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journal or publication title	Bulletin de la Societe franco-japonaise des sciences veterinaires
volume	6
number	2
page range	154-162
year	1995
URL	http://id.nii.ac.jp/1588/00000079/

ROLE OF HEAT SHOCK PROTEINS IN PROTECTIVE IMMUNITY AGAINST INFECTION WITH *TOXOPLASMA GONDII*

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— *Bull. Soc. Fr.-Jpn. Sci. Vét. 6(2): 154 - 162, 1995* —

ABSTRACT

Toxoplasma gondii is an obligate intracellular protozoan parasite and cellular immunity plays a crucial role in protective immune responses against *Toxoplasma* infection. We reported previously that expression of 65-kD heat shock protein (HSP65) in host macrophages closely correlates with protective immunity, while this protein is not expressed in *Toxoplasma* themselves. In this study, we examined the expression mechanism of HSP65 in mice infected with *Toxoplasma gondii*. HSP65 was detected in peritoneal macrophages of BALB/c mice infected 7 days previously by electroblot assay with a specific monoclonal antibody (mAb) against microbial HSP65. This expression was not induced in those of athymic nude mice and SCID mice infected with same protozoa. Treatment of BALB/c mice with anti-Thy1.2 mAb one day before infection led to an almost complete loss of the expression of HSP65. To determine the subsets of T cells responsible for induction of HSP65, mice were depleted of either γ/δ T cells or α/β T cells by treating with corresponding antibodies before infection. From these experiments, γ/δ T cells were shown to be essential for the expression of HSP65. Thus, γ/δ T cells appear to play an important role in protective immunity against infection with *T. gondii* through mediating the expression of HSP65 in host macrophages.

INTRODUCTION

Exposure of cells to a variety of stressful conditions including elevated temperature, stressful chemical intoxication, or infection leads to the transcription of a set of genes and, thus, to the synthesis of a family of polypeptides termed heat shock proteins (HSPs) (12, 17, 20). Immunodominant antigens from a wide variety of bacteria and parasites have been identified by sequence homology as belonging to the family of HSPs (25). Recently, HSPs have receiving the attention of immunologists as target of T cells for specific recognition by antibodies and T cells of the immune system. Among the various HSPs, a 65-kDa mycobacterial HSP has been identified as a target of T cells, including γδ T cells (7, 9, 11, 16). This HSP contains a significant sequence similarity and cross-reactivity with antigens from a variety of other microbes. HSPs in parasite infection appear, on the one hand, to play important roles in adaptation of microorganisms (1, 21). On the other hand, HSPs function as prominent antigenic proteins that can activate the host immune systems (7).

Toxoplasma gondii is an obligate intracellular protozoan parasite found throughout the world. We showed earlier that T cells play an important role in protective immunity against infection with *Toxoplasma* parasites (14). However, we also found that the protective mechanisms involved in resisting infection with a strain of *T. gondii* of low virulence (Beverley strain) differ greatly from those involved in resisting infections with a highly virulent strain (RH strain) by the following phenomena. When mice were immunized with *Toxoplasma* homogenate before infection with a lethal dose of Beverley strain bradyzoites, the mice acquired resistance and survived. By contrast, vaccination with a sublethal dose of live Beverley strain bradyzoites was required for acquisition of resistance to infection with the highly virulent RH strain. These findings seem consistent with our observations

that immunization with *Toxoplasma* homogenate along with complete Freund's adjuvant failed to prevent infection of mice by tachyzoites of the RH strain.

In this paper, we present that HSP65 possessing an epitope located between amino acids 172 and 224 of *Mycobacterium bovis* is expressed in peritoneal macrophages of mice infected with *T. gondii*. The degree of expression of HSP65 correlates with protection that occurred in exposed mice, regardless of differences in virulence or strain specificity of this protozoa or in species of host. Moreover, we investigated the mechanism of induction of host-derived HSP65 in peritoneal macrophages from mice which were immunized with *Toxoplasma* homogenate, or infected with a low virulent strain of *T. gondii*. Our findings provide evidences that T cells, especially γ/δ T cells, are essential for the expression of HSP65 by peritoneal macrophages induced by immunization with *Toxoplasma* homogenate, and for the protective immunity against infection with *T. gondii*.

