Springer Semin Immunopathol (1991) 13: 25–36

Springer Seminars in Immunopathology

Heat-shock proteins and pathogenesis of bacterial infections

Stefan H. E. Kaufmann

Department of Immunology, University of Ulm, Albert-Einstein-Allee 11, W-7900 Ulm, Federal Republic of Germany

Introduction

Infectious disease occurs as an eventual, not mandatory result of the encounter of a microbial pathogen with its mammalian host. Certain bacteria, such as *Corynebacterium diphtheriae* or *Clostridium tetani,* cause disease by a few (or even a single) defined toxins. The pathogenicity of other bacterial infections is more sophisticated. Several extracellular bacteria, such as gram-positive and gram-negative cocci as well as various enterobacteriaceae produce a variety of virulence factors, including toxins, wich allow them to establish stable infection. Although such bacterial components are often directly responsible for disease, in many cases immune mechanisms are involved in pathogenicity. Thus, fever (a general phenomenon of acute systemic infections), septic shock (a fatal consequence of septicaemia by gram-negative bacteria), toxic shock syndrome caused by certain strains of *Staphylococcus aureus,* and local tissue destruction during various infections are ultimately mediated by cytokines such as interleukin 1 and tumor necrosis factor (TNF). In yet another group of bacteria, pathology primarily represents the outcome of an imbalanced host-pathogen relationship. This group of so-called intracellular bacteria includes *Mycobacterium tuberculosis* and *M. leprae, Chlamydia trachomatis, Listeria monocytogenes,* and others. These pathogens often persist in the host for long periods of time without causing clinical disease and it is only later, when the immune response fails to control infection, that clinical disease may develop which then frequently takes a chronic course.

The road from microbial entry to disease can be short or long and winding, and it can be divided into distinct stages including entry into the host, adherence to appropriate host cells, invasion of deeper tissue sites, colonization of an appropriate niche, evasion or avoidance of host-defense mechanisms, infection, and disease. During these stages, bacteria face a variety of insults to which they must rapidly respond and adopt themselves if they want to survive.

Many bacterial pathogens do not live exclusively in the host in which they cause clinical disease and often they shuttle between the infected host and other locations. This is illustrated by pathogens causing gastrointestinal diseases wich

reside in and are taken up via contaminated food or water. It would be uneconomic for such bacteria to express their complete phenotype under such varying conditions [10, 11]. Indeed, bacterial pathogens have chosen an alternative strategy and adopt their phenotype in response to environmental stimuli including starvation, attack by toxic molecules such as reactive oxygen metabolites (ROM), or changes in nutrients, pH, pO_2, or temperature [10, 11, 32, 48]. These assaults occur at various steps from entry into the host to clinical disease.

At least three major systems exist which respond to these stimuli and which are essential for bacterial survival in the host. These are virulence factors, defense against ROM and—the major topic of this treatise—heat-shock protein (hsp) [10, 25, 28, 32, 37, 48].

In the first part of this review the possible role of the three systems in pathogenicity of bacterial disease will be described and their possible relationship discussed. In the second part, we will shift from the predator to the host. During infection, the host also faces several insults. It may have to compete with the pathogen for essential nutrients or the tissue in which bacteria multiply may experience alterations in pO_2 of pH. Defense mechanisms of the host, in an attempt to eradicate the infectious agent, will produce toxic molecules not only harmful for the invader but also for its own tissues. These insults induce hsp synthesis [22, 25].

Sooner or later, the immune system will enter the stage and hsp will become major targets of immunity [25, 28]. Because hsp are highly conserved, the immune system is faced with an antigen which is not only dominant but which also shares a high degree of homology with its counterpart in the host [18, 25]. This extraordinary sequence homology may have consequences for the course of disease since it may deviate the immune response against its own components and further contribute to pathogenesis.

