[Frontiers in Bioscience 7, d43-52, January 1, 2002]

IMMUNOTHERAPY OF CANCER USING HEAT SHOCK PROTEINS

Masoud H. Manjili [1], Xiang-Yang Wang [1], Juneui Park [1], John G. Facciponte [2] , Elizabeth A. Repasky [2] and John R. Subjeck [1]

[1] Department of Molecular and Cellular Biophysics, Roswell Park Cancer Institute, Buffalo, New York 14263, [2] Department of Immunology, Roswell Park Cancer Institute, Buffalo, New York 14263

TABLE OF CONTENTS

1. ABSTRACT

Tumor derived heat shock protein (hsp)-peptide complexes (particularly hsp70 and grp94/gp96) have been demonstrated to serve as effective vaccines, producing anti-tumor immune responses in animals and in man. This approach utilizes the peptide binding properties of stress proteins which are responsible for their functions as molecular chaperones in numerous cellular processes. The present review briefly introduces the reader to the basic stress protein families, i.e. heat shock and glucose regulated proteins, their regulation, compartmentalization and family members. It then introduces the reader to aspects of hsps/grp function and interactions with the host's immune system. An overview of the conventional uses of hsp/grp vaccines as autologous vaccines derived from cancers is presented. We then discuss other stress protein related vaccination approaches. This includes the use of recombinant antigens, both proteins and peptides, naturally complexed to hsp/grps; hsp/grp DNA vaccines, hsp/grp fusion proteins and cell based hsp/grp vaccines. The advantages and disadvantages of each vaccination approach are discussed. Lastly, means of further enhancing the already potent activity of stress protein vaccines are presented, specifically the use of hyperthermia or CTLA-4 blockade as adjuvants.

2. INTRODUCTION

Ever since cancer immunotherapy has emerged, many strategies have been devised to target and destroy tumors by eliciting effective anti-tumor immunity. Cytokine therapy, active specific immunotherapy (vaccination), and adaptive immunotherapy (using tumor specific T cells or monoclonal antibodies) have been developed. A variety of antigenic systems including irradiated tumor cells, tumor antigens, peptide immunization (both protein and genetic vaccination), and anti-idiotype antibodies have been utilized. Different approaches have been explored to stimulate humoral and/or cellular immune responses, and several adjuvants have been tested. With all of these endeavors, immunotherapy of cancer has not been effective for curing human cancers. Under optimal conditions it might only increase the survival time and hopefully improve the quality of life of cancer patients. Cancer immunotherapy requires new approaches as well as finding ways to improve currently used approaches in the hope to cure cancer.

While heat shock proteins (hsps) have been intensively studied at the mammalian level for more than two decades, a new and physiologically significant role for these highly conserved gene products has only become evident in the last several years. Relatively recent studies have demonstrated that stress proteins (often collectively referred to as hsps in this review) play a central and important role in the host's immune response. This is based in part on studies from the early to mid 1990s demonstrating that hsps could function as powerful vaccines. That these vaccines are applicable to the treatment of cancer has opened up a new perspective in cancer immunotherapy. This vaccine strategy has been promising in pre-clinical studies to immunize animals against cancer. Human clinical trials using tumor-derived hsp vaccinations are now underway. This review summarizes some of the basic background on stress proteins and focuses on more recently developed and novel strategies to hsp-based vaccination, highlighting the advantages and disadvantages of each. Several other excellent reviews of this field which emphasize the development, analysis and use of turmor-derived hsp vaccines are also available to the reader (1-6).

3. STRESS PROTEIN FAMILIES AND THEIR IMMUNOGICAL PROPERTIES

3.1. Chaperoning properties

It has been known for the last quarter of a century that heat shock selectively induce the expression of a few proteins called hsps (7-9). While being induced by heat and other stress conditions, it has also been long recognized that hsps are also abundant proteins in the absence of stress and have important roles in regulating various cell functions. Known to act as ‘molecular chaperones’, hsps have been implicated in the folding and translocation of newly synthesized proteins, the assembly and disassembly of protein complexes, the refolding of misfolded proteins and, in special cases, the control of activity of native proteins (10-12). Based on expression levels, the major hsps of mammalian cells are the hsp25, hsp70, hsp90, and hsp110, although other well known hsps are also recognized (e.g. hsp40 and hsp60). These reside in the nucleus, cytosol and, in certain cases the mitochondria. They are strongly induced by heat, ethanol, amino acid analogues, oxidative reagents, recovery from anoxia, heavy metals, and inflammation.

While the above described hsps reside primarily in the cytosol (and nucleus), a second and distinct family of stress proteins resides in the endoplasmic reticulim (ER). These proteins are called glucose regulated proteins (or grps) and differ from hsps in their cellular compartmentalization and regulation. They are referred to as grps based on their induction by glucose starvation. In addition to this, grps are also induced by reducing agents, anoxia, heavy metals, amino acid analogues, some reagents which interfere with calcium homeostasis, and inhibitors of glycosylation. All of these conditions interfere in one way or another with the function of the ER. Importantly, grps are basically hsps with regard to function and sequence homology. Interestingly, the redox balance across the ER membrane (cytosol being reductive and ER oxidative) appears to play a key role in a reciprocal regulation of hsps and grps. For example, grps are induced by anoxia (reductive stress) whereas subsequent reoxygenation (oxidative stress) depresses grp synthesis to control levels while concomitantly inducing hsps (13). The redox states of the cytosol and ER are primary determinants in protein folding in these compartments. The oxidative state in the ER allows the formation of disulfide bond, which are disallowed in the cytosol. Reductive or oxidative stress is believed to interfere respectively with folding in either the cytosol or ER. The grp families are grp78 or BiP (a hsp70 homolog), grp94 (a hsp90 homolog), grp170 (a hsp110 homolog). Grps assist in the folding of secretory proteins and membrane bound proteins such as MHC class I and II (14) and immunoglobulins (15).

The chaperoning mechanism of hsps (and grps) has been most effectively studied by examining the E.coli DnaK protein (which is homologous to mammalian hsp70). It is believed that the chaperoning cycles of DnaK and protein substrate provide the substrate with the chance to fold on its own by preventing incorrect intra- or inter-molecular interaction. The rate of binding and release is regulated so that substrate is bound to a chaperone long enough to prevent aggregation, but not too long to inhibit its proper folding. Quantitative measurements of the kinetics of the chaperoning cycle are not well established (16, 17).

