especially in susceptible animals like mice. The mechanisms for survival of *Toxoplasma* within certain phagocytic cells have been addressed (Wilson *et al.*, 1980). These investigators showed that the survival of *Toxoplasma* within normal mouse peritoneal macrophages can be attributed to the failure of this parasite to stimulate an oxidative burst that normally occurs with phagocytosis of *Candida, Staphylococci* spp, or latex particles. At early phases after infections, infectivity or virulence of intracellular parasites are determined by two factors; one is the ability of parasites to evade host cell surveillance systems as mentioned above, and the other is regulated by natural resistance genes probably expressed in macrophages of insusceptible animals (Blackwell *et al.*, 1991). At late stages, T cells will mainly control infections by activating macrophages or directly destroying phagocytic cells.

The mechanism of HSP induction *in vivo* remains obscure, although there are some reports showing that some cytokines induce the expression of HSPs in mouse macrophages and in other cells (Koga *et al.*, 1989). We speculated that macrophages activated by T cells may kill the infected parasites by means of respiratory burst in cytoplasma, consequently they may be exposed to harmful molecules such as oxygen metabolites following by synthesis endogenous HSP65 to protect themselves from these molecules. Recently, the number of γδ-T cells were found to increase rapidly in peripheral blood of patients with acute *T. gondii* infection (De Paori *et al.*, 1992. Scalise *et al.*, 1992). This subset of T cells

is thought to represent a first line of defense against infection with intracellular pathogens (Hiromatsu *et al.*, 1992). We demonstrated that γδ-T cells have an important role in inducing the expression of HSP65 on host macrophages in mice which have acquired resistance against *T. gondii* infection. Thus, it is not surprising that HSP65 involves in protective immunity against infection with some kinds of pathogen. In $CD4^+$ T cell-deficient LEC rats infected with RH tachyzoites, the expression of HSP65 was very weak but still significant. We cannot determine in rats whether γδ-T cells play a role in the expression of HSP65 and in the protection since we do not have adequate monoclonal antibodies to γδ receptors of rat type T cells. At any rate, the present study suggests that the expression of HSP65 and acquisition of protective immunity against infection with *T. gondii* clearly correlate in different host species irrespective of whether they are resistant or susceptible to *T. gondii* infection.

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