MATERIALS AND METHODS

Animals

Female BALB/c +/+ and BALB/c nu/nu (nude) mice purchased from Japan Shizuoka Laboratory Animal Center (SLC) (Hamamatsu, Japan), and C.B-17 +/+ and C.B-17 scid/scid (SCID) mice purchased from the Central Institute for Experimental Animals (Kawasaki, Japan) were used for experiments at 8 - 10 weeks of age. *Parasites.* A low-virulence Beverley strain and a high-virulence RH strain of T. gondii were used in these studies. Bradyzoites of the Beverley strain were obtained from cysts isolated from the brains of chronically infected mice. The RH strain of T. gondii was maintained by routine passage in mice. Bradyzoites and tachyzoites were routeinly prepared according to described procedures (14).

Infection and immunization

Mice were infected with 1 x 10^4 bradyzoites of Beverley strain or 1 x 10^4 tachyzoites of RH strain. To induce protective immunity against the low-virulence Beverley strain, mice were injected i.p. with 0.2 mg (total protein) of *Toxoplasma* homogenate and challenged i.p. with 1 x 10^4 live bradyzoites of Beverley strain 7 days after this immunization as described (14). *Toxoplasma* homogenate was prepared from RH strain tachyzoites by three cycles of freeze-thawing as described previously (14). To induce protective immunity against a highly virulent RH strain, mice that had been immunized with *Toxoplasma* homogenate and that had survived for 4 weeks after challenge with bradyzoites of the Beverley strain were inoculated i.p. with 1 x 10^4 tachyzoites of RH strain.

Immunoblotting

Protein extracts of PEC homogenates derived from immune mice were mixed with lysate buffer (50 mM Tris-HCl / 1% Nonidet P-40 / 0.1% SDS / 1 mM leupeptin / 100 mM phenylmethyl sulfonyl fluoride / 1 mM pepstatin A / 100 mM EDTA / 150 mM NaCl). Protein concentration was determined with BCA (bicinchoninic acid) protein assay reagent (Pierce, Rockford, IL). The protein samples were suspended and denatured in sample buffer (0.025 M Tris-HCl, pH 6.8 / 2% SDS / 10% (vol/vol) glycerol / 10% (vol/vol) 2-mercaptoethanol / 0.002% bromophenol blue) with boiling at 100 °C for 3 min. The protein was separated by SDS/PAGE (12.5% polyacrylamide) at 200V for 30 min.

Murine mAb IA10, specific for an epitope located between amino acids 172 and 224 of HSP65 derived from *M. bovis*, was provided by J. DeBruyn (Institute Pasteur de Brabant, Belgium), which is known to recognize mammalian HSP65 (11, 15). Blots were prepared by electrophoretic transfer of the proteins to a PVDF membrane (Millipore Co., Bedford, MA), and binding of mAb was assayed with peroxidase-conjugated secondary antibody as described previously (15).

Depletion of T cell subsets

Monoclonal antibodies specific for each T cell subset were harvested as ascites from pristane-primed nude mice with mAb-producing hybridomas in their peritoneal cavity. Antibodies were precipitated from the ascites with 45% ammonium sulfate and dialyzed against PBS. Before use, their total protein was measured. Preliminary

experiments showed that i.v. injection of 0.5 mg of partially purified mAb derived from hybridomas secreting anti-Thy1.2 (30H-12), anti-TCR-γ/δ (UC7-13D5) or anti-TCR-α/β (H57-597) resulted in >95% depletion of the respective T cell subsets in spleens, lymph nodes and PEC of mice as determined by fluorescence-activated cell sorter analysis in a FACScan (Bekton Dickinson, Mountain View, CA). Accordingly, mice were treated i.v. with 0.5 mg of each mAb one day before immunization to deplete these T cell subsets.

Flow cytometric analysis

Flow cytometric analysis with a single laser FACScan (Beckton Dickinson, Mountain View, CA) was performed with the non-adherent PEC, spleen cells or lymph node cells from mice. Cells were stained with appropriate dilutions of the following mAbs for analysis of surface phenotype. Anti-CD3 (145-2C11) conjugated to phycoerythrin (PE) was purchased from PharMingen (San Diego, CA). Anti-Thy1.2 (30H-12), anti-TCR-γ/δ (UC7-13D5) or anti-TCR-α/β (H57-597) were labeled with fluorescein isothiocyanate (FITC). Stained cells were analyzed with gates set to exclude debris, clumps, and dead cells by light scatter gating.