3.2. Peptide binding properties

Hsps and grps are thought to indiscriminately bind a broad range of substrates and yet they perform exact functions in specific cellular pathways. The substrate-binding specificity and interacting co-factor specificity are main determinants of their functional specificities. Several groups have tried to determine whether hsp binding motifs are specific or promiscuous. While the hsps show indiscriminate binding behavior, each hsp also shows some unique specificity. Peptides that bind DnaK, BiP, and mammalian hsp70 were isolated and shown to have different amino acid sequence motifs (18-21). These chaperones also demonstrated different affinities for a single peptide, suggesting that they may have different substrate specificity (19, 22).

3.3. Hsps interact with professional antigen presenting cells (APCs)

While hsps have essential intracellular functions as molecular chaperones, binding and chaperoning both proteins and peptides, they have also been shown to have important extracellular functions which are related to the host's immune response. It has been suggested that antigen presenting cells (APCs) which play a central role in antigen processing, have cell surface receptors specific for hsps (23). For example, Castellino *et al.* (24) demonstrated that hsp70 binds to surface receptors on macrophages prior to internalization. In addition, Binder *et al.* (25) demonstrated that surface binding of hsp70, hsp90 and gp96 is saturable, the hallmark of receptor specificity. Studies by Asea *et al.* (26) also demonstrated that hsp70 binds to the surface of human monocytes, with CD14 being the likely candidate

for the hsp70 receptor. It has been reported that gp96 binds to a macrophage cell surface receptor which has been identified as the α-2 macroglobulin/LDL receptor related protein, α-2MR/LRP or CD91 (25). Additionally, hsp60 may be a ligand for the Toll-like receptor-4 (TLR4) complex on macrophages (26). These observations are consistent with the "danger signal theory" which states that APCs have evolved receptors that detect danger signals (hsps in this case) released during neoplasia, tissue injury, and infection (27). It has been further reported that gp96, hsp90, hsp70 and calreticulin (an ER chaperone) all utilize the same CD91 molecule as a receptor for internalization by APCs (dendritic cells and macrophages). How this is achieved remains a mystery since these proteins fall into three distinct gene families with no common overlapping sequence homology (28). CD91 is a receptor for alpha 2-macroglobulin (alpha 2-M), and it has recently been shown that alpha 2-M like hsps, can function as a T cell adjuvant (29). Thus, CD91 is not only involved in channeling exogenous antigens into the endogenous pathway of antigen presentaion, but also appears to possess a broad specificity to interact with ligands. While the receptors for hsps and how they function requires further elucidation, the intracellular pathway for antigen presentation also remains poorly understood. It has been suggested that the processing of hsp-associated peptides require proteasomes and TAP (30). However, the sequence of the hsp-associated peptide might determine the proteasome and TAP dependency (26).

Beyond the roles that hsps play in antigen presentation, it has been shown that hsps can induce APC maturation. Gp96 can induce an increased level of co-stimulatory molecules such as CD86 (B7-2) and up-regulation of MHC class II molecules on DCs (30-32). Hsp72 was also shown to up-regulate surface expression of MHC class I in B16 melanoma cells (33). Using a mycobacterial hsp65 fusion protein, Cho *et al*. (34) demonstrated that it can directly stimulate DCs to up-regulate surface expression of MHC class I molecules. Moreover, the activated dendritic cells are effective in eliciting CD4 T cell-independent production of CTL. We have also observed that hsp110 and grp170 can up-regulate surface expression of both MHC class I and class II on DCs (unpublished data). Thus, hsps may activate CD8 T cells even in the absence of CD4 T cells by providing the immune system with both signal I (up-regulation of MHC molecules) and signal II (up-regulation of co-stimulatory molecules).

It has also been demonstrated that hsp70 and gp96 can induce APCs to secrete proinflammatory cytokines (26, 31). Preliminary studies in our laboratory have also shown that hsp110 and grp170 can induce secretion of proinflammatory cytokines, IL-6, IL-12, and TNF-α (unpublished data). These cytokines, IL-12 in particular, are important in antigen specific immune responses.

Lastly, several studies have also demonstrated that hsps/grps can act as vaccines when purified from diseased tissues (e.g. cancers). It has been shown that hsps purified from tumors can effectively elicit antigen specific cytotoxic T cell (CTL) responses to the associated peptides which were being chaperoned in the cancer cell by the hsp (described below). These properties of hsps/grps to interact with APCs, is expected to contribute to their ability to function as powerful vaccines. A discussion of this follows.

4. IMMUNOGENICITY OF HSPs

4.1. Tumor-derived hsp vaccines

The concept of hsps as autologous cancer vaccines derives from the identification of hsp70, hsp90 and grp94/gp96 as tumor-specific rejection antigens of chemically induced mouse tumors by Srivastava and colleagues years ago (35-37). It was observed that vaccination with tumor-derived hsps elicited a strong immunity against the syngeneic tumor, but not against antigenically distinct tumors. Although hsp genes from tumors and normal tissues reveal no differences in the predicted amino acid sequences, hsps derived from normal tissues do not induce tumor immunity (38, 39). The immunogenicity of these tumor-derived hsps was found to be due to the individually distinct array of antigenic peptides associated with hsps. This is consistent with the well-recognized capacity of hsps to bind polypeptide chains in response to physiological stress (40).

Immunity against the tumor obtained by vaccination of tumor-derived hsps (hsp70 and grp94) was first shown to occur in preventative immunization settings. It has also been shown that vaccination with these stress proteins can result in the therapeutic benefit for pre-established tumors and metastasis (3, 4). Other stress proteins, specifically calreticulin, hsp110 and grp170 were also found to be able to serve as cancer vaccines (42, 43). So far, tumor immunity elicited by hsps has been demonstrated against a chemically induced tumor, a UV-induced tumor, and spontaneous tumors of different histologic origins such as fibrosarcomas, lung carcinomas, melanomas, colon cancers and prostate cancer (3, 4, 39, 42-44).

The use of tumor-derived hsps as vaccines takes advantage of the peptide binding properties of stress proteins (as briefly outlined above) and purification of an hsp or grp from a tumor is believed to co-purify a specific peptide "fingerprint" of the cancer cell of origin. As a result of these peptides, many of which are expected to be antigenic, the hsp preparation can be used as a multivalent vaccine. Hsp-peptide complexes derived from tumors circumvents the need to identify a large number of CTL epitopes from individual cancers that are normally required to create vaccines. This advantage extends the use of hsp-based immunotherapy to cancers where specific tumor antigens have not yet been characterized. Secondly, this approach reduces the possibility of tumor cells escaping from immunotherapy due to single antigen loss, since a diverse antigenic repertoire would be available in the vaccine. Interestingly, hsps do not elicit an immune response to themselves. These properties make hsp vaccines effective, less toxic, and safe for human use. Success in pre-clinical animal studies has led to phase I clinical trials using tumor-derived grp94/gp96 preparations

as autologous tumor vaccines against renal cancer, melanoma (M.D. Anderson Cancer Center, Houston, TX), pancreatic cancer (Memorial Sloan-Kettering Cancer Center, NY), and gastric cancer (University of Mainz, Germany) (45). The results from these preliminary clinical trials show promising data (30).

