Heat shock protein-peptide interaction as the basis for a new generation of vaccines against cancers and infectious diseases

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Heat shock proteins (HSPs) are associated *in vivo* with the entire repertoire of peptides (antigenic and otherwise) generated within that cell. Immunization with such HSP-peptide complexes is unusually efficient in eliciting cellular immune responses against the antigenic peptides associated with the HSPs. This broad and general principle is the basis for a new generation of vaccines against cancers and infectious diseases and circumvents the need for identification of the T cell epitopes for any given cancer or infectious agent.

1. Background

The observations that inbred mice and rats can be immunized against their own tumours or against tumours of the same genetic background were convincingly made between 1943 and 1962 (Gross 1943; Foley 1953; Prehn and Main 1957; Klein *et al* 1960; Old *et al* 1962; for review, see Srivastava and Old 1988). They provided the underpinnings for the idea of immunogenicity of cancers and by deduction, of the existence of tumour-specific antigens. These studies showed that mice vaccinated with inactivated cancer cells were immune to subsequent challenges of live cancer cells. The phenomenon was shown to be individually tumour-specific, in that mice were resistant specifically to the tumours which were used to immunize them and not to other tumours (Basombrio 1970; Globerson and Feldman 1964), hence the name *individually distinct* tumour rejection antigens. The demonstration of immunogenicity of cancer cells led to a search for the cancer-derived molecules, which elicit resistance to tumour challenges. The general structure of these experiments was to fractionate cancer-derived proteins and test them individually for their ability to immunize mice against the cancers from which the fractions were prepared (see Old 1981; Srivastava and Old 1988; Boon 1992 for other approaches to identification of tumour-specific antigens). The proteins of 96 kDa, 90 kDa and 70 kDa size identified by the method (Srivastava and Das 1984; Srivastava *et al* 1986; Ullrich *et al* 1986; Udono and Srivastava 1993) turned out to be related to a class of proteins known as heat shock proteins (HSPs) or stress-induced proteins (see Lindquist and Craig 1988). Similar to the immunogenicity of intact tumour cells, it turned out that HSPs Gp96, Hsp90 and Hsp70 isolated from tumours were able to immunize and elicit protective immunity specifically against the tumours from which the HSPs were isolated. HSPs isolated from normal tissues were found to be unable to elicit immunity to any cancers tested.

Heat shock or stress-induced proteins are expressed in all living cells and are among the most abundant proteins in living cells. They are expressed at high levels constitutively; however, the expression of some members of the HSP family is induced by stress-causing conditions such as heat shock, glucose deprivation etc. HSPs are generally classified by their molecular weights; thus, Hsp110, Hsp90, Hsp70, Hsp60 and Hsp28 represent HSPs of 110 kDa, 90 kDa, 70 kDa, 60 kDa and 28 kDa sizes respectively. Each such HSP family may have one or more members which are highly homologous to each other; however, there is little homology among different families. Thus, Hsp70 and Hsp90 have no homology among them. Further, each specific HSP family is highly conserved phylogenetically. Thus, Hsp70

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molecules in bacteria and humans are very similar to each other.

The identification of HSPs as eliciting individual-tumour-specific tumour immunity is a curious paradox. HSPs are among the most highly conserved and abundant proteins in living systems; they are found across the phylogenetic ladder from archaebacteria to primates and differ but modestly among different species, let alone within an inbred strain. All in all, they are the most unlikely candidates for tumour-specific antigens.

