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Heat shock proteins in immune response to cancer: The Fourth Paradigm

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Abstract. The involvement of heat shock proteins in immune response is categorized into four distinct paradigms. In the First Paradigm, HSP derived from foreign organisms act as classical foreign antigens, and they elicit immune response to the non-conserved HSP epitopes. The Second Paradigm refers to instances where the host responds to self HSP to which there is no central or peripheral tolerance. The Third Paradigm involves molecular mimicry, where cross-reactivity between an HSP and another protein leads to an immune response to the latter under conditions which elicit an immune response to the former, such as infection with a bacterium whose immunodominant antigen is an HSP. The Fourth Paradigm refers to situations where an HSP-antigen complex elicits an effective response to the antigen and *not* to the HSP. Thus the HSP acts as a carrier for the antigenic peptide. The role of HSP in recognition by $\gamma\delta$ T cells may also fall into this paradigm. In this article, the Fourth Paradigm is considered as a crucial element in the development of vaccines against cancers and infectious diseases, and is analyzed through the prism of the observed association of hsp70 species with antigenic peptides.
Key words. Vaccination; T lymphocytes; infection; peptides; antibodies; $\gamma\delta$ T cells; antigen presentation; cancer.

Introduction

Heat shock proteins (HSP) continue to appear with intriguing regularity among antigens detected by immune response to infectious agents and cancers and autoantigens[56]. These observations have occasionally led to comparison of HSP with superantigens, endotoxins, adjuvants and other such non-specific stimulators of immune responses. This is a misleading simplification of a complex immunological phenomenon. In this article, I propose to formally discriminate among the various paradigms encountered in the roles of HSP in immune response. As this volume is dedicated exclusively to the most pedigreed and, hence, the most venerable HSP (with apologies to democracy), we look at the various paradigms through the prism of hsp70.

The roles of HSP in immune response can be described by four distinct paradigms (fig. 1). In the First Paradigm, the HSP are classical antigens and elicit antibody or cellular responses in a host, simply because they are foreign antigens. Although HSP are highly conserved, there are regions of species-specific differences among corresponding HSPs and they are seen by the immune system as foreign epitopes[6,15,17,23,26,52,53]. In a number of other studies, however, the precise HSP epitopes have not been structurally characterized and compared with the host homologues[4,5,29,36–38,54], making it difficult to determine whether the reactions fall under the First or the Second Paradigm (see below).

The Second Paradigm consists of instances where the immune response is directed to self HSP epitopes[16,18,23,25,33,52]. At first sight, immune response to HSP appears paradoxical, as HSP are the quintessential self molecules and one expects HSP-reactive T cells to be negatively selected during thymic development[27]. However, a closer scrutiny suggests a number of mechanisms which may prevent development of thymic tolerance to HSP. In the first instance, regardless of the cliché that HSP are ubiquitous, a number of HSP may be expressed in a relatively tissue-specific manner and only a sub-set of HSP, such as hsc70, grp78 and gp96/grp94 and a few others, may be expressed ubiquitously. One may reasonably expect thymic tolerance only to this small group of HSP, and indeed, there is little evidence of immune response to these housekeeping self HSP. Further, even among HSP expressed in the thymic epithelium, one can make a case that these would be among the more poorly presented molecules, because of their unusually long half-lives of up to a few days. As anitgen presentation is dependent on protein degradation (see ref. 11), it is conceivable that proteins with longer half lives may be presented poorly. Furtheremore, the HSP which are localized in specific intracellular organelles such as mitochondria may be subject to additional constraints on presentation, of which we are presently unaware.

The recent series of papers demonstrating that nearly all the immunogenic antigens of human melanomas are unaltered, self-differentiation antigens (see ref. 13), has expanded our thinking regarding the reach of negative selection during thymic development; it can be argued that HSP which are not accessible to presentation in the thymic epithelium, for any of the reasons discussed, are indeed differentiation antigens. Therefore, observations of immune response to unaltered, self HSP need not be as surprising as they may appear at first sight. Clearly, the Second Paradigm is relevant to questions of autoimmunity[2,12,55].

