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Role of a 65-kDa Heat Shock Protein in Protective Immunity against *Toxoplasma* Infection

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ABSTRACT

A 65-kDa heat shock protein (HSP65) is present in mouse peritoneal macrophages that have been infected with a low-virulence Beverley strain of *Toxoplasma gondii* or immunized with *Toxoplasma* homogenate, as determined by electroblot assay using a monoclonal antibody specific for microbial HSP65. This HSP65 is, however, not expressed in the infection with the high-virulence RH strain of *T. gondii*. Furthermore, mice previously vaccinated with a sublethal dose of live Beverley strain bradyzoites of *T. gondii*, acquired resistance against infection with the virulent RH strain and they likewise expressed HSP65. Rats are genetically resistant against the infection even with high dose of RH strain, and HSP65 is also expressed in their peritoneal macrophages. These results suggest the important role played by HSP65 in developing effective defense reactions that include effective immune response against infection with *T. gondii in vivo*. We clarified that γδ T cells as well as $CD4^+$ T cells play a crucial role in the expression of HSP65 in macrophages of animals which have acquired resistance against *Toxoplasma* infection. The expression mechanism(s) and the function of HSP65 are still unknown.

INTRODUCTION AND HISTORY

Exposure of cells to a variety of stressful conditions such as elevated temperature, stressful chemical intoxication, or infection leads to the transcription of a highly conserved set of genes and, subsequently, to the synthesis of a family of polypeptides called heat shock proteins (HSPs) (Lindquist 1986; Schlesinger 1986; Pelham 1988). Immunodominant antigens from a wide variety of bacteria and parasites have been identified by sequence homology as belonging to the family of HSPs (Young et al. 1988). Recently, HSPs have attracted 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 (van Eden et al. 1988; Res et al. 1988; Koga et al. 1989; O'Brien et al. 1989; Holoshitz et al. 1989; Haregewin et al. 1989). This HSP contains a significant sequence similarity and cross-reactivity with antigens from a variety of other microbes. Moreover, monoclonal antibodies (mAbs) against this common bacterial HSP have been used to identify a similar molecule of identical size and specificity that is produced by murine macrophages (Koga et al. 1989).

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 (Nagasawa 1984). 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* cell homogenate 7 d before infection with a lethal dose of Beverley strain bradyzoites ($1x10^4$), the mice 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 (Nagasawa et al. 1991). 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 host peritoneal exudate cells (PEC) infected with *T. gondii*. The degree of expression of this apparent HSP correlates with protection that occurred in exposed mice, regardless of differences in virulence or strain specificity of this protozoa or in species of host.

Relationship between the expression of HSP65 on host macrophages and protective immunity in mice infected with *T. gondii*.

The role of HSPs in infection and immunity is gaining much attention, and it has been postulated that HSP response to stress during inflammation actually plays a role in the host defense against certain infections (Polla and Kantengwa 1991). HSPs in parasite infection appear, on the one hand, to play important roles in adaptation of microorganisms. For example, they may play a role in differentiation of parasites and in development of infectivity (Smejkal et al. 1988; Buchmeier and Heffron 1990). Likewise, HSPs function as prominent antigenic proteins that can activate the host immune system (Haregewoin et al. 1989). Whether the host cells stressed by the invading parasites synthesize HSPs, whether and how host HSPs can affect parasite-host interactions, and whether HSPs generated by host cells or parasites participate in protective immunity and /or in the development of autoimmunities are questions that need to be further studied.

Shown in Fig. 1 is the 65-kDa HSP detected by an immuno-histochemical electron microscopy using gold particles. We have reported that HSP65, measured by electroblot assay with specific mAb (IA10), is expressed in peritoneal exudate cells (PEC) of mice infected with a low-virulence strain of *Toxoplasma* (Beverley) but is not expressed if infected with a high-virulence RH strain (Nagasawa et al. 1992). Also, the expression of HSP65 decreases in the PEC lysate from mice infected with the serially passaged tachyzoites of Beverley strain, which in turn, increases in pathogenicity to approximate the RH strain in terms of infectivity. These findings suggest that expression of virulence in these parasites correlates with interaction between the host cells and parasites. This correlation occurs despite major differences between the strains of *Toxoplasma*. It is, therefore, postulated that the low-virulence strain of *T. gondii* with the capacity to persist in host macrophages for prolonged periods may generate production of abundant quantities of host HSP that can then be expressed on the surface of macrophages and, thus, presented