Although the hsp/grp vaccines contain the antigenic peptides, a significant number of self-peptides would also be expected to be chaperoned. This raises the possibility of eliciting auto-immunity. However, no autoimmune reactions or severe side effects of the vaccine have so far been observed in any of the immunized patients (46). Hsps/grps purified from tumor may carry a "fingerprint" of peptides derived from tumor cell proteins, however, only a small percentage of the associated peptides would actually be true tumor antigens. While potentially powerful, the use of this approach clinically may be limited by the quantity of hsp/grp, which can be purified from a patient's surgical tumor specimen. Beyond this conventional approach, other novel antigen-specific vaccine strategies are being developed.

4.2. Hsp-peptide based vaccines

It has been shown that hsp-peptide complexes can be generated *in vitro* and could elicit specific CTL responses without any additional adjuvant. This occurred despite the fact that the quantity of antigenic peptides associated with an immunogenic dose of hsp-peptide complexes was found to be extremely small (~1-2 nanograms) (47-49). Exogenous peptides can compete with the endogenous peptides to anneal with heat-dissociated gp96 as well as hsp110 (unpublished) non-covalently. Such *in vitro* reconstituted vaccines have advantages such as relative ease of construction and production and chemical stability. Moreover, the ability to specifically select peptides (dominant, subdominant and T-helper) allows the design of highly tailored hsp vaccines.

In addition to non-covalent complexes of peptides with mammalian hsps, covalent or non-covalent complexes of mycobacterial hsp65 or hsp70 with peptides can also be used to elicit potent and specific responses to the associated peptides (50, 51). However, peptide-based vaccines also have potential disadvantages. Peptides may also induce T-cell tolerance. Additionally, due to HLA restrictions, peptide antigens must be carefully chosen to match the HLA phenotype of the patient. Another important concern which is often not appreciated is that different hsps appear to have different peptide binding affinities or preferred motifs. Thus, one cannot select an appropriate peptide based on HLA phenotype and assume it will bind to the hsp/grp used (often grp94/gp96). Indeed, the preferred mofit is not known for some hsps/grps (e.g. grp94), further complicating this issue. Future studies will eventually define these motifs for each major hsp/grp. Lastly, considering antigen escape as one of the major pitfalls for this approach, multiple peptides from different tumor antigens can be selected and mixed to generate polyvalent vaccine in order to generate a more vigorous and diverse immune response.

4.3. Hsp-protein based vaccines

The peptide binding properties of hsps has been studied based on their abilities to bind to and stabilize proteins. As discussed earlier, the hsps and grps have been shown to be involved in numerous cellular processes involving binding to full length proteins, hypothetically via the binding to preferred peptide motifs in an exposed length of peptide chain. It is this basic function and not the binding to peptides or immune functions that has driven studies of peptide binding properties. However, with all of interest in hsps and grps as peptide carrying vaccines, the immunotherapy element of the field has missed an important potential application. This new approach uses recombinant hsps non-covalently bound to a well-known, full length, tumor antigen *in vitro* as a vaccine. This employs the natural chaperoning property of the hsp to form a natural hsp-protein antigen complex in vitro, usually by using heat shock as the means for coupling.

This approach provides a highly concentrated vaccine preparation targeting a specific protein antigen. More importantly, the adjuvant activity of mammalian hsps would help to elicit powerful immune responses against the hsp-associated antigen without any additional adjuvant. Since whole proteins generally contain a large reservoir of potential antigenic epitopes available to CD4/CD8 T cells, hsp-protein vaccine may circumvent HLA restriction and provide a universal vaccine relevant to any immune background. Hsp-protein vaccines can also provide several T-cell epitopes for a single allele as well as several T-cell epitopes presented by different MHC class I and II alleles. Thus, such a full-length protein preparation would be "partially polyvalent" as well as being targeted against a specific tumor antigen. Moreover, such a vaccine would not be specific to an individual, as are tumor-derived hsp vaccines (32), but could be applied to any patient with a tumor expressing that tumor antigen. For example, in the case of her-2/neu antigen, a hsp110-her-2 complex vaccine could be used for the treatment of numerous patients with breast cancer as well as prostate, colon, lung and ovarian cancers that express her-2/neu. Lastly, this approach simplifies the vaccine production process since surgical specimens are not required. Preparation of such vaccines would be much less labor intensive than that of tumor-derived hsp and would be available in unlimited quantity. Currently, the vaccine efficiency of hsp110-her-2/neu (specifically the 84 kDa intracellular domain of her2/neu) complex has been investigated in our laboratory. We have observed that this complex is highly potent in eliciting antigen specific cellular and humoral immunity (manuscript submitted).

4.4. Hsp-based DNA vaccines

Another approach to hsp based vaccination is the use hsp-based DNA (52, 53). This approach uses hsps in the form of chimeric DNA through linkage of known antigen genes to hsp, which potentially increases the potency of DNA vaccines by a yet to be identified mechanism. It is suggested that the chimeric hsp-antigen DNA is taken up by numerous cells using a "gene gun" method of delivery. This would include dendritic cells (i.e. Langerhans cells) present in the lower, epidermal layers of

the skin, bypassing the need for receptor-mediated or other forms of endocytosis. One major advantage of DNA vaccines is the ability to endogenously generate a CTL response against the antigen, since it is difficult to induce a CTL response using protein-based vaccines (not inclusive of the hsp vaccines discussed here which can induce strong CTL responses). A second advantage of DNA vaccines is their staibility and ease of preparation. It has been reported that vaccines containing human papilloma virus type 16 E7 and hsp70 fusion genes resulted in preventive and therapeutic effects against an established tumor via CD8-dependent pathways (52). Although the hsp fusion gene vaccine holds promise, safety issues such as the risk of integration of DNA into the host genome and the development of autoimmune disease due to induction of response against transfected cells need to be resolved.