The observations which have helped define and substantiate this paradox are described here, as are the recent results which have now begun to explain it. The specificity of immunogenicity of HSPs derived from tumour cells and the lack of tumour-immunogenicity of HSP preparations derived from normal tissues suggested that HSPs might be hot spots for mutations during malignant transformation such that the HSP genes will show variation between normal tissues and tumours and among tumours. However, sequencing of *gp96* cDNAs from tumours and normal tissues did not reveal any tumour-specific, individually distinct polymorphisms (Srivastava and Maki 1991). This directed our attention to the role of N-linked sugars of Gp96 (there are no O-linked sugars in Gp96). However, the following observations ruled out their role of Gp96 in tumour-specific immunogenicity: (i) tumour cells cultured in the presence of tunicamycin can be used to specifically immunize mice against the tumour. (ii) Gp96 derived from tumour cells grown in the presence of tunicamycin did not bind to Con A-Sepharose and successfully immunized mice specifically against the tumour (unpublished observations). In case of Hsp90 and Hsp70, the question of a role for sugars in the specific immunogenicity does not arise, since they are not glycosylated. It is conceivable that specificity might reside in other post-translational modifications; however, we consider this unlikely. Thus, there appears little reason to believe that specific immunogenicity of HSPs lies in the HSPs *per se*.

2. HSPs chaperone antigenic peptides

The lack of diversity in HSPs led us to consider molecules associated with them. As the Gp96 used to immunize mice is homogeneous by all criteria (i. single band on overloaded silver-stained gels; ii. single amino terminus during Edman degradation; iii. anti-peptide antibody to Gp96 depletes a preparation of its immunogenic activity), our attention was focussed on small moieties. A number of HSPs bind to a wide array of molecules, including peptides (Flynn *et al* 1989, 1991) and we proposed that Gp96 molecules may not be immunogenic *per se*, but may act as carriers of antigenic peptides (Srivastava and Maki 1991; Srivastava and Heike 1991). In view of the predominant localization of Gp96 in the endoplasmic reticulum (ER) (Booth and Koch 1989) we further suggested that Gp96 acts as peptide-acceptor for peptides transported to ER and may be accessory to loading of MHC class I molecules (Srivastava and Maki 1991; Srivastava *et al* 1994).

These ideas have now found a solid degree of experimental support from our and other laboratories (Li and Srivastava 1993; Udono and Srivatsava 1993; Suto and Srivastava 1995; Arnold *et al* 1995, 1997; Nieland *et al* 1996; Blachere *et al* 1997; Lammert *et al* 1997; Peng *et al* 1997; Spee and Neefjes 1997; Tamura *et al* 1997). It is now quite clear from these studies from several laboratories and in a variety of experimental systems that the HSPs are not antigenic *per se*, but act as carriers of antigenic peptides. The idea that HSPs chaperone not only tumour-specific peptides but indeed all peptides generated within a cell, including viral peptides generated during infection, has also been developed and successfully tested (Blachere *et al* 1993; Suto and Srivastava 1995; Nieland *et al* 1996; Heikema *et al* 1997). These results have led to the possibility of development of HSP-peptide complexes as the basis of a new generation of specific vaccines.

3. Unique advantages of HSP-peptide vaccines

HSP-peptide complexes offer unique and unprecedented advantages over other types of vaccines against cancers, infectious and transforming viruses, intracellular bacteria and protozoa:

(i) Knowledge of the antigenic epitopes which elicit immunity is a pre-requisite for all forms of vaccination. HSP-peptide based vaccination circumvents this necessity, as HSPs are naturally complexed with the repertoire of peptides generated in a cell. For this reason it is an ideal means for vaccination against infections for which the protective epitopes are yet undefined, or where a single epitope may not be sufficient for eliciting immunity, or where the infectious agent is so highly variable (in a population, season or individual-specific manner) that the prospect of identifying the immunogenic epitopes for each variant is simply impractical.

(ii) Even in case of infectious diseases where the relevant antigenic epitopes *have been* defined, HSP-based vaccination offers a unique advantage: HSP-peptide complexes can be readily stripped of their natural peptides and these "empty" HSPs can be reconstituted with known, synthetic peptide epitope(s) (Blachere *et al* 1997). The *in vitro* reconstituted non-covalent HSP-peptide complexes elicit potent *T cell response* to the complexed peptide. Further, if the peptide is conjugated covalently to the HSP, the HSP-peptide complexes elicit potent *antibody responses* to the complexed peptide (Lussow *et al* 1991; Barrios *et al* 1992).