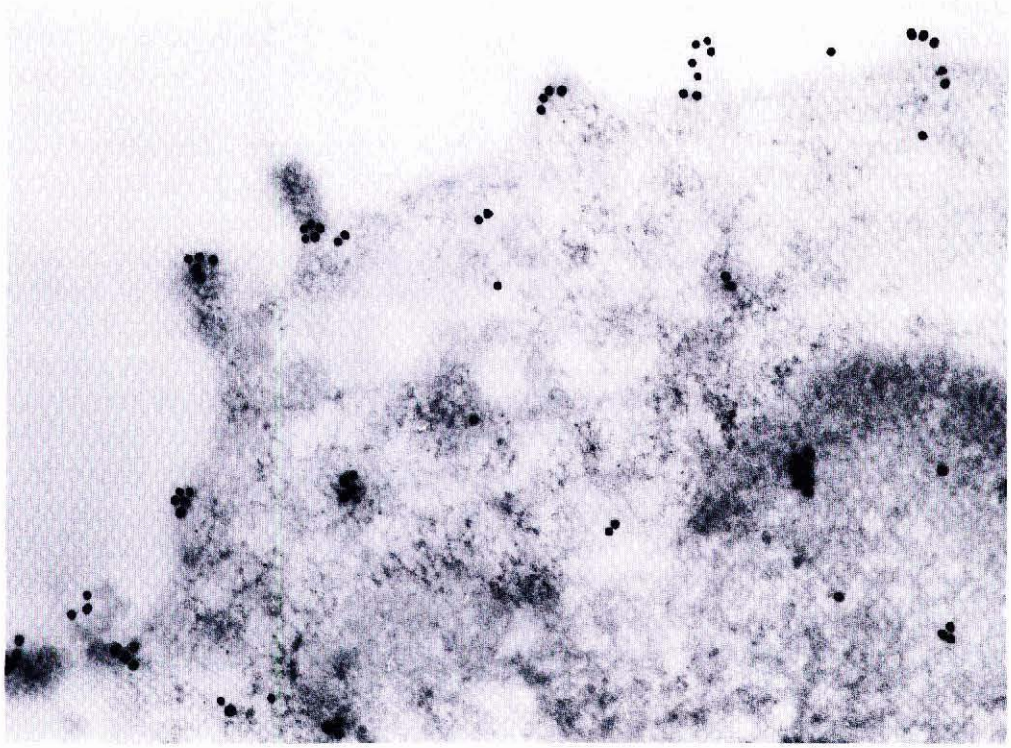

Fig. 1 Immunoelectron micrograph of a peritoneal macrophage from mouse infected with a low-virulence strain of *Toxoplasma* (Beverley strain). Specific gold label is associated with cell surface of macrophage. Magnification = x 54400.

effectively to T cells to induce immunity. By contrast, mice infected with a highly virulent strain of *T. gondii* (RH) seems unable to produce HSP on the macrophages they infect.

Mammalian cells may synthesize HSPs in response to infections and /or physiological stimulation. With regard to phagocytes, these cells protect themselves from noxious molecules that they produce, such as highly reactive oxygen metabolites. Phagocytosis and physiological activators of the oxidative burst induce HSP synthesis in macrophages (Polla and Kantengwa 1991). However, *Toxoplasma* parasites may survive and replicate within macrophages after phagocytosis. The survival of *Toxoplasma* within certain phagocytic cells has been studied by Wilson et al. (1980). These investigators showed that survival of tachyzoites within human monocyte-derived macrophages and normal mouse peritoneal macrophages can be attributed to failure of this parasite to stimulate an oxidative burst that normally occurs with phagocytosis of *Candida, Staphylococcus* spp, or latex particles. During early phase post-infection, infectivity or virulence of intracellular parasites should be defined by two factors. One factor will be determined by parasites themselves as cited above and another factor should be regulated by natural resistance genes expressed in host macrophages (Blackwell et al. 1991). At late stages, T cells will mainly control infections, activating or directly destroying infected macrophages.

In our previous experiments, when mice were immunized with *Toxoplasma* cell homogenate, HSP65 was detectable in PEC from these mice 7 d after immunization but was not detectable from either unimmunized controls or *Toxoplasma* cell homogenate themselves. Furthermore, mice that acquired resistance against a high-virulence RH strain after the resolution of infection with Beverley strain bradyzoites strongly expressed HSP65 in their PEC. We also reported that T cells play a major role in mediating protective immunity against low- and high-virulence strains of *T. gondii* from studies in which T cells were depleted *in vivo* with a mAb against Thy1.2 (Nagasawa et al. 1991). Further evidence comes from the finding that interferon γ released from sensitized T cells acts as a major mediator of the host defenses against *T. gondii* infection (Gazzinelli et al. 1991; Subauste and Remington 1991). Taking these results together, one might suggest that the expression of HSPs in mice immunized by *Toxoplasma* cell homogenate may be attributable to cytokines like interferon γ. Such cytokines may induce T cells to react to and attack macrophages infected with *Toxoplasma* parasites. Indeed, macrophages subjected to interferon γ activation are recognized by class-I-restricted $CD8^+$ T cells raised against 65-kDa HSP (Koga et al. 1989). Perhaps, bradyzoite-immune mice provide a constant source of HSP antigens for T cell stimulation and thus, cannot cause clinical disease. Once activated, macrophages would be expected to rapidly eliminate the highly virulent parasites before clinical disease develops.