4.5. Hsp fusion protein vaccines

Another approach which combines natural hsp-protein complexes and hsp-DNA strategies (discussed in the preceding sections) utilizes chimeric DNA complexes which are then used to generate recombinant proteins in vitro for use as vaccines. It has been demonstrated that a recombinant protein consisting of the HIV-1 p24 antigen fused to the amino-terminus of mycobacterial hsp70 elicited both humoral and cellular immune responses in mice (54). In a separate study, immunization with hsp65/E7 fusion proteins protect mice against challenge with a E7-expressing murine tumor cell line (TC-1 cells), a model of cervical carcinoma (34). These tumor-free animals are also protected against re-challenge with TC-1 cells. In addition, therapeutic immunization with hsp65/E7 fusion proteins induces regression of palpable tumors, confers protection against tumor re-challenge, and is associated with long-term survival (34). The means by which these fusion proteins act to stimulate CTL responses are unknown, but may be a result of strong hsp-specific CD4+ helper cell responses that enhance what might otherwise be a minimal response to the soluble proteins (49, 54-56). It may also be related to the chaperone function of hsps that delivers the fusion protein to intracellular compartments of antigen-presenting cells for processing into short peptides and loading onto MHC classI (57, 58). The latter assumes that the fusion does not interfere with the natural in vivo hsp function. It has recently been demonstrated that hsp70 fusion proteins can elicit a CTL response in the absence of CD4+ T lymphocytes. Additionally, this function resides in a 200-amino acid segment of hsp70, which does not include the peptide binding domain (59). This indicates that hsp70 within a fusion protein acts via maturation of DCs regardless of the peptide binding property.

4.6. Cell-based hsp vaccines

Recently, hsp-peptide complexes were demonstrated to be internalized by antigen presenting cells through receptor mediated endocytosis (24, 57). Adoptive transfer of ex vivo-expanded DCs pulsed with autologous hsps or recombinant hsp vaccines may be used as a cell vaccine strategy for cancer treatment. Hsp vaccines can deliver a tumor antigen directly to functional APCs. This would be beneficial to the patient with impaired immunocompetence, since impaired APC activity could be partially responsible for defective immune responses observed in tumor-bearing hosts (60).

Another cellular vaccine is tumor cells transfected with an hsp gene. It has been shown that a murine macrophage tumor cell line transfected with mycobacterial hsp65 gene abrogated its tumorigenicity in rodent models (61, 62). Furthermore, stable transfection of autologous hsp70 or hsp110 in B16 melanoma and colon-26 tumor cell lines significantly enhanced the immunogenicity of the tumor. Vaccination with these irradiated tumor cells elicited tumor-specific immunity (63). These findings suggest that certain hsps are associated with tumor immunogenicity and that the induction/overexpression of these hsps might help to break tolerance to tumor antigens that otherwise remain immunologically hidden in the progressively growing tumor. Although the mechanisms underlying these observations still remain unclear, gene manipulation of certain hsps may provide a novel approach to boost the immune response and irradiated hsp-overexpressing cells as whole tumor cell vaccines may be used for immunotherapy. A significant limitation in the use of gene modified autologous tumor cells is the need to culture tumor cells *in vitro* for gene transfers and whole cell vaccine could also introduce transforming DNA or potentially immunosuppressive factors.

In addition to the above discussions which have focused on cancer, immunization with hsp-peptide complexes derived from virus-transformed or infected cells was also found to elicit viral immunity (64, 65) and can cross-prime an antiviral response in mice of another haplotype (66, 67). Therefore, while the emphasis has been on cancer, hsp based vaccines could also be valuable in the treatment of infectious diseases.

5. IMPROVEMENT OF HSP-BASED VACCINATION

The approaches to the use of hsps in cancer immunotherapy, described in the preceding sections, are still in various stages of development. Only one has is being actively examined in clinical trials, i.e. tumor derived hsp preparations (3, 36, 39, 41, 46). The examination of some of the other approaches is anticipated, if not already underway at the time of writing. Hsp-based immunotherapy (individual tumor-specific and tumor-associated antigen-specific) may be applicable in a broad range of patients and may also provide benefit as an adjuvant to current standard cancer therapies.

Like other approaches in cancer immunotherapy, hsp vaccines, are not effective in all patients and might only increase the survival time and improve the quality of life. As is the case with cancer immunothearpy in general, hsp-based vaccines may require improvement to generate more potent therapeutic cancer vaccines. Since the immune system consists of several different and interacting elements, a combinational therapy utilizing other approaches to immunomodulation together with hsp-based vaccination may generate a more powerful vaccination approach.

5.1. Hsp-based immunotherapy combined with hyperthermia

Fever-like hyperthermia has been shown to have important stimulatory effects on several immunological endpoints. We have shown that mild, fever-like hyperthermic conditions combined with tumor-derived hsp immunization significantly reduces tumor volumes and enhances the vaccine efficiency on a mole-to-mole basis of the two heat inducible members of this group, hsp110 and hsc70, but not grp170 (42). Okamoto *et al* (68) also demonstrated that tumor-derived cellular lysate enhanced tumor-specific CTL responses when combined with hyperthermia (43 degree for 60 min) and reported that a fraction enriched in hsp70 appeared to be involved. The enhanced equivalent efficiency of hsp vaccines after fever-like hyperthermia could be a consequence of enhanced proteosome activity (i.e., production of new peptides), enhanced peptide binding by the hsps, or other factors like conformational changes. While the mechanism by which mild heating stimulates the activity of hsp110 and hsc70 is unknown, it seems logical that it would be accompanied by an altered spectrum or fraction of hsp, which actually bind peptide.

Whereas it is well known that a prior heat shock can protect cells against inflammatory stress both *in vitro* and in vivo (69, 70), it was shown (71) that induction of a subsequent heat stress in cells 'primed' by inflammation can precipitate cell death by apoptosis. Additionally, hyperthermia may be used as an adjuvant to stimulate other immunological elements that may enhance vaccine potency of hsps. It has been shown that fever-range hyperthermia results in several changes in murine lymphocytes indicative of altered activation levels. These include alteration in the organization of the spectrin-based cytoskeleton and formation of uropods, activation and organization of several protein kinase C isoforms, enhancement of the L-selectin dependent adhesion of lymphocytes to high endothelial venules, and induction of hsp (72-74). There is also a dramatic lymphopenia seen in the blood during and immediately after fever-range whole body hyperthermia treatment in mice (75). Furthermore, hyperthermia has been shown to increase leukocyte infiltration into the tumor site (76). These findings suggest extravasation of lymphocytes from the blood into peripheral tissues directed by hyperthermia. Such leukocyte homing has been suggested to be due in part to the hyperthermia induced alteration in selectin- and integrin-based adhesion (73, 77-79).