The predator

Regulation of virulence gene expression by environmental stress stimuli

Studies carried out during the last couple of years have revealed that many virulence factors such as those required for adhesion to and invasion of mucosal tissues or for survival inside macrophages are only expressed inside the host [10, 32]. As long as bacteria live in the environment they do not need these factors and it would be unnecessary to constitutively express them in an environment which requires very different survival strategies. Only after entry into the host do these virulence factors become essential for survival. To express their virulence factors only when required, bacteria possess specific sensors that allow them to respond to stimuli typical for the new situation [13]. Stimuli causing virulence gene expression include alterations in nitrogen or phosphate concentrations, as well as changes in pH, pO_2, osmolarity, or temperature [4, 10, 13, 31—34, 43]. Many of these stimuli are perceived through specialized two-component sensor systems which transduce the signal to the DNA. Each of the two components represents a bifunctional protein and both are interlinked with each other [13].

A transmembrane protein senses the environment through its sensor part and transmits the signal to the second protein through its cytoplasmic part. The second component which is located in the cytoplasm accepts the signal through its receiver domain and then activates DNA transcription through its regulator part. As a consequence, families of virulence genes can be expressed in a coordinated way.

Such sensors include the PhoR-PhoB system of *E. coli,* which senses limitations in extracellular phosphate; the PhoP-PhoQ system, which regulates *S. typhimurium* virulence in mice and survival in macrophages and which perceives limitations of carbon, nitrogen and phosphorus as well as acidification; the Bvg*C*-Bvg*A*-Bvg*C* system which responds to temperature, nicotinic acid and $MgSO_4$ and controls virulence of *Bordetella pertussis;* and ToxR of *Vibrio cholerae* which activates various virulence factors, including the cholera toxin and pilus expression, and is regulated by changes in temperature, pH, and osmolarity. The single protein ToxR is different in that it senses environmental signals through its extracytoplasmic part and directly interacts with DNA through its cytoplasmic part. It, therefore, does not require the transmitter and receiver domains involved in protein-protein signal transduction in the other systems.

Besides these well-characterized signal transduction systems, other examples are known where temperature changes, as well as other stress stimuli, influence virulence gene expression. These include the recently described regulatory gene prfA which positively regulates expression of listeriolysin, a virulence factor of *L. monocytogenes* which participates in evasion from the endosomal into the cytoplasmic compartment and virF which controls expression of the outer membrane porteins (Yop) related to virulence of *Yersinia* sp. [31, 32].

Microbial defense against ROM

Following colonization of deeper tissue sites, bacterial pathogens will encounter various host-defense mechanisms. Professional phagocytes will enter the site of bacterial replication and engulf bacteria. Subsequently, they will produce toxic effector molecules whose ROM are of both great importance and major interest for this treatise. ROM include H_2O_2, $^{\bullet}OH$, $^1O_2$, O_2^-, and OH^- [14, 20]. H_2O_2 and O_2^- themselves have a lower toxicity for bacteria, but represent major substrates for the more toxic products $^1O_2$, and $^{\bullet}OH$. Furthermore, H_2O_2 participates in the highly microbicidal oxidation of halides. Bacteria which want to survive in the host, therefore, need to defend themselves against ROM. One way would be to sense the onset of ROM production and then rapidly produce detoxifying enzymes [48]. This, indeed, seems to be the case. Major defense molecules include superoxide dismutase which detoxifies O_2^- and catalase which converts H_2O_2 into the harmless H_2O. Expression of these enzymes is controlled by regulators such as soxR or oxyR which respond to O_2^- or H_2O_2, respectively. Although in these cases it is not fully understood how bacteria sense the responsible stimuli and transduce the signals to the DNA, it appears that direct oxidation of sensor molecules induces a conformational change which then provides a control signal at the level of DNA transcription. Consistent with these observations, Fields et al. [8], in screening a battery of *S. typhimurium* transposon mutants which had

lost their virulence for mice as well as their capacity to survive inside macrophages, identified several mutants which were more sensitive to ROM, indicating a relation between resistance to oxidative stress and virulence.