RESULTS

As shown in Fig. 1, all mice which were immunized with *Toxoplasma* cell homogenate 7 days before infection with a lethal dose of Beverley strain bradyzoites ($1x10^4$) acquired resistance and survived. By contrast, vaccination with a sublethal dose of live Beverley strain bradyzoites ($1x10^2$) was required for acquisition of resistance to infection with the highly virulent RH strain. When BALB/c mice were immunized with *Toxoplasma* homogenate, expression of HSP65 was detectable in their peritoneal macrophages from 4 days after immunization, and reached a peak level of expression at 7 to 8 days after immunization (data not shown). Fig. 2 (lane 1) shows the expression of HSP65 in a peritoneal macrophage lysate of mice by western blot assay with a mAb specific for HSP65 7 days after immunization with *Toxoplasma* homogenate. As mentioned above, if mice were immunized with *Toxoplasma* homogenate acquire resistance to infection with a lethal dose of Beverley strain bradyzoites, whereas athymic nude mice and SCID mice did not acquire protective immunity with similar immunization (Fig. 3). Thus, whether peritoneal macrophages from nude and SCID mice injected with *Toxoplasma* homogenate expressed HSP65 was determined. Fig. 2 shows that HSP65 was not detected in peritoneal macrophages from nude mice and SCID mice immunized with a *Toxoplasma* homogenate 7 days previously (Fig. 2, lanes 2 and 3). Thus, T cells appeared to be essential for the expression of HSP65 in peritoneal macrophages from mice immunized with the *Toxoplasma* homogenate, and this expression correlated with protective ability against *T. gondii* infection.

To confirm that T cells are required for the expression of HSP65, we treated mice in vivo with 0.5 mg of purified 30H-12 (anti-Thy1.2) mAb one day before immunization with *Toxoplasma* homogenate. This pretreatment deeply inhibited the expression of HSP65 after immunization. To determine which subset of T cells with α/β or γ/δ T cell receptor was most contributory in the expression of HSP65, BALB/c mice were treated in vivo with 0.5 mg of either anti-TCR-γ/δ (UC7-13D5) or anti-TCR-α/β (H57-597) mAb one day before immunization. FACS analysis showed that percentage of γ/δ T cells was increased in PEC of BALB/c by immunization 5 days previously (8.5%) in comparison with that of naive BALB/c mice (3.8%). T cells with TCR-α/β or TCR-γ/δ were almost completely depleted in the non-adherent PEC, lymph node cells, and spleen cells of BALB/c mice by an intravenous administration of each corresponding antibody (data not shown). The level of HSP65 expressed in peritoneal macrophages was slightly low in immune mice treated with anti-TCR-α/β mAb compared with that in untreated immune mice, and much lower in mice treated with anti-TCR- γ/δ mAb. Furthermore, the expression of HSP65 was completely suppressed by pretreatment with a combination of anti-TCR-α/β mAb and anti-TCR- γ/δ mAb. Moreover, when mice depleted of γ/δ T cells by the administration of mAb, they died most frequently in the early stages. These data are summarized in Tables 1 and 2.

DISCUSSION

The induction of HSP65 closely correlates with the protection against *Toxoplasma* infection. That is, HSP65 is expressed in peritoneal macrophages from mice that have been infected with a low-virulence strain (Beverley strain) of *T. gondii*, as determined by electroblot assay using a mAb specific for microbial HSP65. This HSP is not expressed when infection occurs with a high-virulence RH strain of *Toxoplasma* parasites, but becomes expressed when mice acquire resistance against infection with the RH strain according to the prior vaccination with a sublethal dose of live Beverley strain bradyzoites. Thus, the expression of HSP65 correlates with protective potentials of host mice, regardless of differences in virulence or strain specificity of this protozoa.

In this paper, we showed that the mechanism of expression of HSP65 using mice immunized with *Toxoplasma* homogenate, immunization which induces HSP65 expression in host peritoneal macrophages and gives protective immunity to mice against infection with Beverley strain as reported previously (14). The data presented here demonstrate that γ/δ T cells are most crucial to induce the expression of this protein.

The role of γ/δ T cells in HSP65 expression on the surface of host macrophages is obscure. There are some evidences that a subset of γ/δ T cells recognize HSP65 (7, 9, 16). Some of these γ/δ T cells recognize host-derived HSPs that are cross-reactive with microbial HSPs. From these bases, γ/δ T cells in mice immunized with *Toxoplasma* homogenate might initially recognize a very small amount of parasite-derived HSP or cross-reactive antigen in the homogenate, although we could not detect HSP65 in the homogenate itself by Western blot analysis with monoclonal anti-HSP65 mAb (IA10). Indeed, Western blotting assay and immunogold ultracytochemistry assay using polyclonal anti-HSP65 rabbit serum showed the presence of HSP65 within the homogenate (data not shown).

