

Heat Shock Proteins and The Immune System

There is a large body of scientific evidence demonstrating that Heat shock proteins (Hsp) are very efficient immunogens; inducing strong immune responses. This unique property of Hsp was first discovered when scientists studied the immune responses elicited during infections with microbial pathogens such as bacteria, parasites and fungi (1). It was observed that a large proportion of the immune response was focused on pathogen Hsps. Acting like a “red flag”, Hsps are thought to alert the immune system to the presence of pathogens and trigger a prompt and potent immune response (2). Based on continuing research, Hsps are now understood to be primary targets for immune surveillance not only against infection, but other abnormal situations such as the presence of cancer cells. Indeed, Hsps are thought to function as universal “danger signals” within the body, alerting the immune system to the presence of stressed, infected or diseased tissue (3).

The intense research effort focused on Hsps has further revealed that they can activate both “arms” of the immune system, namely, the innate and adaptive immunity arms. Innate immunity serves as a first line of defense; the activation of this arm results in a non-specific immune response to the foreign invader. In response to signals transmitted by cells of the innate immune system (eg. NK cells, neutrophils, macrophages and dendritic cells), cells of the adaptive immune system (eg. B and T cells) mount a second wave of specific immune responses to eliminate the “invader”. This suggests that Hsps are detected by the body’s natural immune surveillance system, sending signals to multiple cell types which are programmed to defend the body against foreign invaders and to eradicate diseased tissues.

The unique ability of Hsps to activate both innate and adaptive immunity, and the powerful synergies that can result, can be explained by the recent identification of candidate Hsp receptors on dendritic cells and macrophages (4-9). These cell types are referred to as “professional” antigen-presenting cells (APC) because they are ideally suited for initiating antigen-specific immune responses, particularly those mediated by CD4+ and CD8+ T cells. Interestingly, most of the identified Hsp receptors are members of the Toll-like receptor (TLR) or scavenger receptor families. Additional Hsp receptors include the APC co-stimulatory molecule CD40, and CD94 on NK cells. TLR’s and scavenger receptors are key sensors for the innate immune system and are used by APC to detect foreign invaders. Engagement of these innate immunity receptors serves

to activate APC and promotes antigen-specific T and B cell responses by the adaptive immune system.

CoVal™ Fusions: A Platform Technology for Therapeutic Vaccines

With these positive features in mind, scientists have explored the use of Hsp in the development of infectious disease vaccines and cancer immunotherapies, with great success in numerous preclinical animal models (reviewed in 10). Nventa has chosen to exploit the immunostimulatory properties of Hsp in the development of a new technology platform: Hsp fusion proteins. Nventa scientists and collaborators at the Whitehead Institute for Biomedical Research and the Massachusetts Institute of Technology (MIT) have discovered that Hsp fusion proteins represent a broad platform for the creation of therapeutic vaccines. That is, vaccines that are designed to reduce or eliminate existing disease such as chronic viral infections or cancer. This is in contrast to preventative (prophylactic) vaccines, which are given to healthy people prior to exposure to disease-causing pathogens. Hsp fusion proteins are created by standard recombinant DNA techniques, splicing together a Hsp gene with a gene encoding a viral or cancer protein against which an immune response is desired. The fusion protein is produced in the common bacteria *E. coli* by fermentation and purified by standard chromatographic methods. Because the Hsp and the protein antigen are **covalently** linked together in a single molecule, Nventa refers to Hsp fusion proteins as **CoVal™** fusions.

Following parenteral administration, it is thought that the Hsp portion of the **CoVal™** fusion acts like an “address”, directing the Hsp and covalently linked antigen to professional APC. Once bound by the Hsp receptor on the APC surface, the entire fusion protein is engulfed and processed internally for presentation to T cells. Normally, exogenous proteins (such as those present in subunit-type preventative vaccines) are internalized by APC and degraded into small pieces (called peptides) that are then presented on the APC surface bound to class II major histocompatibility complex (MHC) molecules. Processing of antigens by this route, called the class II pathway, activates CD4+ T cells and results in the production of antibodies by B cells. Induction of humoral immunity (antibodies) is the primary mechanism by which many preventative vaccines work.