Bacterial hsp

The third set of bacterial polypeptides induced under stress situations that occur during infection are the hsp themselves. Many of the stress stimuli causing hsp induction in the test tube mimick assaults that arise at different stages of infection [25, 37]. Thus, the microbe entering the host rapidly faces altered pO_2, pH, and temperature levels, and at later stages of infection it will be attacked by host-defense mechanisms, including ROM. These signals are known to induce hsp synthesis. Studies by Fields et al. [8] have provided direct evidence for a role of hsp in bacterial defense against the host. Among a battery of deletion mutants of *S.typhimurium* expressing reduced resistance to intracellular macrophage killing, many were found to be defective in *hsp* gene expression. In another study, this group characterized proteins of *S.typhimurium* which were synthesized at increased levels during macrophage infection [4]. Of 405 proteins identified, 34 proteins showed such an increase and 12 proteins were exclusively expressed in macrophages. Interestingly, half of the proteins were apparently not produced inside nonprofessional phagocytes. Most prominent among the proteins induced inside macrophages were the 60- and 70-kDa bacterial hsp60 and hsp70 homologs, groEL and DnaK. In analyzing hsp induction by oxidative stress in *S.typhimurium* Ames et al. [5, 36] found that pretreatment with H_2O_2 induces the synthesis of some 30 proteins including numerous hsp. Several, though not all of the proteins were also induced by heat. Consistent with these observations, it was recently found that the *htr*A gene encoding an hsp required for in vitro viability at high temperature renders *S.typhimurium* bacteria avirulent for mice [19].

The mechanismus underlying control of hsp synthesis are incompletely understood. It has been known for a long time that in prokaryotic cells σ32 acts as a positive transcription factor for hsp expression [37]. At normal temperature, σ32 has an extremely short half-life; after temperature elevation, however, it stabilizes, and accumulates at higher concentrations, allowing interaction with the promotor controlling *hsp* gene expression. Consequently, increased transcription of *hsp* genes can occur.

How does σ32 sense the temperature shift? Although this question cannot be answered equivocally at the moment, Craig and Gross [6] as well as others recently provided interesting, though speculative, thoughts on how hsp70 itself could serve as the sensor [6]. The major function of intracellular hsp70 is its capacity to interact with unfolded proteins, resulting in the formation of temporary complexes between hsp70 and intracellular proteins. Since the intracellular concentration of hsp70 is limiting, increased concentrations of unfolded proteins will deplete the pool of free and available hsp70. The concentration of free σ32 in turn is negatively regulated by hsp70 in several ways. Thus, reducing the amount of free hsp70 will indirectly increase the concentration of σ32 which is capable of positively interacting with the σ32 element. This finally leads to increased transcription of *hsp* genes.

Relationship between hsp, defense molecules against ROM, and virulence factor expression

Knowing that the expression of virulence factors, defense molecules against ROM and hsp is regulated by similar stress stimuli, one wonders whether these important phenomena share common activation pathways and are regulated coordinatedly. In many cases, no intimate relationship between the three systems has been found. Thus, virulence gene expression is often caused by a shift from 30° to 37° C, whereas hsp expression in many bacteria occurs after temperature increase from 37° to 42° C. Furthermore, hsp expression is a transient event, whereas virulence gene expression seems to be longlasting. In addition *S.typhimurium* mutants, deficient in the phoP gene, which are avirulent for macrophages still express high levels of hsp60 and hsp70 inside macrophages [4].

However, a certain degree of overlap seems to exist. Parsott and Mekalanos [43] described a coordinated, but reciprocal, effect of temperature increase from 22° to 37° C on *hsp* and virulence gene expression in *V.cholerae*. Temperature elevation to 37° C induced expression of the heat-shock gene *htp*G, but decreased cholera toxin expression. Although the biological relevance of this coordination is not understood at all, one might speculate that *V.cholerae*, after entering the gut, first takes care to protect itself by increasing its hsp synthesis and retards toxin production until adopted to the new environment.

Although treatment of bacteria with low doses of H_2O_2 or O_2^- causes both expression of hsp and of ROM detoxifying enzymes, the overlap between the two systems remains unclear. The studies of Morgan et al. [36] revealed that induction of some (though not all) proteins by heat shock depends on oxyR, which regulates expression of ROM detoxifying enzymes. Consistent with these findings, oxyR mutants are more resistant against killing by both, H_2O_2 and by heat shock, and constitutively overexpress certain hsp [5]. These observations strongly suggest a connection between bacterial defense mechanisms against ROM and hsp.

Delineation of the regulatory mechanisms which coordinate expression of hsp, virulence factors and defense against ROM appears to be an interesting novel avenue of research. For a bacterial pathogen such a coordination seems to make sense, since after entering the host it not only has to mobilize the genes required for colonization of the host but also those necessary for protection against aggressive defense mechanisms. In the latter case both specific enzymes such as catalase and superoxide dismutase as well as more nonspecific mechanisms such as hsp seem to contribute to optimum protection.