Cancer gene therapy is another important application of the hsp promoter and hyperthermia when used in combination. One advantage of gene therapy over traditional therapy is the potential to target the expression of therapeutic genes in desired cells or tissues. The hsp promoters can dramaticaly activate gene expression of desirable cytokines following a heat shock. Intratumoral expression of IL-12 under the control of a heat inducible promoter (e.g. hsp70) in combination with hyperthermia has been examined (80). Further, when HIV tat gene is added downstream of a heat shock promoter, IL-2 is induced more than 3 fold after a heat shock of 42 ^{o}C (81).

5.2. Hsp-based vaccine combined with CTLA-4 blockade

Hsps are emerging as key danger signals providing the immune system with both signal I (up-regulation of MHC class I and II molecules) and signal II (up-regulation of co-stimulatory molecules). However, engagement of co-stimulatory molecules with cytolytic T-lymphocyte associated molecule-4 (CTLA-4), a homologue of CD28 that is mainly expressed on activated T cells, may result in T cell anergy thereby preventing the hsp vaccine from being effective enough in immunized individuals.

The CTLA-4 molecule has 20-fold higher affinity than CD28 to bind B7 co-stimulatory molecules (82). Interaction of CTLA-4 with B7-1 and B7-2 co-stimulatory molecules has been shown *in vitro* to prevent T cell proliferation by inhibiting IL-2 production and IL-2R expression and arresting cell cycle progression (83-86). Interestingly, CTLA-4 blockade *in vivo* with anti-CTLA-4 mAb has been shown to have significant anti-tumor therapeutic effects (87-92). Both $CD8^{+}$ and $CD4^{+}$ T cells appear to be involved (91). These observations support a hypothesis that tumor-specific T cells undergo apoptosis through CTLA-4 engagement rendering them unable to kill tumor cells. Blocking CTLA-4 molecules with monoclonal antibodies (mAbs) can prevent this interaction and facilitate CD28/B7 interaction for T cell effector function. This CTLA-4 blockade has been demonstrated to potentiate antitumor immune response against a number of experimental cancers, including murine colorectal carcinoma (51Blim10), fibrosarcoma (Sa1N) (87), mammary carcinoma (SM1), the transgenic adenocarcinoma of mouse prostate (TRAMP)-derived primary prostate cancer cell line, TRAMP-C1 (88, 92) and residual metastatic prostate cancer (89). Up-regulation of co-stimulatory molecules by hsps when combined with CTLA-4 blockade may facilitate B7/CD28 interactions preventing T cell anergy and leading to effective T cell function.

6. PROSPECTIVE

Heat shock or stress proteins have long been recognized as a most highly conserved gene system which is also regulated in a conserved manner. For many years these unique and ancient proteins have been studied as essential components in numerous functions within the cell. These functions, at a fundamental level, are common to all living cells from prokaryotes to the individual cells of complex organisms such as man. It was the long held belief that hsps were entirely intracellular and did not serve any functional extracellular purpose. Studies in the last decade, in particular, have demonstrated that in complex organisms, this is not correct. Indeed, it is now evident that hsps, which are abundant and conserved proteins in all living cells, are deeply involved in the evolution of at least one complex protective response of advanced multicellular organisms, i.e. the immune response. That hsps may also play key roles in other organismal systems essential to the functions of highly integrated and complex cellular assemblies, e.g. man, is also a clear possibility. The present review discusses our knowledge of the roles and uses of

hsps in the immune response and in immunotherapy. The focus here has been on new methods of applying this information. However, as a basic area of biomedical research, this field remains in its infancy and many new, unexpected and exciting discoveries can be expected in the present decade.

7. ACKNOWLEDGMENTS

This work was supported by Roswell Park Cancer Institute Core Grant P30CA16056, Department of Defense Grant 17-98-1-8104 from the Department of Defense, Public Health service Grant GM45944, National Cancer Institute Grant CA71599, and a grant from the Susan G. Komen Breast Cancer Foundation.

8. REFERENCES

1. Srivastava, P. K., A. Menoret, S. Basu, R. J. Binder & K. L. McQuade: Heat shock proteins come of age: Primitive functions acquire new roles in an adaptive world. *Immunity* 8, 657-665 (1998)

2. Blachere, N. E. & P. K. Srivastava: Heat shock protein-based cancer vaccines and related thoughts on immunogenicity of human tumors. *Semin Cancer Biol* 6, 349-355 (1995)

3. Tamura, Y., P. Peng, L. Kang, M. Daou & P. K. Srivastava: Immunotherapy of tumor with autologous tumor-derived hsp preparations. *Science* 278, 117-120 (1997)

4. Nicchitta, C.: Biochemical, cell biological and immunological issues surrounding the endoplasmic reticulum chaperone grp94/gp96. *Curr Opin Immunol* 10, 103-109 (1998)

5. Przepiorka D. & P. K. Srivastava: hsp-peptide complexes as immunotherapy for human cancer. *Mol Med Today* 4, 478-484 (1998)

6. Basu, S. & P. K. Srivastava: hsp: The fountainhead of innate and adaptive immune responses. *Cell Stress & Chaperones* 5, 443-451 (2000)

7. Tissieres, A., H. Mitchell & U. Tracy: Protein synthesis in salivary glands of Drosophila melanogaster: Relation to chromosome puffs. *J Mol Biol* 85, 389-398 (1974)

8. Li, G. & A. Laszlo: Amino acid analogs while inducing hsp sensitize CHO cells to thermal damage. *J Cell Physiol* 122, 91-97 (1985)

9. Hightower, L.: Cultured animal cells exposed to amino acid analogues or puromycin rapidly synthesize several polypeptides. *J Cell Physiol* 102, 407-427 (1980)

10. Gething, M. & J. Sambrook: Protein folding in the cell. *Nature (Lond)* 355, 33-45 (1992)

11. Morimoto, R., A. Tissieres & C. Georgopoulos: The Biology of hsp and Molecular Chaperones. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1994)

12. Gething, M. J.: Role and regulation of the ER chaperone BiP. Review. *Semin Cell Dev Biol* 10(5), 465-472 (1999)

13. Sciandra, J.J., Subjeck, J.R. and Hughes, C.S.: Induction of glucose-regulated proteins during anaerobic exposure and of heat-shock proteins after reoxygenation. *Proc Natl Acad Sci U S A* 81(15), 4843-4847 (1984)