The Third Paradigm of the role of HSP in immune response refers to instances of molecular mimicry be-

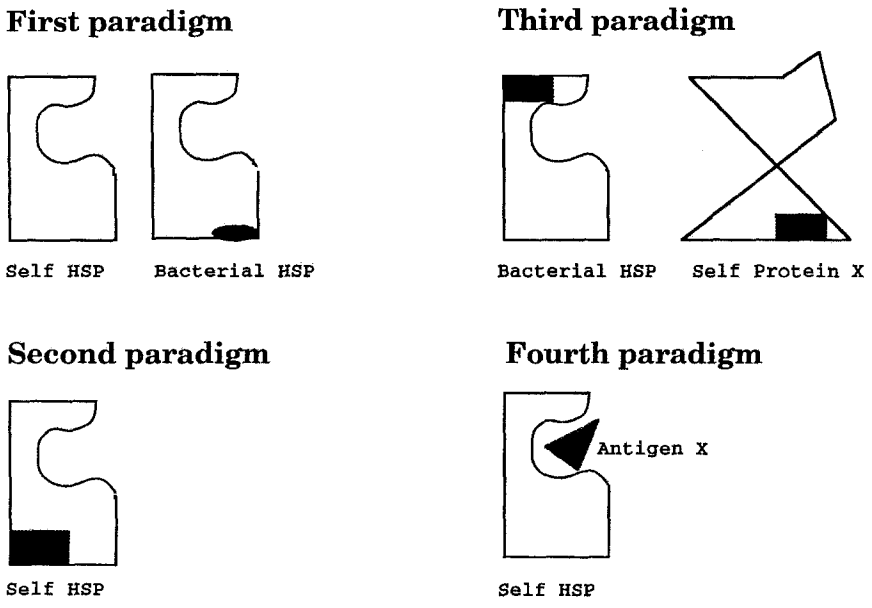

Figure. Schematic representation of the four paradigms in the role of HSP in immune response. Shaded areas represent antigenic epitopes. See text for details.

tween HSP and a non-HSP self protein[14,51]. The prototypical example of this paradigm is the observation of the presence of a cartilage-mimicking T cell epitope on a mycobacterial hsp65 (ref. 51). Thus, when the host is exposed to the mycobacterium and responds to the mycobacterial hsp65, it also responds to a self-cartilage epitope (incidentally shared between the two proteins), thus initiating an autoimmune reaction leading to arthritis. Clearly, molecular mimicry is not restricted to HSP, and therefore this phenomenon is not unique to HSP. Nonetheless, in the light of the relatively high degree of phylogenetic conservation between the HSP of mammals and infectious organisms, mimicry between HSP and other mammalian proteins may have profound implications for the immunology of infection and autoimmunity. However, the generality of this paradigm remains to be demonstrated.

In all of the above paradigms, HSP are the immunogens and the antigens, i.e. they elicit an immune response and are recognized by it. However, there exists a distinct category of observations, where complexes of HSP with other entities are highly immunogenic. The immune response elicited by such complexes is directed not against the HSP, but against the entities complexed to the HSP. We place all such observations under the Fourth Paradigm of the role of HSP in the immune response. As studies from our laboratory first suggested this paradigm[22,43], and have helped to define it, we discuss the Fourth Paradigm at some length.

The Fourth Paradigm

Definition and examples of the paradigm in $\alpha\beta$ T cell responses

The historical development of observations included under the Fourth Paradigm has been discussed elsewhere[40]. Suffice it to say, that apparently homogeneous HSP preparations of mammalian cells are observed to contain non-covalent complexes of HSP and peptides. This observation has been made with three major HSP, hsp70, hsp90 and gp96/grp94 isolated from a wide array of cells, including tumors, normal cells, virus-infected and virally transformed cells, normal tissues, etc.[3,31,42,44,48,49]. While the initial observations were made with the endoplasmic reticular HSP gp96, similar results were obtained with the cytosolic hsp70. Briefly, hsp70 preparations (purified without the use of ATP-agarose chromatography) from Meth A sarcoma were used to immunize mice and were found to elicit protection against Meth A, but not against the antigenically distinct CM4 or CMS5 sarcomas[48]. The ability of hsp70 molecules to bind peptides in vitro, and the lability of this interaction in the presence of ATP, had been demonstrated earlier[9]. Hence, it was logical to treat the immunogenic hsp70 preparations of the Meth A sarcoma with ATP and test whether the presumably peptide-depleted preparations were still antigenically active. The low molecular weight material eluted from hsp70 by treatment with ATP was applied to a C18 reverse phase column and eluted by an acetonitrile gradient. A