The fact that hsp are up-regulated under the stress bacteria face inside the host may also have consequences for rational vaccine development. In studies utilizing recombinant BCG as a viable carrier, expression of foreign vaccine antigens was put under the control of hsp promoters [1, 49]. This made it possible to produce abundant amounts of the desired antigens in vivo, resulting in strong and long-lasting humoral and cellular immune responses. This strategy has already been successfully employed for vaccination with HIV antigens [1, 49].

The prey

Hsp induction: an autoprotective mechanism for host cells?

During infection, not only the pathogen but also the host faces several insults which can lead to increased hsp synthesis. On the one hand such insults can be caused by the microbial organism that produces toxic molecules, competes for important nutrients and changes the pH and pO_2 levels. On the other hand, the host-defense system itself may harm infected host cells and tissue sites in which bacteria replicate. It is, therefore, conceivable that, during infection, host cells are forced to increase their hsp levels. Mononuclear phagocytes, which are potent producers of toxic effector molecules and at the same time serve as a preferred habitat of many intracellular bacteria, may require particularly sensitive hsp expression systems.

Polla [44] and Kantengwa et al. [22] have shown that heat shock renders the premonocytic cell line U937 more resistant to subsequent exposure to H_2O_2, indicating that macrophages protect themselves from their own toxic effector molecules by increasing hsp synthesis. Conversely, these investigators have shown that exogenous H_2O_2 induces hsp synthesis in human monocytes. It is also known that several physiological macrophage activators, such as TNF and 1,25-dihydroxyvitamin D3 in the human system, and interferon-γ (IFN-γ) in the murine system, induce hsp synthesis. Increased levels of hsp70 mRNA were also observed in human monocytes exposed to lipopolysaccharide or streptococci and this stimulation caused resistance to subsequent heat shock [9].

Another situation where hsp induction may be essential, would be the attack of infected host cells by immune effector mechanisms. Although the lysis of infected host cells by killer cells may well contribute to protection against many intracellular bacterial infections, this mechanism can also cause tissue destruction [24]. Cells which perform unique functions and are not easily, if at all, reconstitutable may need to protect themselves from exaggerated host attack. In several systems it was found that stimuli known to inrease hsp synthesis render target cells resistant to subsequent attack by natural killer cells, activated monocytes, specific cytolytic T lymphocytes, or TNF [12, 17, 45, 47, 50]. Thus, lysis by specific cytolytic T lymphocytes is counteracted by pretreatment of target cells with heat or chemical stress inducers. Heat treatment of murine and human cell lines renders them resistant to activated monocytes and to TNF but not to natural or lymphokine-activated killer cells. Also, IFN-γ treatment causes resistance to killer cell-mediated lysis in endothelial cells but not in sensitive tumor cell lines, suggesting that hsp-related resistance is cell type dependent. Consistent with this finding, Steinhoff et al. [47] observed that IFN-γ and heat shock cause resistance to killer cell-mediated lysis in human Schwann cells. Importantly, in this study it was observed that infection with *M. leprae* also renders the Schwann cells resistant to attack by killer cells. This autoprotective effect was only seen after infection with viable *M. leprae* organisms, while pulsing with dead *M. leprae* increased susceptibility to killing. These findings indicate that a naturally occurring infection protects host cells from attack by the immune system. Although a causal link with increased hsp synthesis does not exist, indirect evidence could be

provided. Western blot analyses of *M.leprae*-infected Schwann cells with hsp60-specific antibodies revealed a strong 60-kDa band in *M.leprae*-infected Schwann cells but not in uninfected controls [28]. Furthermore, when newly synthesized proteins were labelled with [^{35}S]methionine and subsequently immunoprecipitated with an hsp60-specific antibody, evidence for increased hsp60 synthesis in *M.leprae*-infected Schwann cells was demonstrable [28]. The mechanisms underlying resistance against killing remain unclear. Evidence has, however, been presented that viable *M.leprae* are capable of evading the endosomal compartment and entering the cytoplasm [35]. Bacterial proteins may then be sensed by hsp70 as being somehow foreign or incorrect and then cause increased *hsp* gene expression as described above [2]. These findings may be relevant to the in vivo situation for the reasons indicated below.