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 (1985), or as a consequence of processing and presentation of foreign antigens for effective immunity (van Buskirk et al. 1989). In the course of characterizing T-cell stimulatory antigens of tubercle or leprosy bacilli, T cells with reactivity to the 65-kDa HSP 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 65-kDa HSP (Kaufmann 1989). Moreover, a significant number of healthy individuals possess T cells specific for the mycobacterial 65-kDa HSP (Munk et al. 1988). Thus, the cellular immune response to the 65-kDa HSP cannot be taken as an identification of immunity to tuberculosis or leprosy, and use of this antigen is inappropriate for diagnosis of these diseases. Still, HSP may contribute to acquired resistance against a variety of intracellular pathogens (Kaufmann 1989). Because of their high degree of conservation in microbes, 65-kDa HSP, as well as other HSPs is likely seen by the immune system quite frequently.

Correlation between the expression of HSP65 and acquisition of protective immunity to *T. gondii* exists across the barrier of species of host

To investigate whether there is a correlation between expression of HSP65 and protection against *T. gondii* is seen regardless of the host species, experiments were designed using rats (Hisaeda et al. in press). Rats completely resolved infection even with a high-virulent strain (RH) of *T. gondii* and HSP65 was strongly expressed on their macrophages, while only 50% of LEC rats with genetically defective $CD4^+$ T cells (Agui et al. 1990) could control the infection and HSP65 was only faintly expressed. Furthermore, all of athymic nude rats died of acute infection and this protein was scarcely expressed on their macrophages (Hisaeda et al. in press). These results indicate that expression of HSP65 closely correlates with protection against *Toxoplasma* infection not only in mice but in rats as well. Moreover, T cells, especially γδ and/or $CD4^+$ αβ T cells, appeared to be important in acquiring host the resistance to *T. gondii,* and play a crucial role in the expression of HSP65.

Role of γδ T cells in protection against *Toxoplasma* infection

Recently, a subset of γδ T cells has been shown to recognize HSP65 (O'Brien et al. 1989; Holoshitz et al. 1989; Haregewoin et al. 1989) and was found to increase rapidly in peripheral blood of patients with acute *T. gondii* infection (De Paoli et al. 1992; Scalise et al. 1992). Thus, it is not surprising that γδ T cells recognizing HSP65 and involved in protective immunity in some kinds of infection including toxoplasmosis. This T cell subset is thought possibly to represent a first line of defense against infection and is probably demonstrable in normal individuals. Furthermore, these HSP65-reactive γδ T