number of distinct peaks absorbing at 210 nm were observed. When the same hsp70 preparation was exposed to 350 mM NaCl and the resulting low molecular weight fraction analyzed, a distinctly different, and much simpler, pattern of peaks was observed. In order to demonstrate that the ATP-eluted peaks truly represent peptides, and that the peptides are derived from cellular proteins, Meth A cells were metabolically labelled for 1 h with ^{35}S methionine, and hsp70 preparations obtained. The low molecular weight fraction was derived by Centricon 10 centrifugation and resolved on a C18 reverse phase column. Fractions were collected and counted. A number of distinct ^{35}S-labeled peaks were obtained, indicating that the radioactivity indeed represented peptides derived from newly-synthesized cellular proteins. Preliminary mass analysis of these peptides by tandem mass spectroscopy indicated that they constitute a heterogeneous group ranging in size from 1000 to 5000 daltons, the bulk of the peptides being between 1600 and 3200 daltons (unpubl. results). Further, in tumor rejection assays, ATP treatment was indeed observed to result in abrogation of immunogenicity of hsp70 (ref. 48). The association of peptides with hsp70 (and indeed other HSP) has been observed in normal tissues as well and is not a tumor-specific phenomenon. However, gp96 and hsp70 preparations derived from normal tissues are unable to elicit immunity to Meth A sarcoma in a range of doses tested[48,49], suggesting the presence of tumor-specific antigenic peptides in HSP preparations from tumors, but not in the preparations from normal tissues.

Thus, vaccination of mice with such HSP-peptide complexes elicits potent T cell responses against the peptides associated with the HSP, but not against the HSP themselves. The specificity of response rules out HSP as antigens and implicates the associated peptides as the antigens. Although a small number of peptides have been characterized by Edman degradation and mass spectroscopy (and a broad effort in this direction is underway), the tumor-specific or virus-specific *antigenic* peptides have not been structurally indentified so far. However, the immunological specificity of the response clearly indicates the tumor or viral specificity of the response.

A number of observations suggest that association of HSP with peptides does indeed occur in vivo and is not an experimental artifact (unpubl.). Firstly, binding of HSP with peptides and dissociation of HSP-peptide complexes in vitro is not a trivial process, and requires an exacting set of conditions[20,48]. It does not happen merely as a result of the presence of HSP and peptides in the same environment. Secondly, addition of a mixture of labelled peptides (derived from partial digestion of metabolically labelled proteins) to the buffer used for cell lysis in the first step of purification of HSP does not lead to association of labelled peptides with HSP. Finally, association of peptides with hsp70 can be monitored in vivo in a kinetic manner by pulse-chase experiments[48]. These observations indicate that the association of peptides with HSP is not a post-cell lysis artifact but occurs in vivo. The peptides are derived from a range of cellular proteins during their proteolytic degradation during antigen processing and other processes. We have recently suggested[20] that the association of peptides with the various HSP serves 1) to shield peptides from their ultimate degradation to single amino acids by cytosolic proteases, 2) to minimize the chances of fortuitous and unproductive coupling with other proteins, 3) to achieve higher local peptide concentrations than might be possible by passive diffusion, and 4) to provide a mechanism for the process to be regulated or modulated. We have also proposed[46] that association of peptides with HSP is an essential mechanism for channeling the peptides on the antigen presentation pathway.