(iii) One of the major conceptual difficulties in cancer immunotherapy has been the possibility that human cancers, like cancers of experimental animals, are antigenically distinct (Globerson and Feldman 1964; Basombrio 1970). The prospect of identification of immunogenic antigens of individual tumours from cancer patients is daunting to the extent of being impractical. The ability of HSPs to chaperone the entire repertoire of antigenic peptides of the cells from which they are derived circumvents this extraordinary hurdle. Thus, patients can be vaccinated with HSP-peptide complexes derived from their own tumours, or cell lines derived from them, without any need for identification of the antigenic epitopes of the patient's tumour. In light of the emerging evidence for existence of "shared" tumour antigens (Houghton 1994; Pardoll 1994), this particular point of advantage for HSP-based vaccines may at first sight, appear less profound than originally imagined; however, closer scrutiny suggests than even if human cancer antigens are cross-reactive rather than individually distinct, HSP-peptide complexes offer a uniquely effective method of vaccination.

(iv) As HSPs are non-polymorphic (i.e., show no allelic diversity, although there are several HSP families), they bind the entire spectrum of peptides regardless of the MHC haplotype of a cell. Thus, an HSP-peptide complex isolated from cells of a given haplotype may be used to vaccinate individuals of other haplotypes (Arnold *et al* 1995; Suto and Srivastava 1995; Nieland *et al* 1996).

(v) Vaccination with HSP-peptide complexes elicits CD8+ T cells without the use of live (attenuated or otherwise) agents and in spite of exogenous administration (Udono *et al* 1994; Blachere *et al* 1997; Tamura *et al* 1997).

(vi) HSP-peptide based vaccines are inherently multivalent because HSPs chaperone, not one or a few but the entire repertoire of epitopes generated in a cell.

(vii) As HSP-peptide complexes can be purified easily to apparent homogeneity, vaccination with such preparations circumvents the risks associated with vaccination with attenuated organisms or undefined biological extracts which contain transforming DNA and immunosuppressive factors such as TGF β.

4. Use of HSP-peptide complexes as cancer vaccines

One of the advantages of HSP-peptide complexes for vaccination against human cancers lies in the possibility that human cancers, like their murine counterparts, are antigenically diverse and individually distinct. In that scenario, it would be practically impossible to identify the antigenic epitopes of individual cancer patients and autologous tumour-derived HSP-peptide vaccines would present an attractive opportunity.

If however, human tumours are antigenically cross-reactive (as is argued by some on basis of the experience with human melanomas), anti-tumour vaccines could be designed simply on the basis of peptide epitopes of known cross-reactive melanoma antigens instead of HSP-peptide complexes isolated from individual tumours. It is the premise of this article that HSP-peptide complexes provide a uniquely effective method of vaccination, regardless of antigenic individuality or cross-reactivity of human tumour antigens. This premise is elaborated as follows.

Firstly, the antigenic cross-reactivity among human melanomas suggests that once a number of shared melanoma antigens are identified, patients can be immunized with synthetic peptides corresponding to the relevant epitopes and that the vaccinated patients will elicit a CD8+ CTL response. Vaccination with peptides under suitable conditions has indeed been shown to elicit CD8+ CTLs in a number of systems (Noguchi *et al* 1994; Schulz *et al* 1991). Such conditions usually include the use of incomplete Freund's adjuvant along with large quantities (~ 100 μg peptide for a 20 g mouse) of peptide. This is clearly incompatible with human use. Alternative approaches, such as addition of a lipophilic tail to the peptides have been employed successfully (Deres *et al* 1989) and could be potentially suited for human applications. In this context, vaccination with *in vitro* reconstituted complexes of human HSPs with the relevant antigenic peptides offers an economical and technologically simple method of vaccination. The ability of HSP-peptide complexes, *administered in saline and without any adjuvants*, to prime naive CD8+ CTLs *in vivo* has been reported recently (Blachere *et al* 1997). The use of human HSPs and synthetic peptides is also attractive from the point of view of circumventing hazards associated with vaccination of patients with chimeric molecular constructs of unknown toxicity.