Several features of the data concerning activation of γ/δ T cells are consistent with the possibility that γ/δ T cells produce cytokines such as IL-2, interferon γ or TNF following specific or non-specific antigen stimulation, resulting in local activation of other lymphoid and non-lymphoid cell types. Further evidence comes from the finding that IL-2 and interferon γ induce the expression of HSP65 in mouse macrophages and other cells (4, 5, 11, 23, 24). Taking these results together, one might suggest that the expression of HSP65 on the surface of peritoneal macrophages in mice immunized with *Toxoplasma* homogenate may be attributable to cytokines which are produced by γ/δ T cells.

The role of HSPs in infection and immunity is receiving much attention, and it has been postulated that HSP responses to stress during inflammation actually play a role in the host defense against certain infections (18). Mounting evidence suggests that γ/δ T cells recognizing HSP65 may represent a first line of defense against certain infections (3, 8) and that γ/δ T cells increase rapidly in peripheral blood of patients with acute *T. gondii* infection (2, 19). We have the evidences that γ/δ T cells increased in PEC of mice at the early stage of infection with a low virulent strain of *T. gondii*. Moreover, treatment of mice with mAb to γ/δ T cells in vivo before infection markedly reduced their survival. Although the exact role of γ/δ T cells for protection against *Toxoplasma* infection have not been defined, there is little doubt that γ/δ T cells which correlates with HSP65 may represent the protection against infection against this protozoa.

HSPs expressed within host cells may also participate in the elimination of pathogens, either by a nonimmunological self-nonself discrimination mechanisms, as hypothesized by Forsdyke (6), or as a consequence of processing and presentation of foreign antigens for effective immunity (22). In the course of infection with tubercle or leprosy bacilli, T cells with reactivity to the HSP65 have frequently been identified. In mice immunized with killed *Mycobacterium tuberculosis*, >10 % of T cells that exhibit reactivity to whole *M. tuberculosis* particles recognize the HSP65 (10). Moreover, a significant number of healthy individuals possess T cells specific for the mycobacterial HSP65 (13), and this protein may contribute to acquired resistance against a variety of intracellular pathogens (10). Because of their high degree of conservation in microbes, HSP65 (as well as other HSPs) is likely seen by the immune system quite frequently.

We showed here that γ/δ T cells participate in the induction of HSP65 on macrophages of hosts which have acquired resistance against toxoplasmosis. However, it still remains undetermined about the mechanisms of expression of HSP65 and the role of this protein in the protection against this infection. Furthermore, it is very

important to elucidate whether γ/δ T cells which induce the expression of HSP65 and those which accumulate recognizing HSP65 involve in the same functional subset, and whether the former and the latter subsets of γ/δ T cells recognize the same epitope of HSP65. Moreover, it is of interest to investigate the relationship between γ/δ T cells and α/β T cells in protective immunity, both of which have an ability to induce this protein.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid from the Ministry of Science, Education and Culture of Japan (Nos. 07670277, 07406014, 06556053). This work also received financial support from the United Nations Development Programme / World Bank / World Health Organization Special Program for Research and Training in Tropical Diseases.

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Table 1. Relationship between the expression of 65 kD-heat shock protein and the protective ability against *Toxoplasma* infection

Host		Infection with	Expression of HSP65	Resistance to infection
mouse	normal	−	−	
	normal	RH strain tachyzoites	−	−
	normal	Beverley strain bradyzoites	+	+
	normal	Beverley strain 1st tachyzoites	±	−
	normal	Beverley strain 6th tachyzoites	±	−
	homogenate-immune	−	+	
	homogenate-immune	Beverley strain bradyzoites	+	+
	homogenate-immune	RH strain tachyzoites	±	−
	bradyzoite-immune	−	+	
	bradyzoite-immune	RH strain tachyzoites	+	+
mouse	+/+	Beverley strain bradyzoites	+	+
	nu/nu	Beverley strain bradyzoites	−	−
	scid/scid	Beverley strain bradyzoites	−	−
rat	+/+	RH strain tachyzoites	+	+
	nu/nu	RH strain tachyzoites	−	−

Table 2. Requirement of T cells for expression of HSP65

Host	Expression of HSP65	Resistance to infection [a]
CB17 scid/scid	−	−
+ fetal thymus graft	+	+
+ fetal liver cell transfer	++	++
BALB/c nu/nu	−	−
BALB/c +/+	++	++
+ anti-TcR $\alpha\beta$	+++	+++ [b]
+ anti-TcR $\gamma\delta$	−	−

a. infection with bradyzoites of the Beverley strain of *T. gondii*
b. especially at the early phase of infection