A characteristic feature of leprosy is its polar form [3]. Whereas in tuberculoid leprosy only few bacilli are found which are confined to distinct lesions primarily in the skin, in lepromatous leprosy multiple bacilli are located in diffuse lesions all over the body. In tuberculoid leprosy strong T cell responses towards antigens of *M.leprae* are observed which are thought to account for its more benign form. In lepromatous leprosy *M.leprae*-specific cellular immune responses are low to absent and this lack may directly relate to the more malignant character of the disease. Throughout the whole spectrum, Schwann cells serve as a major habitat for *M.leprae* and nerve damage represents a major pathogenic feature of leprosy [3]. It appears that *M.leprae* itself does not harm Schwann cells directly to a major extent. Rather, immune mechanisms seem to be responsible for destruction. Schwann cells are of unique importance for the functional integrity of the peripheral nervous system and they can hardly, if at all, be reconstituted. Increased hsp synthesis in response to *M.leprae* infection may, therefore, help to protect this highly susceptible cell type from being destroyed and in this way prevent, or at least, diminish immunopathogenesis.

Hsp60 — a pathogenic antigen?

Tuberculosis and leprosy. Several studies performed in the murine and the human system indicate that hsp60 is a dominant antigen of *M.tuberculosis, M.bovis,* and *M.leprae* [25]. Thus, it has been shown that human CD4 T cells from leprosy patients and tuberculosis patients frequently recognize this protein [7, 25]. Limiting dilution analysis in the murine system showed that approximately 20% of all T cells from *M.tuberculosis*-immune mice which respond to whole M.tuberculosis organisms are specific for hsp60 [27]. Conversely, immunization of mice with hsp60 in Ribi adjuvant induces large numbers of T cells which respond to whole *M.tuberculosis* organisms in vitro [27]. Even in healthy donors T cells specific for hsp60 have been frequently identified [21, 41]. Both, α/β and γ/δ T cells respond to hsp60 [15, 16, 21] and limiting dilution analyses with T cells from healthy donors revealed that the proportion of hsp-specific T lymphocytes among mycobacteria-reactive cells is extremely high [21]. Immunization with recombinant Aro$^-$ *S.typhimurium* expressing hsp60 induces a state of delayed-type hypersensitivity [28]. Attempts to vaccinate mice against tuberculosis using

these constructs, however, gave inconsistent results. Thus, it is possible that hsp60 is not, or is only marginally, involved in protective immunity to tuberculosis. Because hsp60 cognates of high structural homology exist in different bacteria, it is also possible that a baseline immune response to hsp60 already exists in healthy individuals due to previous subclinical infections with various microbes and that this level cannot be further increased by hsp60 vaccination.

Tuberculosis is a chronic infectious disease which primarily affects the lung [26]. In most cases, infection does not directly lead to clinical disease. Rather, bacteria are confined to small lesions by means of a cellular immune response where they persist with reduced metabolic activity. At later time points, however, particularly after weakening of the immune system, bacteria can be reactivated and then cause clinical disease. It has been estimated that approximately 30%—50% of the whole world population is infected with *M.tuberculosis* (and hence is at risk for harboring bacilli). Only a small percentage of this enormous number of people will ultimately suffer from active tuberculosis. It may be that hsp60-crossreactive T lymphocytes contribute to partial protection in the many individuals who carry *M.tuberculosis* without overt disease.

Hsp60 even contains regions shared by bacteria and host cells. For example, the mycobacterial hsp60 and its mammalian counterpart show approximately 60% sequence identity [18]. Such shared regions represent potential targets for an autoreactive immune reponse. Indeed, it has been shown that T cells exist which recognize synthetic peptides representing such shared regions [30, 42]. Munk et al. [41] identified T cells which recognize synthetic peptides representing the most conserved regions of hsp60 [42]. These T cells were class II restricted and probably expressed the CD4 phenotype. Similarly, Lamb et al. [29] identified CD4 T cell clones with specificity for peptides representing semi-conserved hsp60 epitopes. Because such T cells are already demonstrable in healthy individuals, we have to conclude that, under normal conditions, they are harmless. In these studies synthetic peptides were used, and it could be speculated that conserved peptides do not arise through the natural class II pathway of processing. Indeed, short-term T cell lines responding to peptides representing shared epitopes failed to recognize targets pulsed with intact mycobacterial hsp60 protein [41]. However, during infection, such T cells may become activated aberrantly and contribute to pathogenesis.