14. Solheim, J.: Class I MHC molecules: assembly and antigen presentation. *Immunol Rev* 172, 11-19 (1999)

15. Skowronek, M., L. Hendershot & I. Haas: The variable domain of nonassembled Ig light chains determines both their half-life and binding to the chaperone BiP. *Proc Natl Acad Sci USA* 95(4), 1574-1578 (1998)

16. Shigeki, T. & D. McKay: Kinetics of peptide binding to the bovine 70 kDa heat shock cognate protein, a molecular chaperone. *Biochemistry* 35, 4636-4644 (1996)

17. Russell, R., R. Jordan & R. McMacken: Kinetic characterization of the ATPase cycle of the DnaK molecular chaperone. *Biochemistry* 37(2), 596-607 (1998)

18. Blond-Elguindi, S., W. Cwirla, W. Dower, R. Lipshutz, S. Sprang, J. Sambrook & M. Gething: Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell* 75, 717-728 (1993)

19. Gragerov, A. & M. Gottesman: Different peptide binding specificities of hsp70 family members. *J Mol Biol* 241, 133-135 (1994)

20. Rudiger, S., L. Germeroth, J. Schneider-Mergener & B. Bukau: Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. *EMBO J* 16(7), 1501-1507 (1997)

21. Takenaka, I., S. Leung, S. McAndrew, J. Brown & L. Hightower: Hsc70-binding peptides selected from a phage peptide library that resemble organellar targeting sequences. *J Biol Chem* 270, 19839-19844 (1995)

22. Fourie, A., J. Sambrook & M. Gething: Common and divergent peptide binding specificities of hsp70 molecular chaperones. *J Biol Chem* 269, 30470-30478 (1994)

23. Arnold-Schild, D., D. Hanau, D. Spehner, C. Schmid, H.G. Rammensee, S. De La & H. Schild: Cutting edge: receptor-mediated endocytosis of hsp by professional antigen-presenting cells. *J Immunol* 162, 3757-3760 (1999)

24. Castellino F., P. E. Boucher, K. Eichelberg, M. Mayhew, J. E. Rothman, A. N. Houghton, R. N. Germain: Receptor-mediated uptake of antigen/hsp complexes results in major histocompatibility complex class I antigen

presentation via two distinct processing pathways. *J Exp Med* 191(11), 1957-1964 (2000)

25. Binder, R., D. K. Han & P. K. Srivastava: CD91: a receptor for hsp gp96. *Nature Immunol* 2, 151-155 (2000)

26. Asea, A., S. K. Kraeft, E. A. Kurt- Jones, M. A. Stevenson, L. B. Chen, R. W. Finberg, G. C. Koo & S. K. Calderwood: hsp70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* 6(4), 435-442 (2000)

27. Gallucci, S. & P. Matzinger: Danger signals: SOS to the immune system. *Curr Opin Immunol* 13(1), 114-119 (2001)

28. Basu, S., R. J. Binder, T. Ramalingam & P. K. Srivastava: CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin. *Immunity* 14(3), 303-313 (2001)

29. Binder, R.J., Karimeddini, D. and Srivastava, P.K. Adjuvanticity of alpha 2-macroglobulin, an independent ligand for the heat shock protein receptor CD91. *J Immunol* 166(8), 4968-4972 (2001)

30. Singh, M. & D. O'Hagan: Advances in vaccine adjuvants. Review. *Nat Biotechnol* 17(11), 1075-1081 (1999)

31. Singh-Jasuja, H., H. U. Scherer, N. Hilf, D. Arnold-Schild, H. G. Rammensee, R. E. Toes & H. Schild: The hsp gp96 induces maturation of dendritic cells and down-regulation of its receptor. *Eur J Immunol* 30(18), 2211-2215 (2000)

32. Basu, A., G. Chakrabarti, A. Saha & S. Bandyopadhyay: Modulation of CD11C+ splenic dendritic cell functions in murine visceral leishmaniasis: correlation with parasite replication in the spleen. *Immunology* 99(2), 305-313 (2000)

33. Wells, A. D., S. K. Rai, M. S. Salvato, H. Band, M. Malkovsky: hsp72-mediated augmentation of MHC class I surface expression and endogenous antigen presentation. *Int Immunol* 10(5), 609-617 (1998)

34. Chu, N. R., H. B. Wu, T. Wu, L. J. Boux, M. I. Siegel & L. A. Mizzen: Immunotherapy of a human papillomavirus (HPV) type 16 E7-expressing tumour by administration of fusion protein comprising Mycobacterium bovis bacille Calmette-Guerin (BCG) hsp65 and HPV16 E7. *Clin Exp Immunol* 121(2), 216-25 (2000)

35. Srivastava, P. K., A. Deleo & L. J. Old: Tumor rejection antigens of chemically induced sarcomas of inbred mice. *Proc Natl Acad Sci USA* 83, 3407-3411 (1986)

36. Ullrich, S. J., E. A. Robinson, L. W. Law, M. Willingham & E. Appella: A mouse tumor-specific transplantation antigen is a heat shock-related protein. *Proc Natl Acad Sci USA* 83, 3121-3125 (1986)

37. Udono, H. & P. K. Srivastava: hsp 70-associated peptides elicit specific cancer immunity. *J Exp Med* 178, 1391-1396 (1993)

38. Srivastava, P. K.: Peptide-binding hsp in the endoplasmic reticulum: role in immune response to cancer and in antigen presentation. *Adv Cancer Res* 62, 153-177 (1993)

39. Udono, H. & P. K. Srivastava: Comparison of tumor-specific immunogenicities of stress-induced proteins gp96, hsp90, and hsp70. *J Immunol* 152(11), 5398-5403 (1994)

40. Welch, W. J., H. S. Kang, R. P. Beckmann, L. A. Mizzen: Response of mammalian cells to metabolic stress; changes in cell physiology and structure/function of stress protein. *Curr Top Microbiol Immunol* 167, 31-55 (1991)

41. Basu, S. & P. K. Srivastava: Calreticulin, a peptide-binding chaperone of the endoplasmic reticulum, elicits tumor- and peptide-specific immunity. *J Exp Med* 189, 797-802 (1999)

42. Wang, X-Y., L. Kazim, E. A. Repasky & J. R. Subjeck: Characterization of hsp 110 and glucose-regulated protein 170 as cancer vaccines and the effect of fever-range hyperthermia on vaccine activity. *J Immunol* 166, 490-497 (2001)

43. Janetzki, S., N. E. Blachere & P. K. Srivastava: Generation of tumor-specific cytotoxic T lymphocytes and memory T cells by immunization with tumor derived hsp gp96. *J Immunother* 21, 269-276 (1998)