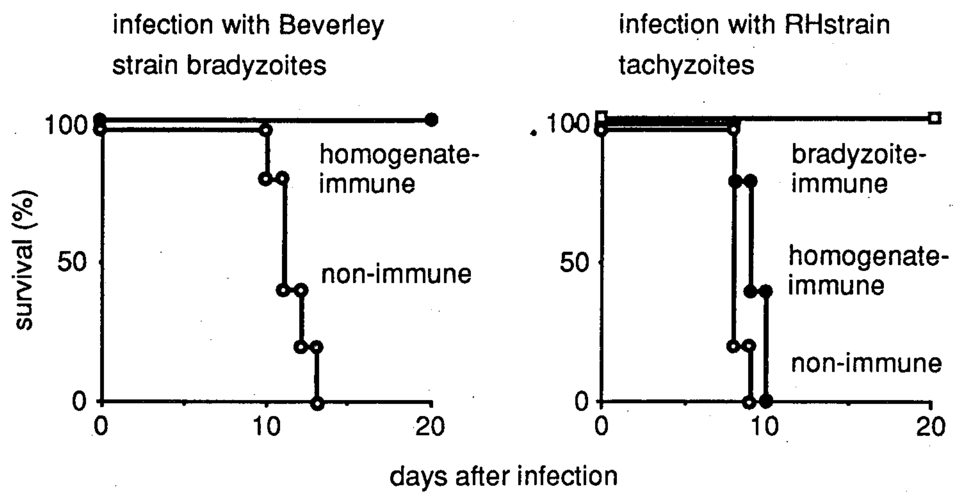

Fig. 1. Effect of immunization on resistance to infection with a low virulent Beverley strain or highly virulent RH strain of *T. gondii.*

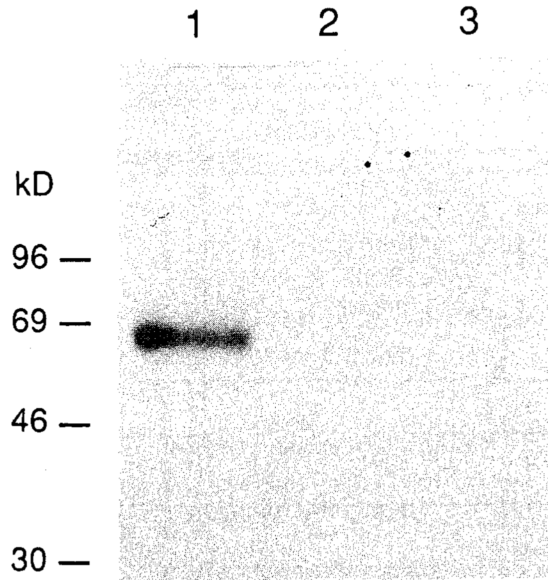

Fig. 2. Importance of T cells for expression of HSP65 in peritoneal macrophages from mice immunized with a homogenate of *Toxoplasma*. Peritoneal macrophage lysates were prepared from BALB/c +/+ mice (lane 1), BALB/c nu/nu mice (lane 2) and C.B-17 scid/scid mice (lane 3) 7 days after immunization with 0.2 mg of *Toxoplasma* homogenate. Each lane was loaded with 10 μg of protein.

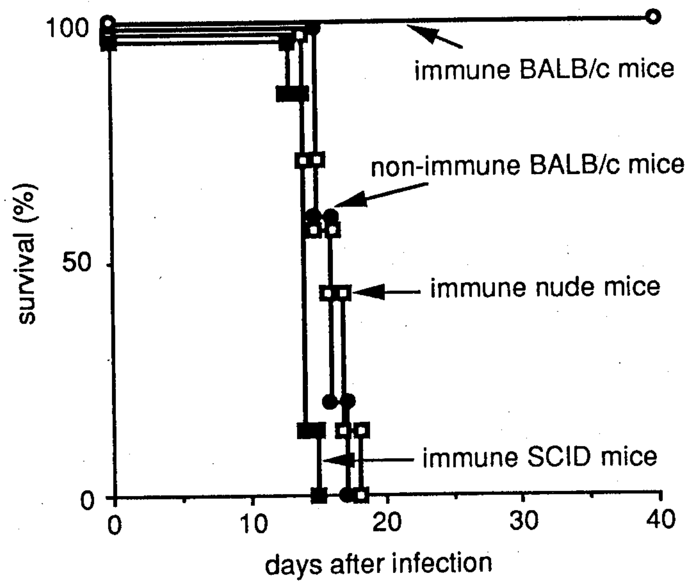

Fig. 3. Effect of immunization on resistance to infection with *Toxoplasma gondii* in mice.