In another study, murine T cells were activated in vitro with tryptic peptides of mycobacterial hsp60 [29, 46]. Subsequently, these T cells were tested in cytolytic assays using autologous macrophages or Schwann cells as targets. As expected, macrophages pulsed with mycobacterial hsp60 peptides were lysed, whereas unpulsed macrophages remained virtually unaffected. Interestingly, macrophages which had been stressed by various stimuli were also recognized and lysed by these T cells. These stimuli include IFN-γ stimulation, heat shock, and infection with cytomegalovirus or mycobacteria. The T cells responsible for lysis were class I restricted and expressed the α/β T cell receptor and the CD8 molecule. Recent evidence suggests that CD8 T cells which recognize stressed macrophages can also be activated in vivo by immunization with intact hsp60 packed in ISCOM (S. Yamamoto and S.H.E. Kaufmann, unpublished). Comparable results were obtained using Schwann cells as targets [46].

These observations could be of relevance to our understanding of the mechanisms underlying nerve damage in leprosy. Although Schwann cell destruction represents the major pathogenic mechanism of tuberculoid leprosy, only a few organisms reside in this cell during this stage of disease. The question arises as to whether a sufficient density of *M. leprae* antigens can accumulate in Schwann cells under these conditions [3, 23]. Perhaps stressed Schwann cells express self-hsp60 peptides in the context of MHC class I molecules and are then recognized by T cells causing immunopathogenesis.

Trachoma

Other situations in which hsp60 may serve as a pathogenic antigen are during ocular and urogenital tract infections caused by *Chlamydia* sp. *C. trachomatis* is an obligate intracellular pathogen with a unique developmental cycle [40]. Bacteria are taken up by host cells as spore-like, metabolically inactive elementary bodies. Once inside the cell, the bacterium is transformed into an intracellular vegetative form. This so-called reticulate body replicates inside a vacuole and later differentiates into an elementary body which is subsequently released from the host cell. Acute genital infections with *C. trachomatis* cause urethritis and cervicitis, and primary infection of the eye leads to a self-limiting conjunctivitis which usually resolves without major consequences. Chronic genital infection may cause infertility and repeated ocular exposure leads to chronic inflammation resulting in blinding trachoma, the primary cause of preventable blindness in developing countries.

C. trachomatis is restricted to humans, while *C. psittaci* is a pathogen of lower mammals. Experimental infection of the guinea pig with *C. psittaci* has provided good evidence that pathogenesis of trachoma is due to a delayed-type hypersensitivity reaction against hsp60 [38, 39]. Morrison et al. [38, 39] found that challenge of *C. psittaci*-infected guinea pigs with a protein of approximately 60 kDa and comprising an epitope shared by *C. trachomatis* and *C. psittaci* causes trachoma. Later, this group demonstrated that the relevant antigen is an hsp60 cognate. Thus far it is not clear whether the disease-causing epitope is shared by bacterial and mammalian hsp60 homologs and, hence, whether pathogenesis represents a true autoimmune response or, alternatively, whether the immune response is directed against an epitope confined to the genus *Chlamydia*. However, it is most likely that hsp60 represent a pathogenic antigen of trachoma.

Concluding remarks

The present treatise attempted to illuminate different levels in the relation between hsp and pathogenicity of bacterial infection. On the side of the predator, hsp are linked with virulence and defense against ROM. On the side of the prey, hsp expression is influenced by inflammatory and immunological signals. During acquired resistance hsp provide a dual link between host and pathogen since, on the one hand, they are dominant antigens of the microbe and, on the other hand, may convert an antibacterial response into one against self. Thus, hsp seem to play a multifactorial, often double-sided role in infection which may be both to the benefit and to the detriment of the host.

Acknowledgements. Work from the author's laboratory cited here received financial support from: SFB 322, EC-India Science and Technology Cooperation Program, UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases, and the A.Krupp award for young professors. The secretarial help of R. Mahmoudi is gratefully acknowledged.

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