44. Vanaja, D. K., M. E. Grossmann, E. Celis & C. Y. F. Young: Tumor prevention and antitumor immunity with hsp 70 induced by 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 in transgenic adenocarcinoma of mouse prostate cells. *Cancer Res* 60, 4714-4718 (2000)

45. Heike, M., A. Weinmann, K. Bethke & P. R. Galle: Stress protein/peptide complexes drive from autologous tumor tissue as tumor vaccines. *Biochem Pharm* 58, 1381-1387 (1999)

46. Janetzki, S., D. Palla, V. Rosenhauer, H. Lochs, J. J. Lewis & P. K. Srivastava: Immunization of cancer patients with autologous cancer-derived hsp gp96 preparations: a pilot study. *Int J Cancer* 88, 232-238 (2000)

47. Blachere, N. E., Z. Li, R. Y. Chandawarkar, R. Suto, N. S. Jaikaria, S. Basu, H. Udono & P. K. Srivastava: hsp-peptide complexes, reconstituted *in vitro*, elicit peptide-specific cytotoxic T lymphocyte response and tumor immunity. *J Exp Med* 186, 1315-1322 (1997)

48. Ciupitu, A-M., M. Petersson, C. L. O'Donnell, K. Williams, S. Jindal, R. Kiessling & R. M. Welsh: Immunization with a lymphocytic choriomeningitis virus peptide mixed with hsp 70 results in protective antiviral immunity and specific cytotoxic T lymphocytes. *J Exp Med* 187, 685-691 (1998)

49. Moroi, Y., M. Mayhew, J. Trcka, M. H. Hoe, Y. Takechi, F. U. Hartl, J. E. Rothman & A. N. Houghton: Induction of cellular immunity by immunization with novel hybrid peptides complexed to hsp70. *Proc Natl Acad Sci USA* 97, 3485-3490 (2000)

50. Barrios, C., A. R. Lussow, J. Van Embden, R. Van der Zee, R. Rappuoli, P. Costantino, J. A. Louis, P. H. Lambert & G. Del Giudice: Mycobacterial heat-shock proteins as carrier molecules. II: The use of the 70-kDa mycobacterial heat-shock protein as carrier for conjugated vaccines can circumvent the need for adjuvants and Bacillus Calmette Guerin priming. *Eur J Immunol* 22, 1365-1372 (1992)

51. Roman, E. & C. Moreno: Synthetic peptides non-covalently bound to bacterial hsp70 elicit peptide-specific T-cell responses in vivo. *Immunol* 88, 487-492 (1996)

52. Chen, C-H., T-L. Wang, C-F. Hung, Y. Yang, R. A. Young, D. M. Pardoll & T-C. Wu: Enhancement of DNA vaccine potency by linkage of antigen gene to an HSP70 gene. *Cancer Res* 60, 1035-1042 (2000)

53. Hsu, K. F., C. F. Hung, W. F. Cheng, L. He, L. A. Slater, M. Ling & T. C. Wu: Enhancement of suicidal DNA vaccine potency by linking Mycobacterium tuberculosis hsp 70 to an antigen. *Gene Ther* 8, 376-383 (2001)

54. Suzue, K. & R.A. Young: Adjuvant-free hsp70 fusion protein system elicits humoral and cellular immune responses to HIV-1 p24. *J Immunol* 156, 873-876 (1996)

55. Horwitz, M. S., L. M. Bradley, J. Harbertson, T. Krahl, J. Lee & N. Sarvetnick: Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nature Med* 4, 781-785 (1998)

56. Könen-Waisman, S., A. Cohen, M. Fridkin & I. R. Cohen: Self heat-shock protein (hsp60) peptide serves in a conjugate vaccine against a lethal pneumococcal infection. *J Infect Dis* 179, 403-413 (1999)

57. Young, D. B., A. Mehlert & D. F. Smith: Stress proteins and infectious diseases. In: Stress proteins in biology and medicine. Eds: Morimoto, R. I., Tissieres, A., Georgopoulos, C. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 131-165 (1990)

58. Schild, H., D. Arnold-Schild, E. Lammert & H-G. Rammensee: Stress proteins and immunity mediated by cytotoxic T lymphocytes. *Curr Opin Immunol* 11, 109-113 (1999)

59. Huang, Q., J. F. L. Richmond, K. Suzue, H. N. Eisen & R. A.Young: Elicitation of CTLs by Mycobacterial hsp70 Fusion Proteins Maps to a Discrete Domain and is CD4+ T Cell-Independent. *J Exp Med* 191, 403-408 (2000)

60. Restifo, N. P., F. Esquivel, A. L. Asher, H. Stotter, R. J. Barth, J. R. Bennink, J. J. Mule, J. W. Yewdell & S. A. Rosenberg: Defective presentation of endogenous antigens by a murine sarcoma. Implications for the failure of an anti-tumor immune response. *J Immunol* 147, 1453-1459 (1991)

61. Lukacs, K. V., D. B. Lowrie, R. W. Stokes & M. J. Colston: Tumor cells transfected with bacterial hsp gene lose tumorigenicity and induce protection against tumors. *J Exp Med* 178, 343-348 (1993)

62. Lukacs, K. V., A. Nakakes, C. J. Atkins, D. B. Lowrie & M. J. Colston: *In vivo* gene therapy of malignant tumors with hsp-65 gene. *Gene Ther* 4, 346-350 (1997)

63. Melcher, A., S. Todryk, N. Hardwick, M. Ford, M. Jacobson & R. G. Vile: Tumor immunogenicity is determined by the mechanism of cell death via induction of hsp expression. *Nat Med* 4, 581-587 (1998)

64. Blachere, N. E., H. Udono, S. Janetzki, Z. Li, M. Heike & P. K. Srivastava: hsp vaccines against cancer. *J Immunother* 14, 352-356 (1993)

65. Heikema, A., E. Agsteribbe, J. Wiscjut & A. Huckriede: Generation of hsp-based vaccines by intracellular loading of gp96 with antigenic peptides. *Immunol Lett* 57, 69-74 (1997)

66. Suto, R. & P. K. Srivastava: A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science* 269(5230), 1585-1588 (1995)

67. Arnold, D., S. Faath, H. Rammensee & H. Schild: Crossing-priming of minor histocompatibility antigen-specific cytotoxic T cells upon immunization with hsp gp96. *J Exp Med* 182, 885-889 (1995)

68. Okamoto, M., K. Tazawa, T. Kawagoshi, M. Maeda, T. Honda, T. Sakamoto & K. Tsukada: The combined effect against colon-26 cells of heat treatment and immunization with heat treated colon-26 tumour cell extract. *Int J Hyperthermia* 16(3), 263-273 (2000)