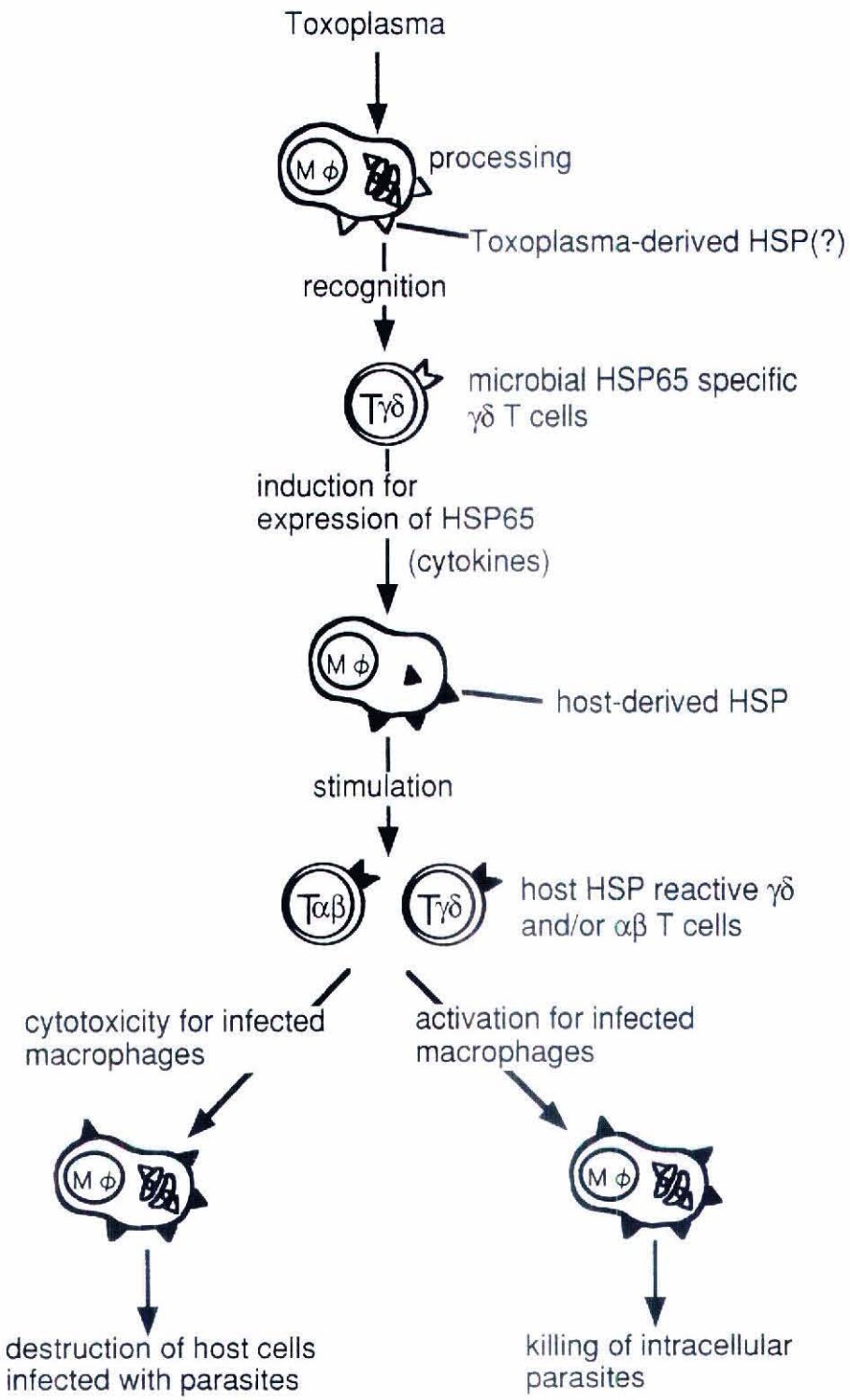

Fig. 2 Hypothetical scheme illustrating the functional potential of HSP-reactive T cells

cells should have been primed previously by contact with many different microbes or by exposure to HSP generated in host cells under various stressful conditions. The role of HSPs as a selective target for γδ T cells remains more speculative than the role of HSPs in other forms of immunologic reactions, particularly nonspecific resistance to parasites.

The expression mechanisms of HSP65 on host macrophages still remain obscure. We, however, demonstrated that T cells, especially γδ T cells, play a crucial role in expressing HSP65 on host macrophages (Nagasawa et al. in press).

Conclusion

Characterization of effector and regulatory functions of HSPs in other hosts and microbial systems should provide an insight into mechanisms of virulence and protective adaptations that control virulence. Thus, it seems likely that HSPs could assume a critical importance in numerous host-parasite relationships, including resistance of host to otherwise destructively virulent parasites. At any rate, it seems clear from a series of our experiments that expression of HSP65 on peritoneal macrophages correlates dramatically with capacity to inhibit destructive consequences of infection with both low-virulence and high-virulence strains of *T. gondii*. In Figure 2, we present hypothetical mechanisms of HSP65 expression on host macrophages by γδ T and/or αβ T cells in animals that have acquired resistance against infection. Although, we could not detect HSP65 in *Toxoplasma* themselves by western blotting with monoclonal anti-HSP65 (IA10) as mentioned above, the same assay with polyclonal anti-HSP65 antibody showed that a very small amount of this peptide exists within parasites. Accordingly, we hypothesize the mechanisms in the expression of host HSP65 and in the protection against *T. gondii* infection as follows. At the first stage after infection with a low-virulence *Toxoplasma*, circulating γδ T cells reactive to microbial HSP65 recognize *Toxoplasma*-derived HSP65 which is presented by host macrophages, and then they accumulate and are activated. At the second stage, they activate macrophages by releasing cytokine(s) like interferon γ and induce the expression of host-derived HSP65 on their cell surface. At the third stage, γδ T cells or αβ T cells reactive to host HSP65 further accumulate and are activated. Such HSP65-reacting T cells directly destroy host macrophages or activate macrophages to kill intracellular *T. gondii*.

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