An extraordinary aspect of immunization with HSP-peptide complexes is their 'specific activity', i.e. immunogenicity per unit quantity of immunogen. Thus, vaccination of mice with 10 μg gp96-peptide preparation from a tumor renders the mice tumor-resistant. Conversion of this value into its molar equivalents is instructive: a 10 μg HSP-peptide preparation contains approximately 6×10^{13} molecules of gp96 (as calculated from Avogadro's number). Preliminary studies show that equimolar quantities of HSP and peptides are present in a given preparation. Thus $\sim 6 \times 10^{13}$ molecules of peptides will be present in this preparation. Of these, one may conservatively expect 0.01% of the peptides to be the specific antigenic peptides, in the presence of an overwhelming background of self antigens (which are ignored by the immune system). Thus, in vaccinating with a 10 μg gp96 preparation derived from a tumor, one is vaccinating with $\sim 10^9$ molecules of tumor-specific antigenic peptide. This number is at the same time too small, and quite large. Too small, in that, assuming an average peptide size of 1 kDa, 10^9 molecules amount to 10 pg of specific antigenic peptide, a quantity too small to be effective in vaccination by itself. On the other hand, 10^9 specific antigenic peptides, presented *efficiently* to the immune system, constitute a potent stimulus as discussed below.

We have shown recently that vaccination with gp96-peptide complexes requires the participation of macrophages or other phagocytic cells[50]. We have explained this requirement on the basis of the premise that macrophages bind to the HSP through a receptor, and once they are bound, the peptide cargo of the HSP is dissociated from the HSP in an intracellular compartment, and is re-presented by the macrophage in the context of its own MHC class I molecules[50]. If one assumes that only 1% of the 10^9 specific antigenic peptide injected is channeled productively, there are still 10^7

specific antigenic peptides available. If these petides are presented by 10^5 specific antigen presenting cells (such as macrophages, dendritic cells or other such cells), each of which presents 100 such peptides along with their MHC class I molecules (which is sufficient for T-cell stimulation), the immune system is faced with a potent stimulus. Thus, while the specific immunogenicity of HSP-peptide complexes is unusually high, the possible mechanisms for it are compatible with existing immunological dogmas.

Fourth Paradigm in antibody responses

Another example of the Fourth Paradigm is the set of observations made by G. Del Guidice and his colleagues, described in detail elsewhere in this volume[7]. Briefly, Del Guidice et al. observed[1,21] that vaccination of BCG-primed mice with *covalent* hsp65-peptide complexes or hsp65-oligosaccharide complexes elicited a potent, long term, T cell-dependent anti-peptide IgG antibody response, without the use of adjuvants. Hsp70 but not hsp18 molecules were effective in similar experiments. Further, in the case of hsp70-antigen complexes, effective responses could be elicited even in mice which had not been previously primed with BCG. It is instructive to compare and contrast our results with those of Del Guidice and colleagues. Clearly, both sets of results fall into the same paradigm. However, during vaccination of several thousand mice and rats over ten years with *non-covalent* HSP-peptide complexes, we have seldom encountered a significant IgG response, although weak IgM responses have been observed[42]. It appears that non-covalent versus covalent coupling of HSP with peptides can determine the outcome of the response in favour of a Th1 or Th2 type response. We are investigating the cellular basis of this dichotomy.