69. Mosser, D. D. & L. H. Martin: Induced thermotolerance to apoptosis in a human T lymphocyte cell line. *J Cell Physiol* 151(3), 561-570 (1992)

70. Li, C. Y., J. S. Lee, Y. G. Ko, J. I. Kim & J. S. Seo: hsp 70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. *J Biol Chem* 275(33), 25665-25671 (2000)

71. DeMeester, S. L., T. G. Buchman & J. P. Cobb: The heat shock paradox: does NF-kappaB determine cell fate? *FASEB J* 15(1), 270-274 (2001)

72. Di, Y. P., E. A. Repasky & J. R. Subjeck: The distribution of hsp70, protein kinase C and spectrin is altered in lymphocytes during a fever-like hyperthermia exposure. *J Cell Physiol* 172, 44-54 (1997)

73. Wang, X-Y., J. R. Ostberg & E. A. Repasky: Effect of fever-like whole-body hyperthermia on lymphocyte

spectrin distribution, protein kinase C activity, and uropod formation. *J Immunol* 162, 3378-3387 (1999)

74. Wang, W. C., L. M. Goldman, D. M. Schleider, M. M. Appenheimer, J. R. Subjeck, E. A. Repasky & S. S. Evans: Fever-range hyperthermia enhances L-selectin-dependent adhesion of lymphocytes to vascular endothelium. *J Immunol* 160(2), 961-969 (1998)

75. Ostberg, J. R. & E A. Repasky: Comparison of the effects of two different whole body hyperthermia protocols on the distributionof murine leukocyte populations. *Int J Hyperthermia* 16(1), 29-43 (2000)

76. Burd, R., T. S. Dziedzic, Y. Xu, M. A. Caligiuri, J. R. Subjeck & E. A. Repasky: Tumor cell apoptosis, lymphocyte recruitment and tumor vascular changes are induced by low temperature, long duration (fever-like) whole body hyperthermia. *J Cell Physio* 177, 137-147 (1998)

77. Evans, S. S., M. Frey, D. M. Schleider, R. A. Bruce, W-C. Wang, E. A. Repasky & M. M. Appenheimer: Regulation of leukocyte-endothelial cell interactions in tumor immunity. In: The Biology of Tumors Eds. Mihich, E. & Croce, C. Plenum Press, New York, NY 9, 273-286 (1998)

78. Evans, S, S., D. M. Schleider, L. A. Bowman, M. L. Francis, G. S. Kansas & J. D. Black: Dynamic association of L-selectin with the lymphocyte cytoskeletal matrix. *J Immunol* 162(6), 3615-3624 (1999)

79. Evans, S. S., W. C. Wang, M. D. Bain, R. Burd, J. R. Ostberg & E. A. Repasky: Fever-range hyperthermia dynamically regulates lymphocyte delivery to high endothelial venules. *Blood* 97(9), 2727-2733 (2001)

80. Lohr, F., Hu, K., Huang, Q., Zhang, L., Samulski, T.V., Dewhirst, M.W. & Li, C-Y. Enhancement of radiotherapy by hyperthermia-regulated gene therapy. *Int J Radiat Oncol Biol Phys* 48(5), 1513-1518 (2000)

81. Gerner, E.W., Hersh, E.M., Pennington, M., Tsang, T.C., Harris, D., Vasanwala, F. & Brailey, J. Heat-inducible vectors for use in gene therapy. *Int J Hyperthermia* 16(2), 171-181 (2000)

82. Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. Freeman, J. M. Green, C. B. Thompson & J. A. Bluestone: CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1(5), 405-413 (1994)

83. Krummel, M. F. & J. P. Allison: CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J Exp Med* 182(2), 459-465 (1995)

84. Walunas, T. L., C. Y. Bakker & J. A. Bluestone: CTLA-4 ligation blocks CD28-dependent T cell activation. *J Exp Med* 183, 2541-2550 (1996)

85. Krummel, M. F. & J. P. Allison: CTLA-4 engagement inhibits IL-2 accumulation and cell cycle progression upon activation of resting T cells. *J Exp Med* 183(6), 2533-2540 (1996)

86. Leach, D. R., M. F. Krummel & J. P. Allison: Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 271, 1734-1736 (1996)

87. Kwon, E. D., A. A. Hurwitz, B. A. Foster, C. Madias, A. L. Feldhaus, N. M. Greenberg, M. B. Burg & J. P. Allison: Manipulation of T cell costimulatory and inhibitory signals for immunotherapy of prostate cancer. *Proc Natl Acad Sci USA* 94(15), 8099-8103 (1997)

88. Kwon, E. D., B. A. Foster, A. A. Hurwitz, C. Madias, J. P. Allison, N. M. Greenberg & M. B. Burg: Elimination of residual metastatic prostate cancer after surgery and adjunctive cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) blockade immunotherapy. *Proc Natl Acad Sci USA* 96(26), 15074-15079 (1999)

89. Yang, Y. F., J. P. Zou, J. Mu, R. Wijesuriya, S. Ono, T. Walunas, J. Bluestone, H. Fujiwara & T. Hamaoka: Enhanced induction of antitumor T-cell responses by cytotoxic T lymphocyte-associated molecule-4 blockade: the effect is manifested only at the restricted tumor-bearing stages. *Cancer Res* 57(18), 4036-4041 (1997)

90. Hurwitz, A. A., T. F. Yu, D. R. Leach & J. P. Allison: CTLA-4 blockade synergizes with tumor-derived granulocyte-macrophage colony-stimulating factor for treatment of an experimental mammary carcinoma. *Proc Natl Acad Sci USA* 95(17), 10067-10071 (1998)

91. Mokyr, M. B., T. Kalinichenko, L. Gorelik & J. A. Bluestone: Realization of the therapeutic potential of CTLA-4 blockade in low-dose chemotherapy-treated tumor-bearing mice. *Cancer Res* 58(23), 5301-5304 (1998)

92. Foster, B. A., J. R. Gingrich, E. D. Kwon, C. Madias & N. M Greenberg: Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. *Cancer Res* 57(16), 3325-3330 (1997)

Key words: Heat Shock Proteins (Hsps), Danger Signal, Antigen Presentation, Chaperon, Vaccine, Immunogenicity, Review

Send correspondence to: John R. Subjeck, Department of Molecular and Cellular Biophysics, Roswell Park Cancer Institute, Buffalo New York 14263 USA. Tel: 716-845-3147 Fax: 716-845-8899 E-mail: john.subjeck@roswellpark.org