However, for situations where a therapeutic immune response is required, such as in the treatment of chronic viral infection or cancer, cellular (ie. T cell) immunity is required. For this reason, technologies which can safely and effectively trigger T cell responses, in particular cytotoxic T lymphocytes (CTL), have long been sought by vaccine developers. CoVal™ fusions are precisely the solution to this problem. Researchers have shown that when administered as purified protein in a saline vehicle, **CoVal™** fusions elicit potent CTL responses. This type of immune response is triggered because, unlike the more conventional

prophylactic vaccine approach, vaccines developed using the **CoVal™** technology enter a pathway in the APC called the class I pathway. In this pathway, peptides derived from the fused antigen are presented on the APC surface in association with class I MHC, resulting in the generation of antigen-specific CD8+ T cells (CTL). The process whereby exogenous antigens are taken up by APC to elicit CTL responses is called cross-priming (or cross-presentation), and represents a critical immune surveillance pathway of dendritic cells (11). Cross-priming is thought to be the primary mechanism by which immune responses are initiated against cells infected by viral pathogens, or against cancer cells, which typically have poor APC function (12, 13). In the absence of cross-priming, such diseased cells would likely escape immune detection.

By utilizing natural cross-priming pathways, CoVal™ fusion immunization results in the induction of CD8+ CTL specific for the antigen present in the fusion. Such CTL recognize and destroy cells which display disease-specific peptide antigens on their surface in association with class I MHC. It is generally accepted that antigen-specific CTL are the most effective cells in the immune system for killing virus-infected or cancer cells.

Publications from Nventa and collaborators have shown that the immunization of mice with a variety of CoVal™ fusions elicits potent antigen-specific immune responses, including CD8+ CTL and production of cytokines such as IL-12, IFN- γ and TNF- α . Summarizing these studies, CTL responses have been achieved: 1) with numerous viral and cancer antigens, 2) in the absence of adjuvant, 3) in the absence of CD4+ T helper cells and 4) in mice of different genetic backgrounds (14-19). In one study, the potency of CTL induction by a CoVal™ fusion was several hundred fold greater than that obtained by immunization with a CTL peptide epitope combined with complete Freund's adjuvant (CFA) (20). In addition, *in vitro* studies have demonstrated that exposure of dendritic cells to CoVal™ fusions results in cross-presentation of antigen to CD8+ T cells and activation of dendritic cells (19, 21). Therefore, the *in vitro* and *in vivo* data are consistent with a mechanism of action involving the targeting of Hsp receptors on dendritic cells resulting in potent antigen-specific CTL induction. These publications provide proof-of-concept for CoVal™ fusions as therapeutic vaccines for diseases caused by human papillomavirus (HPV), influenza, HIV and in cancer immunotherapy.

Nventa's Lead CoVal™ Fusion: HspE7

Preclinical Summary

Nventa's lead product in development, HspE7, is a fusion protein composed of Hsp65 from the bacterium *Mycobacterium bovis* BCG and the E7 protein from

human papillomavirus (HPV) type 16. Hsp65 was chosen based on its potent immunostimulatory properties, while E7 was chosen because it represents a precise target for the immune system to destroy HPV infected cells. The E7 protein from HPV is known to be expressed in HPV infected cells at multiple stages of HPV-associated disease progression.

In preclinical studies conducted at Nventa, HspE7 immunization was shown to trigger robust systemic immune responses in mice to E7, including the induction of CTL and immunomodulatory cytokines such as IFN- γ and TNF- α . We then sought to determine if it was possible to further augment the already potent specific immune response generated using the HspE7 **CoVal™** technology. Current immunological evidence points to the combination of multiple “danger signal” as a way to synergistically boost antigen specific immune responses. Experiments designed to look at the potential synergy of HspE7 in combination with various adjuvants demonstrate that not all combinations resulted in significant enhancement of the already potent HspE7 immune response. Of the combinations tested 2 adjuvants exhibited massive synergistic effects when combined separately with HspE7.

Immunization with this combination resulted in the induction of very high levels of E7-specific immunity. Furthermore, using the murine TC-1 tumor model (a standard model for the evaluation of HPV immunotherapies; E7 protein positive), immunization of mice with the combination induced more durable and far greater levels of tumor protection than had previously been observed with HspE7. On the basis of this very encouraging preclinical data, the combination of HspE7 with an adjuvant is being aggressively pursued for the treatment of a variety of HPV related diseases.

Clinical Summary

HspE7 is being developed for the treatment of diseases caused by human papillomavirus (HPV), including recurrent respiratory papillomatosis (RRP), genital warts (GW), cancer precursors known as cervical and anal intraepithelial neoplasia (CIN and AIN) and HPV-related cancers including cervical cancer and a subset of head and neck cancers. HPV infection is ubiquitous in humans and is estimated to infect over 70% of the sexually active population. HPV transmission is not prevented by condom use and many infections are asymptomatic. HPV causes a spectrum of diseases, ranging from skin warts to cancer. Of the over 100 strains of HPV identified, approximately 30 are mucosotropic, sexually transmitted and are responsible for a variety of pathologic conditions. HPV strains are classified as high or low risk in accordance with their propensity to initiate the development of cancer. Infection with high-risk HPV strains, such as types 16 and 18, is associated with CIN, AIN and cancer, while infection with low risk HPV types, such as types 6 and 11 is associated with GW and RRP. (For a detailed discussion of HPV-associated cancers see, “HPV Cancers” section). Although there is currently no vaccine against HPV infection, prophylactic vaccines against HPV infection have recently completed successful