Fourth paradigm in recognition of targets by $\gamma\delta$ T cells and NK cells

It would be remiss of us not to discuss two other emerging examples of the Fourth Paradigm. Recognition of tumor or other target cells by T cells bearing the $\gamma\delta$ receptors is a continuing enigma in cellular immunology. While the ligands for these supposedly primitive receptors have not been identified so far, there is a tantalizing connection between them and the HSP (see ref. 28). It has been observed repeatedly and in a number of experimental systems that hsp60 and hsp60-derived peptides stimulate polyclonal responses in $\gamma\delta$ T cells of mice and men. Most recently, W. Born and his colleagues have analyzed, extensively and elegantly, the structural requirements for peptides which stimulate a sub-set of murine $\gamma\delta$ T cells[10]. However, these studies do not yet directly address the nature of the $\gamma\delta$ T cell receptor ligand. During our search for the structural basis of specific antigenicity of tumor-derived HSP, we had earlier proposed[43] that HSP directly present peptides to a T cell, in the same manner in which MHC molecules present peptides to $\alpha\beta$ T cells. Subsequently, we considered that model to be unlikely, as specific T cell recognition of tumor cells was blockable by anti-MHC class I antibodies but not by anti-gp96 antibodies. However, recent observations appear to have caught up with our early model. Tamura et al. have shown that cytotoxicity of a double negative T cell line against a tumor cell can be blocked by an anti-hsp70 antibody, but not by an anti-MHC class I or anti-NK antibody[47]. In a distinct vein, G. Multhoff and her colleagues at GSF Institute for Clinical Hematology, Munich, have observed[24] that human tumor cells but not normal cells express an hsp72 molecule on the cell surface and that the cell surface expression of hsp72 correlates with lysability of these tumor cells by NK-like effector cells. The above observations begin to suggest that recognition by at least a sub-set of $\gamma\delta$ T cells and some NK cells occurs through presentation of antigenic peptides by HSP. The observations of antigenic specificity of $\gamma\delta$ T cells and the requirement of antigen processing in the target cells for recognition by $\gamma\delta$ cells in some systems (as described above) are consistent with this possiblity. The lack of requirement for processing in another system suggests the existence of alternative pathways of recognition by the $\gamma\delta$ T cell receptor[39]. The recent demonstration of cell surface expression of a number of HSP is consistent with the possibility of HSP acting as presenting molecules to $\gamma\delta$ T cells and NK cells (refs. 8, 24, 43, 45, 47; A. Altmeyer et al., unpubl.). It is conceivable that, similar to the situation with $\alpha\beta$ T cells, such recognition may be antigen-specific in instances where a specific peptide is recognized in the context of HSP molecules; in other instances, comparable to the allogenic recognition by $\alpha\beta$ T cells, the nature of the peptides may be less germane. Pursuit of these ideas may necessitate a revision in our existing dogmas regarding lack of antigenic specificity of NK and $\gamma\delta$ T cells.

Hsp-peptide as carrier-hapten?

The Fourth Paradigm may appear suspiciously like the classical carrier-hapten model. However, the similarities between the two are skin-deep and a closer scrutiny reveals fundamental differences. Firstly, the hapten molecules are not immunogenic, but only antigenic, whereas the anitgenic peptides associated with HSP are both immunogenic and antigenic. One can effectively immunize with the peptides alone, albeit in much larger quantities, and elicit an antibody or T cell response. The association with HSP 'merely' increases the 'specific immunogenicity' of the peptides. Secondly, there is little evidence that the host responds in any manner to the HSP itself. This is in contrast to the hapten-carrier

system, where there is a clear T cell response to the hapten *and* the carrier, as demonstrated in the classical studies of Ovary and Benacerraf[30] and Raff[35]. Finally, the secret of the extraordinary efficiency of HSP-peptide complexes as immunogens lies in their being efficiently channeled to macrophages or other phagocytic cells. Thus, the HSP act as a delivery system for antigenic peptides, to a crucial component of the immune system. This phenomenon is mechanistically different from the hapten-carrier effect.

Implications of the Fourth Paradigm for antigen presentation by MHC class II molecules and for peptide trafficking

Implications of the Fourth Paradigm for antigen presentation by MHC class I molecules have been discussed at length elsewhere[40]. However, we have recently obtained more sequence information on the nature of anitgenic peptides associated with hsp70 and have found peptides derived from endogenously synthesized as well as exogenous antigens among the peptides characterized (unpubl.). We believe that this result is obtained on account of the microheterogeneity of hsp70 preparations which, although apparently homogeneous, represent a mixture of hsp70 species, which reside in distinct intracellular compartments. The various compartments must feed differentially and specifically into the two major pathways of antigen presentation, thus maintaining a wall between the two. However, biochemical fractionation breaks these barriers, resulting in an intermixing of the different hsp70 pools. Our observation of peptides derived from endogenously synthesized as well as exogenous antigens in the hsp70-associated peptides results from this intermixing. In order to resolve this question, we are isolating individual members of the hsp70 family through high resolution chromatography in order to characterize peptides associated with individual hsp70 species. These studies are expected to provide an insight into the intracellular peptide trafficking pathways and the role of HSP as the highways of such transport. The role of an hsp70 molecule in antigen presentation by MHC class II molecules is also discussed elsewhere in this volume[34]. It is my belief that as we learn more about the two pathways of antigen presentation, the role of HSP as chaperones of antigenic peptides will emerge increasingly as a common theme.