Secondly, vaccination with a given peptide will be effective only for patients with a given HLA allele. If different epitopes from a single molecule are recognized by different HLA alleles (as appears to be the case in case of tyrosinase, Brichard *et al* 1993; Wolfel *et al* 1994), 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. A far more effective and simpler alternative will be to isolate HSP-peptide complexes from human cell lines transfected with the relevant gene under the control of a high expression promoter. The HSP-peptide complexes purified from such transfectants will consist of the entire repertoire of antigenic peptides derived from that particular protein. As HSP-peptide binding is proximal to HLA-peptide binding during antigen processing, there is no HLA restriction in the HSP-bound peptides. Peptides capable

of binding to all possible HLA alleles will be represented among the HSP-peptide complexes.

Thirdly, the methodology of identification of CTL epitopes of human cancers suggests that these epitopes may represent a significantly biased sample of the antigenic repertoire of human cancers. Generation of cell lines is an essential pre-requisite for isolation of CTLs and only a very small proportion of human cancers (less than 2% for breast cancers to about 30% for melanomas) lend themselves to it. Of the tumours from which cell lines are developed, only a small proportion permits generation of CTLs. Thus, the CTL epitopes being identified may represent an atypical and sparse sampling of the cancer antigenic repertoire. Immunogenicity of cancers represents, in all likelihood, the sum total of immunogenicity of a large number of immunogenic epitopes and effective anti-cancer vaccines should include this antigenic multiplicity. HSP-peptide complexes are such a multi-component, multivalent vaccine. If cancer antigens are shared and not individually indistinct, the use of HSP-peptide complexes isolated from tumours becomes even simpler, such that these complexes need not be isolated from cancers of individual patients. Instead, they could be purified from a mixture of human melanomas and be used to vaccinate allogeneic melanomas. The lack of HLA-restriction of HSP-bound peptides (discussed in the preceding section), is a key advantage in this regard. Vaccination with multi-component, multivalent vaccines rather than single or oligo-component vaccines is also necessary for protection against antigenic escape or pre-existing antigenic heterogeneity of human cancers.

These three major considerations indicate that regardless of the cross-reactive or individually distinct nature of human cancer antigens, HSP-peptide complexes offer unique and unprecedented advantages over other existing methods, in vaccination against human cancer.

5. HSP-peptide complexes as vaccines against intracellular infectious agents

Although the ideas discussed here were arrived at through pursuit of cancer immunity, it soon became clear that the peptide-chaperoning role hypothesized for the HSPs is a general one, i.e. valid for any cellular protein and not only for tumour antigens. In that scenario, HSP-peptide complexes isolated from virus-infected cells would be expected to elicit T cell response and immunity against the cognate virus. We and others have tested this idea in a number of viral systems including the influenza virus, Simian Virus 40, and vescicular stromatitis virus. Gp96 preparations were obtained from flu(PR8)-infected Balb/c fibroblasts and used to immunize Balb/c mice. T cells generated from these mice and stimulated *in vitro* with flu-infected cells showed significant antigen-specific, MHC class I restricted CTL activity (Blachere *et al* 1993; Heikema *et al* 1997). In other studies, mice were immunized with Gp96 derived from SV40 transformed PSC3H cells, or with non-SV40-transformed C3H fibroblasts. MLTCs generated from these mice and stimulated with PSC3H cells were tested on PSC3H and non-SV40 transformed fibroblasts. Class I restricted cytotoxicity against PSC3H was seen in MLTCs generated from mice immunized with PSC3H Gp96 but not the fibroblast-derived Gp96 (N E Blachere and P K Srivastava, unpublished). Similarly, mice immunized with Gp96 purified from VSV-infected cells showed antigen-specific, MHC I-restricted CTL response to VSV (Suto and Srivastava 1995). As further proof of these ideas, we have recently demonstrated that one can complex HSPs to synthetic peptides *in vitro* and use such complexes to elicit antigen-specific T cell responses. These studies indicate that the ability of HSPs to chaperone a broad array of peptides can be used effectively not only against cancers but against infectious agents as well. These should include intracellular agents such as viruses, rickettsiae, intracellular bacteria such as mycobacteria, and certain protozoan parasites.

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