clinical testing and are likely to enter the marketplace over the next few years. These prophylactic (preventative) vaccines are designed to prevent initial infection with HPV and accordingly, vaccination programs will likely target adolescents prior to the onset of sexual activity. However, these prophylactic vaccines are predicted to be ineffective against pre-existing HPV infections as the target antigen is no longer expressed at later stages of infection. Thus, despite the introduction of new prophylactic vaccines, HPV infection and HPV-induced cancer is expected to remain entrenched in the adult population for many years to come.

HspE7 treatment: eradication of HPV-infected cells

HPV infects cells in the basal epithelium, presumably via minor skin abrasions. This deepest epithelial cell layer, the source of renewing skin cells, acts as a virus reservoir supporting persistent HPV infection. HPV replication and antigen expression is linked to the differentiation pathway of skin cells (keratinocytes) in their upward migration to the skin surface. Hence, not all HPV-infected cells are visible as abnormal lesions. This is why surgical, ablative or topical treatment of visible HPV lesions is associated with disease recurrence. In contrast, induction of systemic E7-specific immune responses by HspE7 has the potential to target and destroy E7-expressing HPV-infected cells. Based on preclinical research, one of the primary immune effectors in this response are E7-specific CTL. If the virus reservoir can be recognized by the immune system, the source of recurrent disease can be attacked. Another potential benefit of HspE7 immunization is induction of E7-specific memory T cells, which may provide long-term protection against HPV-associated disease.

Active Regimen Identified

In clinical trials conducted in patients with different HPV-associated diseases, namely, RRP, GW and AIN, an active treatment regimen has been identified for HspE7. This regimen consists of three doses of 500 mcg given subcutaneously at monthly intervals. These patient groups suffer from distinct HPV-associated diseases in different anatomic locations, such as the respiratory tract and the anogenital region. Nventa is in the process of determining the best dosing regime for its combination product. Clinical trials will commence in the near future to address safety and dosing.

Favorable Safety Profile

Phase I, II and III studies with HspE7 alone in human volunteers and in patients have resulted in a safety database numbering nearly 400 subjects. Inspection of this database reveals a favorable safety profile in both children and adults. The predominant adverse experience is a reaction at the site of injection, typical of

other vaccinations. This reaction is mild to moderate in severity, appears within hours to a day, and resolves within one to two days without treatment. This reaction occurs in the great majority of subjects. A minority of patients had a mild to moderate systemic reaction including fever and asthenia, symptoms also observed with other vaccinations. Dose limiting adverse experiences have not been identified.

RRP- shortest path to the market

RRP is a rare disease consisting of warts in the upper airways that can affect infants, children and adults. In 1995, there were approximately 21,000 active and new cases of RRP in the U.S. (22) with more recent estimates closer to 25,000 (23). RRP is caused by infection with HPV, primarily types 6 and 11. These are the same HPV types associated with genital warts (GW) and it is known that children born to mothers with GW are at increased risk for RRP. In addition, intrauterine transmission of HPV has also been suggested in RRP. In children, the disease causes respiratory obstruction around the vocal cords that can be life-threatening and must be treated by surgical shaving of the warts under general anesthesia. RRP is an insidious, highly recurrent disease that can persist for many years and even decades. The average child with RRP undergoes five surgeries per year and some children undergo as many as 200 procedures during their lifetime. As a result, RRP places a heavy psychological burden on the patient and their family in addition to a large economic burden on the health care system.

In a RRP phase II trial targeting children requiring frequent surgery, treatment with HspE7 met its targeted primary endpoint of lengthening the time between surgeries post-therapy. This study evaluated the length of time between surgeries following treatment with HspE7 compared to the baseline inter-surgical interval established for each patient during the months preceding treatment. In the overall population of 27 patients, the first post-treatment interval increased 93% over baseline ($p < 0.02$) and the annualized surgical rate was reduced significantly ($p < 0.003$). In addition, the median interval of all surgeries reported following treatment suggests that the patient population experienced 87 fewer surgeries during the first year post-treatment. Using the Derkay-Coltera Score, which is a standard clinical tool to measure the extent of RRP disease, HspE7-treated children showed a significantly decreased Score by study end ($p < 0.04$).