Implications of the Fourth Paradigm for vaccination against cancers

The observations that inbred mice can be immunized against their own tumors or against tumors of the same genetic background were convincingly made between 1943 and 1962 (see ref. 44). These experiments formed the foundation for the idea of immunogenicity of cancers and of the existence of tumor-specific antigens. These studies showed that mice vaccinated with inactivated cancer cells are immune to subsequent challenges with live cancer cells. The phenomenon was shown to be individually tumor-specific, in that mice were immune specifically to the tumors which were used to immunize them and not to other tumors. One of the major conceptual difficulties in cancer immunotherapy has been the possibility that human cancers, like cancers of experimental animals, are also antigenically distinct. The prospect of identification of immunogenic antigens of individual tumors from cancer patients is daunting to the extent of being impractical. In this context, the ability of tumor-derived HSP-peptide complexes to elicit tumor-specific immunity, points to a practical solution: HSP preparations derived from surgically resected tumors can be used to vaccinate patients, without requiring prior knowledge of the antigenic epitopes of a particular tumor. Thus, although T cell epitopes of mouse tumors are unknown, it is possible to vaccinate against them effectively, using HSP-peptide complexes derived from them. Further, as a number of HSP can now be purified rapidly, a means of preparing customized, patient-specific cancer vaccines is within reach. However, the idea that human tumors, like their murine counterparts, are antigenically distinct, is presently under siege (see ref. 32). Identification of CTL epitopes of a number of human melanomas as shared, unmutated differentiation antigens has kindled the hope that human tumor antigens are cross-reactive and that customized, patient-specific vaccines will not be necessary. In the happy event that sets of immunoprotective shared antigens are identified for tumors of different lineages, vaccination of patients with peptides or peptide-based constructs derived from the appropriate differentiation antigens would appear to be a method of choice. In spite of the obvious appeal of this approach, it raises a number of logistical questions. Clearly, vaccines will have to contain epitopes derived from a number of antigens. For any given antigen, vaccination with a given peptide will be effective only for patients with a given HLA allele. If different epitopes from a single molecule, such as tyrosinase, are recognized by different HLA alleles (see ref. 32), a cocktail of peptides will have to be used for vaccination of a general population. Even for a given patient, a cocktail may have to be used, as humans are outbred and possess several restriction elements. In the light of this, peptide-based vaccines will possibly have to be inordinately large chimaeric constructs. Isolation of HSP-peptide complexes from allogeneic human lines expressing the appropriate antigens is a more practical alternative. HSP are non-polymorphic and their association with peptides is proximal to the association of MHC class I with peptides in the antigen presentation pathway[46]. Thus, peptides associated with an HSP are not selected for the HLA specific-

ity and represent a collection of epitopes (or epitope-precursors) corresponding to all possible HLA specificities. Vaccination with such complexes derived from an allogeneic tumor will immunize patients regardless of their HLA haplotype. Thus, it was predicted that HSP-peptide complexes can be effective in cross-priming across the HLA barrier and that matching of a patient's HLA phenotype with that of a tumor vaccine is unnecessary[46]. It would appear therefore that regardless of whether human tumor antigens turn out to be individually specific, cross-reactive, or both, vaccination with HSP-peptide complexes provides a means of circumventing many of the hurdles associated with other peptide-based approaches.

The inherently multivalent nature of HSP-peptide complexes is an added advantage in vaccination. Individual human cancers are heterogeneous with respect to most parameters and antigenicity is unlikely to be an exception. Vaccination of patients with one or few peptides is expected to lead to selective elimination of tumors and outgrowth of immunological escape variants. Vaccination with HSP-peptide complexes makes immunological escape virtually impossible because the vaccines contain not one or a few but the entire antigenic repertoire of that tumor. Finally, vaccination with HSP-peptide complexes has been shown to elicit long-lasting T cell immunity[48]. Recent results show that antigen-specific T cells elicited by vaccination with HSP-peptide complexes are radiation-resistant and display characteristics of memory T cells (S. Janetzki, N. E. Blachere and P. K. Srivastava, unpubl. data). This attribute of HSP-peptide complexes fulfills a crucial requirement for a suitable vaccine. Altogether, studies of the Fourth Paradigm hold out some unique opportunities for development of a new generation of vaccines against cancers.

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