Genital Warts- a large marketplace for HspE7

Genital warts (GW) is one of the most common sexually-transmitted diseases with an estimated prevalence of 1.4 million (24) and yearly incidence of between 500,000 to 1 million (25) in the U.S. alone. GW is caused by infection with HPV, primarily types 6 and 11. In two clinical studies, it has been shown that up to 80% of patients with GW, including patients with difficult, internal warts and patients with extensive and treatment-refractory warts, experience clearance of their

warts following treatment with HspE7. In contrast to surgical treatments that may clear simple warts immediately, the therapeutic effect of HspE7 requires a longer period of time, e.g. up to nine months or longer for complete wart clearance. This interval is presumably a reflection of the time required for systemic immunity to eradicate all wart lesions. However, clearance, once achieved by HspE7 treatment, is durable and few patients achieving complete resolution of warts experience recurrence during the 24 months post-treatment observation. This observation stands in contrast to other surgical, ablative or topical treatments, where longitudinal studies show rates of recurrence of up to 50% and more. Another important feature of HspE7 treatment is the clearance of multiple GW in many patients and in some cases clearance of warts with a large surface area. In the reported trials with HspE7, complete response means clearance of all visible GW. This contrasts with some clinical studies of surgical/ablative/topical treatments, where efficacy is reported for treated warts only and excludes untreated warts that may be present.

Precancerous Dysplasias

Well more than a million patients in the United States each year receive the diagnosis of cervical or anal intraepithelial neoplasia (CIN or AIN respectively). Collectively, these cancer precursors are called dysplasias. When possible, these lesions are surgically removed in order to prevent the progression to cancer. Surgery is difficult in certain settings, namely anal dysplasia, cervical dysplasia recurrent after initial surgery, and dysplasia in HIV+ patients. These special populations have great medical need, because no satisfactory therapy exists for this condition. There are a number of advantages for the Company in pursuing these indications for HspE7. These include the conduct of potentially fewer, smaller and less costly clinical trials, opportunities for FDA orphan drug designation and fast track status which may enable faster routes to market, and potential for premium product pricing. The Company is intending to pursue cervical dysplasia in women who have suffered recurrence after standard surgical therapy and either anal or cervical dysplasia in HIV+ patients. Currently, exploratory trials are underway or planned in each of these settings; these trials are being sponsored by collaborators including the U.S. National Cancer Institute (NCI). The initial results of these trials will form the basis of the Company's decisions regarding the most promising avenues for supplementary regulatory filings for HspE7.

Cancer

HPV is associated with a number of cancers, such as cancer of the uterine, cervix and head and neck cancer. HspE7 is of great interest in this setting, since, like all cancers, these diseases have limited therapeutic options and great unmet medical need. The Company is seeking collaborators to partner in the

development of these indications (For a detailed discussion of HPV-associated cancers, see “HPV Cancers” section).

HspE7- A Broad Spectrum Therapeutic Vaccine for HPV

HspE7 contains the E7 protein from HPV type 16, a high-risk strain which is frequently present in precancerous dysplasias like CIN and AIN, in anogenital cancers (e.g. cervical cancer) and head and neck cancer. However, from the Company’s previous clinical studies, it is evident that patients with RRP and GW, which are caused primarily by HPV types 6 and 11, also respond to HspE7 treatment. These clinical data indicate that therapeutic immunization with HspE7 can treat diseases associated with HPV types other than type 16. These observations are consistent with the induction of cross-reactive T cell responses, such as CTL, following HspE7 immunization. Given our understanding of the mechanism of action of HspE7 in mice (18) and an analysis of the predicted HLA-binding peptides from HPV types 16, 6, 11 and others, our clinical data suggests that HspE7 has the potential to be a broad-spectrum therapeutic vaccine active in the treatment of diseases caused by a variety of HPV types.

Summary

The biology and mechanism of action (MOA) of HspE7 suggest that this therapeutic should lend itself well to combination with specific adjuvants. We have looked at combining HspE7 with adjuvants that very effectively sense intracellular viral and bacterial infection via the use of intracellular receptors. Adjuvants that exhibit activity based on intracellular receptors are thought to specifically initiate immune responses that generate CD4 and CD8 T cells. These immune effector mechanisms target and kill viral or bacterial infected cells to prevent spread of the pathogen. Similarly, MOA studies have shown that HspE7 also functions to generate similar responses which are hallmarked by the ability to potently generate CD8 T cell responses capable of lysing E7 positive cells. It is therefore not surprising that the combination of these two moieties result in levels of immune response far exceeding those observed for each component separately. It is important to restate the fact that combination of HspE7 with the adjuvant does not alter the proposed MOA of HspE7 alone, it only serves to make this HPV specific therapeutic more potent.

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